A Facile Synthesis of Azido-Terminated Heterobifunctional Poly(ethylene glycol)s for "Click" Conjugation

Shigehiro Hiki† and Kazunori Kataoka*,†,‡,§

Department of Materials Engineering, Graduate School of Engineering, and Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan, Core Research Program for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Tokyo, Japan, and Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Received April 27, 2007; Revised Manuscript Received July 26, 2007

New azido-terminated heterobifunctional poly(ethylene glycol) (PEG) derivatives having primary amine and carboxyl end groups, (Azide-PEG-NH_2 and Azide-PEG-COOH, respectively) were synthesized with high efficiency. An α-allyl-ω-hydroxyl PEG was prepared as the first step to Azide-PEG-X (X = NH_2 and COOH) through the ring-opening polymerization of ethylene oxide (EO) with allyl alcohol as an initiator, followed by two-step modification of the hydroxyl end to an azido group. To introduce primary amino or carboxyl functional groups, amination and carboxylation reactions of the allyl terminal ends was then conducted by a radical addition of thiol compounds. Molecular functionalities of both ends of the PEG derivatives thus prepared were characterized by ^1H, ^{13}C NMR, and MALDI-TOF MS spectra, validating that the reaction proceeded quantitatively. The terminal azido functionality is available to conjugate various ligands with an alkyne group through the 1,3-dipolar cycloaddition reaction condition ("click chemistry").

INTRODUCTION

Poly(ethylene glycol) (PEG) conjugation chemistry, termed "PEGylation", has widely been used for the modification of biomolecules such as peptides, proteins, and enzymes (*1–3*), as well as of nanocarriers for drug delivery systems (DDS) such as liposomes and nanoparticles (*4, 5*). The advantages of PEGylation include the improvement of pharmaceutical and pharmacological properties, e.g., increased water solubility and circulatory half-life in vivo, the reduced antigenicity and immunogenicity, and the tolerance of biomolecules against degradation (*1–3*). Actually, several PEG-conjugated proteins have already been available as clinical therapeutics (*6*). Methoxy-terminated monofunctionalized PEG derivatives have mainly been used in these systems. Of further interest from the viewpoint of constructing multifunctionalized biopharmaceutics with PEGylation is the introduction of reactive groups at the α-chain end of PEG to prepare α,ω-heterobifunctional derivatives (heterobifunctional PEG). Heterobifunctional PEG may be useful to install ligands and probes at the distal end of PEGylated liposomes (*7*), polymeric micelles (*8*), synthetic polymers (*9*), and metallic colloids (*10*). So far, several types of heterobifunctional PEG derivatives with reactive groups, such as aldehyde, primary amine, mercapto, and maleimide, have been developed to install a variety of ligands (*8, 11–17*). Nevertheless, to expand the heterobioconjugation through PEG linker into a various combination of ligands and substrates, further improvement in the design of heterobifunctional PEG, particularly from the viewpoint of versatility, is crucial.

Generally, several features such as high yields, chemoselectivity, and the mild reaction condition in aqueous media are indispensable for the bioconjugation. In this regard, the 1,3-dipolar cycloaddition reaction between alkyne and organic azides, yielding the corresponding 1,2,3-triazoles in the mild aqueous condition, has recently received considerable attention in wide areas of chemistry (*18–20*) and has been recognized as particularly useful in the field of bioconjugate chemistry (*21–24*). This Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction (termed "click chemistry") is highly chemoselective and can be performed under mild conditions in aqueous buffers with wide range of pH values. Therefore, the combination of heterobifunctional PEGs with "click chemistry" is a promising approach for bioconjugation (*25*). Indeed, the introduction of biological ligands by "click chemistry" onto the liposomal surface (*26*) and the Au nanoparticle surface (*27*) through the heterobifunctional oligo(ethylene glycol) spacers was recently reported, indicating the high utility of "click"-based bioconjugation.

Nevertheless, little attention has been placed on the novel and versatile method to prepare the azido-terminated heterobifunctional PEGs with a wide range of molecular weight useful for "click"-based bioconjugation. Although, azido-containing oligo(ethylene glycol) derivatives (e.g., N_3-$(CH_2CH_2O)_2$-$CH_2CH_2NH_2$) have been commercialized (*28*), the azido-terminated heterobifunctional PEGs having high molecular weight useful in the bioconjugate chemistry field are not yet available.

Here, we wish to communicate the new synthetic route of azido-terminated heterobifunctional PEGs possessing primary amino and carboxyl groups (Azide-PEG-NH_2 and Azide-PEG-COOH, respectively) with controlled molecular weight through the ring-opening polymerization of ethylene oxide from allyl alcohol as an initiator followed by a quantitative modification of α- and ω-end groups (Scheme 1).

EXPERIMENTAL PROCEDURES

Materials. Ethylene oxide (EO; Sumitomo Seika Chemical, Japan) was dried over calcium hydride and distilled under an argon atmosphere. Allyl alcohol, tetrahydrofuran (THF), *N,N-*

* To whom corresponding should be addressed. Professor Kazunori Kataoka. Tel: +81-3-5841-7138. Fax: +81-3-5841-7139. E-mail: kataoka@bmw.t.u-tokyo.ac.jp.

† Graduate School of Engineering, The University of Tokyo, and CREST.

‡ Graduate School of Medicine, The University of Tokyo.

§ Center for NanoBio Integration, The University of Tokyo.

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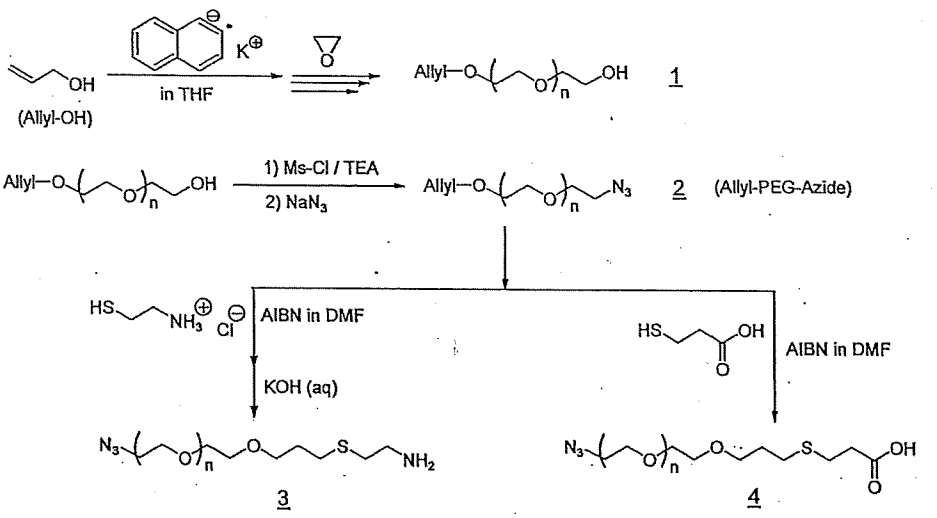
Scheme 1. Synthetic Route to α-Amino-ω-azido-PEG (3) and α-Carboxy-ω-azide-PEG (4)



dimethylformamide (DMF), triethylamine, 2,2′-azobisisobutyronitrile (AIBN), and methanesulfonyl chloride were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and purified by conventional methods. Potassium naphthalene was prepared as a THF solution according to a previous paper (*29*), whose concentration was determined by titration. 2-Aminoethanethiol hydrochloride, mercaptopropionic acid, sodium azide, and other reagents were used as received.

Synthesis of α-Allyl-ω-hydroxyl PEG (1). Allyl alcohol (2.3 mmol, 135 μL) and potassium naphthalene (2 mmol) in 6.6 mL of THF were added to dry THF (75 mL) in a 200 mL flask equipped with a three-way stopcock under argon atmosphere to form potassium allyl alcoholate as an initiator. After stirring for 10 min, liquid EO (302 mmol, 13.3 g) was added to the solution via a cooled syringe. The mixture was allowed to react for 2 days at 25 °C followed by pouring into diethyl ether to precipitate the polymer. The recovered polymer was dried in vacuo and then freeze-dried from benzene. The yield of obtained polymer after purification was 91% (12.1 g). GPC: Number-average molecular weight (M_n) = 5500, M_w/M_n = 1.03. ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm): 3.63 (m, O-(C$\underline{H}_2$)$_2$-O), 4.02 (d, C$\underline{H}_2$ of allyl group), 5.17–5.30 (dd, C$\underline{H}_2$ of allyl group), 5.87–5.96 (m, C$\underline{H}$ of allyl group).

Synthesis of α-Allyl-ω-azide PEG (2). The azide end group was introduced by the mesylation of the hydroxyl terminus and subsequent substitution with sodium azide, referring to the literature methods (*30*).

Freeze-dried polymer **1** (Allyl-PEG-OH) (2.20 g, 0.40 mmol) from benzene was dissolved in anhydrous THF (15 mL) followed by triethylamine (162 mg, 1.60 mmol) addition. The mixture was then added to a solution of methanesulfonyl chloride (160 mg, 1.40 mmol) in THF (8 mL) under Ar stream, and stirred overnight at room temperature. After the reaction, THF was partially evaporated under reduced pressure. The residue was redissolved in water (40 mL) and extracted with dichloromethane (5 × 100 mL). The organic layers were combined and dried over Na_2SO_4. After filtration and concentration, the polymer was recovered by precipitation into ether and dried in vacuo, yielding a white solid.

This mesylated polymer (620 mg, 0.113 mmol) was dissolved in DMF (12 mL), followed by sodium azide (521 mg, 8.01 mmol) addition, and was stirred for 2 days at 30 °C. Dichloromethane (100 mL) was then added, and the reaction mixture was washed five times with water and brine. The organic layer was dried over Na_2SO_4, filtered, concentrated, and then reprecipitated into ether. The recovered polymer was freeze-dried from benzene (508 mg, 82%). ^{1}H NMR (300 MHz, $CDCl_3$, δ in ppm): 3.46 (t, CH_2-C$\underline{H}_2$-N_3), 3.63 (s, O-(C$\underline{H}_2$)$_2$-O), 4.02 (d, C$\underline{H}_2$ of allyl group), 5.17–5.30 (dd, C$\underline{H}_2$ of allyl group), 5.87–5.96 (m, C$\underline{H}$ of allyl group).

Synthesis of α-Amino-ω-azide PEG (3). Amination of the allyl terminus of polymer **2** was conducted by the radical addition reaction of 2-aminoethanethiol hydrochloride (*31*). Polymer **2** (Allyl-PEG-azide, 502 mg, 0.091 mmol) was freeze-dried from benzene and mixed with the solution in DMF (5 mL) containing 213 mg of 2-aminoethanethiol hydrochloride (1.87 mmol, 20 equiv) and 15 mg of AIBN (0.091 mmol, 1 equiv). The reaction mixture was stirred at 65 °C for 24 h under argon atmosphere. The polymer was precipitated twice in a large excess of ether. The resulting white product was dissolved into methanol, and 5.1 mg (1 equiv) of potassium hydroxide dissolved in water was added. The mixture was stirred for approximately 4 h. Then, methanol was partially evaporated and diluted with water (30 mL), and extracted by dichloromethane (5 × 80 mL). The combined organic layer was dried over Na_2SO_4, filtered, and concentrated to 1/100 of the initial volume. The polymer was reprecipitated from an excess volume of ether and freeze-dried from benzene to lead to a white powder (397 mg, yield = 79%).

