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ARTICLE

Biodegradable Main-Chain Phosphate-Caged Fluorescein Polymers for the Evaluation of Enzymatic Activity

Kazuo Tanaka, Narufumi Kitamura, and Yoshiki Chujo*

Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

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ABSTRACT: We describe here the biodegradable polymers consisted of the tandemly linked phosphate-caged fluorescein in the main chains. The caged-fluorescein monomer with the phosphoramidite groups at both hydroxyl groups was synthesized, and the polycondensations were executed in the presence of the diol derivatives which were promised to be the reactivity-tunable linker. After oxidation and the alkaline treatment for the deprotection, less-emissive polymeric products were obtained. The resulting polymers can release fluorescein by the digestion with alkaline phosphatase. We demonstrated that the enzymatic activity of the cell lysate can be quantified from the increase of the magnitude of fluorescence emission with the synthetic polymers.

Introduction

Fluorescence measurements have been powerful tools for the rapid monitoring of detailed behaviors of fluorescent molecules *in vivo*.[1] Combining with target-responsive probes, the static or kinetic information about specific biological events can be obtained from vital organs.[2] Polymeric probes for visualizing biological functions have several advantages. Multiloaded signal units in the sole probe molecule can enhance the detection sensitivity, and the biodistribution and responsiveness to the target can be tailored by modulating the length, size, structure and morphology of the polymer chains.[3]

Fluorescein is a conventional fluorophore for biological researches because of its convenient wavelength for biological measurement, high extinction coefficient, and high fluorescence quantum yield in water. To suppress background noise caused from the unreacted probes, various kinds of the regulation methods or principles for emission intensity of fluorescein have been established such as by energy transfer between quencher molecules,[4] regulation of electronic properties,[5] caging modification,[6] and their combinations.[7] In particular, the phosphate caged-fluorescein derivatives are expected to be a compatible marker for detecting mercury ion, superoxide, and DNA as well as the phosphatase activities.[8] On the other hand, the poor solubility in solvents limits to be applied to the polymerization reactions for preparing the polymeric probes.

Herein, we report the biodegradable polymers which possess the tandemly linked phosphate-caged fluorescein in the main chain. The phosphoramidite modification, which is promised to be a phosphate-caged moiety, can improve the solubility of fluorescein in the organic solvents and make possible to use the fluorescein unit as a monomer in the polycondensation. The fluorescence emission of diphosphorylated fluorescein was extremely abandoned in the main chain of the polymers. These polymers can release fluorescein by the digestion with an alkaline phosphatase (AP), and subsequently, strong fluorescence emission can appear. In addition, the digestion speed can be tuned by the substitution groups at the linker moiety between the caged fluorescein in the main chain. Finally, we demonstrate that the polymeric probe was available to determine the AP activity of the cell lysate.

*Corresponding author. E-mail: chujo@chujo.synchem.kyoto-u.ac.jp. Fax: +81-75-383-2605. Telephone: +81-75-383-2604.

Results and Discussion

To overcome the solubility problem of fluorescein for applying to the polycondensation reaction, we designed the compound **1**, as a monomer (Scheme 1). Disubstituted fluorescein derivatives at 3- and 6-positions of the xanthene ring have been known to less emissive molecules due to the formation of the spiro-type skeleton, and the intrinsic strong emission should be recovered after eliminating these functional groups.[6] The diphosphoramidite groups in the monomer **1** are promised to the phosphate-caged fluorescein after oxidation at the phosphine atom and following deprotection of cyanoethoxy groups. The monomer **1** can be isolated as a colorless powder from conventional silica gel column chromatography in relatively high yield.[9] Fortunately, the monomer **1** showed good solubility and stability in various kinds of organic solvents for subsequent polycondensation.

The polymerization reactions were executed with 0.1 M of the monomer **1** and an equivalent amount of 1,2-diol derivatives as a comonomer in the presence of tetrazole as an activator of phosphine atoms in acetonitrile at 25 °C under Ar conditions. The reaction was monitored with GPC profiles in chloroform.[10] The polymeric products were appeared after 1 h, and the reaction reached a plateau at 3 h. Oxidation of phosphine atoms carried out at 25 °C for 1 h after the addition of two equivalent amounts of mCPBA to the phosphine atoms in chloroform. From ^{31}P NMR measurements, the oxidation proceeded immediately, and less significant chain scission was observed from GPC profiles. The deprotection was executed at 30 °C in the methanolic solution containing saturated potassium carbonate. After the incubation for 30 min, the mixture was evaporated, and the resulting yellow precipitation was obtained by the addition of chloroform. The potassium salts of the desired polymers were obtained.

