Molecular Design of Reactive Amphiphilic Phospholipid Polymer for Bioconjugation with an Enzyme

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ABSTRACT: Water-soluble amphiphilic phospholipid random copolymers such as poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-*random*-*n*-butyl methacrylate (BMA)) and block-type polymer, poly(MPC-*block*-BMA) (Mw = 1.0×10^4) with a carboxylic group on a terminal, were designed by photoinduced living radical polymerization. These polymers and poly(MPC) were conjugated to an enzyme, papain. The effects of BMA units in the polymers on the enzymatic activity and stability were investigated. The modification degrees of papain with poly(MPC-*random*-BMA) and poly(MPC-*block*-BMA) were 16–19% versus the total number of amino groups in native papain. The remaining α-helix content of papains conjugated with poly(MPC-*random*-BMA) and poly(MPC-*block*-BMA) was maintained, but the remaining activity of the papains decreased with an increase in the composition of BMA units in the polymers. These activities were small compared to the papain conjugated to poly(MPC). However, the papains conjugated with poly(MPC-*random*-BMA) with 50% BMA units and poly(MPC-*block*-BMA) with 5% BMA units in the near side of the papain maintained much higher enzymatic activity for 28 days at 40°C compared with the papains conjugated with the other polymers. Moreover, the α-helix content of the papains conjugated with the polymers was maintained at the initial level for 28 days at 40°C. We concluded that the MPC polymers, which contain BMA units, could effectively enhance the stability of papain by controlling the sequence of monomer units and the composition of BMA units in the polymer. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 95: 615–622, 2005

Key words: bioconjugation; enzyme; iniferter; living radical polymerization; phospholipid polymer

INTRODUCTION

Bioconjugations between enzymes and synthetic polymers are excellent methods to enhance enzymatic functions and stability. In this step, the synthetic polymers do not show any adverse effects to enzymes after conjugation. Also, the synthetic polymers require the following features: the structure including end groups is defined, the molecular weight distribution is narrow, the polymer has functional groups, and the polymer is water-soluble. Poly(ethylene glycol) (PEG) was widely studied for the bioconjugation polymer.[1–3] Although PEG has the previous features, the chemical structure of PEG is quite simple, so further modification is difficult. The diversity of the synthetic polymers for bioconjugation is important.

We newly focused on the use of vinyl polymers as bioconjugation polymers. Generally, the vinyl polymers, which have reactive groups, have been synthesized by conventional free-radical polymerization using a chain-transfer agent[4–7] or copolymerization with monomers that have reactive groups.[8,9] Using these methods, it is difficult to control both the molecular weight of the polymer and the molecular weight distribution. To control the molecular weight distribution and the molecular weight, and to easily adapt a reactive end group, we used a photoinduced living radical polymerization method.[10]

We have been recently developing bioconjugation with bioinspired water-soluble phospholipid polymer as a "nanoscaled molecular device."[11,12] The phospholipid polymers composed of 2-methacryloyloxyethyl phosphorylcholine (MPC) units have excellent biocompatibility and thrombogenecity as biomaterials.[13] In this study, a novel MPC polymer with the reactive end group on one terminal was synthesized using photoinduced living radical polymerization. This polymer was used as the polymer for bioconjugation. In our previous reports, the poly(MPC) chain could stabilize the enzyme by controlling the molecular weight of the poly(MPC) and the modification degree to the enzyme.[11,12] Moreover, the enzymatic activity and stability of the enzyme conjugated with poly(MPC) were excellent in comparison to that of the PEG-conjugated enzyme.

The maintenance of the enzymatic activity and stability is related to the mobility of the enzymes; that is,

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the enzymatic activity and stability are maintained by decreasing the molecular mobility of the enzymes. The diffusion of the enzymes is decreased by polymer conjugation. The molecular mobility of the enzymes is decreased by the introduction of hydrophobic units. These hydrophobic units combined strongly with water. Therefore, the molecular mobility of the enzyme is restrained by decreasing the exchange rate of water around the hydrophobic units. In this study, various compositions of MPC units and monomer unit sequence, that is, random copolymer, poly(MPC-*random*-BMA) and block-type polymer, poly(MPC-*block*-BMA), were synthesized and conjugated to the enzyme. The effects of the BMA unit composition and the polymer structure on the enzymatic activity and stability were investigated.