Synthesis of α-Carboxy-ω-azide PEG (4). The carboxylation reaction of allyl terminus of polymer **2** was performed in the same manner as described above for the preparation of polymer **3**. Typically, 409 mg of Allyl-PEG-azide, in 4 mL of DMF, was reacted with 159 mg of 3-mercaptopropionic acid (1.50 mmol, 20 equiv) in the presence of 12.2 mg of AIBN (1 equiv). After the reaction, the solution was precipitated into ether twice, and polymer **4** was obtained as a white powder (317 mg, yield = 77%).

Polymer Analysis. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-AL 300 spectrometer (JEOL, Tokyo, Japan) at 300 and 75.45 MHz, respectively. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Number-average molecular weight (M_n) and molecular weight distribution (M_w/M_n) were determined using a GPC (TOSOH HLC-8220) system equipped with two TSK gel columns (G4000HHR and G3000HHR) and an internal refractive index (RI) detector. Columns were eluted with DMF containing lithium chloride (10 mM) at a flow rate of 0.8 mL/min and temperature of 40 °C. Molecular weights were calibrated with poly(ethylene glycol) standards (Polymer Laboratories, Ltd., UK). MALDI-TOF-MS spectra were recorded using Bruker

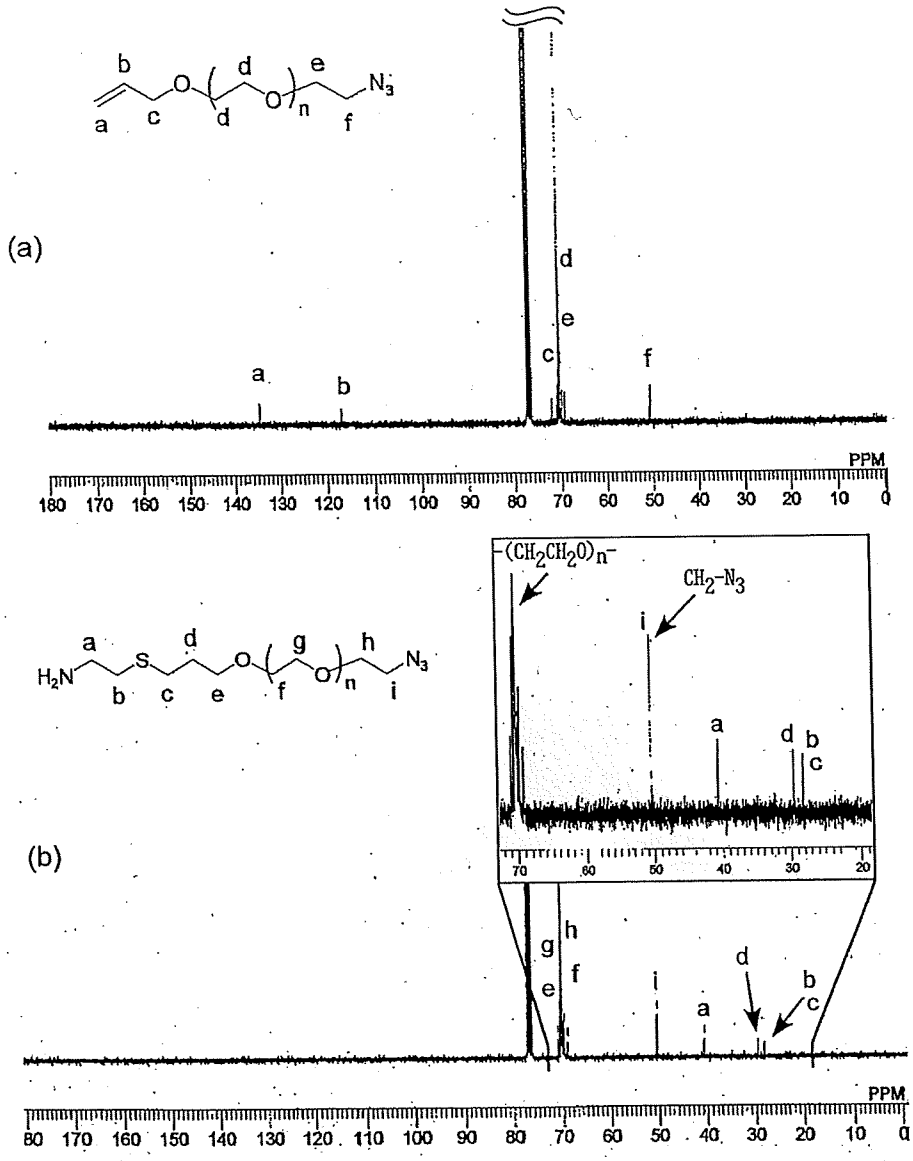


Figure 1. (a) ^{13}C NMR spectrum of Allyl-PEG-Azide ($CDCl_3$ at 20 °C). (b) ^{13}C NMR spectrum of Azide-PEG-NH_2 ($CDCl_3$ at 20 °C).

REFLEX III. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix for the ionization operated in the reflection mode.

RESULTS AND DISCUSSION

There are several reports on the preparation of oligo(ethylene glycol)s having azido and amino groups from homotelechelic oligo(ethylene glycol)s as the starting materials (*32, 33*). Nevertheless, the synthetic methods involve several complicated reactions as well as separation steps such as chromatographic isolation due to the difficulty of selective monoprotection of the hydroxyl termini. Thus, the application of these synthetic approaches of end-group modification is not expedient for a large-scale synthesis of heterobifunctional PEGs having high molecular weight, giving an impetus to develop a novel synthetic route to the azide-functionalized heterobifunctional PEG with quantitative yields and high specificity under mild reaction conditions.

In this study, allyl alcoholate was selected as an initiator for anionic polymerization of EO, because the allyl end group of the PEG chain allows several kinds of chemical modifications such as addition reactions. To prepare α-allyl-ω-hydroxyl PEG (**1**), the anionic polymerization of EO initiated with potassium allyl alcoholate was carried out in THF solution for 48 h. After precipitation into ether, the product was obtained as white powder. The MW of the polymer **1** (Allyl-PEG-OH) determined from GPC [M_n(GPC) 5500] was close to that from the initial monomer/initiator ratio [M_n(calcd) 5780], indicating an effective initiation efficiency of potassium allyl alcoholate without detrimental side reactions.

α-Allyl-ω-azide PEG (**2**) was obtained through the two-step modification, the mesylation and the subsequent substitution by sodium azide, of hydroxyl function as described in the Experimental Procedures. In the ^{13}C NMR spectrum (Figure 1a), the signals of the carbons of the allyl group are detected at δ 134.7 ppm (a), 117.0 ppm (b), and 72.1 ppm (c), respectively. In addition to these signals, the methylene unit adjacent to the azido group ($CH_2\underline{C}H_2N_3$) was observed at 50.5 ppm (f). Moreover, no signal of the methylene carbon adjacent to the hydroxyl terminus ($CH_2\underline{C}H_2OH$ δ = 61.5 ppm) (*34*) was detected.

Figure 2a shows the MALDI-TOF MS spectra of α-allyl-ω-azide PEG (**2**). In this analysis, although the major peaks of the polymer were detected as the species derived from loss of N_2 from azido group which ascribed to fragmentation due to

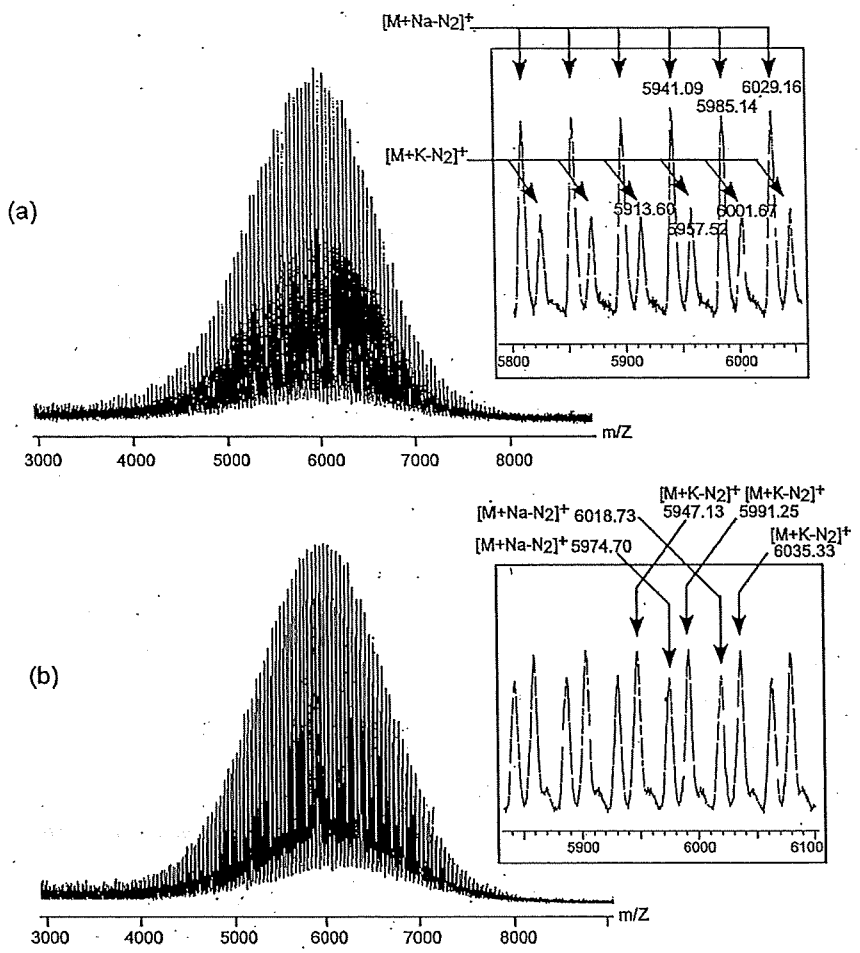


Figure 2. MALDI-TOF Mass Spectrum of Allyl-PEG-Azide (a) and Azide-PEG-NH_2 (b).

the higher laser power during MALDI experiment (*35*), only parent ions of each polymer molecule as planned are observed, without the signal of the side reaction product. From the expanded view of the spectrum in Figure 2a, the mass of the products appears to be around 5900 (M_n = 5670; M_w/M_n = 1.03), which is in a good accordance with the GPC results (M_n = 5500). These data demonstrated that α-allyl-ω-hydroxyl PEG (**1**) was successfully transformed to α-allyl-ω-azide PEG (**2**). Note that there was a report on the chain degradation of tosylate-activated PEG derivatives (*36*). Nevertheless, no sign of degradation was found in the present case of mesylate-activated PEG samples by MALDI-TOF MS spectroscopy, indicating that essentially no degradation of PEG occurred in the activation route via mesylation.

