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Facile and Efficient Chemoenzymatic Semisynthesis of Fc-Fusion Compounds for Half-Life Extension of Pharmaceutical Components

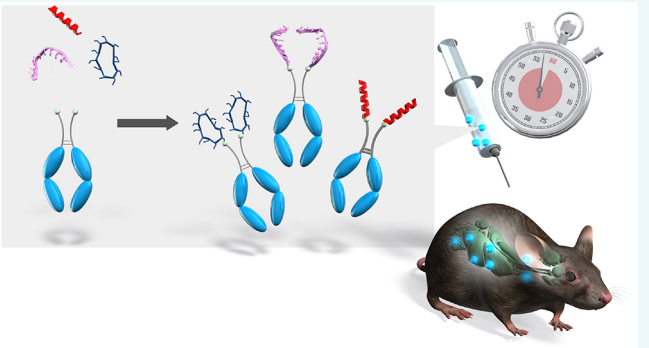
Shigeo Hirasawa,*[,†,§] Yoshiro Kitahara,[‡] Yoriko Okamatsu,[‡] Tomohiro Fujii,[‡] Akira Nakayama,[‡] Satoko Ueno,[†] Chiori Ijichi,[‡] Fumie Futaki,[‡] Kunio Nakata,[‡] and Masumi Taki*[,§]

[†]Research Institute for Bioscience Products and Fine Chemicals and [‡]Institute of Innovation, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki, Kanagawa 210-8681, Japan

[§]Department of Engineering Science, Graduate School of Informatics and Engineering, The University of Electro-Communications (UEC), 1-5-1 Chofugaoka, Chofu, Tokyo 182-8585, Japan

Supporting Information

ABSTRACT: The formation of Fc-fusions, in which biologically active molecules and the Fc fragment of antibodies are linked to each other, is one of the most efficient and successful half-life extension technologies to be developed and applied to peptide and protein pharmaceuticals thus far. Fc-fusion compounds are generally produced by recombinant methods. However, these cannot be applied to artificial middle molecules, such as peptides with non-natural amino acids, unnatural cyclic peptides, or pharmaceutical oligonucleotides. Here, we developed a simple, efficient, semisynthetic method for Fc-fusion production involving our previously developed enzymatic N-terminal extension reaction (i.e., NEXT-A reaction) and strain-promoted azide−alkyne cycloaddition, achieving quantitative conversion and high selectivity for the N-terminus of the Fc protein. An Fc-fusion compound prepared by this method showed comparable biological activity to that of the original peptide and a long-circulating plasma half-life. Thus, the proposed method is potentially applicable for the conjugation of a wide range of pharmaceutical components.



INTRODUCTION

Middle molecules have attracted substantial attention as a promising molecular modality in recent drug discovery research. These molecules, which are placed between small synthetic molecules and large proteins, are anticipated to combine the high efficacy of the former with the high specificity of the latter.[1−4] For example, peptides are efficacious and specific signaling molecules that bind to cell surface receptors.[2] These compounds have already been applied to cancer treatments, metabolic disorders, gastrointestinal diseases, and respiratory indications.[1] Of particular note are recent improvements in the molecular design of peptide pharmaceutical candidates. In addition to traditional lead optimization from a large pool of natural peptides, high-throughput screening using chemical peptides and phage-display libraries has broadened the capability of discovering promising peptide candidates. The species in peptide libraries have been varied by the introduction of non-natural amino acids, which are typically assigned by artificially expanded genetic codes.[5−7] In addition, cyclic peptides are recognized as new, promising modalities that can improve potency and specificity because they are less prone to proteolysis and have an entropy advantage in receptor binding compared with flexible linear peptides.[8]

Oligonucleotides have also attracted attention as new molecular modalities and promising pharmaceutical candidates.[3,4] Therapeutic oligonucleotides are classified into antisense oligonucleotides, aptamers, immunostimulatory oligonucleotide adjuvants, small interfering RNAs, and microRNAs depending on their mode of action.[4] Various antisense oligonucleotides have been under clinical development in hematology and oncology, and the therapeutic targets of such molecules are being extended to inflammatory, cardiovascular, and neurological diseases.[3] Oligonucleotide aptamers, which are also called "chemical antibodies", bind to specific targets and can be usually obtained from an in vitro selection process, namely, systematic evolution of ligands by exponential enrichment (i.e., SELEX).[9−11] The ease of this process makes oligonucleotide aptamers viable antibody alternatives in an increasing number of applications, including therapeutics, targeted drug delivery systems, and diagnostics.

