Facile Method of Protein PEGylation by a Mono-Ion Complex
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Supporting Information

ABSTRACT: Diethylaminoethyl end-modified poly(ethylene glycol) (DEAE-PEG) has been synthesized for the noncovalent PEGylation of proteins. The resulting DEAE-PEG and catalase formed an ion complex, that is, a protein mono-ion complex (MIC). The formation of the protein MIC was confirmed by native poly(acrylamide) gel electrophoresis and gel-filtration chromatography. The resulting catalase MIC preserved the catalase activity, confirmed by monitoring the O2 concentration with a Clark-type oxygen electrode, in spite of MIC formation. The catalase activity of the protein MIC was protected in the presence of a protease, trypsin, or 10% fetal bovine serum. Furthermore, less change in the circular dichroism measurements of the catalase MIC was observed as compared to those of a catalase–PEG conjugate (covalent PEGylation), suggesting less influence of the protein conformation. Consequently, the formation of the MIC is considered to be a facile method of protein PEGylation.

INTRODUCTION

Protein PEGylation is the process of attaching one or many poly(ethylene glycol) (PEG) chains to a protein by chemical modification via covalent bonding.1 PEGylation is progressing in the field of biomaterial as well as polymer chemistry and is applied for the pharmacokinetics of the target molecule to enhance its half-life in the blood by the stealth property of PEG.2–4 The formation of covalent bonds for PEGylation is well-known.5–9 Common chemical modifications of the amino groups of proteins,10 and site-selective PEGylation to the N-terminal amino group,11 two thiol groups in the protein disulfide bridge,12 or unnatural amino acids inserted by fused protein13 have widely been reported. However, any covalent PEGylation affects the primary structure of the proteins. A few noncovalent PEGylation mechanisms have also been reported, that is, through hydrophobic interactions14,15 or coordination complex formation,16 as well as through recently discovered unique methods.17–19

Recently, we have established the concept of “mono-ion complex” (MIC) formation with plasmid DNA (pDNA) by monocationic PEG for in vivo diffusive gene delivery.20,21 The pDNA MIC is formed between a phosphate group of pDNA and only one terminal cationic group of the PEG chain. These studies have led us to prepare “protein MIC” using monocationic PEG. The resulting protein MIC is expected to be used for the facile method of protein PEGylation. In this study, we have chosen catalase (molecular weight = 230 kDa; pl = 6.0–6.2) as the protein. This article described the synthesis of a new monocationic PEG, MIC formation between a protein and the monocationic PEG, the effect of the resulting MIC formation on the protein structure and its enzymatic activity, and the stability of the MIC in the presence of protease or fetal bovine serum (FBS); detailed studies on the mechanism of MIC formation are outside the scope of the present study.

RESULTS AND DISCUSSION

MIC Formation between Diethylaminoethyl End-Modified PEG (DEAE-PEG) and Catalase. To form an MIC with the carboxyl group of a protein, we have synthesized DEAE-PEG. As shown in Figure 1, to synthesize DEAE-PEG, the carboxyl group of 3-(diethylamino)propionic acid was activated in N,N-dimethylformamide (DMF) using N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC). The active ester was then reacted with aminopropyl PEG, resulting in DEAE-PEG. The 1H NMR spectrum of DEAE-PEG showed characteristic chemical shifts derived from a

Figure 1. Synthesis scheme of DEAE-PEG.
diethylaminoethyl group (6H, terminal methyl protons) and propyl PEG (3H, terminal methoxy protons) (see Figure S1 in the Supporting Information (SI)). The yield is 46%. Thus, we succeeded in synthesizing DEAE-PEG.

To examine whether the resulting DEAE-PEG formed an MIC with catalase, as shown in Figure 2, we performed native poly(acrylamide) gel electrophoresis (native-PAGE). In the presence of DEAE-PEG, a band with a high molecular weight appeared. The intensity of the bands that appeared depended on the mixing amount of DEAE-PEG. The bands that appeared were not observed when DEAE-PEG and catalase were mixed under salt conditions (in PBS(−) and not in H2O; results not shown). From these results, although further confirmation is necessary, it was postulated that MIC formation between DEAE-PEG and catalase may occur.

Furthermore, to confirm the MIC formation of catalase with DEAE-PEG, we carried out gel-filtration chromatography (GFC) of the sample that showed the highest band intensity in Figure 2. As shown in Figure 3, although the main elution time seems to be indistinguishable, the GFC profile of catalase in the presence of DEAE-PEG was obtained earlier than that in the presence of catalase alone. Especially, earlier elution from 6.8 to 7.2 min appeared. Taking these results into account, we have succeeded in the formation of an MIC with catalase using DEAE-PEG, that is, in “protein PEGylation by MIC”.