To prepare α-amino-ω-azide PEG, the radical addition reaction of 2-aminoethanethiol hydrochloride to an allyl end group of polymer (**2**) was examined according to the previous method with a slight modification (*31*). The reaction was carried out under the varying ratios of [allyl-PEG-azide]$_0$/[$HSCH_2CH_2NH_3^+Cl^-$]$_0$/[AIBN]$_0$ (=1/5/1, 1/7.5/1, 1/10/1, and 1/20/1 mol equiv) in DMF at 65 °C. The resulting polymer was precipitated twice in ether in order to remove excess of reagents. After the subsequent aqueous workup with potassium hydroxide, the amino-terminated PEG sample was obtained (Scheme 1). The ^{1}H NMR spectrum of the polymer obtained at the conditions of [allyl-PEG-azide]$_0$/[$HSCH_2CH_2NH_3^+Cl^-$]$_0$/[AIBN]$_0$ = 1/10/1 and 1/20/1 revealed the complete disappearance of the signals assigned to the allyl protons at 4.02 (CH_2=CHC$\underline{H_2}$), 5.17–5.30 (C$\underline{H_2}$=$CHCH_2$), and 5.87–5.96 ppm (CH_2=C$\underline{H}CH_2$). Concomitantly, the new signals were clearly observed at 1.82 ($CH_2C\underline{H_2}CH_2S$), 2.60 ($SC\underline{H_2}CH_2NH_2$), 2.62 ($C\underline{H_2}SCH_2CH_2NH_2$), and 2.86 ppm ($CH_2SCH_2C\underline{H_2}NH_2$) corresponding to the resulting structure via the radical addition of 2-aminoethanethiol to allyl group. A decreased ratio of thiol compound in the initial reaction mixture to less than 10 equiv ([allyl-PEG-azide]$_0$/[$HSCH_2CH_2NH_3^+Cl^-$]$_0$/[AIBN]$_0$ = 1/5/1 and 1/7.5/1) resulted in the incomplete amination judged from the observation of the remaining ally protons in the ^{1}H NMR spectra of the obtained polymer samples, suggesting that 10 mol excess equiv of thiol group may be required for the complete amination of allyl group settled at the PEG chin end. No prohibitive effect was observed even at 20 equiv of thiol to allyl groups. In the ^{13}C NMR spectra of the sample obtained at [allyl-PEG-azide]$_0$/[$HSCH_2CH_2NH_3^+Cl^-$]$_0$/[AIBN]$_0$ = 1/20/1 (Figure 1b), no carbon signal corresponding to allyl function remained. The signals detected around δ 40.7 (a), 29.6 (d), and 28.4 ppm (c,d) can be assigned to the $\underline{C}H_2NH_2$, $CH_2\underline{C}H_2CH_2S$, and $CH_2CH_2\underline{C}H_2S\underline{C}H_2$ of the end groups, respectively. Note that no side reaction of the azido group such as a radical scavenging and a subsequent decomposition with denitrogenation occurred, because the signal corresponding to the azido group was detected as intact.

The quantitative conversion of the end allyl group to the primary amino group was also monitored by the MALDI-TOF MS analysis. Figure 2b shows the MALDI-TOF MS spectrum of the reaction product after the radical addition reaction of 2-aminoethanethiol hydrochloride ([allyl-PEG-azide]$_0$/[$HSCH_2CH_2NH_3^+Cl^-$]$_0$/[AIBN]$_0$ = 1/20/1). As mentioned above, the mass distribution of the obtained polymer after the radical addition was the same as that before the reaction, indicating the occurrence of no side reaction. The major series of the molecular masses of the product is expressed in the following equation.

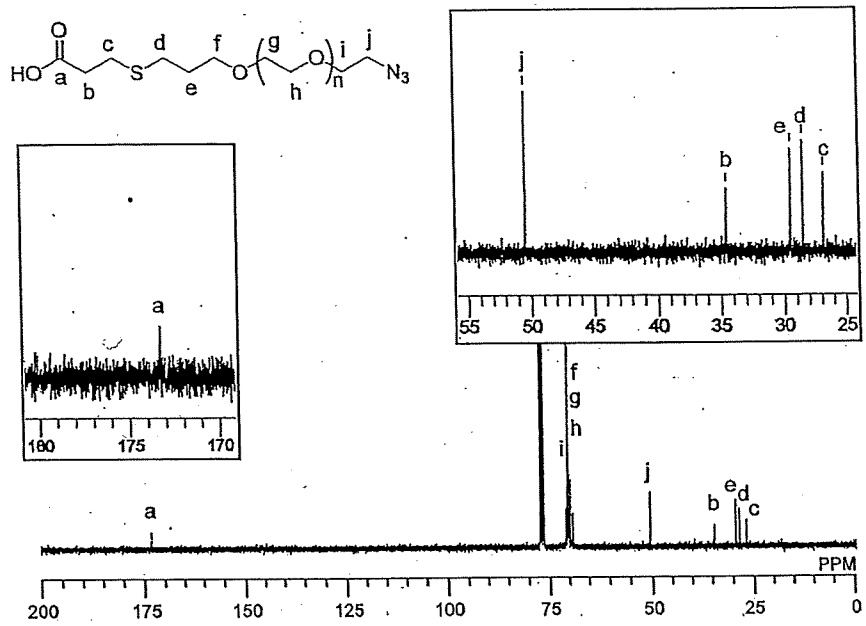


Figure 3. ^{13}C NMR spectrum of Azide-PEG-COOH ($CDCl_3$ at 23 °C).

MW_{MS} = 44.053n (EO) + 70.07 (Azide) + 134.22 ($OCH_2CH_2CH_2SCH_2CH_2NH_2$) + 22.99 (sodium).

These results strongly supported the statement that the novel heterobifunctional PEG possessing an azido group at one end and a primary amino group at the other end was quantitatively prepared.

To demonstrate the versatility of this synthetic route with azido-containing PEG, the carboxylation of α-allyl-ω-azide PEG (2) was examined under the same conditions as the amination reaction ([allyl-PEG-azide]$_0$/[$HSCH_2CH_2COOH$]$_0$/[AIBN]$_0$ = 1/20/1). From the ^1H NMR analysis of the resultant polymer, the signals assigned to the allyl group completely disappeared after the reaction, while the methylene protons of the 3-mercaptoacetic acid unit were clearly observed at 1.84 ($CH_2C\underline{H}_2CH_2S$), 2.60 ($SC\underline{H}_2CH_2COOH$), 2.62 ($C\underline{H}_2SCH_2CH_2COOH$), and 2.79 ppm ($CH_2SCH_2C\underline{H}_2COOH$), respectively. In addition, the structure of the resulting polymer after the carboxylation reaction was confirmed by ^{13}C NMR. The corresponding carbon signals of the terminal groups appeared at δ 173.0 ((a) $CH_2\underline{C}OOH$), 50.4; ((j) $\underline{C}H_2N_3$), 34.5; ((b) $\underline{C}H_2COOH$), 29.6; ((e) $CH_2\underline{C}H_2CH_2S$), 28.5; ((d) $CH_2CH_2\underline{C}H_2S$), and 26.9; ((c) $S\underline{C}H_2CH_2COOH$) ppm, respectively, and no signal corresponding to allyl group was detected (Figure 3). These results indicate the successful preparation of heterobifunctional PEG possessing azido and carboxyl groups at the chain ends.

In conclusion, the facile and quantitative synthesis of azido-containing heterobifunctional PEGs via EO polymerization was demonstrated in this study. These azido-containing PEGs can be used to Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction (click chemistry) to introduce versatile biofunctional ligands into the azido terminus, and are thus relevant to a wide range of applications in the field of bioconjugate chemistry. We focused here on the PEG with MW of 5000 because of its versatility in bioconjugate applications, including liposome modification, polymeric micelle formation, and protein conjugation. Basically, chemistry developed here may be applicable to PEG with even higher MW, because the reaction scheme may not be directly influenced by the MW. Nevertheless, the quantitative conversion is still in an issue for the higher MW PEG due to the limited availability of the chain end possibly buried in the coil of PEG strands. We feel that this issue may be better addressed in a future study directly focusing on the reactivity of the allyl group located at the chain end of PEG with different molecular weights.

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Self-Assembled Nano-Bioreactor from Block Ionomers with Elevated and Stabilized Enzymatic Function

Akifumi Kawamura,† Atsushi Harada,*,† Kenji Kono,† and Kazunori Kataoka*,‡,§

Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan, Department of Materials Engineering, Graduate School of Engineering, and Center for NanoBio Integration, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. Received January 24, 2007; Revised Manuscript Received July 7, 2007

Core-cross-linked polyion complex (PIC) micelles entrapping trypsin in the core were prepared by mixing trypsin and poly(ethylene glycol)-*block*-poly(α,β-aspartic acid) in aqueous medium, followed by the introduction of glutaraldehyde cross-linkages. Trypsin incorporated into the core-cross-linked micelles showed high storage stabilities, and the initial enzymatic activity of trypsin was maintained even after standing for one week at ambient temperature. Further, stable compartmentalization of trypsin into the core-cross-linked micelles led to a unique modulation in the enzymatic functions including an improved thermal tolerability with an increased maximum reaction rate compared to native trypsin.

INTRODUCTION

Immobilized enzymes have been utilized in numerous applications including bioreactors, biosensors, and therapeutics (*1*–*5*). The major advantage of immobilized enzymes is an improvement in the storage and operational stabilities, which is especially crucial for proteolytic enzymes showing autolysis reaction. Conventionally, solid and porous matrices, including agarose, cellulose, and silica, have been used to immobilize enzymes through physical adsorption or covalent attachment. Also, water-soluble conjugates of enzymes with various polymers have been prepared (*6*–*9*). A novel approach to the immobilization of enzymes at the nanometric scale is the supramolecular assembly of nanoreactors. Noteworthy in this regard is the core–shell-type polyion complex (PIC) micelles formed from block ionomers. Charged enzymes can be incorporated into PIC micelles driven by electrostatic interaction in aqueous medium (*10*–*13*). Indeed, egg white lysozyme with cationic character was successfully incorporated into the core of PIC micelles through complexation with poly(ethylene glycol)-*block*-poly(α,β-aspartic acid) (PEG-PAA). Lysozyme-incorporated PIC micelles showed unique features including the on–off switching of an elevated enzymatic reaction synchronized with an external electric field. However, in the case of entrapping proteolytic enzymes (e.g., trypsin) in the micelles, autolysis was facilitated due to an increase in the local concentration of the enzyme. This problem has been recently overcome by the introduction of cross-linking into the core of PIC micelles (*14*). The tolerability of trypsin in PIC micelles, which is a typical proteolytic enzyme that selectively cleaves the bond adjacent to Lys and Arg, was dramatically improved by introducing cross-linking in the core of the micelles. Glutaraldehyde was used as a cross-linking reagent of the PIC micelles prepared by mixing trypsin and PEG-PAA. The core-cross-linked PIC micelles had a hydrogel core of nanoscopic size, formed by the cross-linking of trypsin and PAA, and an outer PEG shell layer. The core-cross-linked micelles might provide unique features in the field of nanoscopic enzymatic reactions.

Here, we studied in detail the enzymatic function of the core-cross-linked PIC micelles entrapping trypsin. Note that the Schiff base linkages in the cross-linked PIC micelles may undergo reductive amination to form alkylamine linkages. The amidase activity of entrapped trypsin in the micelle was evaluated by colorimetric assays using L-lysine *p*-nitroanilide as a substrate, both before and after the reductive amination. A remarkable improvement in the storing stability of trypsin was observed as a result of the compartmentalization of the enzyme into the cross-linked core of the PIC micelles. Further, an appreciable increase in the optimal temperature as well as a significantly improved tolerance to urea in the enzymatic reaction were achieved in the cross-linked micelles.