A major obstacle to the therapeutic application of middle molecules is their short circulating plasma half-life.[12−14] To improve the half-life, three types of approach can be applied

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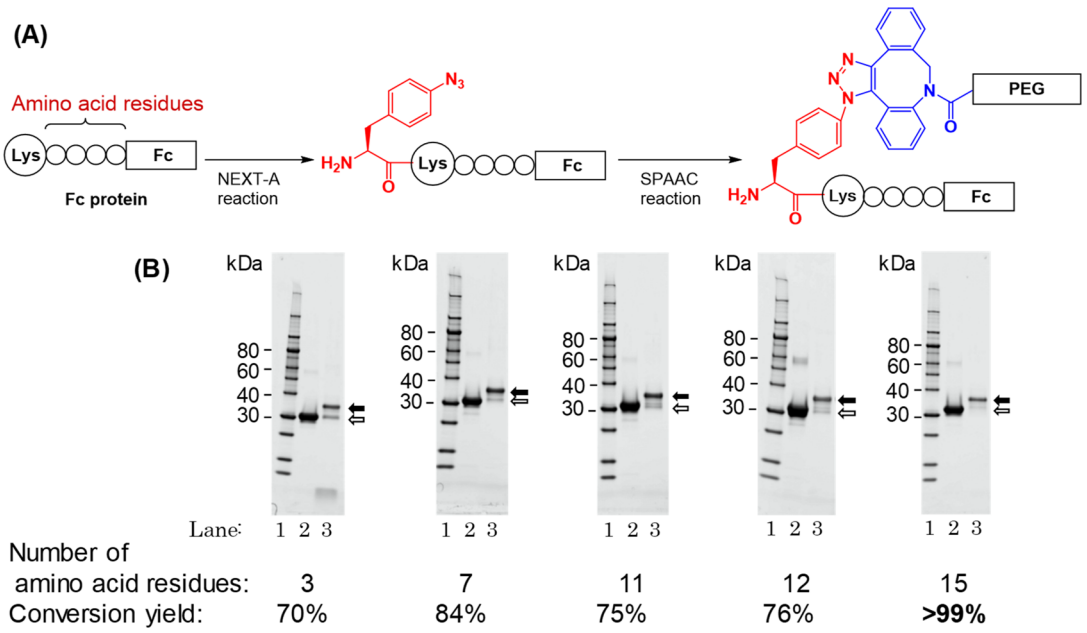

Figure 1. Optimization of the number of amino acids at the N-terminus of the Fc protein. (A) Reaction scheme for the bioconjugation of Fc proteins with different chain lengths via the N-terminal extension (NEXT-A) and strain-promoted azide−alkyne cycloaddition (SPAAC) reactions. (B) SDS-PAGE of the reaction mixtures after SPAAC. Lanes 1, 2, and 3 show the molecular-weight marker, starting Fc protein, and reaction mixture, respectively. The solid and hollow arrows indicate PEG-Fc conjugates (reaction product) and residual starting Fc protein, respectively.

independently or in combination: (1) protection from biodegradation in the bloodstream, (2) avoidance of renal clearance, and (3) recycling via neonatal Fc receptor (FcRn) binding.[15] The first approach can be achieved by improving the molecular structure. In the case of peptides, modification of the amino acid sequence and introduction of non-natural amino acids to the protease cleavage site have been successful.[16,17] In the case of oligonucleotides, degradation can be avoided by including a non-natural phosphorothioate backbone and modifying the sugar structure.[18] The second approach depends on the size of the pharmaceutical molecule; particularly, compounds with molecular weights smaller than 30 kDa usually possess a short half-life.[19] Therefore, several types of modification to increase molecular weight have been applied.[12−14] A typical approach is poly(ethylene glycol)-conjugation (i.e., PEGylation), which is a well-established method of improving the pharmacokinetic properties of peptides and proteins. Indeed, many PEGylated pharmaceuticals have been approved and clinically applied. However, the generation of anti-PEG antibodies inside patients potentially decreases their therapeutic response.[20] Thus, alternative technologies, such as conjugation with unstructured protein polymers, namely, extended recombinant polypeptides (i.e., XTEN)[21] or those with proline/alanine-rich sequences (i.e., PASylation),[22,23] are being investigated. Finally, the last approach involves the bioconjugation of middle molecules to the Fc fragment of immunoglobulins G1, G2, or G4, or albumin, which have naturally long half-lives compared with other serum proteins.[24] In addition to their large molecular size, their excellent half-life can be attributed to the recycling of these proteins through FcRn binding. Consequently, Fc- and albumin-fusions have much longer plasma half-lives than the original peptides.[15]

