J|A|C|S ARTICLES

Published on Web 04/20/2009

Regulation of Lysozyme Activity Based on Thermotolerant Protein/Smart Polymer Complex Formation

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Abstract: Proteins have evolved to acquire highly specialized biological functions and are ideal for various applications in both medicine and biotechnology, although denaturation is one of the major problems in protein chemistry. Here, we show a novel strategy for the regulation and preservation of the enzymatic activity even after heat treatment by the complex formation with a cationic smart copolymer, poly(*N*,*N*-diethylaminoethyl methacrylate)-graft-poly(ethylene glycol) (PEAMA-*g*-PEG). PEAMA-*g*-PEG suppressed the enzymatic activity of lysozyme completely without any conformational change, indicating complex formation and the capping of the active site of lysozyme by PEAMA-*g*-PEG. The addition of an anionic polymer, poly(acrylic acid) (PAAc), recovered the inhibited enzymatic activity of the lysozyme/PEAMA-*g*-PEG complex completely. Surprisingly, even after heating the lysozyme with PEAMA-*g*-PEG for 20 min at 98 °C, the addition of PAAc recovered 80% enzymatic activity of lysozyme. Circular dichroism (CD) spectral analysis clearly indicated that the irreversible inactivation of lysozyme induced by the heat treatment was suppressed by the complex formation with PEAMA-*g*-PEG.

Introduction

Proteins regulate a variety of biochemical reactions with high specificity under physiological conditions. In general, attractive and repulsive forces based on noncovalent interactions, such as the van der Waals force, hydrogen bonds, hydrophobic effects, and electrostatic interactions, play an important role in maintaining the protein structure, thereby inducing sophisticated protein functionalities. There may be no single dominant factor determining protein stability and function; nevertheless, intermolecular electrostatic interactions have attracted special attention among the noncovalent interactions.

In nature, there are many proteins that work under high-temperature conditions, for example, the hyperthermophilic proteins that exist in hyperthermophiles, exhibit hyperthermostability, and can function even in hot springs. Indeed, the CutA1 protein from *Pyrococcus horikoshii* has an extremely high denaturation temperature (T_d), nearly 150 °C.[1] The thermostabilization mechanism of hyperthermophilic proteins has been investigated on the basis of the effect of the core hydrophobicity,[2] hydrogen bonds,[3] packing density,[4] and electrostatic interactions.[1,5,6] In the case of CutA1 proteins, the number of ion pairs in CutA1 from *T. thermophilus* (T_d = 112.7 °C at pH 7.4) was drastically lower than that from *P. horikoshii* (T_d = 148.5 °C at pH 7.4). This result suggests that the contribution of the intermolecular electrostatic interactions plays a key role in stabilizing the protein.

In this article, we present a novel technique for the regulation of enzymatic activity and the prevention of the heat-induced inactivation of enzymes using an artificial smart polymer. Our

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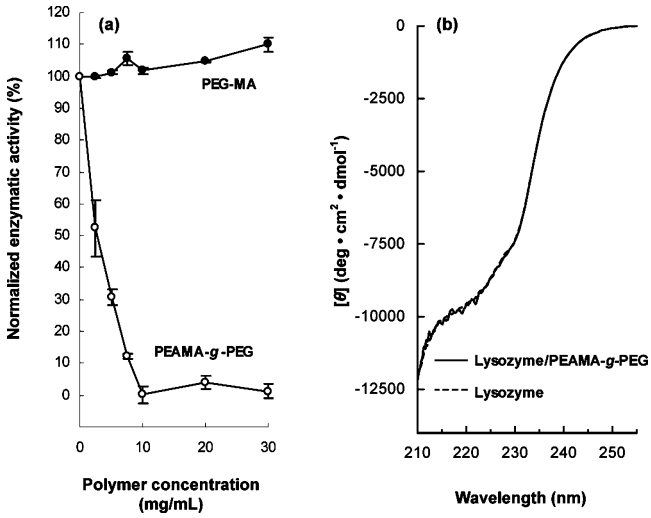