EXPERIMENTAL PROCEDURES

Materials. Poly(ethylene glycol)-poly(α,β-aspartic acid) block copolymer [PEG-PAA; 12 000 g/mol of PEG Mw; 68 of polymerization degree in PAA segment] was synthesized as described previously (*10*). Bovine pancreas trypsin, L-lysine *p*-nitroanilide and 3-methyl-2-benzothiazolinone hydrazone were purchased from Sigma (St. Louis, MO) and used without further purification. Sodium cyanotrihydroborate ($NaBH_3CN$), $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, and 70% glutaraldehyde were reagent grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Core-Cross-Linked PIC Micelles Entrapping Trypsin in the Core. Given amounts of trypsin and PEG-PAA were separately dissolved in sodium phosphate buffer (10 mM; pH 7.4) at 4 °C. The solutions were mixed at the optimum mixing ratio for PIC micelle formation, i.e., the ratio of the number of Asp residues in PEG-PAA against the total number of Lys and Arg residues in trypsin to be 0.75. The mixed

* To whom corresponding should be addressed. Prof. Kazunori Kataoka, Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku Tokyo 113-8656, Japan, Tel: +81-3-5841-7138, Fax: +81-3-5841-7139, e-mail: kataoka@bmw.t.u-tokyo.ac.jp. Dr. Atsushi Harada, Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan, Tel & Fax: +81-72-254-9328, e-mail: harada@chem.osakafu-u.ac.jp.

† Osaka Prefecture University.

‡ Graduate School of Engineering, The University of Tokyo.

§ Center for NanoBio Integration, The University of Tokyo.

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solution was stored at 4 °C for 30 min, and then 70% glutaraldehyde solution was added to prepare cross-linked micelles with varying cross-linking ratios (GR). The cross-linking ratio (GR) was defined as the residual molar ratio of aldehyde group to lysine in the solution. The cross-linked PIC micelle solution was then kept at 4 °C for 30 min. In order to remove excess glutaraldehyde, the cross-linked PIC micelle solution was dialyzed against buffer. The removal of free glutaraldehyde was confirmed from the absence of aldehyde species in the dialysate, as determined by colorimetric assay using 3-methyl-2-benzothiazolinone hydrazone. Then, $NaBH_3CN$ was added to the solution to convert the Schiff base linkages to alkylamine linkages by reductive amination. The reductive amination step was also confirmed by colorimetric assay using 3-methyl-2-benzothiazolinone hydrazone.

Dynamic Light Scattering Measurements. Dynamic light scattering (DLS) measurements were carried out using a DLS-700 spectrometer (Otsuka Electronics Co., Ltd., Japan) equipped with an Ar ion laser ($\lambda = 488$ nm) at 25 °C. The detection angle was fixed at 90°. The average diameter and polydispersity index were obtained by the cumulant method, and the size distribution was obtained by histogram analysis.

Evaluation of Amidase Activity of Trypsin. The amidase activity of trypsin was evaluated using L-lysine *p*-nitroanilide as a substrate. The reaction rates of the native trypsin and trypsin incorporated into the core-cross-linked micelles were determined by monitoring the change in the absorbance at 410 nm, where the wavelength for the extinction coefficient of *p*-nitroaniline after mixing of the trypsin solution and substrate solution is 8800 cm^{-1} M^{-1}. The initial reaction rate was determined from the slope of the change in the absorbance at 410 nm during the period between 150 and 250 s after mixing.

RESULTS AND DISCUSSION

The cross-linkages in the core of PIC micelles, which were prepared from trypsin and PEG-PAA, were introduced using glutaraldehyde as cross-linking reagent for the stabilization of the micellar structure. There is known to be two types of cross-linkages in the cross-linking reaction of amine compounds by glutaraldehyde: One is the Schiff base linkage formed between the primary amino group of the Lys residue in trypsin and the aldehyde group of glutaraldehyde, and the other cross-linkage involves the formation of a quaternary pyridinium structure (see Supporting Information). We have recently confirmed the formation of the quaternary pyridinium structure for lysozyme-entrapped PIC micelles cross-linked using glutaraldehyde by spectroscopic analysis (*15*). Further, the quaternary pyridinium structure might also be formed in trypsin-entrapped PIC micelles cross-linked by glutaraldehyde. Reduction of the Schiff base and pyridinium structure using $NaBH_3CN$ as a reductant results in the formation of saturated alkylamines, further stabilizing the micelle structure due to the formation of the stable covalent bonds. The nonreacted aldehyde groups in the core-cross-linked PIC micelles were also reduced to hydroxyl groups by $NaBH_3CN$ treatment, which was confirmed by using 3-methyl-2-benzothiazolinone hydrazone as an aldehyde-detecting reagent. Figure 1 shows the size distribution of PIC micelles before and after the reductive amination. Unimodal size distribution was maintained even after this treatment. Also, the reduction process induced no change in the average diameter and polydispersity index (μ_2/Γ^2), which were determined by the cumulant analysis of dynamic light scattering measurements (69.6 nm, $\mu_2/\Gamma^2 = 0.04$ for the original sample; and 68.9 nm, $\mu_2/\Gamma^2 = 0.06$ for the sample after the treatment).

As an evaluation of the stabilized enzymatic function, the storage stability of the amidase activity of trypsin was studied by using L-lysine *p*-nitroanilide as a substrate. Figure 2 shows

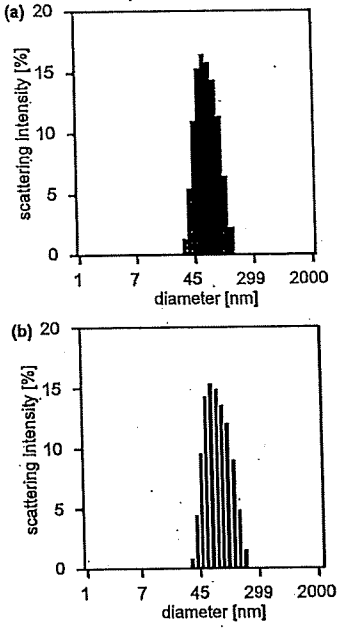

Figure 1. Size distributions of core-stabilized micelles before (a) and after (b) reductive amination, as measured by dynamic light scattering (GR = 100; detection angle 90°; temperature 25 °C).

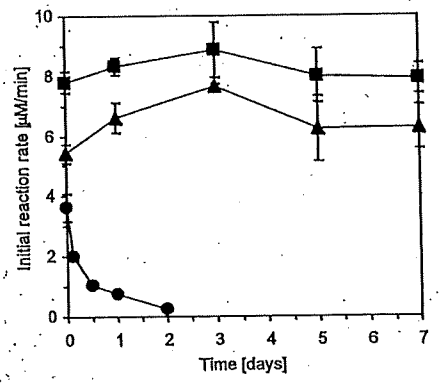

Figure 2. Time course of the enzymatic reaction rate of native trypsin (●) and trypsin incorporated into nonreduced (▲) and reduced (■) micelles at 25 °C. (Trypsin concentration 16.8 μM; substrate concentration 5 mM. The plots are presented as the average of three experiments ± SD.)

the time-dependent change in the initial reaction rate for both native trypsin and trypsin incorporated into the nonreduced and reduced micelles. The initial reaction rate of native trypsin immediately decreased due to autolysis, and there was no detectable activity after 2 days incubation. On the other hand, the trypsin incorporated into the nonreduced and reduced core-cross-linked micelles maintained a constant reaction rate even after 1 week incubation at 25 °C, indicating that the autolysis of trypsin was effectively prevented due to being incorporated into the core-cross-linked micelles. In the cross-linked core, the migration of trypsin molecules was highly restricted, thereby inhibiting their mutual contact. As a result, the autolysis of trypsin was effectively inhibited. There was no difference in the storage stability of trypsin activity between the nonreduced and reduced micelles. This effect will be discussed later on the basis of the enzymatic reaction constants. Further, it should be noted that the reaction rate of the trypsin incorporated into the core-cross-linked micelles was appreciably higher than that of native trypsin, suggesting that the amidase activity of trypsin might increase through the entrapment into the core of the

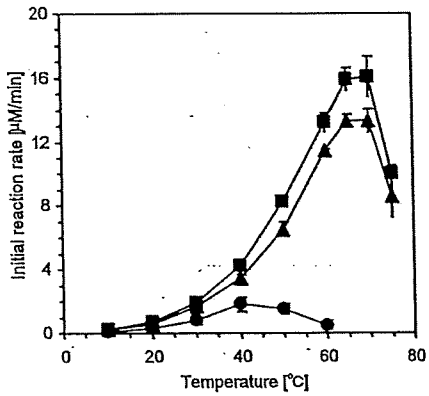

Figure 3. Temperature dependence of the enzymatic reaction of native trypsin (●) and trypsin incorporated into nonreduced (▲) and reduced (■) micelles. (Trypsin concentration 4.2 μM; substrate concentration 1.25 mM. The plots are presented as the average of three experiments ± SD.)

micelle. For the stabilized enzymes physically or chemically immobilized in polymer networks, the decreased enzymatic activity through the immobilization was often observed, which was induced by the restriction of the mobility of enzyme as well as by the limited solute transfer (*16*, *17*). No decrease in enzymatic activity of trypsin in the micelles might be due to the unique characteristics of core-cross-linked micelles including small size and core–shell structure with enzyme molecules stably entrapping into the core surrounded by a biocompatible and hydrophilic shell layer, providing a uniform distribution of stabilized enzymatic nanoreactor and maintenance of substrate diffusion.

The entrapment of trypsin into the micellar cross-linked core induced a substantial shift in the optimal temperature of the enzymatic reaction, as compared to native trypsin. Figure 3 shows the temperature dependence of the enzymatic reaction rate of both the native trypsin and the trypsin incorporated into the nonreduced and reduced micelles. In the case of native trypsin, a maximum reaction rate was observed at 40 °C, and this reaction rate gradually decreased in the higher-temperature region due to thermal denaturation. Obviously, the optimal temperature of the trypsin activity increased from 40 to 65 and 70 °C, respectively, for the nonreduced and reduced micelles. Various types of trypsin stabilized by polymers have been evaluated on their thermal stability, and the highest optimal temperature of stabilized trypsin in such a way was 65 °C reported by Fernandez et al. (*18*, *19*). In their study, trypsin conjugated with the derivatives of cyclodextrin showed an increased optimal temperature of trypsin, but the autolysis reaction was not completely prevented. The significant increase in the optimal temperature up to 70 °C obtained in the present study of the PIC micelle system might be due to the effective stabilization of the trypsin molecule by the introduction of the cross-linked structure in the matrix of the micellar core. In the core of the cross-linked micelles, cross-linkages were formed not only between the trypsin–trypsin molecules, but also between the primary amino groups at the ω-end of the PAA segments and the aldehyde groups in glutaraldehyde, i.e., trypsin–trypsin and trypsin–polymer cross-linkages, forming a hybrid nanogel structure. Consequently, the thermal-denaturing temperature of trypsin shifted to a higher temperature through the immobilization into the nanogel network, thus avoiding the thermal unfolding of the protein structure. It is worth noting that, in the immobilized trypsin in the micellar core, the spatial arrangement of the Asp-His-Ser catalytic triad in the trypsin molecule should be maintained even at 70 °C.

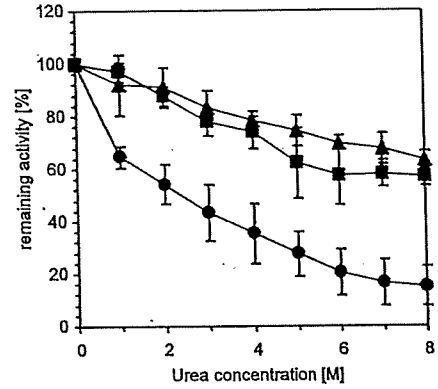

Figure 4. Change in relative remaining activity with urea concentration for native trypsin (●) and trypsin incorporated into nonreduced (▲) and reduced (■) micelles. (Trypsin concentration 16.8 μM; substrate concentration 5.0 mM; The initial reaction rates of native trypsin and trypsin incorporated into nonreduced and reduced micelles were 4.3 ± 0.1, 7.3 ± 0.7, and 8.9 ± 0.9 μM/min, respectively. The plots are presented as the average of three experiments ± SD.)