The chemical structure was investigated with ^{1}H NMR and MALDI−TOF−MS measurements.[11,12] Compared to the ^{1}H NMR integral ratios between the methylene and aromatic rings,

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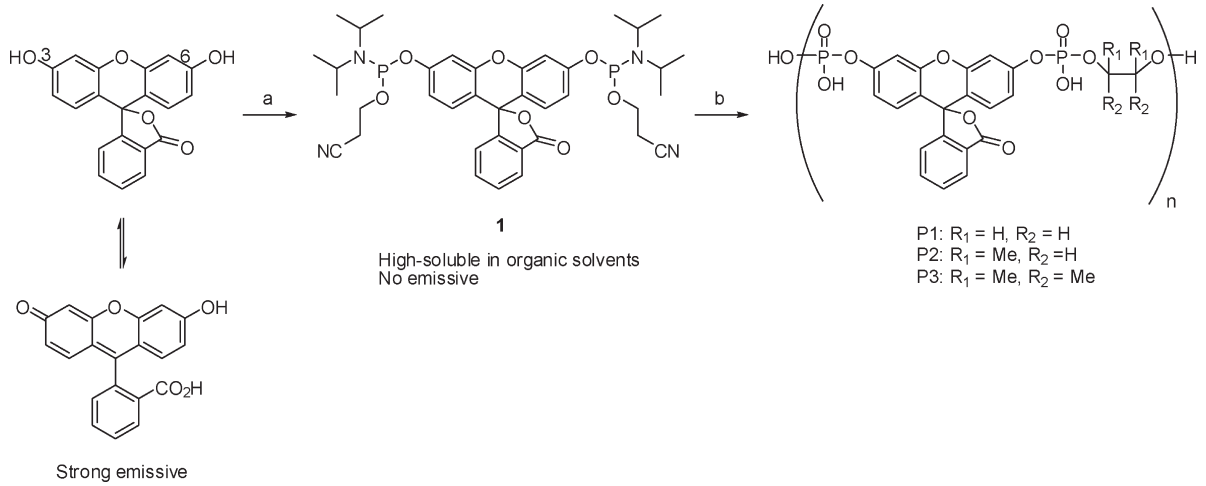
Scheme 1. Synthetic Scheme of the Fluorescein Monomer and Biodegradable Polymers[a]



[a] Reagents and conditions: (a) 2-cyanoethyl tetraisopropyldiphosphoramidite, tetrazole, acetonitrile 25 °C, 3 h, 63%; (b) diol compounds, tetrazole, acetonitrile, water, 25 °C, 3 h.

Table 1. Properties of the Polymeric Probes Composed of Various Linkers

	before treatments		after oxidation and deprotection		
linker	M_n[a]	PDI[a]	M_n[b]	PDI[b]	yield (%)
ethylene glycol	5300	1.30	1720	1.40	21
butanediol	4500	1.20	1680	1.36	15
pinacol	6300	1.22	c	c	c

[a] M_n and polydispersity index (PDI = M_w/M_n) of the polymers were determined by the GPC analysis using polystyrene standards in chloroform. [b] M_n and PDI were determined by the GPC analysis using poly(ethylene glycol) standards in water. [c] Polymeric products were not observed from GPC profiles.

both comonomers were incorporated into the polymeric product with approximately same equivalent. It was confirmed that deprotection of cyanoethoxy groups proceeded, and less impurities such as tetrazolinium salts, unreacted mCPBA, or leaving groups after the deprotection were included in the product in ^{1}H NMR spectra. Phosphorylated ethylene glycol ends and free fluorescein ends were observed from the negative mode MALDI−TOF−MS spectrum. These data proposed that the detachment of the monophosphorylated fluorescein and the internal cyclization could occur at the end of the polymer in the alkaline treatment for the deprotection.