MATERIALS AND METHODS

Materials

The MPC was synthesized by the method previously reported.[14] BMA was purchased from Kanto Chemicals (Tokyo, Japan). 4-(*N,N*-Diethyldithiocarbamoylmethyl) benzoic acid (BDC) was synthesized by condensation between 4-chloromethylbenzoic acid and sodium *N,N*-diethyldithiocarbamate.[12] 8-Anilino-1-naphthalene sulfonic acid sodium salt (ANS) was purchased from Tokyo Kasei (Tokyo, Japan). Papain was purchased from Worthington Biochemical Corp. (Lakewood, NJ). Other organic reagents and solvents were purified by the usual method.

Synthesis of poly(MPC) and poly(MPC-random-BMA) with carboxyl group

The BDC was dissolved in tetrahydrofuran (THF) (30 mL). MPC and BMA were dissolved in ethanol (150 mL). After being purged with Ar gas, the solutions were poured into a glass tubing for mixing, and then the tubing was sealed. The polymerization was carried out by photoirradiation using a high-mercury lamp (Riko, Chiba, Japan) at room temperature for 5 h. The mixture was poured into a large amount of chloroform to purify the poly(MPC) (PMPC-COOH) and poly(MPC-*random*-BMA) (rPMB-COOH) by precipitation. The obtained polymers were dried under reduced pressure for 2 days. The chemical structure of PMPC-COOH and rPMB-COOH was confirmed by ^{1}H-NMR (500 MHz, JEOL α-500, Tokyo, Japan, in ethanol-d_6): PMPC-COOH, δ1.29 (d, 6H, -*N*-C*H*$_3$), 1.87–1.93 (d, 3H, α- C*H*$_3$), 3.21–3.30 (d, 9H, -N(C*H*$_3$)-), 3.60–3.73 (d, 4H, -C*H*$_2$-N-), 4.07–4.32 (d, 4H, -O-C*H*$_2$-C*H*$_2$-P), 7.89 (d, 6H, *benzyl*). The molecular weight of the PMPC-COOH and rPMB-COOH was determined by gel permeation chromatography (Jasco, Tokyo, Japan; flow rate, 0.5 mL/min, detector, RI; eluent, 0.1 mol/L LiBr aqueous solution; Column, Shodex OHpak SB-803 (HQ)) with well-defined PEO as reference samples (PEO Standards, Tohso, Tokyo, Japan).

Synthesis of poly(MPC-block-BMA) with a carboxyl group

Two kinds of poly(MPC-*block*-BMA) were synthesized from prepolymers with a carboxyl group, PMPC-COOH and PBMA-COOH. The PMPC-COOH and BMA were dissolved in ethanol/dioxane (1/1 = v/v). The polymerization and purification were carried out by the same procedures as the PMPC-COOH preparation, then the poly(MPC-*block*-BMA) was obtained, and the carboxyl group was located on the MPC sequence side (bMPMB-COOH). To obtain PBMA with a carboxyl group, BMA and BDC were dissolved in THF. Photoinduced polymerization was carried out. This solution was evaporated and poured into a large amount of methanol to purify the PBMA by precipitation. The obtained polymer was dried under reduced pressure for 2 days. The MPC and PBMA were then dissolved in ethanol/dioxane (1/1 = v/v). After the photoirradiation for a given period, the solution was evaporated about three times and poured into a large amount of acetone to purify the poly(MPC-*block*-BMA) by precipitation. The obtained polymers were dried under reduced pressure for 2 days. This polymer was dissolved in water and dialyzed (molecular cutoff = 2×10^3) against pure water at room temperature for 3 days to eliminate unreacted MPC. This solution was freeze-dried and poly(MPC-*block*-BMA) was obtained, locating the carboxyl group on the BMA side, (bBPMB-COOH).