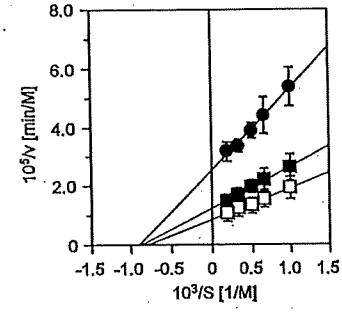

Figure 5. Lineweaver–Burk plots of native trypsin (●) and trypsin incorporated into the nonreduced (■) and reduced (□) micelles. (Trypsin concentration 16.8 μM; temperature 25 °C; $n = 3$.)

The stabilized spatial arrangement of the catalytic triad in the core-cross-linked micelles was also supported by the results of the tolerated trypsin activity against urea addition. Figure 4 shows the change in the relative remaining activity, defined as the ratio of the initial enzymatic reaction rate in the presence and absence of urea. Urea is well-known as a strong breaker of hydrogen bonding, as well as a disruptor of hydrophobic interaction, including enzyme denaturation in a concentration dependent manner. Indeed, the relative remaining activity of native trypsin gradually decreased with increasing urea concentration, and became lower than 50% at 3.0 M urea. On the other hand, even at 8.0 M urea, the trypsin incorporated into the core-cross-linked micelles maintained more than 60% of its relative remaining activity, indicating that the spatial arrangement of the catalytic triad in the cross-linked micelles was preserved even at high urea concentration. These results shown in Figures 3 and 4 suggest that the original tertiary structure of the trypsin molecule in the micelles might be effectively stabilized through chemical cross-linking.

As seen in Figures 2 and 3, the trypsin incorporated into the core-cross-linked micelles always showed higher initial reaction rates compared with native trypsin, suggesting that the enzyme incorporated into core-cross-linked micelles exhibits apparently enhanced activity. In order to evaluate the difference in the amidase activity of native trypsin and trypsin in the nonreduced and the reduced micelles, enzymatic reaction constants were determined by preparing Lineweaver–Burk plots (Figure 5). The good linear relations observed between the reciprocals of the initial reaction rate and the substrate concentration in the Lineweaver–Burk plots was confirmed not only for native

Table 1. Enzymatic Reaction Constants of Native Trypsin and Trypsin Incorporated into the Core-Cross-Linked Micelles

	k_{cat} [min^{-1}]	K_m [mM]	k_{cat}/K_m [M^{-1} min^{-1}]
native trypsin	0.236 (1.0)[a]	1.11 (1.0)[a]	213 (1.0)[a]
trypsin in nonreduced micelle	0.482 (2.0)[a]	1.14 (1.0)[a]	423 (2.0)[a]
trypsin in reduced micelle	0.689 (2.9)[a]	1.23 (1.1)[a]	560 (2.6)[a]

[a] These values were relative values against the values of native trypsin.

trypsin, but also for the trypsin incorporated into the reduced and nonreduced micelles. This linear relation indicates that the enzymatic function of trypsin incorporated into the core-cross-linked micelles can be kinetically analyzed on the basis of the Michaelis–Menten equation. The obtained enzymatic reaction constants, the catalytic rate constant (k_{cat}), the Michaelis constant (K_m), and k_{cat}/K_m, were summarized in Table 1. It was observed that the K_m values remained almost unchanged following the incorporation of trypsin into the core-cross-linked micelles, indicating that the affinity between trypsin and the substrate did not change. On the other hand, the k_{cat} values of the core-cross-linked micelles were approximately two to three times higher than that of native trypsin. The higher reaction rates of trypsin in the core-cross-linked micelles, as compared to those of native trypsin, were ascribed to an increase in the k_{cat} values. It is well-known that trypsin has a catalytic site constituted of the Asp-His-Ser triad (*20*–*22*). In this catalytic site, the nucleophilicity of the hydroxyl group of Ser increases through a proton-transfer process in the Asp-His dyad, in which the carboxylate group of Asp stabilizes the imidazolium ion of the His residue. We recently found that a part of the carboxylate group of the Asp residue in PEG-PAA stabilized an imidazolium ion of the His residue in the catalytic triad, thus accelerating the catalytic reaction of trypsin (*23*). This effect might also be induced in the core-cross-linked micelles as a means to increase the catalytic constant, as compared to native trypsin.

CONCLUSION

Core-cross-linked PIC micelles, prepared from trypsin and PEG-PAA, were effectively formed in both a reduced and a nonreduced state, both entrapping trypsin in the core. Both a remarkable improvement in the storing stability and a significant increase in the optimal temperature of the trypsin activity, as well as the marked resistance to denaturation by urea, were all confirmed as effects of the incorporation of trypsin into the core-cross-linked micelles. Note that there were few reports on the immobilization method of enzymes which attained increases in both the enzymatic activity and the optimal temperature. Most of the conventional enzyme-immobilized systems showed an apparent decrease in enzymatic activity due to the mass-transfer effect attributed to the restricted mobility of the enzyme as well as to the limited diffusion of the solute molecules (*16*, *17*). In the case of the core-cross-linked micelles, the enzymes were fixed to each other by the cross-linking reagent to form a nanoscopic hydrogel domain consisting of several tens of enzyme molecules. Since the size of the domain was sufficiently small, the diffusion of the substrate might not be the rate-determining step of the enzyme reaction. As a result, the mass-transfer effect was not significant for the core-cross-linked micelles. This is also an advantage of the cross-linked PIC micelle systems when compared to the conventional immobilization of enzymes in gel matrices with significantly larger dimensions. These core-cross-linked micelles incorporating enzymes represent a new entity of supramolecular nanobioconjugates, which are expected to find significant application in both the medical and bioengineering fields.

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Supporting Information Available: The quaternary pyridinium structure in the core-cross-linked micelle. This material is available free of charge via the Internet at http://pubs.acs.org.

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Study of the quantitative aminolysis reaction of poly(β-benzyl L-aspartate) (PBLA) as a platform polymer for functionality materials

Masataka Nakanishi [a], Joon-Sik Park [b], Woo-Dong Jang [c], Makoto Oba [d], Kazunori Kataoka [a,b,*]

[a] *Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan*
[b] *Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-0033, Japan*
[c] *Department of Chemistry, College of Science, Yonsei University, 134 Sinchondong, Seodaemun-gu, Seoul 120-749, Republic of Korea*
[d] *Department of Clinical Vascular Regeneration, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan*

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Dedicated to Professor Teiji Tsuruta on the occasion of his 88th birthday (Beiju).

Abstract

A facile and quantitative aminolysis of poly(β-benzyl L-aspartate) (PBLA) as well as the solution properties of the prepared cationic polyaspartamide were investigated in this study. The reaction was found to proceed in good yield without undesired side reactions via the formation of a succinimide intermediate in the polymer backbone, which was efficiently converted to polyaspartamide accompanying the α,β isomerization of the main chain. The polarity of solvents and the secondary structure of the polymer strand were closely related to each other in terms of reactivity and stereoselectivity. The aminolysis of PBLA treated with one equivalent amine against benzyl ester groups resulted in the complete conversion at 35 °C in random-coil solvents within 1 h. The racemization that accompanied this reaction was observed in random-coil solvents, but was efficiently suppressed in helicogenic solvents, with 95% of the optical purity maintained in CH_2Cl_2. In addition, the quantitative introduction of *N*,*N*-diisopropylethylenediamine (DIP) led to the formation of cationic polyaspartamide, poly[*N*-(*N*′,*N*′-diisopropylaminoethyl)aspartamide] (PAsp(DIP)), which showed pH and thermo-sensitivities in aqueous media. This systematic investigation of the aminolysis of PBLA with DIP demonstrates the feasibility of a PBLA-aminolysis system for designing functionalized polyaspartamides which can be useful as biomaterials.

Keywords: PBLA; Aminolysis; Quantitative side-chain reaction; Succinimide; Suppression of racemization; pH and thermo-sensitivities

* Corresponding author. Address: Department of Materials Engineering, School of Engineering, The University of Tokyo, Japan. Tel.: +81 3 5841 7138; fax: +81 3 5841 7139.
E-mail address: kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka).

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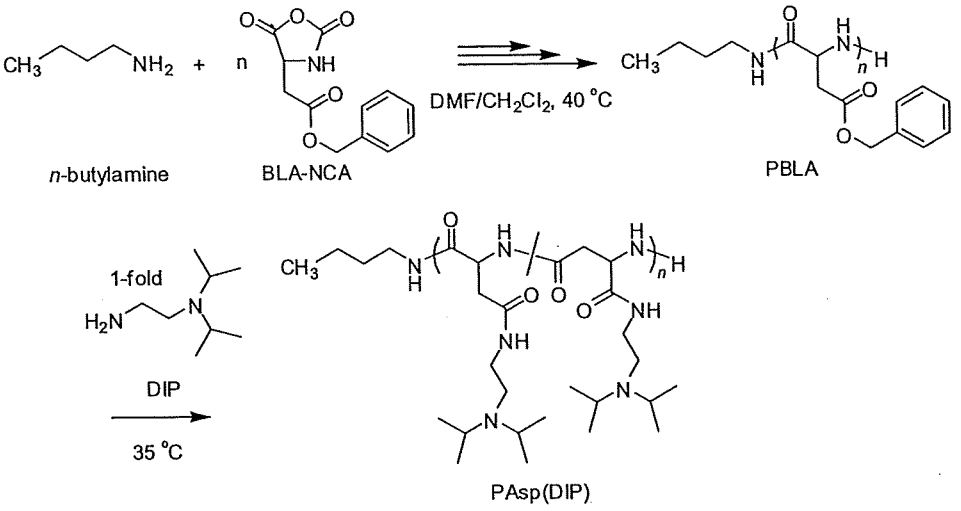


Scheme 1. Synthetic procedures of PBLA and PAsp(DIP) by the successive aminolysis reaction of PBLA.

1. Introduction

Chemical modification by the side-chain reaction of polymer is a convenient way to prepare a variety of functionalized derivatives from a single platform polymer [1–3]. For example, the precursor polymers bearing functional groups such as active ester [4–9], (meth)acryl chloride [10–12], and alkyne [13,14] are typical representatives with a potential for further functionalization or versatile modification according to particular applications. As a rule, the side-chain reaction was considered a facile synthetic route to obtaining polymer analogues with a constant degree of polymerization (DP) and molecular weight distribution (MWD) from a single platform polymer. Thus, the use of precursor polymer as a common intermediate allows for combinatorial strategies, feasible for evaluating and optimizing the correlation between the polymer structure and function.