Conventional Fc-fused therapeutic peptides are currently produced as genetic fusion proteins. Thus, the molecular platform of an Fc-fusion, prepared by recombinant technology, is limited to conventional peptides composed of the canonical 20 amino acids. Here, to expand the application of this molecular platform, we developed an alternative method for generating Fc-fusions composed of artificial pharmaceutical components found in most emerging middle molecules. The new semisynthetic method uses our previously developed N-terminal extension (NEXT-A) reaction with leucyl/phenylalanyl-tRNA-protein transferase[25] and aminoacyl-tRNA synthetase.[26−28] Importantly, the Fc-fusions produced by our alternative platform achieved the half-life extension while retaining the biological potency of the original middle molecule component.

RESULTS AND DISCUSSION

Semisynthetic Studies of Fc-Fusion Compounds. The design of our Fc-fusion compounds is based on the conjugation of pharmaceutical components at the N-terminus of an Fc protein to maintain biological function, especially FcRn binding ability. Therefore, to introduce a reactive group for conjugation at the N-terminus, the NEXT-A reaction developed by our group was applied.

An Fc protein is a large molecule possessing a complex three-dimensional conformation. Steric hindrance often interferes with the reactivity of a functional group near a large protein, which makes the precise conjugation of the protein with a middle molecule challenging. Thus, the effect of chain length on the reactivity of the N-terminus of an Fc protein was investigated (Figure 1). Five Fc proteins with N-terminal Lys (Lys-Fc) at varying chain lengths from the Fc fragment were successfully prepared via native chemical ligation between a peptide thioester and Fc protein with N-terminal Cys.[29] During the expression of the latter, large amounts of deficit Fc protein at the N-terminal Cys or Cys-Pro were also generated; however, these impurities could be removed by cation exchange chromatography of Lys-Fc (Supporting Information). Subsequently, the reactivities of

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

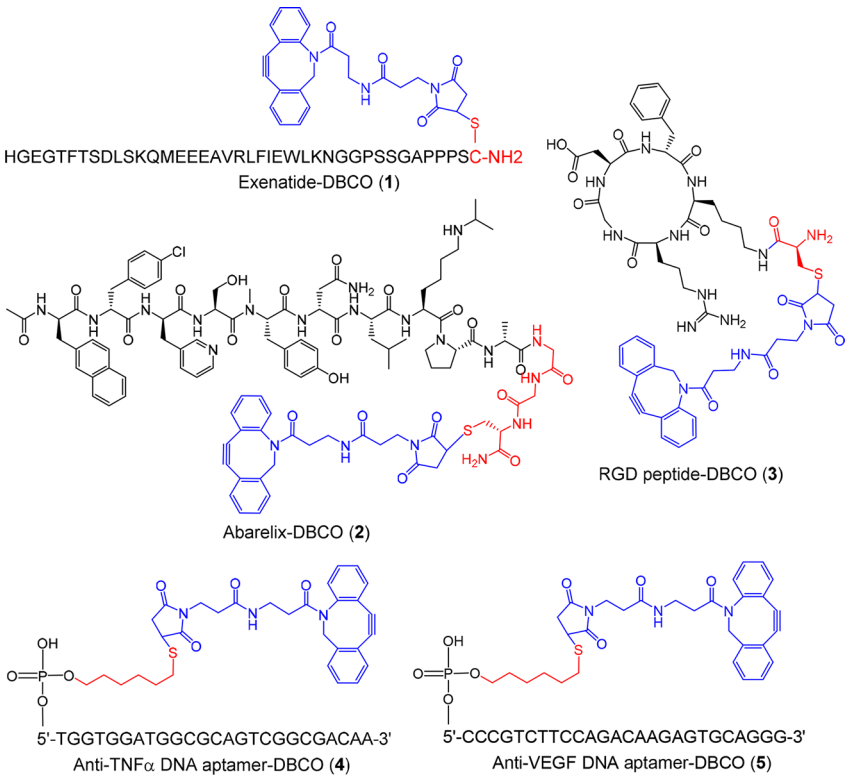

Figure 2. Structures of active pharmaceutical components to which the Fc-fusion semisynthetic method was applied. The structures of the original pharmaceutical component, linker, and conjugation component are shown in black, red, and blue, respectively (DBCO, dibenzocyclooctyne; anti-TNFα, anti-tumor necrosis factor alpha; anti-VEGF, anti-vascular endothelial growth factor).