Evaluation of solution state of polymers

To evaluate the dissolving state of poly(MPC-*block*-BMA), the formation of the hydrophobic domain in an aqueous medium was evaluated using fluorescence probe as an indicator. The polymers were dissolved in 1.0×10^{-5} mol/L ANS aqueous solution. The concentrations were adjusted from 3.0×10^{-5} to 1.0×10^{1} mg/mL. Fluorescence measurements were carried out, and the excitation wavelength was 350 nm with a spectrofluorometer (FP-6500, Jasco).

Conjugation of polymers with papain

The carboxyl group at the terminal of the polymers was conjugated to the amino groups of papain after the carboxyl group was activated. Initially, 0.30 mol of the polymer was dissolved in 30 mL of 0.1 mol/L, pH 7.0 phosphate-buffered solution (PBS). Afterward, 0.016 mol of water-soluble carbodiimide was added into the solution and stirred for 2 h. The solution including activated polymer was added into 70 mL

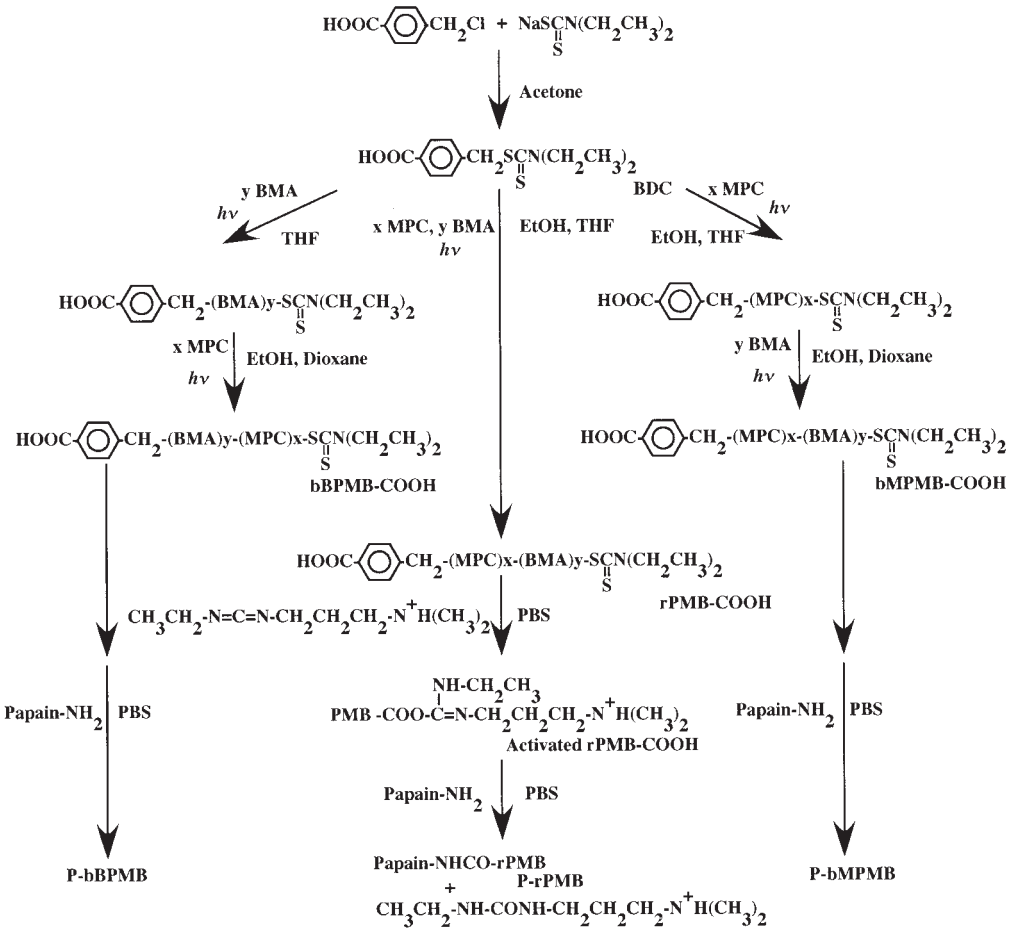

Figure 1 Scheme for the synthesis of various polymer-conjugated papains.