**Catalytic Activity of the Catalase/DEAE-PEG MIC.** Despite PEGylation by the MIC, the resulting catalase should preserve its enzyme activity. Therefore, as shown in Figure 4, we measured the catalase activity of the resulting catalase/DEAE-PEG MIC. The concentration of O2, which was produced from H2O2 by the catalase reaction, was monitored in the solution of the catalase/DEAE-PEG MIC. The O2-production profile of the MIC was almost the same as that of native catalase as well as a catalase—PEG conjugate. According to this result, PEGylation by the MIC preserved the catalase activity.

To examine the stability of the resulting catalase/DEAE-PEG MIC for in vivo administration, as shown in Figure 5, we monitored the O2 production by the MIC in the presence of a protease, trypsin. O2 production of the catalase/DEAE-PEG MIC was the almost same as that of the catalase—PEG conjugate and was higher than that of native catalase in the presence of trypsin. Because the O2-production profile of the MIC was the almost same as that of native catalase in the absence of trypsin (Figure 4), the decrease in the O2 production of native catalase in the presence of trypsin is considered to be due to the digestion of catalase by trypsin. Furthermore, from native-PAGE analysis, the band of the catalase/DEAE-PEG MIC, as well as that of the catalase—PEG conjugate, was preserved in the presence of trypsin, although the band of native catalase was weakened (see Figure S2). On the basis of these results, the catalase in the catalase/DEAE-PEG MIC as well as in the catalase—PEG conjugate is considered to be protected from digestion by trypsin. In addition, in the presence of 10% FBS, the O2-production profile of the catalase/DEAE-PEG MIC was the almost same as that of the catalase—PEG conjugate and was higher than that of native catalase (Figure 6). Collectively, these results indicate that...
PEGylation by MIC is the facile method for protection of proteins from digestion by proteases.

Finally, as shown in Figure 7, we examined the effect of MIC formation on the conformation of catalase by circular dichroism (CD) measurements. The CD spectrum of the catalase/DEAE-PEG MIC was almost the same as that of native catalase (Figure 7A). Conversely, the CD spectrum of the catalase–PEG conjugate was especially disordered from 250 to 280 nm (near-ultraviolet) as compared to that of native catalase (Figure 7B). The disordered near-ultraviolet CD spectrum may be attributed to the induced interaction of aromatic side chains.22 Hence, although a recent crystal structure of a PEGylated protein demonstrates that the protein structure was not affected by the PEG,23 the covalent PEGylation is considered to affect the conformation of the protein in aqueous solution as well as the primary structure.

Furthermore, in the presence of DEAE-PEG, the CD spectrum of a typical polypeptide with carboxyl groups, poly(l-glutamic acid), showed a helix-characteristic CD band (Figure S3).24 Meanwhile, in the absence of DEAE-PEG, the CD spectrum of poly(l-glutamic acid) showed a CD band from the polypeptide chain in a random coil (Figure S3).23 The difference in the CD spectra is direct evidence of the ionic interaction between the DEAE group of DEAE-PEG and the carboxyl group of poly(l-glutamic acid). Hence, the DEAE group of DEAE-PEG is considered to interact with the carboxyl group of catalase.

■ CONCLUSIONS

DEAE-PEG has been synthesized for the noncovalent PEGylation of proteins. The resulting DEAE-PEG and catalase formed a protein MIC. The resulting catalase MIC preserved the catalase activity despite MIC formation. The catalase activity of the protein MIC was protected in the presence of a protease, trypsin, or 10% FBS. Furthermore, as compared with the catalase–PEG conjugate (covalent PEGylation), the catalase MIC is less influenced by protein conformation. Consequently, the formation of an MIC is considered to be a facile method of protein PEGylation.

■ EXPERIMENTAL SECTION

Materials. Aminopropyl PEG (weight-average molecular weight, \( M_w = 2000 \) g mol\(^{-1}\)) was purchased from NOF Corporation (Tokyo, Japan). 3-(Diethylamino)propionic acid hydrochloride was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO). All other chemicals were of special grade and were used without further purification.

Synthesis of DEAE-PEG. The typical procedure is as follows (Figure 2): 3-(Diethylamino)propionic acid hydrochloride was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO). All other chemicals were of special grade and were used without further purification.