Figure 1. Changes in the normalized enzymatic activity of lysozyme in the presence of (●) PEG-MA and (○) PEAMA-*g*-PEG, as a function of the polymer concentration. (b) CD spectra of lysozyme and the lysozyme/PEAMA-*g*-PEG complex at 25 °C. The samples contained 0.5 mg/mL lysozyme and 10 mg/mL PEAMA-*g*-PEG. The enzymatic activities were measured at room temperature and pH 7.0.

strategy is based on the stabilization of the enzyme through intermolecular electrostatic interactions with a synthetic smart copolymer that has both a polyionic and a hydrophilically polynonionic segment. We anticipated that the polyionic chain would interact with the enzyme through electrostatic force, thereby efficiently increasing the stability of enzyme, while the hydrophilically polynonionic chain would interact with the enzyme through repulsive force, thereby increasing the solubility and the dispersion stability of the enzyme/polymer complex. During the experimental trials, we found that the positively charged copolymer, poly(*N*,*N*-diethylaminoethyl methacrylate)-*grafted*-poly(ethylene glycol) (PEAMA-*g*-PEG), inactivated the enzymatic activity of lysozyme almost completely. The addition of poly(acrylic acids) (PAAc) recovered the inhibited enzymatic activity of lysozyme in the lysozyme/PEAMA-*g*-PEG complex to the same level as that of native lysozyme, and, surprisingly, 80% of the enzymatic activity was also recovered when the PAAc was added to the complex even after the heat treatment at 98 °C for 20 min. The circular dichroism (CD) analysis indicates that no large conformational changes in lysozyme occurred as a result of heat treatment with PEAMA-*g*-PEG. These facts suggest that PEAMA-*g*-PEG blocked the negatively charged active site of lysozyme effectively, preventing the heat-induced denaturation of lysozyme under the present experimental conditions.

Results and Discussion

Hen egg white lysozyme was used as the model protein in this study. Figure 1a shows the enzymatic activity of lysozyme in the presence of two synthetic polymers, α-methoxy-ω-methacryloyl PEG (PEG-MA) and PEAMA-*g*-PEG. In the case of PEG-MA, a slight increase in enzymatic activity was observed in the PEG-MA concentration range of 0−30 mg/mL. It is known that the nonionic polymer PEG can interact with lysozyme,[7,8] and the data in Figure 1a indicate that the interaction between lysozyme and PEG had almost no effect on the enzymatic activity of lysozyme under the present experimental conditions.[9] It was rather surprising for us that the polycation PEAMA-*g*-PEG showed a significant effect on the enzymatic activity of lysozyme when PEAMA-*g*-PEG was mixed with the lysozyme. The enzymatic activity completely disappeared in the presence of PEAMA-*g*-PEG at concentrations above 10 mg/mL (0.12−3.9%), suggesting the strong interaction between lysozyme and PEAMA-*g*-PEG. To obtain information on the conformational change in the complexation step of lysozyme with PEAMA-*g*-PEG, a CD spectrum of the lysozyme and its polymer complex was obtained. The far-UV CD spectrum of a protein generally reflects its secondary structure.[10] As shown in Figure 1b, there was no difference in the CD spectra between lysozyme and the lysozyme/PEAMA-*g*-PEG complex, indicating no substantial changes in the secondary structure of lysozyme in the complexation step with PEAMA-*g*-PEG. According to the results of the X-ray crystallography, lysozyme is composed of 129 residues and has a positive net charge of +9 at pH 7.0 (pI = 11),[11] of which 8 are negatively charged (2 glutamic acids and 6 asparagine acids) and 17 are positively charged (6 lysines and 11 arginines). Because it is well-known that some negatively charged polymers interact with lysozyme effectively,[12,13] it is quite unexpected that this positively charged polymer inhibited the enzymatic activity of lysozyme substantially. The active site of lysozyme is located between α-domain and β-domain, which have four negatively charged residues as shown in Figure 2a,[14] where the lower amino-terminal domain (residues 40−88) consists of some helices and is mostly antiparallel β-sheet, while the second domain is made up of residues 1−39 and 89−129 and its secondary structure is largely α-helical. Because PEAMA-*g*-PEG has a positively charged segment at neutral pH, it is suggested that the electrostatic interaction between the negatively charged residues in the active site and the amine segments of PEAMA-*g*-PEG leads to the inhibition of the substrate attack into the active site of lysozyme, resulting in a decrease in lysozyme activity. It was also assumed that the PEG chain on the surface of the lysozyme/PEAMA-*g*-PEG complex prevented the contact with the substrate, resulting in a marked suppression of the lysozyme activity. These results indicate that PEAMA-*g*-PEG interacted with lysozyme effectively, resulting in the complete inactivation of its enzymatic activity, but that the structure of lysozyme was maintained in the lysozyme/PEAMA-*g*-PEG complex as shown in Figure 2b.

