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Separation of mono- and di-PEGylate of exenatide and resolution of positional isomers of mono-PEGylates by preparative ion exchange chromatography

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ABSTRACT

Exenatide is a synthetic version of the 39-mer peptide of Exendin-4, which is an FDA-approved therapeutic against Type II diabetes mellitus. However, exenatide has a very short in-serum half-life and PEGylation have been performed to improve its in-serum stability. PEGylation often yields multivalent binding to non-specific residues, and the desired species should be carefully separated by chromatographies. In this study, we first devised an aqueous-phase, two-step PEGylation process. This consists of thiolation of Lys 12 and 27 residues followed by attachment of PEG-maleimide (10 kD) to thiol groups. This process yields various species: mono-PEGylates with positional isomers, di-PEGylate, and other higher MW substances. A prep-grade cationic exchange chromatography (HiTrap SP) at pH 3.0 partially separated mono- and di-PEGylates based on the molar ratio of conjugated PEG and peptide and thus molecular weight of the conjugates. To further investigate the chromatographic separation of positional isomers of mono-PEGylates, we prepared two kinds of exenatide analogs by point mutation; K12C and K27C. Each analog was mono-PEGylated with very high yield (>95%). When a mixture of the two positional isomers of mono-PEGylates was applied to HiTrap SP chromatography, K12C-PEGylate and K27C-PEGylate eluted separately at 0.22 M and 0.33 M NaCl, respectively. When the proportions of acid and its conjugate base of the amino acid residues adjacent to the PEGylation site at pH 3.0 were analyzed, K27C-PEGylate shows stronger positive charge than K12C-PEGylate, and we propose the residence time difference between the two mono-PEGylates could be due to the charge difference. ELISA result shows that the immuno-binding activity of both analogs and their mono-PEGylates are well maintained. Furthermore, both mono-PEGylates of the analogs show higher than 50-fold improved anti-trypsin stability. We expect that mono-PEGylates of the exenatide analogs are alternatives to the conventional C40-PEG.

1. Introduction

Exenatide is a synthetic version of a natural peptide, Exendin-4, which was discovered from the saliva of *Heloderma suspectum* known as Gila monster [1]. It consists of 39 amino acids and its pI value is approximately 4.7, and its water solubility is about 10 mg/ml (per Abcam's product datasheet). Exenatide is the first peptide drug mimicking glucagon-like polypeptide-1 (GLP-1) properties and was approved by the US FDA (Food Drug Administration) in 2005 for treatment of Type 2 diabetes mellitus [2]. Exenatide is known to enhance glucose-dependent insulin secretion from β-cells, shield β-cells from apoptosis, elongate β-cell proliferation, improve postprandial glycemic control, reduce gastric emptying rate, and reduce food intake in both in-vitro and in-vivo models [3–6]. However, exenatide has serious limitation of a very short in-serum half-life due to apparent kidney clearance. Therefore, patients must subcutaneously inject exenatide twice daily for the desired therapeutic effects [2,4]. A slow releasing microsphere format was developed and approved by the US FDA in 2012 for once a week injection. However, chemical modification of exenatide is still needed to further improve its stability for a more extended release format of the peptide.

PEGylation is chemical modification of a protein or peptide through covalent attachment of active polyethylene glycol (PEG) molecules. It has been widely used to enhance both the pharmacokinetic and pharmacodynamics properties of peptides/proteins [7–11]. This technique is known to improve solubility, increase apparent size to decrease renal filtration, improve in-serum sta-

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bility against proteolytic enzymes, shield antigenic epitopes, and ultimately enhance the in-serum half-life of proteins [8]. Various PEGylation strategies have been developed. These methods continue to grow to the extent that several PEGylated proteins/peptides have been on the market while others are in the clinical stages. We hope that PEGylated exenatide increase the half-life in systemic circulation and improve its pharmacokinetic and pharmacodynamics.

Mono-PEGylation, in which one PEG molecule is attached to a specific residue of a target protein/peptide, is desirable for quality homogeneity as well as from a regulatory standpoint. However, PEGylation frequently results in random and multiple attachments, which might mask or interfere with the active sites causing a dramatic decrease in bioactivity [12–14]. To solve this problem, solid-phase PEGylation employing reductive amination conjugation was attempted using an ion exchange matrix as the solid phase [15].

To recover a purified form of mono-PEGylate from a mixture of mono- and multi-PEGylates as well as unreacted PEG and proteins/peptides, several types of chromatography should be explored, including size-exclusion, reverse-phase, hydrophobic interaction, and ion exchange chromatography. Among these, ion exchange chromatography (IEC) is the most widely used, at least for initial separation [16–18]. PEGylation often results in a "charge-shielding effect", in which steric hindrance from an attached moving PEG chain could reduce the electrostatic interaction between a PEGylated proteins/peptides and a ligand [19–21]. If there is more than one PEGylation site in a peptide/r protein, positional isomers of the PEGylate can be generated. The chromatographic resolution of a positional isomer from others would then be challenging, especially in a preparative scale.

This study consists of two parts: In the first part, we report that a preparative-grade cationic exchange chromatography could partially separate the mono- and di-PEGylates of exenatide, while anionic exchanger could not resolve them. In the second part, we demonstrate that positional isomers of exenatide mono-PEGylates could be fully resolved by a preparative-grade cationic exchange chromatography, probably because of electric charge difference in the amino acid residues located in the vicinity of the PEG conjugation sites.