Polymerization reactions with ethylene glycol derivatives, which are promised to introduce the substitution groups at the linker moiety, were executed. The results obtained from the GPC analysis in chloroform before oxidation and in water after deprotection are summarized in Table 1. The molecular weights and the PDI values showed similar. On the other hand, the stability of the polymer under alkaline condition was reduced by the incorporation of the methyl groups at the linker moiety. The synthetic polymer consisted of butanediol was gradually decomposed at pH 8.0, and in the case of the pinacol linker, the polymeric product was degraded after the deprotection even with shorter incubation time.[13] The hydrolysis at the phosphate linkers under alkaline conditions could be enhanced by the introduction of methyl groups at the linker moiety.

Calf intense alkaline phosphatase which is a class of low-specific exonuclease was used to evaluate the kinetics in the enzymatic reactions. The solutions contained 100 μg/mL of the polymers (monomer standard) at pH 6.0 were incubated at 37 °C, and the digestion rate was monitored with the increase of the magnitude of the fluorescence intensity at 515 nm assigned as the emission band from fluorescein (Figure 1). From the

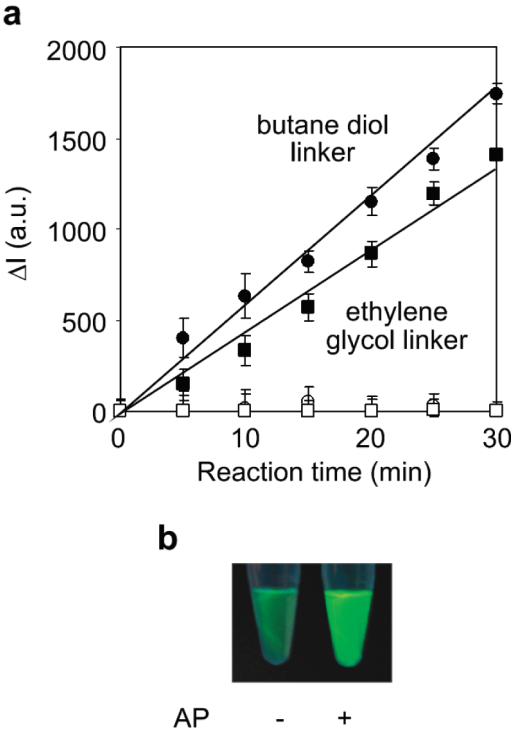
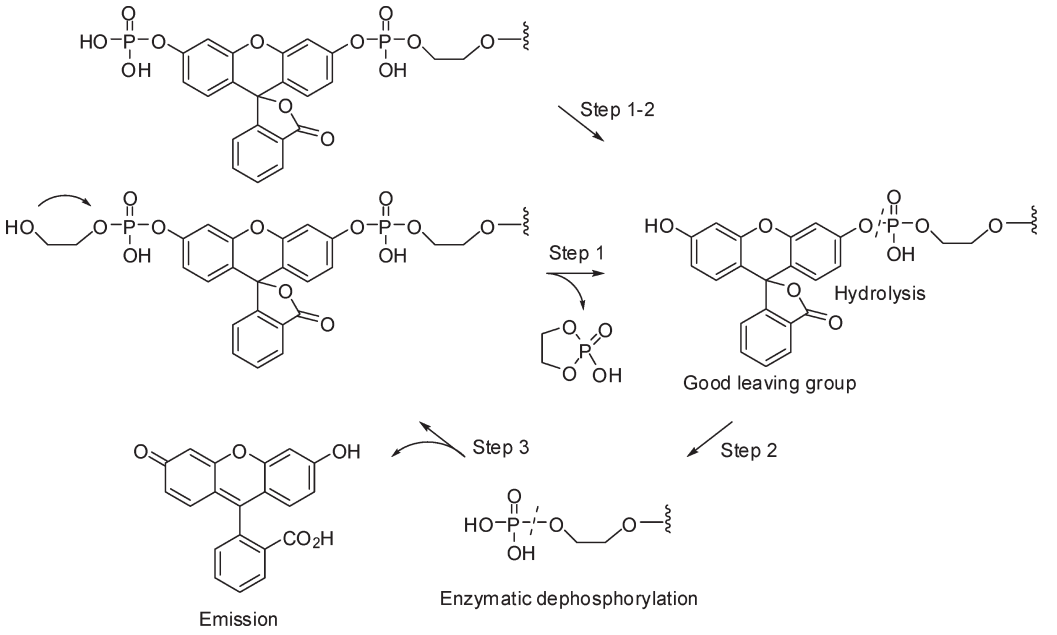