To evaluate the electrostatic interaction between lysozyme and PEAMA-*g*-PEG, an exchange reaction with the anionic polymer PAAc was carried out, and the variations in the enzymatic activity of the lysozyme/PEAMA-*g*-PEG complex were monitored. It is widely known that oppositely charged macromolecules strongly interact through multiple electrostatic

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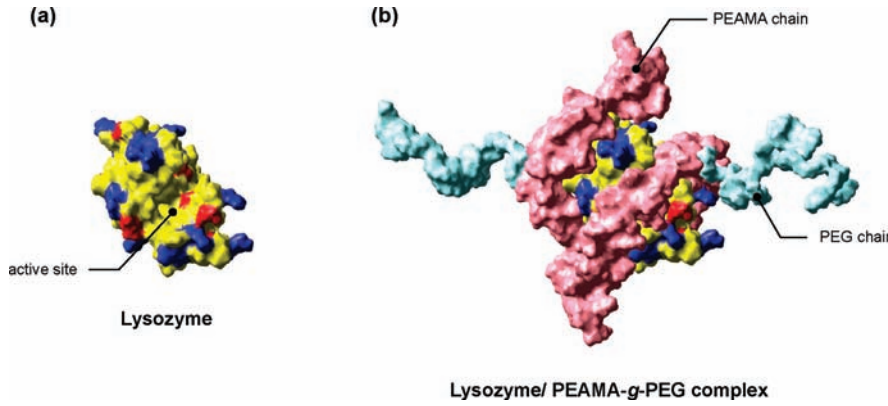

Figure 2. Molecular models of (a) lysozyme and (b) lysozyme/PEAMA-*g*-PEG complex. These models were constructed using MacroModel ver. 9.0. (a) The molecular surfaces of lysozyme with mapping of the charged areas, where negatively charged areas and positively charged areas are rendered red and blue, respectively. (b) The molecular surface of the lysozyme/PEAMA-*g*-PEG complex, which was constructed by molecular mechanics calculation using OPLS 2003 force field, where PEAMA and PEG chain are rendered pink and turquoise, respectively. The details of the molecular modeling were described in the materials and method section.

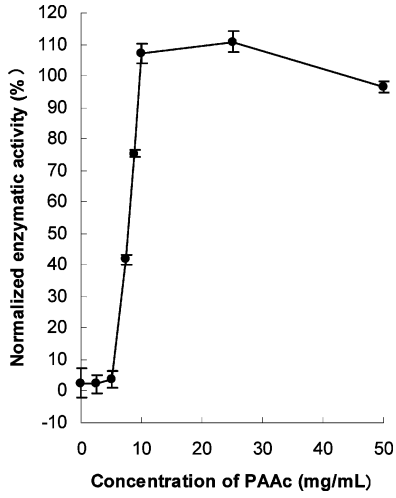

Figure 3. Recovery of the enzymatic activity of the lysozyme/PEAMA-*g*-PEG complex upon the addition of PAAc. All samples contained 0.25 mg/mL lysozyme with 25 mg/mL PEAMA-*g*-PEG, and measurements were carried out at room temperature and pH 7.0.