PBS containing 100 mg of papain and reacted at 4°C for 24 h. To remove the by-product, unreacted papain, and polymer, the solution was dialyzed (molecular cutoff = 5×10^4) against the water at 4°C for 24 h. After the dialysis, the polymer-conjugated papain solution was purified by ultrafiltration 30 times. Finally, the papains conjugated with PMPC-COOH, rPMB-COOH, bMPMB-COOH, and bBPMB-COOH were obtained by freeze-drying as P-PMPC, P-rPMB, P-bMPMB, and P-bBPMB, respectively. The synthetic route of P-PMPC and P-PMB is schematically illustrated in Figure 1.

The modification degree of the polymers against the total number of amino groups of papain was quantitatively determined by the 2,4,6-trinitrobenzene sulfonic acid method to count the residual amino groups of the papain after conjugation.

The α-helix content of the native papain, P-PMPC, and P-PMB was determined by measuring with a circular dichroism spectropolarimeter (J-720W, Jasco). The measurements were carried out at 40°C, and the concentration of the papain was 3.10×10^{-8} mol/L.

Measurement of enzymatic activity

The enzymatic activity of the native papain, P-PMPC, and P-PMB was determined using benzoyl-L-arginine-ethylester (BAEE) as the substrate. BAEE was dissolved in 0.1 mol/L, pH 6.1 PBS and the native papain, P-PMPC, and P-PMB/0.1 mol/L, pH 6.1 PBS that contains 1 m*M* EDTA, and then 5 m*M* L-cysteine was added. The reaction rate was determined by UV absorbance at 254 nm with a UV-vis photospectrometer (UV-650, Jasco). The reaction was carried out at 40°C. The concentration of the native papain, P-PMPC, and P-PMB was adjusted in 18 unit/L.

RESULTS AND DISCUSSION

Characterization of MPC polymers with carboxyl group on a terminal

The synthetic results of the polymers are summarized in Table I. The molecular weight could be regulated about 1.0×10^4 based on the feeding ratio of the monomers and iniferter. Moreover, the molecular weight distribution of the polymers was narrow

TABLE I
Characterization of PMPC-COOH, rPMB-COOH, bMPMB-COOH, and bBPMB-COOH

Code	Monomer/iniferter	BMA mole fraction in feed	BMA mole fraction[a]	Mw[b]	Mw/Mn[b]
PMPC-COOH	33.6	0	0	1.1×10^4	1.35
rPMB-COOH5	35.4	0.05	0.05	1.0×10^4	1.45
rPMB-COOH25	38.9	0.25	0.22	1.0×10^4	1.44
rPMB-COOH50	47.9	0.50	0.55	1.2×10^4	1.40
bMPMB-COOH5	35.4	0.05	0.06	1.1×10^4	1.39
bMPMB-COOH25	38.9	0.25	0.27	1.1×10^4	1.45
bMPMB-COOH50	47.9	0.50	0.47	1.0×10^4	1.49
bBPMB-COOH5	35.4	0.05	0.09	1.2×10^4	1.41
bBPMB-COOH25	38.9	0.25	0.26	1.0×10^4	1.47
bBPMB-COOH50	47.9	0.50	0.52	1.0×10^4	1.47

[a] Determined by ^{1}H-NMR.
[b] Determined by GPC with PEO standards.

(Mw/Mn = 1.35−1.49) compared with a conventional free-radical polymerization. The ^{1}H-NMR spectra data corresponded to the structures of the polymers. Thus, these polymers were better adapted to conjugate with papain.

