Preparation of Zwitterionic Polymethacrylamide Modified with L-Lysine and Its Effect on Fibrinolytic Activity

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Zwitterionic polymethacrylamides, poly(N^{α}-methacrylamide-L-lysine) [poly(α-LysMA)] and poly(N^{ε}-methacrylamide-L-lysine) [poly(ε-LysMA)] were prepared to examine their bioactivity by an evaluation of fibrinolytic activity and a binding assay using resonant mirror biosensor (IAsys). Poly(α-LysMA) enhanced the fibrinolytic activity by plasminogen/tissue-type plasminogen activator while no enhancement was observed in the case of poly(ε-LysMA). A strong interaction between Plg to poly(α-LysMA) was also observed by IAsys when compared with poly(ε-LysMA).

Anticoagulant biomaterials have been demonstrated by immobilizing antithrombogenic or fibrinolytic substances.[1,2] We assumed that a polymer having a strong affinity for plasminogen (Plg) could be used to construct an anticoagulant surface on the polymer by the selective adsorption of Plgs from blood, because it has been shown that the binding Plg increases the hemocompatibility of biomaterial surfaces.[3] Specific interactions between the Plg and the polymer immobilized on a material surface, therefore, would effectively prevent the formation of stable fibrin clots without their subsequent interference in the biomaterial functions.

Plg is converted to the fibrinolytic enzyme, plasmin, by the action of tissue-type plasminogen activator (t-PA), which in turn digest fibrin to fibrin degradation products.[4] Plg sequence contains prominent lysine binding site (LBS), which is an important factor of fibrinolytic activity.[5,6] The interaction between Plg and L-lysine analogs enhances Plg conversion to plasmin and fibrinolytic activity.[3,7]

On the other hand, zwitterionic poly[2-(methacryloyloxy)-ethyl phosphorylcholine] has already been established as a biomedical material.[8,9] We have also reported that the polymers consisting of zwitterionic *O*-methacryloyl-L-serine segments have a potential function as a biomaterial.[10–12]

In this study, in order to develop a new class of an antithrombogenic polymer showing fibrinolytic activity by specifically adsorbing Plg from serum proteins in blood without adsorption and denaturation of the serum proteins except for fibrinolytic proteins on its immobilized material surfaces based on the zwitterionic polymer structure, poly(N^{α}-methacrylamide-L-lysine) [poly(α-LysMA)] and poly(N^{ε}-methacrylamide-L-lysine) [poly(ε-LysMA)] were prepared with L-lysine in the side chain. In order to know their fibrinolytic activiy and specific interaction to Plg before their immobilization on biomaterials, the effect of poly(α-LysMA) or poly(ε-LysMA) on fibrinolytic activity in Plg/t-PA system was examined by enzymatic analysis using chromogenic plasmin substrate, H-D-Val-Leu-Lys-*p*-nitroaniline (S-2251: Daiichi Pure Chemicals, Japan), and interaction analysis of Plg to poly(α-LysMA) and poly(ε-LysMA) was also carried out using the IAsys resonant mirror biosensor (Affinity Sensors, Saxson Hill, Cambridge, UK).

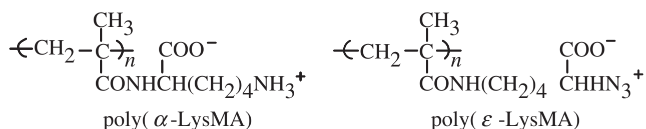

Figure 1. Structure of polymers.

The zwitterionic poly(α-LysMA) and poly(ε-LysMA) were synthesized as following reactions: N^{α}-methacrylamide-N^{ε}-*t*-butoxycarbonyl-L-lysine *t*-butyl ester (Boc-LysMA-OtBu)[13] and N^{α}-acetyl-N^{ε}-methacrylamide-L-lysine methyl ester (Ac-LysMA-OMe)[13] were polymerized radically with 2,2′-dimethylazobisisobutyrate (MAIB). The poly(α-LysMA) and poly(ε-LysMA) were obtained by deblocking with 80 vol % trifluoroacetic acid aq. and 2 M (1 M = 1 mol·dm^{-3}) NaOH aq., respectively[13] (Figure 1).