the different Lys-Fc proteins in the sequential incorporation of (4-azido)phenylalanine at the N-terminus via the NEXT-A reaction and strain-promoted azide−alkyne cycloaddition (SPAAC) with mPEG-dibenzocyclooctyne (DBCO) were investigated. Lys-Fc proteins with a shorter chain produced moderate yields, and residual starting materials were observed. On the contrary, Lys-Fc with a chain length of 15 amino acids resulted in a quantitative conversion, that is, no unreacted Lys-Fc was observed (Figure 1). These results suggest that a chain length of 15 amino acids between the N-terminal Lys and folded Fc protein is necessary for a good contact between the reactant and the reaction site, i.e., the N-terminal Lys.

Bioconjugation via copper-catalyzed azide−alkyne cyclization and S-alkylation between an Fc protein with the appropriate reactive group and peptide were additionally examined; however, only SPAAC resulted in quantitative conversion (Supporting Information).

Using the Lys-Fc with a spacer of 15 amino acids, described above, Fc-fusions with biologically active middle molecules were prepared. As a representative peptide middle molecule, we chose Exenatide, which is a well-known glucagon-like peptide-1 (GLP-1) receptor agonist for type 2 diabetes therapy.[30] Peptide **1** (Figure 2), which exhibits the Exenatide motif, was prepared by solid-phase peptide synthesis followed by Michael addition of DBCO-maleimide. An Fc protein with an incorporated azide group (N_3−Fc) was prepared via the NEXT-A reaction. Conjugation between peptide **1** and N_3−Fc via SPAAC yielded the Exenatide-Fc fusion compound as a homodimeric product, and no residual N_3−Fc was observed (Figure 3A). Exenatide-Fc was purified by size-exclusion chromatography to 87% of the isolated yield. The detailed structure of Exenatide-Fc was unambiguously identified by

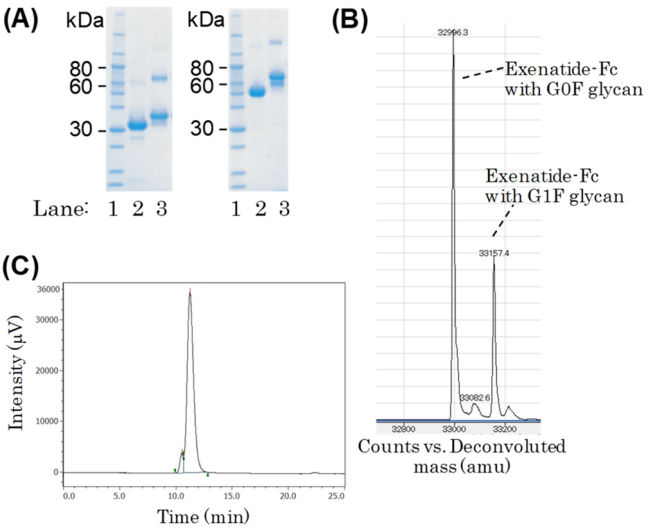

Figure 3. Detailed identification of Exenatide-Fc. (A) SDS-PAGE of the reaction mixture of strain-promoted azide−alkyne cycloaddition under reducing (left) and nonreducing (right) conditions. Lanes 1, 2, and 3 show the molecular-weight marker, N_3−Fc, and reaction mixture, respectively. (B) Deconvoluted mass spectrum of purified Exenatide-Fc under reducing condition. (C) Size-exclusion chromatogram of Exenatide-Fc at 280 nm.

mass spectrometry, including its two glycoforms, G0F and G1F glycan (Figure 3B). The sequential conversion proceeded under mild reaction conditions; therefore, the homodimeric structure of Lys-Fc was retained by Exenatide-Fc and generation of aggregates was minimal (Figure 3C). A vast excess of reagents or fragments is usually necessary to complete

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

bioconjugation with a large protein such as an Fc protein.[29,31] However, our semisynthetic method only required 2 equiv of peptide **1** to complete SPAAC (Supporting Information).

