Bioconjugate Chemistry

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Novel Protein PEGylation Chemistry via Glutalaldehyde-Functionalized PEG

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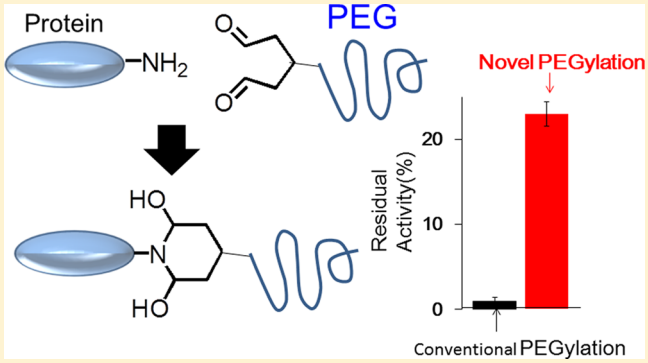
Supporting Information

ABSTRACT: Several PEGylated proteins have been approved as therapeutic drugs. In many cases, PEGylated protein has been synthesized by the conjugation reaction between PEG possessing activated ester and amine(s) in the protein. This reaction, however, often causes inactivation of PEGylated proteins. In this report, we present a novel chemistry which enables the PEGylation of proteins under the mild reaction condition. PEGylated lysozyme prepared by the method developed increased the biological activity of the PEGylated lysozyme more than 20 times compared with the PEGylated lysozyme prepared by the conventional method.



INTRODUCTION

PEGylation is recognized as a promising means of enhancing the therapeutic efficacy of proteins in clinical settings.[1] The advantages of PEGylation include increasing the size of drug molecules with consequently reduced kidney filtration, an increase in solubility, protection from enzymatic digestion, and reduction of protein immunogenicity.[2] A variety of proteins such as peptides,[3] enzymes,[4] antibodies, and antibody fragments[5] have been modified with PEG. Several PEGylated drugs have been approved by the Food and Drug Administration (FDA) and several more are being tested in clinical settings.[6]

Several factors affect the biological activity of a PEGylated protein. Increased number of the PEG on a protein often causes significant deactivation of the product. For example, 20−70% of native interferon-beta-1b (IFN-β-1b) antiviral activity was retained in mono-PEGylated IFN-β-1b, but the activity was greatly reduced or disappeared almost completely in multi-PEGylated IFN-β-1b.[7] PEGylation chemistries and reaction conditions also affect activity. Among PEGylation technologies, most common strategy is a reaction through the amide bond formation in which PEG(s) possessing activated ester react with amine(s) in a protein.[1] This reaction proceeds under mild, aqueous conditions and has been recognized as the gold standard of PEGylation reactions since last several decades. However, basicity of amine(s) in a protein significantly reduced after the amide bond formation, resulting in the significant inactivation of native proteins, especially in the case that the basicity of the amine is critical for retention of biological activity. Another approach is an alkylating PEGylation. PEG possessing aldehyde, for example, affords the stable PEGylated protein by cyanoborohydrate reduction of Shiff base.[1] This reaction, however, requires acidic condition and relatively long reaction time to reach completion, resulting in the inactivation of proteins. Accordingly, alternative methods for protein PEGylation that give rise to high biological activity under mild reaction condition are highly desired. Herein, a new PEGylation reaction exploiting glutalaldehyde as a key reactive moiety is proposed. Remarkably, the PEGylated protein produced via the developed method exhibits much higher biological activity than the PEGylated protein produced by the conventional method.

EXPERIMENTAL SECTION

Materials. Tetrahydrofuran (THF), benzene (reagent-grade; Kanto Chemical Co., Inc., Tokyo, Japan), and ethylene oxide (EO; 100%; Sumitomo Seika Chemicals Co., Ltd., Hyogo, Japan) were purified conventionally. A THF solution of potassium naphthalene was prepared by a method described in the previous paper.[8] PEG (5 kDa) possessing an activated ester (PEG-NHS) was purchased from NOF. PEG possessing two PEG chains and an activated ester (2-arm PEG-NHS) was prepared according to the previously reported method.[9]

Analysis. SEC analysis and MALDI-TOF MS analysis was carried out by TOSOH HLC-8220: TSKgel column (SuperHZ3000 and SuperHZ4000) and ultrafleXtreme (Bruker Daltonics), respectively.