Dissolving state of the MPC polymers in aqueous medium

It is well known that the maximum fluorescence wavelength of ANS ($\lambda_{em\ (max)}$) shifts to the low wavelength with increasing the polarity of the surrounding environment. Figure 2 shows the relationship between the concentration of the polymers and the $\lambda_{em\ (max)}$ of ANS in the polymer aqueous solutions at 25°C. The $\lambda_{em\ (max)}$ of ANS in every polymer solution, where the polymer concentration was 1.0×10^1 mg/mL, shifted about 40 nm compared to the low polymer concentration, 3.0×10^{-5} mg/mL. These results indicated that the hydrophobic domains were formed by polymer aggregation or the increase in solution viscosity. Table II shows $\lambda_{em\ (max)}$ of ANS in every polymer aqueous solution, the concentration of which was 1.0×10^1 mg/mL at 40°C. When the temperature of the solutions changed from 25 to 40°C, the viscosity of the solutions decreased about half, and the hydrophobic interactions became much stronger. If the $\lambda_{em\ (max)}$ of ANS increased at 40°C, the shift of the $\lambda_{em\ (max)}$ of ANS at 25°C was mainly caused by the increase in the viscosity. If the $\lambda_{em\ (max)}$ of ANS decreased or did not change at 40°C, the shift of the $\lambda_{em\ (max)}$ of ANS at 25°C was mainly caused by the formation of the hydrophobic domain. The $\lambda_{em\ (max)}$ of ANS in the PMPC-COOH solution increased when the temperature was increased from 25 to 40°C. On the other hand, the $\lambda_{em\ (max)}$ of ANS in other polymers, which contained BMA, did not change. These results indicated that the shift of the $\lambda_{em\ (max)}$ of ANS in PMPC-COOH solution

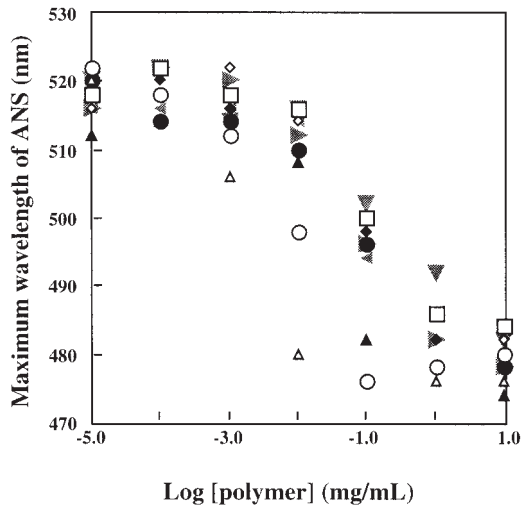

Figure 2 Relationship between various polymer concentrations and maximum wavelength of ANS in water at 25°C. (□) PMPC-COOH, (◇) rPMB-COOH5, (○) rPMB-COOH25, (△) rPMB-COOH50, (◆) bMPMB-COOH5, (●) bMPMB-COOH25, (▲) bMPMB-COOH50, (▼) bBPMB-COOH5, (▶) bBPMB-COOH25, (◀) bBPMB-COOH50.

TABLE II
Maximum Wavelength of ANS in Various Polymer Solutions at 25 and 40°C

Code	Maximum wavelength at 25°C (nm)	Maximum wavelength at 40°C (nm)
PMPC-COOH	484	492
rPMB-COOH5	482	482
rPMB-COOH25	480	480
rPMB-COOH50	476	476
bMPMB-COOH5	482	484
bMPMB-COOH25	478	478
bMPMB-COOH50	474	478
bBPMB-COOH5	482	484
bBPMB-COOH25	478	478
bBPMB-COOH50	478	478

Note. [Polymer] = 1.0×10^1 mg/mL.

TABLE III
Characterization of Various Polymer-Conjugated Papains

Code	Modification degree (%)	Remaining activity (%)	Remaining α-helix content (%)
P-PMPC	19	41	20
P-rPMB5	19	35	23
P-rPMB25	18	32	24
P-rPMB50	16	24	23
P-bMPMB5	18	33	23
P-bMPMB25	18	33	23
P-bMPMB50	17	12	14
P-bBPMB5	18	30	24

at 25°C can be attributed the increase in the viscosity, while the shift of the $\lambda_{em\ (max)}$ of ANS in other polymer solutions at 25°C results from the formation of the hydrophobic domain.

When the polymers are reacted with the enzyme, it is important that the reactive group in the polymer chain can easily attack the enzyme easily. In the case of rPMB-COOH and bMPMB-COOH, the hydrophobic BMA units existed randomly and at the opposite side of the carboxyl group, respectively. That means that the reactive carboxyl group in the polymer chain faced the outer portion of the polymer aggregates even when the polymer chain aggregate by hydrophobic interactions. However, in the case of bBPMB-COOH, the BMA units exited near the carboxyl group. Thus, when the polymer chain formed the aggregates, the polymer chain hid the carboxyl group. In the case of bBPMB-COOH25 and bBPMB-COOH50, the strong hydrophobic domain was formed. Therefore, it was difficult to conjugate papain with these polymers under this preparative condition. We are investigating new preparative conditions and will report on them in a forthcoming paper.