We also verified the general application of this method to a wide range of compounds containing non-natural peptides and oligonucleotides. As representative examples of non-natural peptides, we chose Abarelix,[32] a gonadotropin-releasing hormone antagonist containing various types of non-natural amino acids, and cyclic RGD peptide,[33,34] which has been investigated as an adhesive molecule against integrin $\alpha_v\beta_3$. Fc-fusions of these peptides cannot be prepared by traditional recombinant technology. However, with our method Abarelix-DBCO (**2**) and cyclic RGD peptide-DBCO (**3**) generated the corresponding Fc-fusions as the sole products (Figure 2 and Supporting Information). These Fc-fusions were purified by Protein A affinity chromatography, and the mass numbers of the products obtained by mass spectrometry agreed well with theoretical values (differences between measured and theoretical mass numbers were 0.7 and 1.2 for Abarelix-Fc and cyclic RGD peptide-Fc, respectively; for details, see Supporting Information). In protein preparation, it is quite difficult to remove impurities with properties similar to those of the product (e.g., unreacted Fc); therefore, quantitative conversion for conjugation with an Fc protein is desirable.

Next, we prepared oligonucleotide-Fc fusions. The anti-tumor necrosis factor alpha[35] and anti-vascular endothelial growth factor[36] DNA aptamers were converted to DNA aptamer-DBCOs **4** and **5**, respectively. These compounds were also similarly and easily conjugated to an Fc protein to generate the corresponding Fc-fusions (i.e., oligonucleotide-protein chimeric molecules). SDS-PAGE analysis of these products revealed increased molecular weights appropriate for the addition of the DNA aptamer (Figure 2 and Supporting Information). Moreover, the bands of the adducts were stained by both protein- (SYPRO Ruby) and DNA-detecting (SYBR Safe) reagents, suggesting the generation of DNA aptamer-Fc fusions. Conventional antibody drugs are usually discovered based on the immunological response of immunized animals; therefore, antibodies from highly toxic or nonimmunogenic antigens are difficult to obtain.[37] Because DNA or RNA aptamers can be produced in an artificial manner (i.e., SELEX),[37] the semisynthetic production of DNA aptamer-Fc fusions can create antibody-like molecules without depending on conventional immunization methods for the discovery of antibodies.

Pharmacokinetic Studies of Exenatide-Fc. The purpose of applying the Fc-fusion molecular platform is to impart the long half-life of antibodies to other pharmaceutical compounds. In addition to avoiding renal clearance through their large molecular size, Fc-fusion molecules can undergo recycling through FcRn binding. Thus, the pharmacokinetics of an Fc-fusion compound prepared by the developed semisynthetic method was evaluated using Exenatide-Fc fusion derived from the Lys-Fc directly expressed by Expi293F as a proof of concept. The pharmacokinetic properties of Exenatide and our Exenatide-Fc fusion were examined using male C57BL/6J mice. The mice were given a single dose of Exenatide or Exenatide-Fc via intraperitoneal administration, and the plasma samples were then analyzed for the presence of these compounds by ELISA. The Fc-fusion compounds prepared using the semisynthetic method are homodimers and consequently possess two pharmaceutical components. Thus, the molar concentration of Exenatide-Fc was based on the monomer to facilitate comparison with that of Exenatide. Exenatide underwent fast clearance with a calculated half-life of 0.9 h based on few data points (Figure 4). This result was

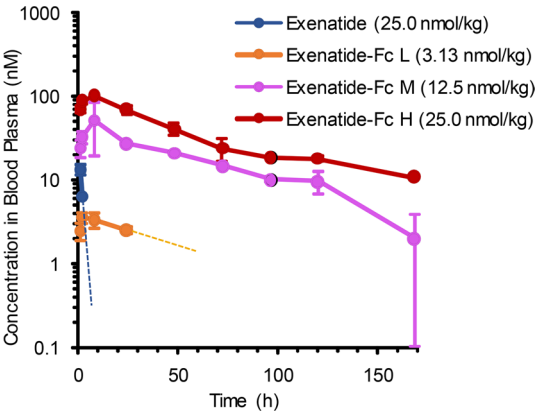

Figure 4. Plasma concentrations of Exenatide and Exenatide-Fc after intraperitoneal administration. The doses were as follows: Exenatide (25.0 nmol/kg), Exenatide-Fc L (3.13 nmol/kg), Exenatide-Fc M (12.5 nmol/kg), and Exenatide-Fc H (25.0 nmol/kg). The data are presented as mean ± standard deviation (n = 2−3 for each group), and the error bars that are not visible are smaller than the size of the marker. For Exenatide and Exenatide-Fc L, the final clearance rates are represented by dotted lines because the plasma concentrations promptly fell below the quantification limit.