Received: April 4, 2013
Revised: September 25, 2013
Published: October 17, 2013

Synthesis. *Compound 2.* Compound **1** was synthesized according to a previously reported method.[10] Compound **1** (0.50 mmol) was dissolved in dry tetrahydrofuran (THF) (30 mL). Potassium naphthalene (1.00 mmol) in dry THF was added as a base to the solution under a nitrogen atmosphere to deprotonate an initiator. The mixture was stirred for 10 min and ethylene oxide (110 mmol) that was cooled below 0 °C was then added to the solution via a cooled syringe, and the mixture was stirred for 2 d at room temperature. The solvent was removed by evaporation, and the residue was dissolved in a small amount of methanol. Samples were precipitated into cooled 2-propanol to obtain the polymer as a white precipitate. The recovered polymer was dried in vacuo and then freeze-dried from 1,4-dioxane. The yield of the obtained polymer after purification was 93%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.53 (2H, s), 3.83−3.45 (976H, m), 3.36 (4H, s), 2.76 (2H, br), 2.18 (4H, s).

Compound 3. Sodium hydride (0.60 mmol) was added to a solution of compound **2** (0.10 mmol) in dry THF (15 mL) at 0 °C under a nitrogen atmosphere, and stirred at room temperature for 30 min. Methyl iodide (8.00 mmol) was added to the solution, and the mixture was stirred for 24 h at room temperature. Next, the solution was filtered through a pad of Celite, and the resulting filtrate was evaporated. The residue was purified by the method described in the synthesis of compound **1**. The yield of the obtained polymer after purification was 85%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.54 (2H, s), 3.83−3.45 (976H, m), 3.38 (6H, s), 3.36 (4H, s), 2.18 (4H, s).

Compound 4. Compound **3** (0.01 mmol) was dissolved in a mixture of acetone (3 mL), H_2O (3 mL), and acetonitrile (3 mL). 4-Methylmorpholine *N*-oxide (0.50 mmol) and osmium oxide-immobilized catalyst I (Wako) (10 mg) were added to the solution. The mixture was stirred at room temperature for 48 h and then filtered and evaporated. The residue was purified by the method described in the synthesis of compound **1**. The yield of the obtained polymer after purification was 91%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.92−3.90 (2H, br), 3.83−3.45 (976H, m), 3.38 (6H, s), 3.35 (2H, s), 3.26−3.28 (2H, m), 3.21 (2H, s), 1.85−1.65 (4H, m).

Compound 5 (PEG-GALD). A solution of $NaIO_4$ (0.02 mmol) in H_2O/methanol (1:4, v/v) was prepared and compound **4** (0.02 mmol) was subsequently added with stirring for 1 h. Next, the polymer was extracted with CH_2Cl_2, dried in vacuo, and then freeze-dried from 1,4-dioxane to afford glutaraldehyde-functionalized PEG (PEG-GALD). The yield of the obtained polymer after purification was 49%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.78 (2H, t, J = 2.16), 3.83−3.45 (976H, m), 3.51 (4H, s), 3.38 (6H, s), 2.57 (4H, d, J = 2.16).

Reaction of PEG-GALD with 2-Aminoethanol. PEG-GALD (20 mg) was added with 2-aminoethanol (100 μL) in a phosphate buffer (50 mM, pH 7.0). Samples were precipitated into cooled 2-propanol. The recovered product was dried in vacuo and then freeze-dried from 1,4-dioxane.

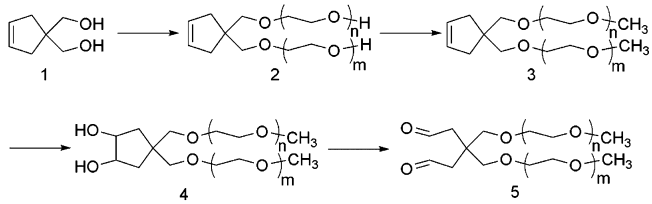
Preparation and Purification of mono-PEGylated Lysozyme Conjugate. PEG-GALD was added to a lysozyme solution in a phosphate buffer (50 mM, pH 7.0) in a molar ratio of 5:1 (PEG:lysozyme) at 4 °C and reacted for 6 h. Mono-PEGylated lysozyme was purified by cation exchange chromatography (TOSOH TSK-gel SP-5PW) and deionized using an ultrafree centrifugal filter device with a 5-kDa MWCO membrane. PEGylation reaction of lysozyme with PEG-NHS or 2-arm PEG-NHS was carried out and mono-PEGylated lysozyme was purified by the same method.