There have also been many examples in bio-related fields where poly(amino acid)s with high biocompatibility and low toxicity [15–18] were chemically modified to increase their feasibility by binding hydrophobic drugs [19], a hydrophilic ethylene glycol segment [20] and pilot molecules [21] into the side chain. Although there have been several studies on side-chain modification using poly(lysine) or poly(glutamate/aspartate) as a platform polymer, the side-chain reaction of these poly(amino acid)s does not proceed quantitatively. The conversion of all the flanking moieties of the precursor polymers requires extreme conditions such as a high concentration of reactant, high temperature and long reaction time, leading to side reactions such as the decrease of molecular weight (MW) by the cleavage of the amide linkages in the main-chain, as in the case of the aminolysis of Poly(γ-benzyl L-glutamate) (PBLG) [22,23]. Alternatively, poly(succinimide) has been investigated as a more active precursor to preparing the isomeric library of polyaspartamide by the quantitative introduction of the functional groups [24–29]. However, the synthesis of this active precursor has some drawbacks, including a long time reaction, high reaction temperature, and coloring of the obtained product [30,31], limiting the resultant polymer under control. Namely, the polymers are still highly heterogeneous, not only in terms of DP and MWD but also in terms of optical purity and composition of the functional units in the side chain [32,33].

In this regard, we have recently established that the flanking benzyl ester groups of poly(β-benzyl L-aspartate) (PBLA) undergo a quantitative aminolysis reaction with various primary amine compounds, thus offering a variety of polyaspartamides useful for designing polymeric micelles and vesicles as a biomaterial application [34–39]. Although it has been suggested that the mechanism of this unique aminolysis reaction is involved with the succinimidyl ring formation, the details have not yet been clarified. Thus, it is critical to obtain insight into the reaction mechanism, particularly from the standpoint of kinetics, and to identify the detailed structure of polyaspartamide in order to assess its feasibility for use as biomaterials. To this aim, we investigated the mechanism and kinetics of the aminolysis reaction of PBLA, focusing on both the α to β transition

and racemization relevant to the solvent effect and the higher-ordered structure of polymer strands.

In this study, the cationic polyaspartamide was synthesized by reacting PBLA with *N,N*-diisopropylethylenediamine (DIP), and its solubility behavior responsive to pH and temperature was investigated. DIP was selected as a nucleophile to study the aminolysis mechanism and the environmentally responsive properties of polyaspartamide. The primary amino group of DIP was converted to the amide group after the aminolysis of PBLA, and the *N,N*-diisopropylaminoethyl group of DIP was responsible for pH- and thermo-sensitivities (Scheme 1). Among several alkyl groups as a hydrophobic moiety, the isopropyl group was selected with the expectation that a sharp transition of the polymer in aqueous media, like the typical thermoresponsive polymer, poly(*N*-isopropylacrylamide) [40], would occur. In addition, the tertiary amino group with two neighboring isopropyl groups was also selected not only for the hydrophobic-hydrophilic balance between the ionic segment and alkyl segment, but also to exclude undesired cross-linking during the aminolysis. Concerning the kinetics, in general the reactivity of side chains can be controlled not only by the polarity of the solvent but also by the polymer conformation depending on the solvation of side groups, steric hindrances, and the distance of the vicinal groups. From this point of view, we conducted this study in a comparable solvent system for the purpose of evaluating each effect of solvent polarity and polymer conformation on the kinetics and racemization.

2. Experimental

2.1. Materials

β-Benzyl L-aspartate *N*-carboxy-anhydride (BLA-NCA) was obtained from Nippon Oil and Fats (Tokyo, Japan). *N,N*-Dimethylformamide (DMF), 1,4-dioxane (dioxane), dimethylsulfoxide (DMSO), dichloromethane (CH_2Cl_2), and chloroform ($CHCl_3$) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and were purified by distillation according to the conventional procedure [41]. *N,N*-Diisopropylethylenediamine (DIP), *n*-butylamine and triethylamine (TEA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and were distilled from calcium hydride under reduced pressure. The other chemicals were used as received.

2.2. Method

The ^{1}H NMR spectrum was recorded on a JEOL EX 300 spectrometer (JEOL, Tokyo, Japan) at 300 MHz. Chemical shifts were reported in parts per million (ppm) downfield from tetramethylsilane. MW and MWD were estimated using a gel-permeation chromatography (GPC) (TOSOH HLC-8220) system equipped with two TSK gel columns (TSK-gel Super AW4000 and Super AW3000) and an internal refractive index (RI) detector. The columns were eluted with *N*-methyl-pyrrolidone (NMP) containing lithium bromide (50 mM) (0.3 ml min^{-1}) at 40 °C. MW were calibrated with poly(ethylene glycol) standards (Polymer Laboratories, Ltd., UK). The IR spectra were obtained with an IR-550 JASCO spectrophotometer. Gas chromatography (GC) was carried out with GC17A (SHIMADZU, Tokyo, Japan) gas chromatograph equipped with a 30-m long, 250 μm i.d., open tubular column, DB-1 (SHIMADZU GLC, Tokyo, Japan), and a flame ionization detector. C-R7A (SHIMADZU, Tokyo, Japan) was used for instrument control and data acquisition. The carrier gas was hydrogen. The pressure at the head of the column was 400 kPa, and the linear velocity at the end of the column was 41 cm s^{-1}. The sample was injected onto the column at a split ratio of over 25:1. The injection port temperature was 200 °C.

2.3. Synthesis of poly(β-benzyl L-aspartate) (PBLA)

To obtain PBLA (Scheme 1), BLA-NCA (2.49 g, 10 mmol) was polymerized in the mixture of DMF (10.0 mL) and CH_2Cl_2 (100 mL) at 40 °C by the initiation from the terminal primary amino group of *n*-butylamine (14.6 mg, 200 μmol). PBLA was purified by precipitation in ether (3 L) three times, and was confirmed to have a unimodal MWD (M_w/M_n: 1.07) by GPC measurement (Fig. 1C). The DP of PBLA was calculated to be 52 based on ^{1}H NMR spectroscopy (Fig. 1A).

2.4. Synthesis of poly[N-(N′,N′-diisopropylaminoethyl)aspartamide] (PAsp(DIP))

Lyophilized PBLA (202 mg, 20 μmol) was dissolved in DMSO (10 mL), followed by the reaction with 1-fold DIP (1 equiv to the residual benzyl ester group in PBLA, 144.3 mg, 1 mmol) under mild anhydrous conditions at 35 °C for 1 h to obtain

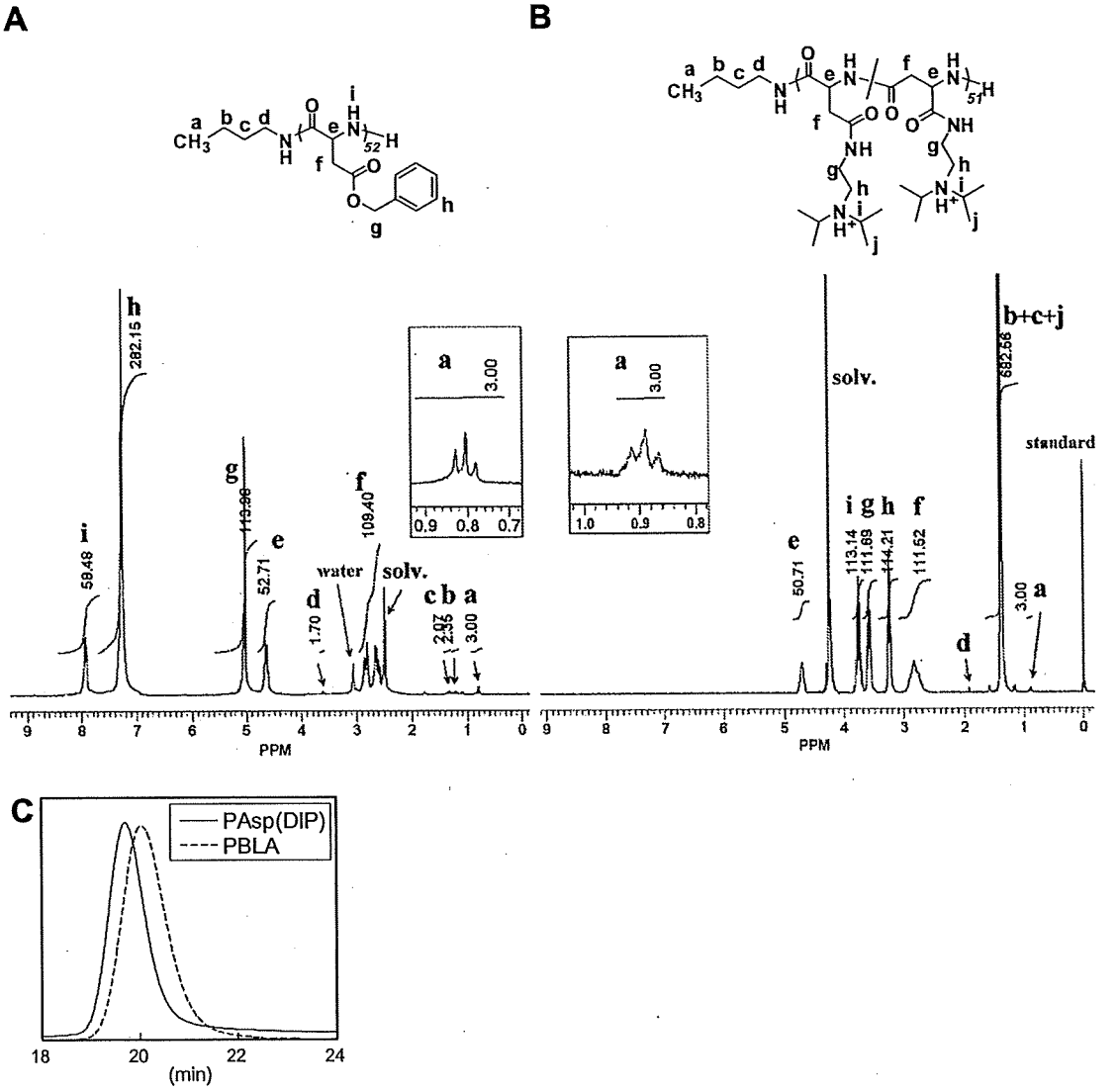


Fig. 1. ^{1}H NMR spectra of (A) PBLA in DMSO-d_6 at 50 °C and (B) PAsp(DIP) synthesized in DMSO in D_2O at 80 °C. (C) GPC diagrams of PBLA and PAsp(DIP) (PEG standard, eluent: NMP (containing 50 mM LiBr), temperature 40 °C, RI detection).

PAsp(DIP). After the reaction, the reaction mixture was slowly added dropwise into a cooled aqueous solution of acetic acid (10% v/v, 40 mL) and dialyzed against an aqueous solution of 0.01 N HCl three times and distilled water one time (molecular weight cut off: 3500 Da). The final solution was lyophilized to obtain the polymer in the chloride salt form with a yield of 92% (221 mg). In addition to the chloride salt form, the unprotonated PAsp(DIP)s were obtained as follows. The reaction mixture was purified by dialysis against DMSO three times and methanol three times. The solution was evaporated *in vacuo* and the polymer was dissolved in benzene, followed by lyophilization. The unprotonated PAsp(DIP) was then obtained as a white powder with a yield of 90% (215 mg). Similarly, the aminolysis of PBLA with DIP was carried out at 35 °C in NMP (reaction time: 1 h), DMF (reaction time: 1 h), $CHCl_3$ (reaction time: 200 h) and CH_2Cl_2 (reaction time: 160 h), respectively. In the case of dioxane, the solution of PBLA in dioxane was at first completely dissolved at 50 °C and then the temperature was allowed to decrease the temperature to 35 °C, followed by the reaction with an equivalent DIP for 260 h. All the yields were approximately 90%. The completion of the aminolysis reaction was confirmed by GC.