2. Materials and methods

Chemically synthesized exenatide (MW 4187 Da) with the following amino acid sequence, HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH_2, was purchased from CS Bio Co. (Menlo Park, CA, USA). Two types of exenatide analogs (point-mutated), K12C and K27C (MW 4162 Da), in which a lysine residue at the 12th or 27th position was replaced with cysteine, were synthesized and purchased from GL Biochem Ltd. (Shanghai, China). Lys12 and 27 were chosen for thiolation and point mutation because they contain the reactive amine group frequently used for PEGylation. PEG maleimide (PEG-MA, MW 10 kD) was purchased from NOF Corporation (Tokyo, Japan). Ellman's reagent and 2-iminothiolane used for exenatide thiolation were purchased from Pierce (Rockford, IL, USA). The buffer and SDS-PAGE chemicals were from Invitrogen Co. (Seoul, Korea), and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). Ion exchange chromatography columns (HiTrap Q and SP) were purchased from GE Healthcare (Piscataway, NJ, USA).

2.1. Thiolation of exenatide (Exn-SH)

Most of the previous work on exenatide PEGylation involved genetic addition of a cysteine residue to the C-terminus serine, followed by conjugation of a PEG-MA molecule to the thiol group in the presence of dimethylsulfoxide (DMSO). The C-terminus was chosen for the modification to maintain the bioactivity of exenatide. If the first two N-terminus residues were removed, its affinity to the GLP-1R agonist decreased 100-fold, and if the first eight residues were removed, its affinity was reduced 370-fold [22,23].

In this study, an aqueous-phase, two-step PEGylation method was used. This involved: (1) thiolation of exenatide's amine groups (Lys12 and Lys27) with 2-iminothiolane followed by; (2) PEGylation of the thiol group by PEG-MA. Both steps occur in an aqueous buffer without using an organic solvent. Exenatide was dissolved in 50 mM sodium phosphate buffer (pH 8.0) containing 10 mM EDTA to 0.24 mM (or 1 mg/ml) concentration. In the same buffer, 2-iminothiolane solution was prepared at 14 mM (2 mg/ml) concentration. The two solutions were mixed at a molar ratio of 1 (exenatide) to 10 (2-iminothiolane). After 2 h incubation at room temperature, the mixture was purified by dialysis (MWCO 2 kD) against 10 mM PBS (pH 7.0) containing 10 mM EDTA. The concentration of free thiol groups was determined by Ellman's reagent method.

2.2. PEGylation of exn-SH and exenatide analogs (K12C and K27C) with PEG-MA

Fig. 1 depicts the PEGylation schemes used in this study. For PEGylation of thiolated exenatide (Exn-SH), its solution was mixed with a PEG-MA solution (10 mM PBS buffer (pH 7.0) containing 10 mM EDTA) at a molar ratio of 1 (exenatide) to 3 (PEG-MA) at room temperature for overnight. The same PEGylation process was applied to the point-mutated analogs, i.e., K12C and K27C. PEGylates were analyzed by SDS-PAGE using a 12% Bis-Tris gel with Coomassie Blue staining. PEG was also stained to monitor the presence of PEG in PEGylates. PEG staining procedure was as follows [24]: After washing the gel with deionized water, it was placed into 20 ml of 5% barium chloride solution for 10 min. Then, it was rinsed by deionized water for 10 min and placed into 20 ml of 0.1 N titrisol iodine solution (Merck Millipore, Darmstadt, Germany) for another 10 min. Titrisol was washed off with deionized water.

2.3. Separation of PEGylates (Exn-SH-PEG, K12C-PEG, and K27C-PEG) by preparative-grade ion exchange chromatography

The PEGylates were purified by prep-grade cation and anion exchange chromatography (HiTrap SP and HiTrap Q, 1 ml column) using AKTA prime plus system (GE Healthcare, Piscataway, NJ, USA) and Biologic LP system (Bio-Rad, Hercules, CA, USA). For the SP column, 10 mM citric acid buffer at pH 3.0 (Buffer A) was used as an equilibration buffer. After sample loading, the column was washed with 5 column volumes (CV) of Buffer A. Elution was performed with 30 CV of Buffer A containing 1 M NaCl (Buffer B). For elution, the buffer composition linearly was changed from 0% to 50% or 60% of Buffer B for 30 min. For the Q column, 10 mM Tris-HCl (pH 9.5) was used for Buffer A and the same buffer containing 0.5 M NaCl was used for Buffer B. A step gradient from 0% to 70% Buffer B was applied. The flow rate was set at 1 ml/min, and all experiments were performed at room temperature. Detection was at 280 nm.

2.4. Immunobinding assay

In vitro immunoreactivity of the analogs and their mono-PEGylates to anti-exenatide IgG (from rabbit) were measured by competitive enzyme immunoassay (Catalog #EK-070-94 Exendin-4 EIA kit, Phoenix Pharmaceutical Co., Burlingame, CA, USA). Results were compared with those of native, unmodified exenatide. Based on the known concentrations of native exenatide, curve-fitting software (Origin Pro 8) capable of 4 parameter logistics was used to