The dissolving state of the MPC polymer in an aqueous medium is illustrated in Figure 2.

Conjugation of polymers to papain

The conjugation of polymers to papain was carried out by the reaction between the carboxyl group in the terminal of the polymer chain and the amino groups of the papain. The modification degree, the remaining α-helix content and the remaining enzymatic activity after the bioconjugation are summarized in Table III. The modification degrees of the papain conjugated with the polymers were almost the same values, 16–19%. Additionally, the remaining α-helix contents of the almost polymer-conjugated papain did not decrease compared with native papain, but that value of P-PMPC and P-bMPMB50 decreased compared with native papain. These results indicated that the secondary structures of the papain conjugated with the polymers were maintained after bioconjugation. In the case of P-bMPMB50, the hydrophobic interaction induced a change in the secondary structure. The remaining activities of the papains conjugated with the polymers having BMA units decreased with an increase in the BMA units and were slightly low compared to the value of P-PMPC. Especially, the value of P-rPMB50 was significantly lower as 12%. These results were reasonable because the diffusivity of the substrate at the surrounding of papain conjugated with the MPC polymers with BMA units was restrained with an increase in the BMA units and when many BMA units existed on the bulk side.

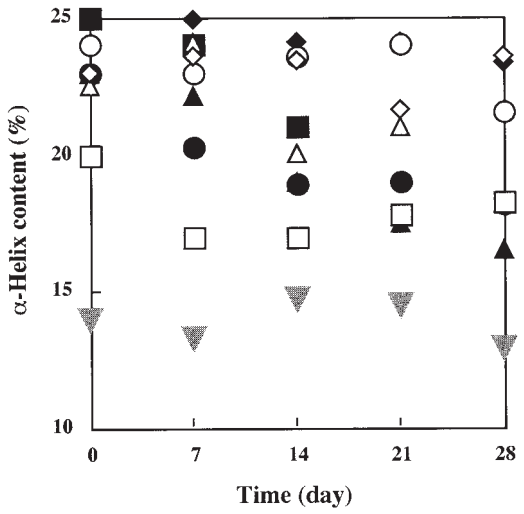

Figure 3 Storage time dependence of α-helix content of papain at 40°C. (□) P-PMPC, (◇) P-rPMB5, (○) P-rPMB25, (△) P-rPMB50, (◆) P-bMPMB5, (●) P-bMPMB25, (▲) P-bMPMB50, (▼) P-bBPMB5, (■) native.

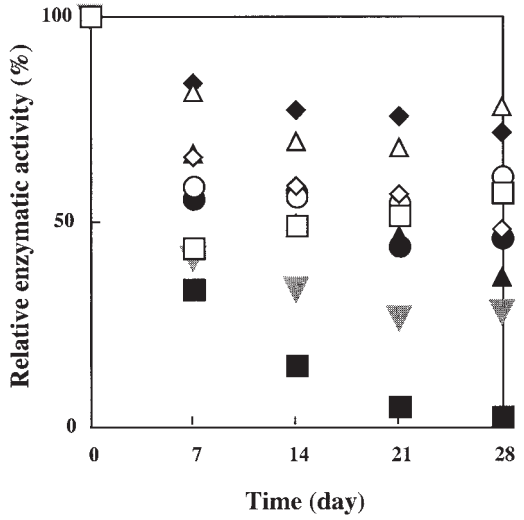

Figure 4 Relative enzymatic activity of papain at 40°C. (□) P-PMPC, (◇) P-rPMB5, (○) P-rPMB25, (△) P-rPMB50, (◆) P-bMPMB5, (●) P-bMPMB25, (▲) P-bMPMB50, (▼) P-bBPMB5, (■) native.