2.5. Reaction velocity measurements

The aminolysis of PBLA was carried out using an equivalent DIP at 35 °C in a comparable solvent sys-

tem. The conversion of the BLA residue into the aspartamide residue was calculated from both the remaining amount of DIP and the amount of the benzyl alcohol. The remaining amount of DIP was measured using GC with the internal reference method using *n*-decane as internal standard. PBLA (202 mg) was reacted with 1-fold DIP (144 mg) in 10 mL of solvent. The rate of debenzylation was determined by comparison of the intensity of CH_2 of the leaving benzyl alcohol with that of benzyl ester based on ^{1}H NMR spectroscopy using DMSO-d_6 and CD_2Cl_2 as solvent. PBLA (20.2 mg) was reacted with DIP (14.4 mg) in 1 mL of solvent at 35 °C during the ^{1}H NMR measurement.

2.6. Optical rotation measurements

The specific optical rotation measurements of the polymer samples were carried out in CH_2Cl_2 and DMSO respectively, using a digital polarimeter DIP-370 (JASCO) at a 546 nm wavelength, with a cell of 100 mm length and an integration time of 30 s. The concentration of polymer was adjusted to 1.0 wt% for all the measurements. $[\alpha]_D$ was measured at definite time intervals after adding 1-fold DIP. The measurement was done 20 times for each sample to obtain the average value.

2.7. Analysis of aspartamide enantiomers

The D/L-aspartamide ratios were determined by high performance liquid chromatography carried out by the HiPep Laboratories (Kyoto, Japan) using the enantiomer labeling method (ELAB). This analysis was conducted using a fully automated D/L and quantitative amino acid analyzer, a Shimadzu-CAT Model DLAA-1, which consists of an automated derivatizer with a robot arm, Autoderivat 100/2 of CAT, and a gas chromatograph, Shimadzu Model GC/DLAA with an auto injector, AOC/DLAA, in combination with a chromatographic data processor, Shimadzu Chromatopac C-R4A [42].

2.8. Potentiometric titration and transmittance measurements

PAsp(DIP) (30 mg) prepared in DMF was dissolved in 50 mL 0.01 N HCl and titrated with 0.01 N NaOH added in quantities of 0.063 mL after the pH values were stabilized (minimal interval: 30 s), using an automatic titrator (TS-2000, Hiranuma, Kyoto, Japan) for the titration and transmittance measurements. The pH values and transmittance were measured at 10 °C, 20 °C, 30 °C, 40 °C and 50 °C. The α/pH curves were determined from the titration curves obtained. Each calibration was carried out at the same temperature as each measurement.

3. Results and discussion

3.1. Preparation of PAsp(DIP)

PBLA is known to form the left-handed α-helix in apolar solvents such as dioxane [43], $CHCl_3$ [44], CH_2Cl_2 [45] and the random-coil in polar solvents such as DMF [46] and DMSO [43]. Because of this, these five random-coil and helicogenic solvents with various dielectric constants were selected for this study.

From the ^{1}H NMR measurement, the DP of the PAsp(DIP), comparing the peak intensity ratio of the CH_3 of the *n*-butyl group (**a**) with α-C*H* of PAsp(DIP) (**e**) was calculated to be 51, and it was confirmed that PAsp(DIP) synthesized in DMSO has a unimodal MWD (M_w/M_n: 1.08) by GPC measurement, thus indicating that the aminolysis proceeded quantitatively without causing any cleavage of the main chain (Fig. 1). Similarly, it was confirmed that all the PAsp(DIP)s prepared in other solvents had almost the same MW and a unimodal MWD (Table 1). Therefore, this result demonstrated that the aminolysis of PBLA was a useful side-chain exchanging reaction avoiding the side reaction of the cleavage of the main chain in the appropriate condition.

3.2. Reaction velocity

A significant difference was found in the reaction rate between polar random-coil solvents and apolar helicogenic solvents, as shown in Fig. 2. The reaction was much faster in random-coil solvents than in helicogenic solvents. From a practical point of view, it is worth mentioning that aminolysis with a 1-fold amine is completed after 1 h at 35 °C in polar solvents. In addition, although no difference was found in reactivity among random-coil solvents, the reaction was clearly faster in helicogenic solvents with the increasing dielectric constant of the solvent, suggesting that the rate of the aminolysis reaction of PBLA also depends on the polarity of the solvents. The results of the kinetic studies show that the aminolysis of side-

Table 1
Analytical data of M_w/M_n and L-isomer (%) of PAsp(DIP) synthesized in various solvents

Polymer solvent	PBLA	PAsp(DIP)				
		Dioxane	$CHCl_3$	CH_2Cl_2	DMF	DMSO
ε[a]		2.2	4.8	9.1	37	47
M_w/M_n[b]	1.07	1.08	1.07	1.08	1.09	1.08
L (%)[c]	99.9	83.8	89.0	94.5	72.7	73.6

[a] Indicates the dielectric constant of the solvent.
[b] Determined by GPC.
[c] Determined by ELAB method carried out by HiPep laboratories.

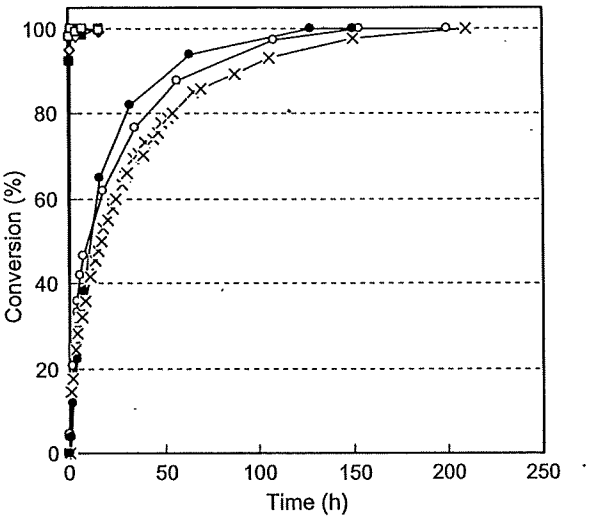
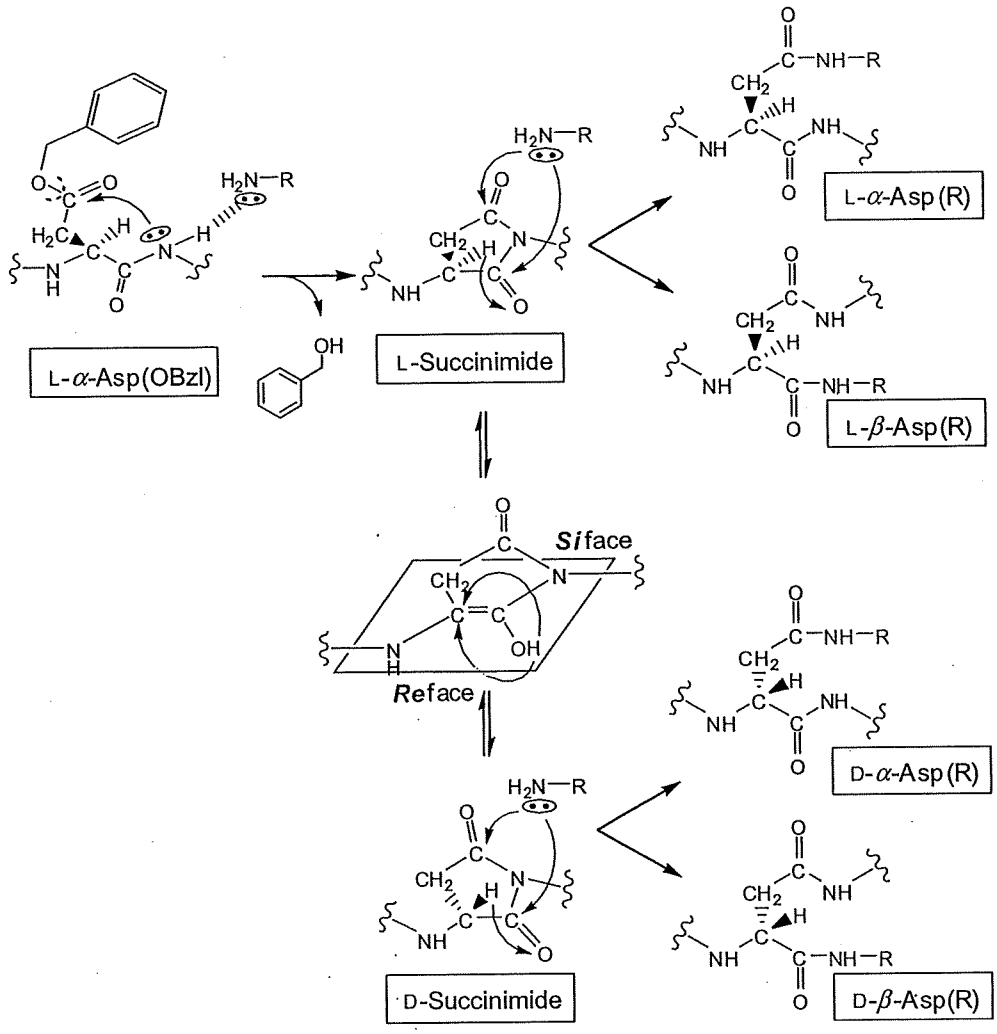


Fig. 2. Time profile of the conversion rate calculated from the remaining DIP in DMSO (□), DMF (◇), CH_2Cl_2 (○) and $CHCl_3$ (×) and the leaving benzyl alcohol in DMSO-d_6 (■) and CD_2Cl_2 (●) in the condition where PBLA reacts with 1-fold DIP at 35 °C.

chain esters of PBLA can be greatly affected by both the conformation of the polymer strand and the polarity of the solvents.

The kinetics data described above suggest that there could be an active intermediate to have this reaction progress rapidly and quantitatively, because the aminolysis of esters, in other words, a way of directly transforming esters to amides, usually requires stoichiometric amounts of promoters or metal mediators [47]. It has been reported that a large amount of primary amines was required to modify all the flanking esters in the side chain of PBLG by aminolysis, and that main-chain scission caused due to the aminolysis of the amide linkage in the main chain by the remaining primary amines [22,23]. In contrast, the stoichiometric aminolysis reaction of PBLA resulted in the prompt and complete conversion in polar and apolar solvents under a mild condition. Of interest is the significant difference in the reaction rate between PBLA and PBLG, because the difference between their primary structures is the presence or absence of γ-CH_2 in the side chain. Blout et al. [46] reported the formation of poly(succinimide), an active precursor polymer, from PBLA when PBLA was treated with catalytic amounts of base in DMF or DMSO. The formation of poly(succinimide) was determined by isolation and comparison with the infrared spectra reported in the literature [46]. However, the treatment of PBLG under identical conditions showed no evidence of cyclization to poly(glutarimide) [46]. Therefore, the mechanism by which α to β isomerization of aspartic acid occurs was focused here in order to understand the mechanism by which the aminolysis of PBLA occurs. Moreover, there have been many reports showing that the racemization of aspartic acid and asparagine residues was accelerated via succinimide intermediates [48–53]. From this result, it is highly expected that the racemization occurs when the succinimide formation occurs.