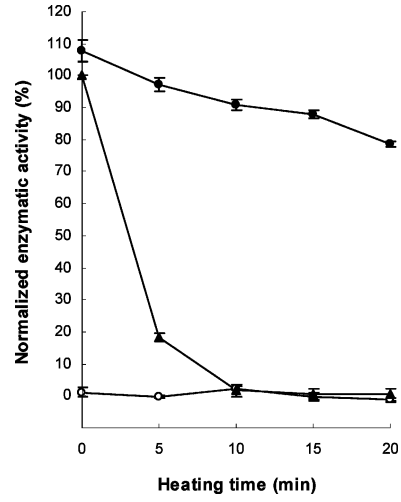

Figure 4. Changes in the enzymatic activity of lysozyme and the lysozyme/PEAMA-*g*-PEG complex as a function of the heating time. (▲) Lysozyme with heating, (○) lysozyme/PEAMA-*g*-PEG complex with heating, and (●) lysozyme/PEAMA-*g*-PEG complex with heating followed by the addition of PAAc. The concentrations of lysozyme, PEAMA-*g*-PEG, and PAAc were 0.5, 10, and 10 mg/mL, respectively. Heat treatment was carried out at 98 °C for 20 min, and then measurements were carried out at room temperature and pH 7.0.

interactions to form a polyion complex (PIC).[15−17] Figure 3 shows the recovery of the enzymatic activity of the lysozyme/PEAMA-*g*-PEG complex as a result of the addition of PAAc. It was observed that the enzymatic activity of the complex completely recovered to the level of the native lysozyme (107 ± 3.17%) upon the addition of 10 mg/mL PAAc and remained constant at almost 100% up to 50 mg/mL PAAc. These results indicate that the lysozyme was released from the lysozyme/PEAMA-*g*-PEG complex due to the PIC formation of PEAMA-*g*-PEG with PAAc. Surprisingly, during the course of our experiment, we found that the enzymatic activity of the heated-treated lysozyme/PEAMA-*g*-PEG complex also recovered when PAAc was added to the complex solution. Figure 4 shows the change in the enzymatic activities of native lysozyme and the lysozyme/PEAMA-*g*-PEG complex as a function of the heating time at 98 °C. The inactivation of the enzymatic activity through heat treatment is due to the denaturation of the enzyme;[18] the enzymatic activity of lysozyme decreased with increasing heating time, and heat treatment at 98 °C for 10 min suppressed the enzymatic activity completely. In the case of the lysozyme/PEAMA-*g*-PEG complex, the activity remained at almost zero in the heating time interval from 0 to 20 min because of the complexation of the active site in lysozyme with the graft copolymer. However, the enzymatic activity of the heat-treated lysozyme/PEAMA-*g*-PEG complex recovered after the addition of PAAc. For example, after 20 min of heating at 98 °C, lysozyme did not show any enzymatic activity, whereas the lysozyme/PEAMA-*g*-PEG showed a recovery of enzymatic activiy to 78 ± 0.86% of the level of native lysozyme upon the addition of 10 mg/mL of PAAc. It was revealed that PEAMA-*g*-PEG was very efficient in preventing the heat-induced inactivation of lysozyme, and, interestingly, PAAc significantly