Polyacrylamide Gel Electrophoresis. Gradient acrylamide gels (8−25%) were used in all experiments. Prior to loading on the gel, samples were incubated with buffer containing 25% Tris/HCl (1 M, pH 6.8), 4% SDS, 23% glycerin, 1% phenol blue, and 2% dithiothreitol and boiled for 3 min. After electrophoresis, the gel was put in perchloric acid (0.1 M) for 15 min, and then 5% barium chloride solution and 0.1 M iodine solution were added to stain PEG band. The staining solution was washed with water and put into a Coomassie solution to stain proteins.

RESULTS AND DISCUSSION

A novel PEG derivative was designed and synthesized for use in the protein PEGylation reaction. The synthesis procedure is summarized in Scheme 1. Compound **1**, which was synthesized

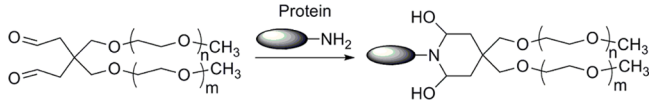
Scheme 1. Synthesis of Glutaraldehyde-Functionalized PEG (PEG-GALD)



as previously reported,[8] was used as an initiator for the polymerization of ethylene oxide to afford the PEG derivative **2** (M_n: 9.50 × 10^3 and *MWD:* 1.19 determined by SEC diagram) The hydroxyl end of the PEG molecule was capped with CH_3I (Figure S2) to protect PEG chain from undesired oxidation reactions. The osmium oxidation of compound **3** (M_n: 1.00 × 10^4 and *MWD:* 1.13 determined by SEC diagram) afforded the diol compound **4** (M_n: 9.32 × 10^3 and *MWD:* 1.14 determined by SEC diagram); subsequent oxidation by sodium periodate afforded compound **5**. From the SEC diagram, the number-average molecular weight (M_n) and the molecular weight distribution (*MWD*) were determined to be 1.03 × 10^4 and 1.23, respectively, which are consistent with the values determined from the NMR data ($M_{n(\mathrm{NMR})}$ = 1.09 × 10^4), assuming one glutalaldehyde per PEG derivative (Figure S4). Glutalaldehyde reportedly exists as a mixture of the monomer, oligomer, and higher polymeric species.[11] The successful synthesis of compound 5 (PEG-GALD) indicates that the oligomeric reactions of glutalaldehyde were inhibited because of the steric hindrance from the PEG chains. The synthesized PEG derivative possesses two PEG chains, resulting in the formation of the branched form of the conjugate after the protein PEGylation reaction. Branched forms of PEGylated proteins have been frequently explored in drug development, because the branched products possess higher biological activities and stabilities and reduce immunogenicity more effectively than their linear PEGylated counterparts.[12]

The expected reaction between PEG-GALD and a protein is shown in Scheme 2. The detailed structure of the product