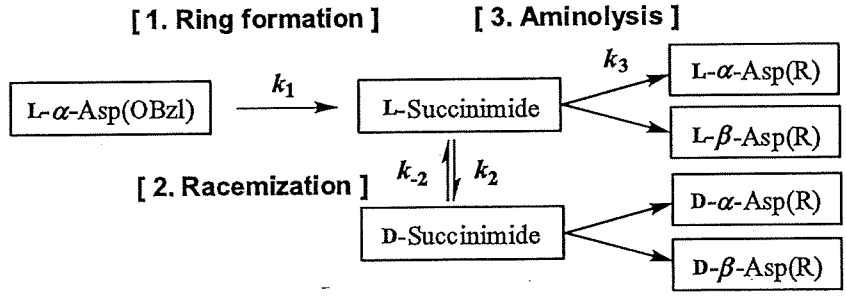
For the reasons mentioned above, it is estimated that there are three stages: (1) ring formation, (2) racemization, (3) aminolysis in the present reaction of PBLA (Scheme 2). The reaction rate constants of each stage were defined as k_1, k_2 and k_3, respectively. In the first stage, the aminolysis of PBLA starts with the activation of the nitrogen atom in the main chain by an amine as a weak base coordinating the proton of the amide group, and then the nucleophilic attack by the activated nitrogen on the carbon atom of the carbonyl group in the side chain occurs to form the succinimidyl ring. The eliminated proton which recombines with the benzyloxyl group is released as benzyl alcohol, accompanying the regeneration of amine. Therefore, the ring formation is expected to be a catalytic reaction. In the second stage, the proton in the α-position is easily eliminated with ease in the succinimidyl ring, and then racemization proceeds by the keto–enol tau-



Scheme 2. Mechanism of aminolysis reaction of PBLA.

tomerism. In the third stage, an amine undergoes a nucleophilic attack to one of two carbonyl groups in the succinimidyl ring, which is efficiently converted to the isomerization to form the α,β-aspartamide.

Therefore, the aminolysis of PBLA by less than a 1-fold amount of DIP in DMSO($-d_6$) or CH_2Cl_2 (CD_2Cl_2) was performed to confirm whether aminolysis proceeds via the formation of the succinimide



intermediate or not. In addition, the degree of racemization after the aminolysis of PBLA with 1-fold DIP was analyzed.

3.3. Identification of intermediate structure and kinetics

3.3.1. Identification of intermediate structure and kinetics in DMSO

The confirmation of an intermediate structure was done in the condition where PBLA reacted with 0.5-fold DIP in DMSO-d_6 using ^{1}H NMR spectroscopy recorded after 0.5 h, 1 h and 6 h (Fig. 3). It was determined that a 0.5-fold amount of DIP was added to this system by comparing the intensity of CH_3 (**j**) of DIP with that of CH_2 (**c**) of the benzyl group of PBLA. The sharp peak of CH_2 (**f**) of the leaving benzyl alcohol appeared at 4.5 ppm, and the intensity increased promptly in association with the separation of the peak (**d**) due to the benzyl alcohol. The integration value of CH_2 (**f**) of the benzyl alcohol became ca. 2 at 1 h. This is consistent with the complete disappearance of the peak corresponding to CH_2 (**c**) of the benzyl group of PBLA within 1 h. Thus, it was confirmed that the debenzylation was completed within 1 h. It was also confirmed by GC that all the DIP were consumed after 6 h.

Comparing the spectrum of PBLA (A) with that of the reactant at 0.5 h (B) in Fig. 3, it is worth noting the substantial shift of the α-C*H* (**a**) peak at 4.7 ppm. Concomitantly, the peak assigned to β-CH_2 seems to shift from 2.6 and 2.8 ppm (**b**) to 2.7 and 3.2 ppm (**l**), respectively, suggesting the substantial change in the main chain structure. Furthermore, the appearance of new peaks at 5.3 (**k**) and 5.1 (**m**) ppm was clearly observed. For further analysis, each intensity and the summation of the two peaks (**k**) and (**m**) from (B) to (D) in Fig. 3 were compared. According to the reported chemical shift values of poly(succinimide) [54], the peaks (**k**) and (**l**) were assigned to α-C*H* and β-CH_2, respectively, of the succinimide ring produced in the main chain. In accordance with the gradual decrease in the peak intensity of peak (**k**) with time, an alternative increase in the intensity for peak (**m**) was observed, suggesting the progress of the aminolysis reaction. Note that the summation of the peak intensities of (**k**) and (**m**) always took the constant value of 1.1 after 1 h. Therefore, it is reasonable to conclude that the peak (**m**) is assigned to α-C*H* of polyaspartamide. The time-trace of the ^{1}H NMR spectra revealed that the aminolysis reaction successively occurred after the prompt progress of succinimide formation ($k_1 > k_3$) in DMSO.

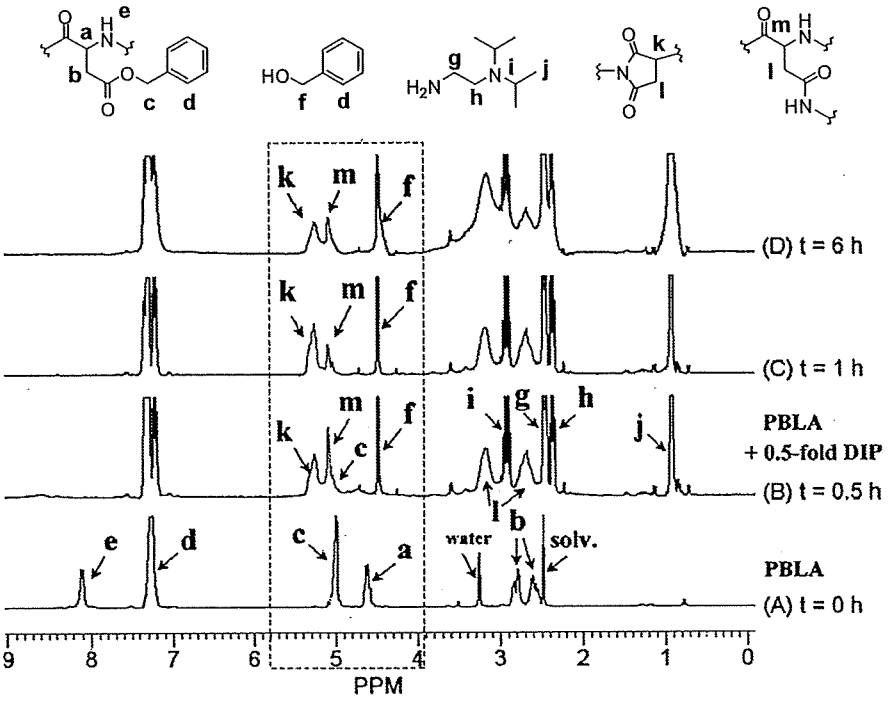


Fig. 3. Time-trace of ^{1}H NMR spectra of PBLA reacting with 0.5-fold DIP in DMSO-d_6 at 35 °C.

Next, an IR measurement was performed to directly detect the succinimide structure in the condition where PBLA reacted with 0.005-fold DIP in DMSO at 35 °C for 30 min. In the IR spectrum of the product, the amide I, amide II and ester peaks almost disappeared and the imide peaks of the succinimidyl ring appeared clearly at 1717 cm^{-1} and 1800 cm^{-1}, as shown in Fig. 4A and B. The IR spectrum of the product was very similar to that of the model imide, *N*-ethyl-succinimide (data not shown) and similar to the spectrum of Poly(succinimide) which has been reported [46]. Therefore, it is reasonable to conclude that DIP catalytically transduces the BLA residue to succinimide, an active intermediate, which then quantitatively converted to aspartamide, and that aspartamide includes β-isomer.

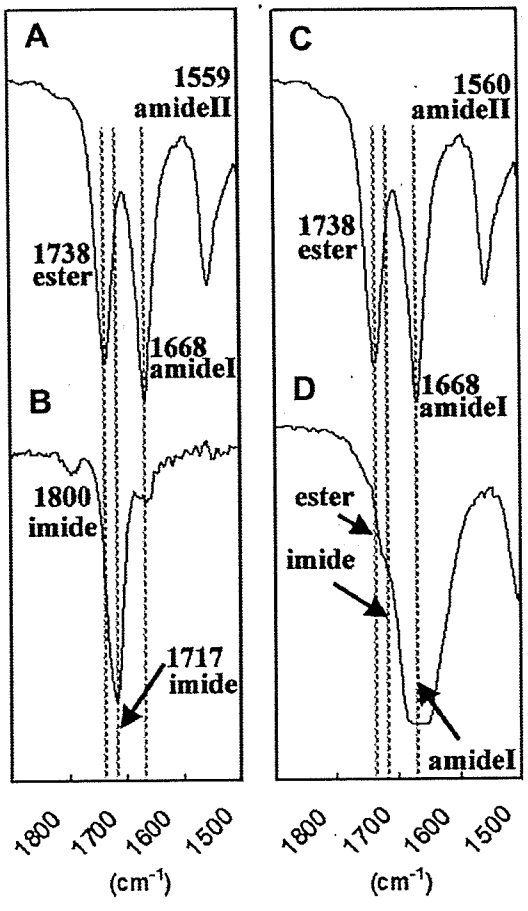


Fig. 4. Infrared absorption spectra with assignments at a range of 1800–1500 cm^{-1} of (A) PBLA, (B) PBLA reacting with 0.005-fold DIP in DMSO for 30 min, (C) PBLA reacting with 0.005-fold DIP in CH_2Cl_2 for 24 h, and (D) PBLA reacting with 1-fold TEA in CH_2Cl_2 for 12 h.

3.3.2. *Identification of intermediate structure and kinetics in CH_2Cl_2*

Similarly, the identification of an intermediate structure in CH_2Cl_2 was carried out to explore the mechanism involved in the quantitative aminolysis reaction in apolar solvents. The ^1H NMR spectra were obtained in the condition where PBLA reacted with 0.5-fold DIP in CD_2Cl_2 at 35 °C as shown in Fig. 5. The sharp peak of CH_2 (**f**) of the leaving benzyl alcohol appeared at 4.6 ppm with a gradual increase in the intensity, with the reaction time occurring more slowly than that in the DMSO-d_6 system. Eventually, 47% of benzyl alcohol was eliminated in 237 h. Nevertheless, no peak corresponding to succinimide as an intermediate structure was found in the spectra from (A) to (E), unlike in the DMSO-d_6 system. An IR measurement was carried out in the condition where PBLA reacted with the catalytic amount of DIP for 24 h in CH_2Cl_2. The spectrum obtained after 24 h was quite similar to that of PBLA (Fig. 4C) without any sign of succinimide ring formation. Further analysis was then carried out using triethylamine (TEA) as a weak base, which can act as a catalyst leading to formation of the succinimide ring, yet cannot undergo an aminolysis reaction because of the lack of a primary amino group. Eventually, the solution of PBLA reacting with an equivalent TEA for 24 h became turbid, and its IR spectrum clearly included the band assignable to the imide structure (Fig. 4D), indicating the formation of a succinimide structure.

Note that poly(succinimide) is known to be insoluble in CH_2Cl_2 [46], being consistent with the precipitate formation in the reaction mixture of PBLA with TEA in CH_2Cl_2. In the reaction system with DIP, the produced succinimide moiety promptly reacted with the primary amino group of DIP to form an aspartamide with a flanking diisopropylaminoethyl group because the succinimidyl ring formation was the rate-limiting step ($k_1 < k_3$), eventually maintaining the polymer solubility in CH_2Cl_2.

3.4. *Stereoselectivity*

A measurement of the specific optical rotation was conducted to analyze the racemization and conformational change of the polymer strand during the aminolysis of PBLA. Notably, a significant difference in $[\alpha]_D$ was observed between the polymers dissolved in DMSO and CH_2Cl_2 (Fig. 6). $[\alpha]_D$ of the polymer in DMSO immediately became almost