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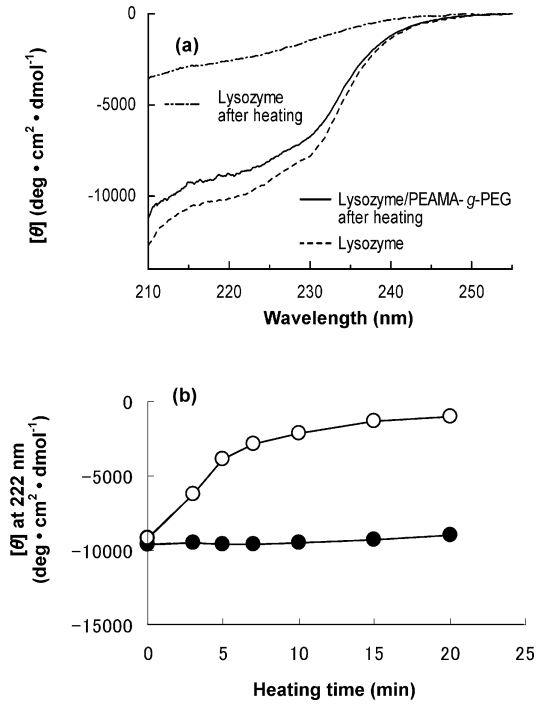

Figure 5. (a) CD spectra of lysozyme, lysozyme after heating, and the lysozyme/PEAMA-*g*-PEG complex after heating at 98 °C for 20 min. (b) Changes in the molar ellipticity at 222 nm in the CD spectra of (○) lysozyme and (●) the lysozyme/PEAMA-*g*-PEG complex as a function of the heating time at 98 °C. The samples contained 0.25 mg/mL lysozyme and 10 mg/mL PEAMA-*g*-PEG. Measurements were carried out at 25 °C and pH 7.0.

restored the enzymatic activity of heat-treated lysozyme. The electrostatic repulsive force between lysozyme and PEAMA-*g*-PEG in the complex plays an important role for the observed recovery of the lysozyme activity by PAAc.

To elucidate the conformational change of lysozyme in the lysozyme/PEAMA-*g*-PEG complex, the CD spectra of lysozyme and the lysozyme/PEAMA-*g*-PEG complex before and after heat treatment were measured. Figure 5a shows the CD spectra of lysozyme and the complexes after heating at 98 °C for 20 min. A drastic change in the CD spectrum of lysozyme was observed after heating, whereas, in the case of lysozyme/PEAMA-*g*-PEG, only slight changes in the secondary structure of lysozyme were observed after heating. The molar ellipticity at 222 nm originates from the α-helix of the protein, and lysozyme has a residue molar ellipticity of about −10000 in this region. Figure 5b shows the change in the residue molar ellipticity at 222 nm in the CD spectra as a function of the heating time at 98 °C. With increasing heating time, the molar ellipticity of lysozyme increased strongly and finally reached about −1000, suggesting the collapse of the α-helix and the heat-induced denaturation of lysozyme. It is noteworthy that the molar ellipticity of lysozyme in the lysozyme/PEAMA-*g*-PEG complex did not change at all in the heating time interval from 0 to 10 min. These results indicate that the heat-induced irreversible inactivation of lysozyme hardly occurred when lysozyme complexed with PEAMA-*g*-PEG. To the best of our knowledge, although there are several additives that prevent protein aggregation[19,20] and the regulation of enzymatic activity,[21−25] we successfully regulated the enzymatic activity and the thermotolerant states of lysozyme for the first time using the PEAMA-*g*-PEG with PAAc system.

Conclusions

The smart copolymer, PEAMA-*g*-PEG, prevented the heat-induced irreversible inactivation of lysozyme and the complete inactivation of its enzymatic activity. It was also interesting to note that PEAMA-*g*-PEG preserved the enzymatic activity of lysozyme at temperatures around the boiling point of water and that PAAc easily restored activity 80% of the lysozyme activity. Further refinement of the polyion and hydrophilic polynonion chains in this smart copolymer will lead to an improvement in the efficiency of the recovery of the lysozyme activity, and lysozyme may demonstrate its efficaciousness at high temperatures. Binding behavior between polymer and protein characterized by structure retention offers new prospects for protein stabilization and delivery. This same strategy might be extended to regulate the enzymatic activity of other enzymes or the binding affinity of proteins to polymers, DNA, or other proteins.