The reaction of S-2251 and Plg (SIGMA, USA) by t-PA (SIGMA, USA) was carried out in the presence of poly(α-LysMA) or poly(ε-LysMA) in Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) buffer (pH = 7.4) in a manner similarly to the paper.[14] As a result, poly(α-LysMA) enhanced Plg activation

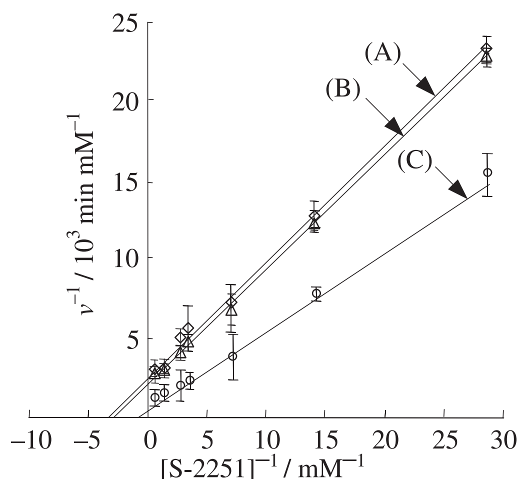

Figure 2. Lineweaver-Burk plots for the enzymatic reaction of Plg/t-PA and S-2251 in the presence of L-lysine, poly(ε-LysMA), or poly(α-LysMA) in Tris buffer solution (pH = 7.4; the pH of Tris solution (50 mM) was adjusted with 0.1 M HCl) at 37 °C, [Plg] = 340 µg mL^{-1}, [t-PA] = 80 ng mL^{-1}, [S-2251] = 0.035 ~ 2.0 mM, [L-lysine] = 5.0 mM, [poly(ε-LysMA)] = [poly(α-LysMA)] = 5.0 mM for ε-LysMA or α-LysMA units, v represents the reaction velocity; L-lysine (A; ◇), poly(ε-LysMA) (B; △), and poly(α-LysMA) (C; ○)

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Table 1. Kinetic parameters for the reaction of Plg/t-PA and S-2251 in Tris buffer solution (pH = 7.4) at 37 °C.

	K_m	V_{max}	k_{cat}/K_m
	mM	mM min^{-1}	mM^{-1} min^{-1}
L-Lysine	3.2×10^{-1}	4.4×10^{-4}	4.0×10^{-7}
poly(α-LysMA)	2.4	4.5×10^{-3}	8.8×10^{-7}
poly(ε-LysMA)	3.4×10^{-1}	4.5×10^{-4}	4.1×10^{-7}

by t-PA, while free L-lysine or poly(ε-LysMA) did not (Figure 2). Michaelis constant (K_m), maximum reaction velocity (V_{max}), and the enzymatic efficiency (k_{cat}/K_m) of plasmin converted from Plg by t-PA were calculated from Lineweaver-Burk plots for the reaction of S-2251 in the presence of poly(α-LysMA) or poly(ε-LysMA). Poly(α-LysMA) showed approximately a 8.0-fold increase in K_m, and approximately a 10.1-fold increase in V_{max}, and approximately a 2.2-fold increase in k_{cat}/K_m, respectively, in comparison with free L-lysine. However, poly(ε-LysMA) did not affect these parameters (Table 1). It is considered that a higher Plg conversion to plasmin by t-PA bearing LBS was mediated by poly(α-LysMA).

Furthermore, in order to know the binding behavior between poly(α-LysMA) and Plg, the binding assay was examined using IAsys resonant mirror biosensor, where Plgs were immobilized on a three dimensional carboxymethyl dextran surface.[15] The binding of poly(α-LysMA) or poly(ε-LysMA) to Plg was recorded, and the dissociation of poly(α-LysMA) or poly(ε-LysMA) with phosphate buffer saline (PBS; pH = 7.4) was carried out. The response after adding poly(α-LysMA) increased rapidly, while a similar increase was not observed for poly(ε-LysMA). After the measurement of the association phase, a part of the bound poly(α-LysMA) was abruptly dissociated by successive addition of PBS. Binding parameters were calculated from the association (Figure 3) and dissociation phases of the reaction curve on each concentration (5, 10, 20, and 40 mM for α-LysMA or ε-LysMA units) using the Fast Fit Software (Affinity Sensor). The association constant (k_a), the dissociation constant (k_d), and the affinity constant (K_D) between Plg and poly(α-LysMA) were 3.2×10^{-1} (M^{-1}s^{-1}), 1.0×10^{-4} (s^{-1}), and 3.1×10^{-3} (M), respectively. On the other hand, little interaction of poly(α-LysMA) was observed with serum albumin,

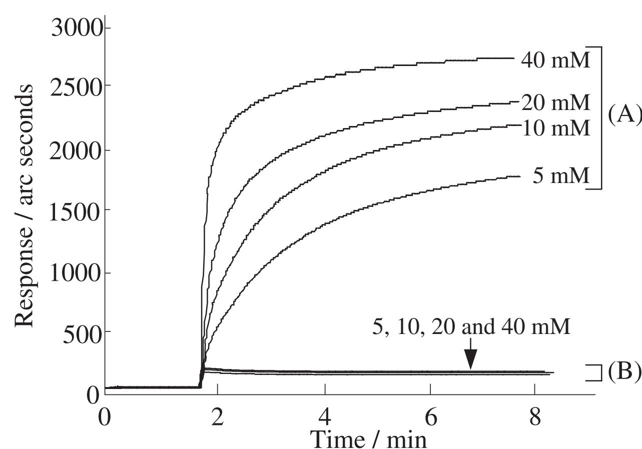

Figure 3. Binding assay between Plg and poly(α-LysMA) or poly(ε-LysMA) using IAsys resonant mirror biosensor in PBS at 25 °C; (A): poly(α-LysMA), (B): poly(ε-LysMA).