Figure 1. Fluorescence increase from the solutions containing the synthetic polymers. (a) Time-courses of the emission change at 515 nm from the reaction solutions containing the polymers composed of the ethylene glycol linker (square dots) and the butanediol linker (circular dots) with (black dots) or without (clear dots) AP (200 U) in 50 mM sodium phosphate buffer (pH = 6.0) at 37 °C. The errors represent the standard deviation calculated from the three data sets. (b) Picture of the solutions after 30 min incubation in the absence (left) or presence (right) of AP.

measurements of the time-course of the intensity changes with the solutions containing AP, the signal increase was observed during the incubation. On the other hand, the reaction solutions without AP showed less significant change of fluorescence intensity. These results indicate that the synthetic polymers possess biodegradability.

The reaction rates were enhanced by the introduction of methyl groups into the linker moiety. The polymer with the butanediol linker (52 nM/min) can be digested 1.3-fold faster

Scheme 2. Biodegradation of Synthetic Polymers



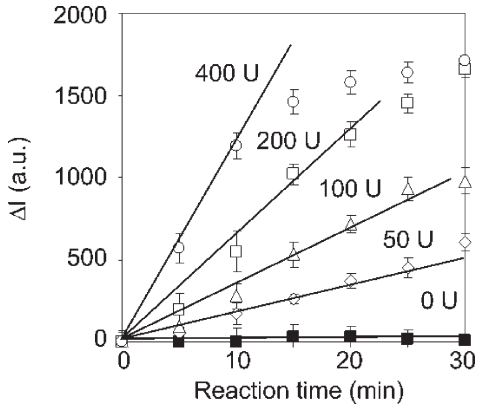


Figure 2. Time courses of fluorescence changes at 515 nm of the solution with variable concentrations of AP. The errors represent the standard deviation calculated from the three data sets.

than that with the ethylene glycol linker (41 nM/min). Previous reports suggested that the substitution of the alkyl groups on the glycol moiety should be favorable for the formation of the five-membered products via the cyclization including phosphate because of higher heat of formation.[14] In addition, the hydrolysis of secondary alcohols can proceed faster than that of primary alcohols in the deprotection. It is assumed that the acceleration of the internal cyclization (step 1) and the hydrolysis (step 2) illustrated in Scheme 2 could contribute to enhancing the polymer digestion with the linker modification.

We executed reactions with variable concentrations of enzyme to investigate the kinetics of the digestion by AP. The linear increases of the fluorescence signals were observed from the polymer solutions, corresponding to the enzyme concentrations (Figure 2). On the other hand, we attempted to analyze the kinetics of enzymatic digestion by Lineweaver−Burk plots, and significant values, however, were not determined. According to the proposed degradation mechanism illustrated in Scheme 2 consisting of intramolecular cyclization of the linker moiety (step 1), the fluorescein releasing (step 2), and the enzyme-catalyzed hydrolysis of phosphate groups at the end of polymer chains (step 3), those respective three steps could be responsible for the digestion rates of the polymers.