Experimental Section

Materials. Poly(ethylene glycol) monomethyl ether (MeO-PEG-OH, M_n = 5000 g/mol) was purchased from Fluka (Germany) and used as received. Commercial tetrahydrofuran (THF; Kanto Chemical, Tokyo, Japan) and methacrylic anhydride (Aldrich Chemical, Milwaukee, WI) were purified by conventional methods.[26] 2-(*N*,*N*-Diethylamino)ethyl methacrylate (EAMA) was obtained from Wako Pure Chemical Industries (Osaka, Japan) and was purified by distillation before use. Hen egg white lysozyme was obtained from Sigma Chemical Co. (St. Louis, MO). *Micrococcus lysodeikticus* for activity assay and PAAc (M_n = 5000 g/mol) were purchased from Wako (Osaka, Japan). Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals were of the highest analytical grade commercially available and were used as received without further purification. The water used in this study was purified using a Milli-Q system (Nihon Millipore Co., Tokyo, Japan).

Synthesis of PEG-MA Macromonomer. α-Methoxy-ω-methacryloyl poly(ethylene glycol) (PEG-MA) macromonomer was synthesized through a modification reaction of MeO-PEG-OH using methacrylic anhydride catalyzed by NaH in THF as in the following procedure: 0.25 g of NaH (10.5 mmol) purified by hexane and 5 g of MeO-PEG-OH (1 mmol) were dissolved in 50 mL of THF at room temperature. 0.23 g of methacrylic anhydride (0.22 mL, 1.5 mmol) was added to the solution under nitrogen atmosphere with vigorous stirring. Next, the mixture was allowed to react for 10 h at room temperature. Subsequently, the reactant solution was poured into a large amount of cold iso-propylalchol (IPA) to precipitate the product and finally freeze-dried with benzene to obtain as a white powder. The final product was characterized by SEC and ^{1}H NMR measurements (Figures S1 and S2). The characteristics of this polymer were summarized in Table S1.

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Synthesis of PEAMA-*g*-PEG. The synthesis of PEAMA-*g*-PEG was carried out as the following procedure: 1 g of PEG-MA (0.21 mmol) was dissolved in 30 mL of THF, and 0.16 g of AIBN (1 mmol) was added to the solution followed by nitrogen bubbling for few minutes to remove soluble oxygen. 3.68 g of EAMA (4 mL, 20.1 mmol) was added to the solution under nitrogen atmosphere and reacted for 24 h at 60 °C. Subsequently, the reactant solution was poured into a large amount of cold IPA to precipitate the product. The precipitate was then freeze-dried in benzene to obtain a white powder. To remove the unreacted PEG-MA, purification by high performance liquid chromatography (HPLC) was performed based on SEC using JASCO-BORWIN HPLC system (Japan Spectroscopic Co., Tokyo, Japan) with Shodex GPC KF-5003 column (50 mm ID × 300 mm L) and THF with stabilizer, which were used as the stationary and mobile phases, respectively. The injection samples were prepared at a concentration of 100 mg/mL to the respective mobile phase solvents. The flow rate of the mobile phase was 8 mL/min. After HPLC purification, obtained samples were dialyzed to eliminate the stabilizer. The purified graft copolymer was analyzed by SEC and ^{1}H NMR measurements (Figures S1 and S3). The characteristics of this polymer were summarized in Table S2.