Scheme 2. Reaction between PEG-GALD and a Primary Amine



dx.doi.org/10.1021/bc400169s | Bioconjugate Chem. 2013, 24, 1824−1827

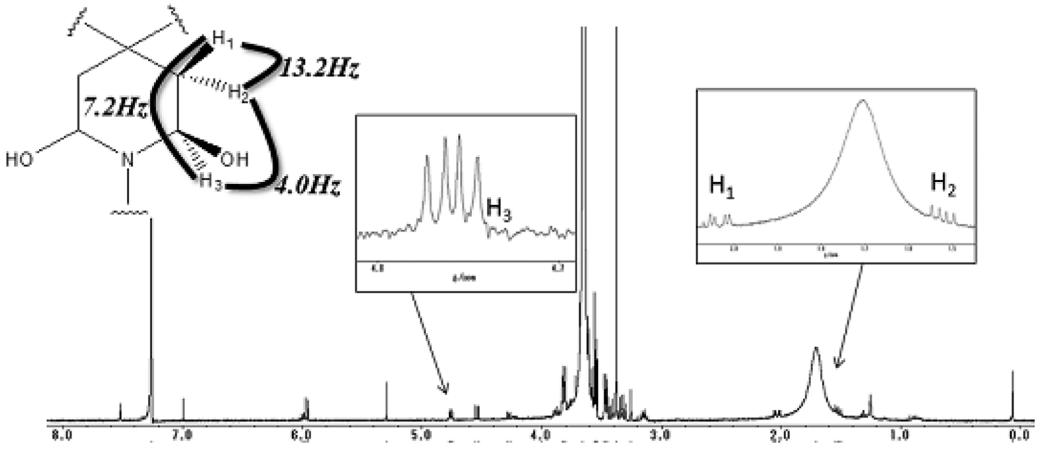


Figure 1. Detailed ^{1}H NMR analysis of the structure resulting from the reaction of PEG-GALD with 2-aminoethanol. ^{1}H NMR coupling constants indicate the six-membered ring formation.

following PEGylation was explored by analyzing the reaction product formed from PEG-GALD and 2-aminoethanol (as a model of protein) by NMR (Figure 1) and MALDI-TOF MS (Calcd.: 4567.41, Found: 4569.96 $[M+H]^{+}$). The results indicate that the conjugation reaction proceeded via the formation of a six-membered ring, as anticipated. The reaction shown in Scheme 2 is an alkylation reaction of a primary amine, indicating the retention of the basicity of the amine even after conjugation. Accordingly, this method is useful for conjugation reactions in which the basicity of the amine is critical for retention of biological activity. It should be noted that this alkylation reaction does not require the use of additional chemical reagents such as $NaBH_3CN$, which must be used in the conjugation reaction with monoaldehyde-PEG;[13] further, no small molecules are liberated during the conjugation reaction, thus simplifying the purification process.

Inactivation of the protein by PEGylation is particularly detrimental for small proteins such as cytokines, which act on large molecules. In this study, lysozyme was chosen as a model protein because of its relatively small size (14.4 kDa); the large peptidoglycan on the bacterial cell wall was selected as a substrate. PEGylation of the lysozyme was carried out in a phosphate buffer (50 mM, pH 7.0). PEG-GALD (5 kDa) was added to a lysozyme solution in a molar ratio of 5:1 (PEG:lysozyme) and the mixture was maintained at 4 °C for 6 h. The products were purified by cation exchange chromatography (Figure 2a) and subjected to SDS-PAGE analysis (Figure 2b) and MALDI-TOF MS analysis (Figure 2c). Gel was stained with barium chloride solution and iodine solution to stain PEG band and then stained with Coomassie solution to stain proteins (Figure 2b). These results indicated successful preparation and purification of the mono-PEGylated lysozyme. The yield of mono-PEGylated lysozyme was 18.2%. Resulting PEGylated lysozyme was stable under the aqueous solution for more than 2 month, indicating the six-membered structure described in the Figure 1 was stable against hydrolysis.

The activity of the obtained mono-PEGylated lysozyme was evaluated (Figure 3) and compared to the mono-PEGylated lysozyme prepared by the conventional method, in which PEG possessing an activated ester (PEG-NHS) was reacted with an amine. Conventional PEGylation resulted in the complete loss of the native lysozyme activity (Figure 3a), consistent with many previous studies.[14,15] The activity of the branched form of the PEGylated lysozyme, in which the lysozyme was reacted with PEG possessing two PEG chains and an activated ester