consistent with previously reported terminal half-life (1.7−1.9 h).[38,39] In contrast, Exenatide-Fc displayed a significant increase in terminal circulation half-life (40.4−87.8 h, 24−52-fold vs Exenatide), which was highly comparable with those obtained using other half-life extension technologies.[38−40] Additionally, the maximum concentration and area under the curve of Exenatide-Fc evidenced a linear dose-dependent relationship. This remarkable half-life extension can be explained by the recycling mechanism involving FcRn binding, as indicated by the favorable binding of Exenatide-Fc to human FcRn observed in ligand binding analyses (Supporting Information).[41−43]

Evaluation of In Vitro and In Vivo Biological Activities of Exenatide-Fc. Peptides conjugated to a large protein such as an Fc protein often have reduced activity because the protein inhibits their binding.[44]

Thus, we confirmed the retention of the native biological activity of Exenatide after conjugation with an Fc protein. Because Exenatide is a GLP-1 agonist, the biological activity of Exenatide-Fc was evaluated using the human GLP-1 receptor expressed in *Xenopus laevis*.[45] As in the evaluation of the pharmacokinetic property, the molar concentration of Exenatide-Fc was based on the monomer. As shown in Figure 5, Exenatide and Exenatide-Fc presented similar GLP-1 activities with EC_{50} values of 1.1 and 1.5 nM (as a half-molecule), respectively, suggesting that Exenatide-Fc retained a GLP-1 binding activity comparable to that of the original peptide. In the molecular design of Lys-Fc, enzyme reactivity in the NEXT-A reaction was improved by securing the distance between the peptide attachment site and Fc. This distance is also expected to minimize inhibition of the binding of the conjugated peptide to the GLP-1 receptor.

Exenatide-Fc also displayed potent suppression of blood glucose level, comparable to that of Exenatide in vivo (Figure 6). The suppressive effect of a single administration of

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

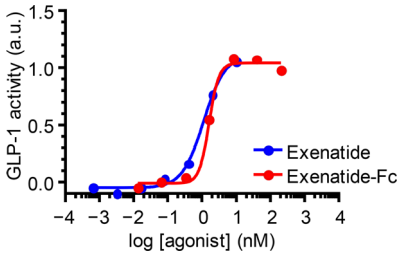

Figure 5. GLP-1 binding activities of Exenatide and its Fc-fusion in the human GLP-1 receptor expressed in *X. laevis*.

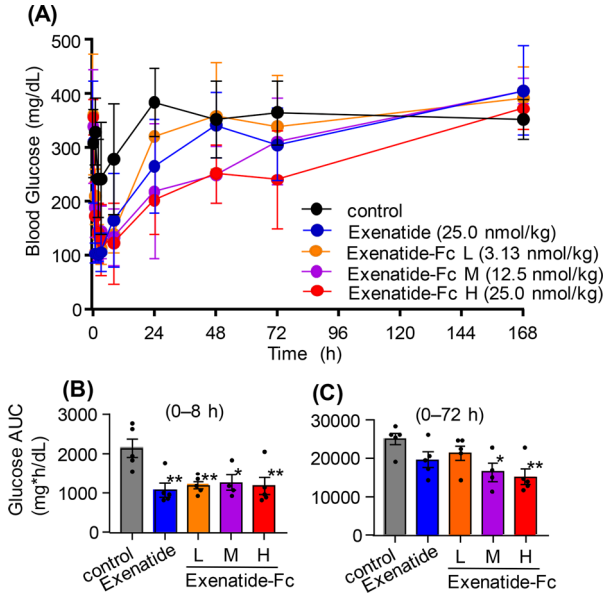

Figure 6. Pharmacodynamics of Exenatide or Exenatide-Fc in *db*/*db* mice. (A) Blood glucose concentration−time relationship after single intraperitoneal administration of Exenatide or Exenatide-Fc. Area under the curve (AUC) of blood glucose (B) from 0 to 8 h and (C) from 0 to 72 h. For all experiments, the doses were as follows: Exenatide (25.0 nmol/kg), Exenatide-Fc L (3.13 nmol/kg), Exenatide-Fc M (12.5 nmol/kg), and Exenatide-Fc H (25.0 nmol/kg). The data are presented as mean ± standard error of the mean (n = 4−5 for each group). *p < 0.05 and **p < 0.01 vs control by Dunnett's test.