Enzymatic Activity Measurements. The enzymatic activity of lysozyme was measured on the basis of bacteriolysis reaction with *M. lysodeikticus*.[27,28] Lysozyme concentration was determined by measuring the absorbance at 280 nm with an appropriate blank, using an extinction coefficient of 2.63 mL mg^{-1} cm^{-1}.[29] *M. lysodeikticus* suspension (substrate solution) was prepared by mixing 0.5 mg/mL of *M. lysodeiktictus* with 50 mM sodium phosphate buffer at pH 7.0. All of the stock solution for additives and protein was dissolved in 50 mM phosphate buffer solution (pH 7.0). A 10 μL aliquot of the sample was added to a 1490 μL of *M. lysodeikticus* solution, and the decrease in turbidity of the solution was monitored at 600 nm for 60 s using a UV−vis spectrophotometer model V-550 (Japan Spectroscopic Co., Tokyo, Japan) at room temperature. The absorbance decay plots from 10 to 20 s were fitted to a linear equation, and then the enzymatic activities were determined from the slope of the fitted line.[30]

CD Spectra Measurements. Far-ultraviolet CD spectra were monitored using a Jasco spectropolarimeter (JASCO J-720W, Jasco Ltd., Tokyo, Japan). A cuvette with 0.1 cm path length was used, and the photomultiplier voltage did not exceed 600 V in the measurements. The PEAMA-*g*-PEG before HPLC purification was used for CD measurements, because PEG-MA had almost no effect on the complex formation of PEAMA-*g*-PEG and lysozyme as shown in Figure 1. The results are expressed in terms of residue molar ellipticity.

Molecular Modeling. All molecular surface structures described in Figure 2 were constructed using integrated molecular modeling package Maestro ver.7.0 and Macromodel ver. 9.0.[31] The X-ray crystallography data of lysozyme were obtained from the Protein Data Bank (PDB entry 1UIH). The structure of lysozyme was shown as a molecular surface, where the surface areas for positively and negatively charged amino residues were rendered blue and red, respectively. Molecular modeling for constructing the lysozyme/PEAMA-*g*-PEG complex was demonstrated on the basis of the molecular mechanics calculation using OPLS 2003 force field.[32] Extended cutoff values for nonbonded interactions were used, and solvent effects were considered as an aqueous generalized Born/surface area (GB/SA) solvent model.[33] All calculations were demonstrated with frozen structure of lysozyme, because the CD spectrum of the lysozyme/PEAMA-*g*-PEG complex showed almost no change in that of lysozyme (Figure 2b). First, as an initial structure, the 10 units of PEAMA that possessed the five positively charged units were generated and placed close to lysozyme manually. Next, conformational search calculation based on Monte Carlo Multiple Minimum (MCMM) was demonstrated. The most stable structure was generated as a lysozyme/PEAMA(10 units) complex, where 10 units of PEAMA were docked into the active site of lysozyme. The 20 units of PEAMA that possessed the 10 positively charged units were linked to the PEAMA segments in the lysozyme/PEAMA(10 units) complex, and energy minimization was carried out. After three cycles of the same operating procedure, the energy-minimized structure of lysozyme/PEAMA(90 units) was generated. The stochastic molecular dynamics simulation (300 K, 1.5 fs time set up, SHAKE[34] applied to all bonds involving hydrogen atoms, and 52 ns simulation time) of PEG chain (3000 molecular weight) was preliminarily demonstrated, and two shapes of PEGs were extracted from the generated structures after 50 ns. The two extracted PEG chains were linked to the PEAMA segment in the energy-minimized structure of lysozyme/PEAMA(90 units) manually, and energy minimization was carried out. Finally, the energy-minimized structure of lysozyme/PEAMA(16k)-*g*-PEG(3k) was obtained, and its molecular surface structure was generated by Maestro ver.7.0.

Acknowledgment. This research was partially supported by a Grant-in-Aid for Scientific Research (A), No. 18200033, and a Grant-in-Aid for Scientific Research on Innovative Areas, No. 20106011, from the Ministry of Education, Science, Sports, and Culture.

Supporting Information Available: Size exclusion chromatogram and ^{1}H NMR spectrum of PEG-MA and PEAMA-*g*-PEG including their characteristics. This material is available free of charge via the Internet at http://pubs.acs.org.

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