γ-globulin, and fibrinogen in the same manner of IAsys measurements. It is considered that zwitterionic poly(α-LysMA) enhances a Plg activation by t-PA through binding between poly(α-LysMA) and Plg. The Plg activation by t-PA in the presence of poly(α-LysMA) in detail and the evaluation of hemocompatibility are now under consideration.

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13 To a $CHCl_3$ solution (150 mL) of N^{ε}-*t*-butoxycarbonyl-L-lysine *t*-butyl ester (1.5×10^{-2} mol) and triethylamine (2.3×10^{-2} mol) was added dropwise a $CHCl_3$ solution (30 mL) of methacryloyl chloride (1.8×10^{-2} mol) 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-LysMA-OtBu was purified by passing through basic alumina column with the mixed solvent of dichloro-methane and hexane (50/50 vol %) (yield; 84%). ^{13}C-NMR ($CDCl_3$, TMS): δ 18.5 (–$\underline{C}H_3$), 22.4 (–$CH_2\underline{C}H_2$-CH_2CH_2NH–), 28.0 (–$C(\underline{C}H_3)_3$), 29.4 (–$C(\underline{C}H_3)_3$), 32.3 (–$\underline{C}H_2CH_2$-$\underline{C}H_2CH_2NH$–), 40.2 (–$CH_2CH_2CH_2\underline{C}H_2$-NH–), 52.5 (–$\underline{C}H$<), 78.5 (–$\underline{C}(CH_3)_3$), 82.0 (–$\underline{C}(CH_3)_3$), 119.8 ($\underline{C}H_2$=C<), 139.5 ($CH_2$=$\underline{C}$<), 155.9 (–NH$\underline{C}$OO–), 167.8 (–$\underline{C}$ONH–), 171.6 (–$\underline{C}$OO–). Anal. Calcd for $C_{19}N_2H_{34}$: C, 61.64; N, 7.57; H, 9.19. Found: C, 61.81; N, 7.46; H, 9.38%. Poly(Boc-LysMA-OtBu) was obtained by radical polymerization of Boc-LysMA-OtBu (1.3×10^{-2} mol) with MAIB (1.3×10^{-4} mol) as an initiator in 15 mL of tetrahydrofuran to give a white powdery polymer (yield; 87 %). The reaction mixture was evaporated in reduced pressure, and purified by removing unreacted Boc-LysMA-OtBu with hot hexane. The deprotection was carried out in 80 vol % trifluoroacetic acid aq. with stirring at 50 °C for 12 h. After removing the solvent under reduced pressure and washing with diethyl ether, the resulting polymer was neutralized with NaOH aq., and then purified by dialysis against water using a seamless cellulose membrane (Cellu-Sep™, USA; MWCO3500). Zwitterionic poly(α-LysMA) was obtained by freeze drying. The number average molecular weight (Mn) of poly(α-LysMA) by gel permeation chromatography (GPC) (columns; TOSOH, Japan; TSKgel α-4000, α-3000, and α-2500, eluent; ammonium acetate buffer at pH 5.5, calibrated with standard polyethyleneglycol) was 6.7×10^4. The specific rotation ($[\alpha]_D{}^{25}$) in PBS (pH = 7.4) was +3.1 degree. N^{α}-acetyl-N^{ε}-methacrylamide-L-lysine methyl ester (Ac-LysMA-OMe) was obtained in a manner similar to Boc-LysMA-OtBu. Anal. Calcd for $C_{13}N_2H_{22}$: C, 57.79; N, 10.37; H, 8.15. Found: C, 57.60; N, 9.93; H, 8.32. The poly(Ac-LysMA-OMe) and poly(ε-LysMA) were also prepared in a mannar similar to poly (Boc-LysMA-OtBu) and poly(α-LysMA), respectively. The poly(Ac-LysMA-OMe) was deblocked with 2 M NaOH aq. at room temperature for 24 h, where the yield of poly(ε-LysMA) was 73%. The Mn of poly(ε-LysMA) by GPC was 12.3×10^4. The $[\alpha]_D{}^{25}$ in PBS (pH = 7.4) was +5.2 degree.
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