Exenatide or Exenatide-Fc on model mice with type 2 diabetes, *db*/*db* mice,[46−48] was evaluated. Immediately after administration, Exenatide and Exenatide-Fc caused the same degree of suppression of blood glucose level (Figure 6B). Moreover, the result suggests that both Exenatide molecules in a single Exenatide-Fc fusion had the same pharmacological activity as Exenatide alone. Notably, the suppressive effect of Exenatide disappeared 2 days after administration, whereas that of Exenatide-Fc at the same dose was retained after 3 days (Figure 6A). Thus, the area under the curve for Exenatide from 0 to 72 h was not significantly different from that of the control group, although that for Exenatide-Fc showed a significant difference (p < 0.01 vs control by Dunnett's test, Figure 6C). This difference indicates that the half-life extension of Exenatide-Fc leads to a long-acting property.

CONCLUSIONS

We developed an efficient semisynthetic method, which combines the chemoenzymatic NEXT-A reaction with SPAAC, to produce Fc-fusion compounds. This method achieved quantitative bioconjugation between any type of pharmacological middle molecule and the N-terminus of an Fc protein. SPAAC reactivity at the N-terminal azide of the Fc protein was improved by optimizing the spacer length between the azide group (i.e., peptide attachment site) and original N-terminus of the highly folded Fc protein.

Evaluation of the biological activity of Exenatide and Exenatide-Fc revealed a comparable potency. In many cases, the biological activity of peptides decreases following conjugation to large molecules such as Fc proteins. In this study, we believe that the appropriate spacer length between the peptide attachment site at the N-terminus and complicatedly folded, large Fc protein not only improved the reactivity in the conjugation reaction, but also allowed for the biological activity of the pharmaceutical peptide to be retained. In addition, due to dimeric structure of the Fc-fusion, an avidity effect may also compensate for its biological activity.

The long half-life of antibodies compared to that of small molecules is attributed to a combination of two mechanisms: (1) avoidance of renal clearance due to large molecular size and (2) recycling via FcRn binding. From this viewpoint, the semisynthesis of Fc-fusions is an appropriate method to impart the long half-life of antibodies to artificial Fc-fusion molecules, as it the mild reaction conditions reduce the risk of impairing binding affinity to FcRn. Therefore, the Fc-fusion platform described herein can be universally applied to various kinds of pharmaceutical components.

MATERIALS AND METHODS

General Procedure. Leucyl/phenylalanyl-tRNA-protein transferase,[49,50] doubly mutated aminoacyl-tRNA synthetase,[26,51] and transfer-RNA^{Phe}[26,52] were prepared according to previously reported methods.

Mass spectra were obtained using the 1290 Infinity II LC and 6545XT AdvanceBio LC/Q-TOF systems (Agilent Technologies, Inc.) equipped with a PLRP-S column (1000A, 5 μm, 2.1 mm I.D. × 50 mm, Agilent Technologies, Inc.). Linear gradient elution was performed using 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile as the mobile phase. Protein quantification was performed by the direct A280 method based on calculated ε values using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). SDS-PAGE analysis was performed using the Mini-PROTEAN TGX precast protein gel (4−20%, Bio-Rad) with XL-Ladder Broad (APRO Life Science Institute, Inc.) as the molecular-weight marker and Biosafe Coomassie Brilliant Blue G-250 dye (Bio-Rad) to stain protein samples unless otherwise specified. Analytical size-exclusion chromatograms were obtained using the Superdex 75 10/300 GL column (10 mm I.D. × 300 mm, GE Healthcare Life Sciences). PBS was used as the eluent, and chromatograms were monitored at 280 nm using a UV detector.