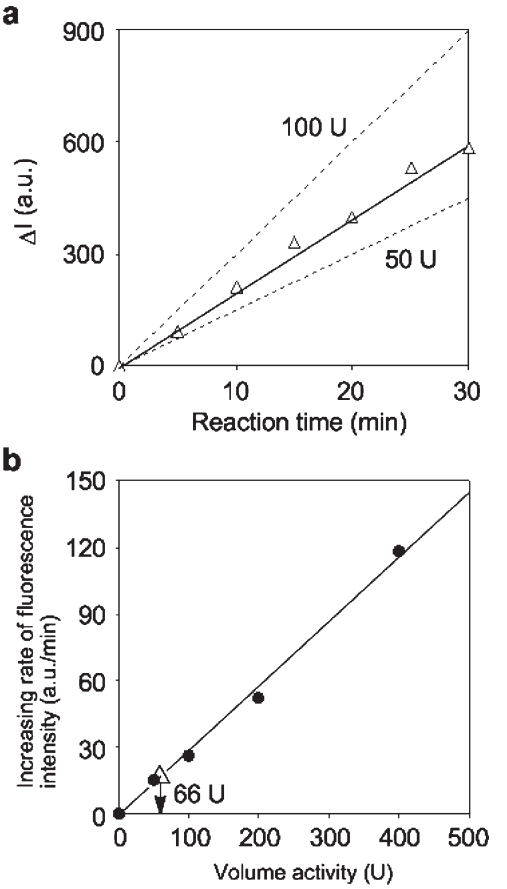


Figure 3. Determination of AP activity in the HeLa cell lysate. (a) The increase of fluorescence intensity from the reaction solution incubated at 37 °C. Detail conditions are described in the Experimental Section. Dashed lines represent theoretical values with respective enzyme concentrations. (b) Fitting of the obtained value (clear triangular dot) to the standard line according to the results of Figure 2.

To investigate the biodegrability in the presence of the miscellaneous molecules and to demonstrate feasibility of the quantitative analysis of the probes under biomimetic conditions, the

reactions were carried out with the HeLa cell lysate. Initially, the reactions were determined from the hydrolysis rate of *p*-nitrophenyl phosphate, and the AP activity was determined as 61 U/mL. With our probes, the linear increase of the magnitude of the signal intensity was observed, and the enzymatic activity of the cell lysate was evaluated to be 66 U/mL from the slope fitted to the standard line (Figure 3). These data suggest that the polymeric probes can be used for quantitative assay with biological samples.

Conclusion

In conclusion, we synthesized the biodegradable polymers consisted of the phosphate-caged fluorescein, and the digestion speeds of the polymers can be controlled by the introduction of methyl groups on the comonomers. The enzymatic reactions can be monitored by the increase of the magnitude of the fluorescent emission from the released fluorescein molecules after enzymatic digestion, and we presented that the enzymatic activity of the cell lysate can be quantified. In principle, the concentration of the polymer terminals can be maintained through the digestion. Therefore, the main-chain type caged-polymers can gradually release signal molecules, and this can be applied for drug sustained-releasing as well as for quantifying biological reactions described here. In addition, our polymers can be developed for color tuning by using other fluorescein derivatives and sensing not only pH via hydrolysis but also other kinds of enzymes such as nucleases and proteases by the modification with the linker. It is expected that our strategy should be a good platform for constructing advanced biomaterials.

Experimental Section

General. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were measured with a JEOL EX-400 (400 MHz for ^{1}H, 100 MHz for ^{13}C, and 162 MHz for ^{31}P) spectrometer. Coupling constants (J value) are reported in hertz. The chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual chloroform (δ = 7.24 in ^{1}H NMR, δ = 77.0 in ^{13}C NMR) as an internal standard. The chemical shifts are expressed in ppm downfield from 85% phosphoric acid (δ = 0.00 in ^{31}P NMR) as an external standard. Emission spectra of the samples were monitored by a Perkin-Elmer LS50B at 25 °C using 1 cm path length cell. The excitation bandwidth was 0.1 nm. The emission bandwidth was 0.1 nm. Fourier transform infrared (FTIR) spectra were recorded on a Perkin-Elmer 1600 infrared spectrometer. Gel permeation chromatography (GPC) was carried out on a UV-8020 and RI-8020 (TSK-GEL g-3000) using chloroform and water as an eluent after calibration with standard polystyrene and poly(ethylene glycol), respectively. MASS spectra were obtained on a JEOL JMS-SX102A and an Applied Biosystems Voyager Elite. Masses of the polymers were analyzed with a MALDI−TOF mass spectroscopy (acceleration voltage 21 kV, negative mode) with 2′,3′,4′-trihydroxyacetophenone (THAP) as a matrix. Calf intense alkaline phosphatase (CIAP) was purchased from Invitrogen Corporation (USA).