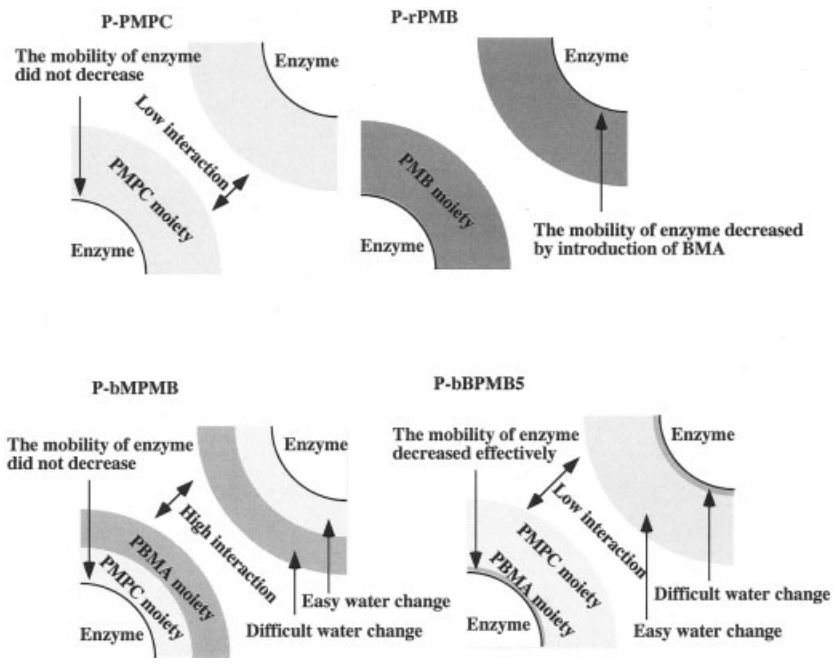

Figure 5 Schematic illustration of the dissolved condition of various polymer-conjugated papains.

Stability of polymer-conjugated papain

Figure 3 shows the change in the α-helix content of the native papain and papain conjugated with the MPC polymers when they were stored at 40°C. The α-helix content represents a parameter of the change in the secondary structure of the enzyme. The α-helix content is decreased by the conformation change in proteins that were induced by the mobility of the proteins or the change in the environment of the proteins such as temperature and pH. The α-helix content of the native papain was almost 25%, but it gradually decreased and could not be detected after 14 days. This phenomenon indicated that the secondary structure of the native papain completely collapsed under this condition. On the other hand, the α-helix contents of the papain conjugated with the MPC polymers, except for P-PMPC and P-bMPMB50, were almost maintained at the initial level for 28 days. The α-helix content of P-PMPC and P-bMPMB50 decreased after bioconjugation and that of P-bMPMB25 gradually decreased with time. The α-helix content of the papain conjugated with PMPC-COOH decreased with time, but when the hydrophobic units were introduced into the polymer for bioconjugations under a condition such that many hydrophobic units did not exist on the bulk side, the stability of the papains conjugated with MPC polymers increased.

Figure 4 shows the enzymatic activity profile of the native papain and the MPC polymer-conjugated papain when they were stored at 40°C. The enzymatic activity of the native papain decreased with the storage period. This result corresponded to the change in the α-helix content of the native papain with time. The enzymatic activity of P-PMPC was maintained at 50% of the initial enzymatic activity. This is inhibition of the self-digestion between the bioconjugates and structure change in papain by conjugation with the PMPC chains. The time dependence of the enzymatic activity of P-rPMB5 showed the same tendency as that of P-PMPC. The effect on the introduction of hydrophobic units was not apparent. However, by increasing the composition of the BMA units in rPMB, the P-rPMB maintained high enzymatic activity for 28 days. In the case of P-bMPMB, the enzymatic activity was the lowest in comparison with the P-PMPC and P-rPMBs. The time dependence of the enzymatic activity of P-bMPMB5 showed the same tendency as that of P-PMPC. However, by increasing the composition of the BMA units in bMPMB, the P-bMPMB could not maintain the enzymatic activity. These results corresponded to the results of the α-helix content of the P-bMPMB. In the case of P-bBPMB5, the enzymatic activity was maintained over 75% of the initial level for 28 days. The dissolution state of the papain conjugated with the MPC polymers is schematically illustrated in Figure 5 and summarized in Table IV.