Figure 6. Time-course of \( \text{O}_2 \) production from \( \text{H}_2\text{O}_2 \) catalyzed by the catalase/DEAE-PEG MIC (closed circle), native catalase (open square), and catalase–PEG conjugate (gray triangle) in 50 mM phosphate buffer (pH 7.4) in the presence of 10% FBS. \( \text{O}_2 \) production was compared at the same concentration of catalase (100 \( \mu \)g/mL).

Figure 7. CD spectra of native catalase (dashed line), (A) the catalase/DEAE-PEG MIC (solid line), and (B) the catalase–PEG conjugate (dotted line).

1\(^{\text{H}}\) NMR Spectroscopy. The polymer (6 mg) was dissolved in 700 \( \mu \)L of \( \text{D}_2\text{O} \) (99.8 atom % deuterium; Acros, NJ). 1\(^{\text{H}}\) NMR spectra (500 MHz) were obtained on a Bruker AV500 spectrometer (Billerica, MA).
Native-PAGE. Various amounts of DEAE-PEG (5–100 μg, 2.4–48 nmol) and catalase (5 μg, 0.02 nmol) were mixed in 18 μL of H2O. The resulting mixture, as well as free catalase (no PEG), was incubated at 37 °C for 24 h, followed by freeze-drying. Then, 18 μL of PBS(−) was added to the dried sample, mixed with loading buffer (2 μL), and loaded onto 8% polyacrylamide gel. The prepared gel was run using buffer (pH 8.3) comprising 50 mM Tris and 38 mM glycine. Electrophoresis was performed at room temperature for 1 h, and the current was kept at 20 mA. Coomassie brilliant blue was used to observe the catalase-containing bands. After washing with 7% acetic acid solution, the catalase-containing bands were visualized.

GFC. DEAE-PEG (1000 μg, 480 nmol) and catalase (50 μg, 0.2 nmol) were mixed in 180 μL of H2O. The resulting mixture was incubated at 37 °C for 24 h, followed by freeze-drying. Then, 250 μL of PBS(−) was added to the dried sample. GFC was carried out using a JASCO PU-980 pumping system (Tokyo, Japan) at a flow rate of 1.0 mL/min with a Shodex OHpak SB-804 HQ column (Showa Denko K. K., Tokyo, Japan). The aqueous solution containing PBS(−) was used as the mobile phase. The resulting samples in PBS(−) were mixed with loading buffer (2 μL) and 100 μL FBS. Then, the catalase activity was measured by monitoring current was kept at 20 mA. Coomassie brilliant blue was used to detect the catalase-containing bands by Coomassie brilliant blue. After washing with 7% acetic acid solution, the catalase-containing bands were visualized.

Measurement of Catalase Activity. DEAE-PEG (100 μg) and catalase (5 μg) were mixed in 18 μL of H2O. For the synthesis of a catalase–PEG conjugate, monomethoxy-NHS-activated ester/carbonate-PEG (100 μg) and catalase (5 μg) were mixed in 18 μL of H2O. The resulting mixture was incubated at 37 °C for 24 h, followed by freeze-drying. The resulting sample was dissolved in 900 μL of 50 mM phosphate buffer (pH 7.4), followed by mixing with 100 μL of 10 mM H2O2 in the absence or presence of 2.5 mg/mL trypsin or 10% loH2O. The resulting mixture was incubated at 37 °C for 24 h. The final concentration of catalase was adjusted to 50 μg/mL (0.2 μM). The CD spectrum of the resulting sample was measured with a Jasco J-820 spectropolarimeter (Tokyo, Japan).

CD Measurements. DEAE-PEG (100 μg) and catalase (50 μg) were mixed in 180 μL of H2O. For the synthesis of a catalase–PEG conjugate, monomethoxy-NHS-activated ester/carbonate-PEG (100 μg) and catalase (50 μg) were mixed in 18 μL of H2O. The resulting mixture was incubated at 37 °C for 24 h, followed by freeze-drying. The final concentration of catalase was adjusted to 50 μg/mL (0.2 μM). The CD spectrum of the resulting sample was measured with a Jasco J-820 spectropolarimeter (Tokyo, Japan).

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00462.
1H NMR spectrum of DEAE-PEG, native-PAGE analysis in the absence or presence of trypsin, CD spectra of poly(γ-glutamic acid) in the absence or presence of DEAE-PEG (PDF)

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS

PVIm, poly(1-vinylimidazole); PVIm-R, alkylated poly(1-vinylimidazole); PVIm-Me, methylated poly(1-vinylimidazole); PVIm-Et, ethylated poly(1-vinylimidazole); PVIm-Bu, butylated poly(1-vinylimidazole); pDNA, plasmid DNA; PVIm-R, Zn, Zn2+-chelated poly(1-vinylimidazole)

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