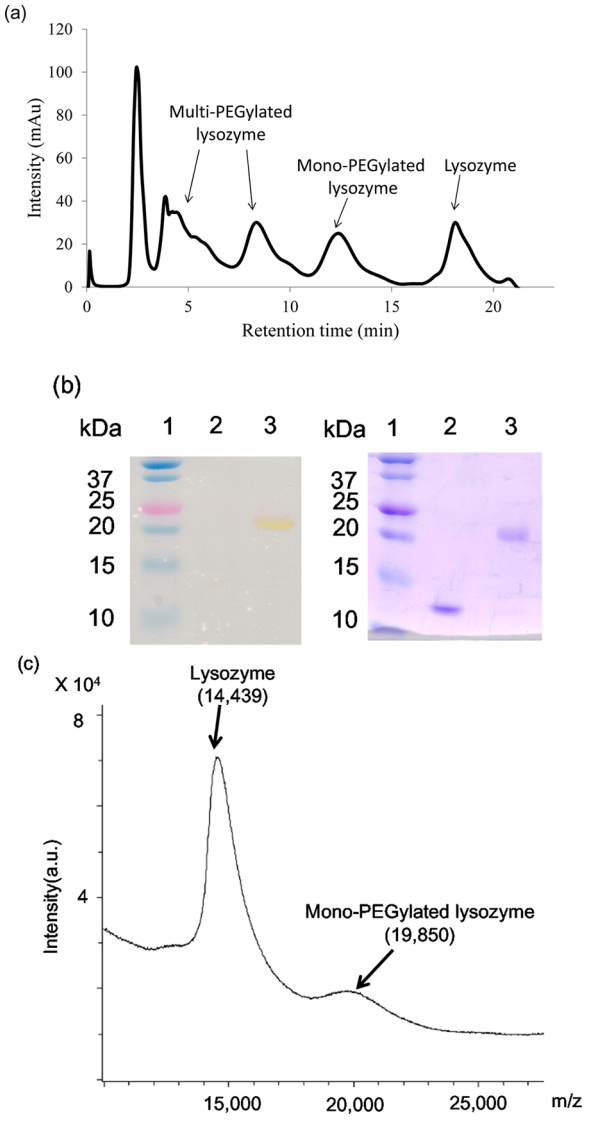


Figure 2. (a) Chromatographic profile of the reaction mixture. (b) Gels stained with barium iodide (left) and Coomassie blue (right). Lane 1: marker, lane 2: lysozyme, lane 3: purified PEGylated lysozyme. (c) MALDI-TOF MS spectra of purified mono-PEGylated lysozyme was identified as the sodium adduct. 2,5-Dihydroxybenzoic acid was used as a matrix.

dx.doi.org/10.1021/bc400169s | Bioconjugate Chem. 2013, 24, 1824−1827

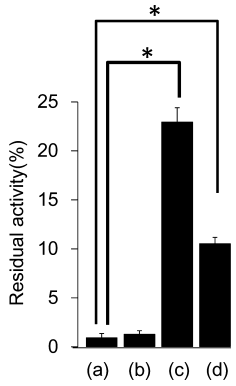


Figure 3. Residual enzymatic activities of PEGylated lysozymes. (a) mono-PEGylated lysozyme with PEG-NHS (5 kDa); (b) mono-PEGylated lysozyme with 2-arm PEG-NHS (10 kDa); (c) mono-PEGylated lysozyme with PEG-GALD (5 kDa); (d) mono-PEGylated lysozyme with PEG-GALD (10 kDa). PEGylated lysozyme compared to the original activity (*$P < 0.01$).

(2-arm PEG-NHS), was also evaluated, because in certain cases, branched PEGylated proteins have been reported to possess higher activities compared to their linear congeners.[12] In the case of the lysozyme, however, the branched PEGylated lysozyme that was prepared from PEG possessing an activated ester also underwent a large loss of the original activity (Figure 3b). In contrast, the branched PEGylated lysozyme prepared by the system proposed in this study exhibited much higher activity, because the modification of the lysozyme with 5 and 10 kDa PEG resulted in more than 20% (Figure 3c) and 10% (Figure 3d) retention of the original enzymatic activity, respectively. Detailed mechanistic analysis of the developed PEGylation technology is currently being conducted in our laboratory.

In summary, a novel PEGylation reagent was developed, which enables the PEGylation of proteins via alkylation reaction under mild reaction conditions. The PEGylated protein prepared by the method developed in this study exhibited much higher biological activity than the PEGylated protein prepared by the conventional method. This novel PEGylation technique is expected to be useful for development of the next-generation therapeutic protein drugs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra and SEC diagram of compounds are provided. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

SEC, size exclusion chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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