Interactions between bioconjugates

In the case of P-PMPC and P-bBPMB5, the PMPC segment in the polymer faced the bulk waterside. Therefore, the interactions between these bioconju-

TABLE IV
Dissolution State of Papain Conjugated with MPC Polymers

Code	Interaction of bioconjugates	Diffusion of bioconjugates	Mobility of enzyme	Enzymatic activity and stability
P-PMPC	−	+	+	−
P-rPMB	+	−	−	+
P-bMPMB	++	−	+	−
P-bBPMB5	−	+	−	+

gates were low compared with native papain. It is well known that the PMPC does not affect the network formation of water molecules by them even when it is dissolved in water. Association between PMPC chains hardly occurred in the aqueous medium. Thus, papain covered with the PMPC segment, which faced the aqueous medium, could not aggregate each other. The PMPC segment can prevent the aggregation, and PMPC shows excellent biocompatibility. On the other hand, in the case of P-bMPMBs, the hydrophobic BMA units were concentrated and faced the bulk waterside. The hydrophobic interaction between these bioconjugates may be higher compared with that of native papains. The interactions became stronger with increasing composition of the BMA units in the polymers. When the rPMBs conjugated to papain, the hydrophobic BMA units randomly located to the bulk waterside randomly. The hydrophobic interactions increased with increasing composition of the BMA units in the polymers.

Diffusion of bioconjugates

In the case of P-PMPC and P-bBPMB5, the diffusivity of the bioconjugates was decreased by the effect of increasing the molecular weight by the bioconjugations. In the case of P-bMPMBs and P-rPMB, the diffusivity of the bioconjugates was decreased by the effect of increasing the molecular weight by the bioconjugations and the aggregation, which was induced by the hydrophobic interaction of the BMA units in the polymers. The hydrophobic interaction became strong with increasing the composition of the BMA units in the polymers.

Mobility of the enzyme

In the case of P-PMPC and P-bMPMB, the mobility of the enzymes did not decrease compared with that of native papain. It is known that the PMPC chains do not disturb the hydrogen bonding between the water molecules surrounding the polymer chains, other water-soluble polymers affect the state of the water even in the case of PEG.[15] In the case of P-rPMBs, the mobility of the enzyme decreased with increasing the composition of the BMA units in the polymers. The hydrophobic BMA units near the enzyme caused the displacement of water molecules. The same effect occurred in the case of P-bBPMB5.

Enzymatic activity and stability

In the case of P-PMPC, the PMPC chains conjugated with the enzyme provided good circumstances for the prevention of denaturation of the enzyme. The interaction between the bioconjugates was reduced; however, the mobility of the enzyme did not decrease, so the enzymatic activity was maintained only at 50% of the initial level. In the case of P-rPMB, the positive effect of the conjugated polymers on the mobility of the enzyme by the introduction of the hydrophobic units was overlapped by the negative effect of the conjugated polymers on the interaction between the bioconjugates. These effects were increased with increasing BMA units. Thus, with an increase in the composition of the BMA units, the enzymatic activity and stability of P-rPMBs increased. In the case of P-bMPMBs, aggregation of the bioconjugates may be induced by hydrophobic segment in the polymer chains conjugated with the enzyme. Thus, the enzymatic activity and stability were decreased. In the case of P-bBPMB5, the arrangement of the hydrophobic BMA units near the enzyme induced lowering.

The mobility of the enzyme was effectively decreased in only a few hydrophobic BMA segments facing the enzyme side. The MPC segment which faced the bulk waterside weakened the interaction between the bioconjugates; therefore, the enzymatic activity and stability were maintained.

CONCLUSIONS

For the preparation of bioconjugates, amphiphilic phospholipid polymers with a reactive end carboxylic group were synthesized by photoinduced living radical polymerization. The composition of the hydrophobic units in the polymer and the sequence of monomer units were regulated by the introduction of hydrophobic units in the polymers for bioconjugation, The papain conjugated with these amphiphilic phospholipid polymer chains could be maintained high

enzymatic activity and stability compared to PMPC conjugated bioconjugate.

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