Fluorescein Monomer (1). To a solution of fluorescein (332 mg, 1 mmol) and tetrazole (154 mg, 2.2 mmol) in dichloromethane (100 mL) was added 2-cyanoethyl tetraisopropyldiphosphoramidite (700 μL, 2.2 mmol), and the mixture was stirred for 2 h at ambient temperature. The reaction mixture was concentrated and purified by column chromatography on silica gel, eluting with hexane−ethyl acetate (1:1) to give **1** (464 mg, 63%) as a white solid. ^{1}H NMR (CD_2Cl_2, 400 MHz), δ: 8.00 (1H, d, J = 7.3 Hz), 7.70 (2H, t, J = 7.4 Hz), 7.64 (2H, t, J = 7.4 Hz), 7.17 (1H, d, J = 7.3 Hz), 6.99 (2H, d, J = 8.0 Hz), 6.76−6.69 (4H, m), 3.92 (4H, t, J = 6.9 Hz), 3.77−3.71 (4H, m), 2.68 (4H, t, J = 6.3 Hz), 1.22 (24H, d, J = 19.7 Hz). ^{13}C NMR (CD_2Cl_2, 100 MHz), δ: 169.38, 156.64, 153.30, 152.41, 135.45, 130.13, 129.35, 126.91, 125.18, 124.22, 117.94, 116.36, 113.47, 107.69, 82.87, 59.31, 44.14, 24.64, 20.69. ^{31}P NMR (CD_2Cl_2, 162 MHz), δ: 146.19.: LRMS (NBS): [(M + H)$^+$] calcd 733, found 733.HRMS (NBS): [(M + H)$^+$] calcd 733.2920, found 733.2930.

General Procedure for the Polymerization. To a solution of **1** (146 mg, 0.2 mmol) and tetrazole (35 mg, 0.5 mmol) in acetonitrile (1 mL) were added diol compounds (0.2 mmol), and the reaction mixture was stirred at room temperature under Ar atomsphere. The polymerization was monitored with GPC, and the results are shown in Table 1 in the content. After stirring for 12 h, the reactions were quenched by the addition of the chloroform solution containing 4 mL of 0.2 M *m*-chloroperbenzoic acid (mCPBA). After stirring for 30 min, the mixtures were evaporated, and then the deprotection was executed at 30 °C in the methanolic solution containing saturated potassium carbonate. The mixture was evaporated, and the resulting yellow precipitation was dissolved in methanol and reprecipitated by the addition of chloroform. The potassium salts of the desired polymers were obtained as a white powder.

Enzymatic Reactions with Synthetic Polymers. Synthetic polymers (100 μg/mL) were incubated in 500 μL of the reaction solutions containing CIAP (2.5 U) in 50 mM sodium phosphate, 25 mM Tris−HCl, and 0.05 mM EDTA (pH = 6.0) at 37 °C. The reaction was terminated by adding 50 μL of 10 mM EDTA to the reaction mixture, and then emission spectra were measured. The digestion rates were monitored with the increase of the magnitude of the fluorescence intensity at 515 nm assigned as the emission from fluorescein.

Preparation for HeLa Cell Lysate. HeLa cells (1.0×10^6 cells) were cultured in a dish (90% confluent in ϕ100 mm dishes) and washed twice with ice-cold PBS(−). The cell lysate was then harvested with 2 mL of ice-cold CelLytic M Cell Lysis Reagent (SIGMA-Aldrich, Inc. St. Louis, MO), kept at ambient temperature for 15 min, and centrifuged at 12,000 rpm for 5 min to remove the cell debris. The enzymatic volume activity (U/mL) of the supernatant was measured from the hydrolysis rate of *p*-nitrophenyl phosphate. The solutions (100 μL) containing 1 μL of the cell lysate, 6.7 mM *p*-nitrophenyl phosphate, and 2.0 mM magnesium chloride in 50 mM sodium phosphate buffer (pH = 6.0) were incubated at 37 °C, and the absorption changes at 405 nm were monitored. The volume activity was estimated from the fitting of the standard curve with CIAP.

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Supporting Information Available: Figures showing the 1H NMR spectra of **1** and the , GPC profiles, negative mode MALDI−TOF−MS of the polymer containing ethlene glycol, and time courses of the emission changes and a table of assignments of MS values in the MALDI−TOF−MS spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) The ^{1}H NMR spectrum of the monomer is shown in Figure S1 in the Supporting Information.

(10) GPC profiles in chloroform are shown in Figure S2 in the Supporting Information.

(11) The ^{1}H spectrum of the polymer containing ethylene glycol is shown in Figure S3 in the Supporting Information.

(12) The negative mode MALDI−TOF−MS of the polymer containing ethylene glycol is shown in Figure S4, and the assignments are summarized in Table S1 in the Supporting Information.

(13) See in Figure S5 in the Supporting Information.

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