Semi-Synthesis of Exenatide-Fc. *Expression of Lys-Fc.* Expi293F cells (Thermo Fisher Scientific) were grown in serum-free Expi293F expression medium (Thermo Fisher Scientific) with 8% CO_2 at 37 °C. The recombinant plasmid encoding Lys-Fc (Supporting Information) was inserted into the pcDNA 3.4 TOPO vector (Thermo Fisher Scientific) and transiently transfected into a 4 L suspension of the Expi293F cell culture. After 6 days of incubation, the cell culture supernatant was centrifuged at 6000 rpm for 25 min using the GL-10MD centrifuge (Hunan Xiangyi Laboratory Instrument Development Co., Ltd.) and then filtered. The filtered cell

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

culture supernatant was adjusted to pH 7.20−7.40 with 1 M Tris-HCl (pH 9.0) and purified by a Monofinity A Resin prepacked column (5 mL, GenScript). The purified fraction was immediately neutralized with 1 M Tris-HCl (pH 9.0), and the buffer was exchanged with HyClone PBS solution (pH 7.2, Thermo Fisher Scientific) using the HiPrep 26/10 desalting column (GE Healthcare Life Sciences) to generate 30 mL of Lys-Fc solution in PBS. The concentration of Lys-Fc, determined by the Bradford assay with BSA as the standard, was 8.80 mg/mL. Lys-Fc was analyzed by ESI-TOFMS under reducing condition. ESI-TOFMS: Calcd for $C_{1248}H_{1948}N_{320}O_{400}S_8$ 28091.2 (with G0F glycan) and $C_{1254}H_{1958}N_{320}O_{405}S_8$ 28253.4 (with G1F glycan); found, 28088.5 and 28250.5 (after deconvolution).

Preparation of N_3−Fc. The following were added to a reaction vessel (Celstar spinner flask, 25 mL): 1.07 mL of stock solution A (100 mM $MgCl_2$ and 10 mM spermidine in 500 mM HEPES buffer, pH 7.6) and 1.07 mL of stock solution B (25 mM adenosine triphosphate and 200 mM KCl in water). Subsequently, 547 μL of $tRNA^{Phe}$ solution (0.195 OD/μL), 2.40 mL of doubly mutated aminoacyl-tRNA synthetase solution (17.8 μmol/L), 1.09 mL of leucyl/phenylalanyl-tRNA-protein transferase solution (39.0 μmol/L), 1.71 mL of 25.0 mM 4-azidophenylalanine solution, and 1.50 mL of water were added. Lys-Fc (1.29 mL, 15.5 mg/mL in PBS) was then added, and the solution was mixed for 23 h at 37 °C. The reaction mixture was purified by the HiTrap rProtein A FF column (1 mL, GE Healthcare Life Sciences) using PBS for capture and 0.10 M citric acid buffer (pH 2.7) for elution, and the fraction was neutralized with 1.0 M Tris (pH 9.0). Subsequently, the buffer was exchanged with 0.10 M sodium phosphate buffer (pH 7.0) using an Amicon Ultra-4 centrifugal filter unit (10K MWCO, Merck Millipore) to generate 1.50 mL of N_3−Fc solution (12.5 mg/mL, 93% yield). N_3−Fc was analyzed by ESI-TOFMS under reducing condition. ESI-TOFMS: Calcd for $C_{1257}H_{1956}N_{324}O_{401}S_8$ 28279.4 (with G0F glycan) and $C_{1263}H_{1966}N_{324}O_{406}S_8$ 28441.6 (with G1F glycan); found, 28273.7 and 28435.7 (after deconvolution).

Preparation of Exenatide-DBCO. Exenatide-Cys-NH_2 was prepared by solid-phase peptide synthesis using the Rink Amide-ChemMatrix resin (0.263 mmol scale, Matrix Innovation, Inc.) according to the general Fmoc method[53] and cleaved from the resin using TFA/triisopropylsilane/water (95:2.5:2.5, v/v/v). The resulting solution was added to cold diethyl ether, and the precipitate was washed five times with the same solvent. The precipitate was air-dried and purified by preparative HPLC to yield a 5-TFA salt of the Exenatide-Cys-NH_2 peptide (39.5 mg, 3.1% yield).

Next, 5-TFA salt (30.2 mg, 6.21 μmol) in 0.1 M sodium phosphate buffer (pH 7.0) and 264 μL of DBCO-maleimide (40 mM in DMSO, 10.6 μmol, Tokyo Chemical Industry) were added to 3.26 mL of the Exenatide-Cys-NH_2 solution, and then mixed for 5 h at 25 °C. The buffer of the reaction mixture was exchanged with water by repeated ultrafiltration with an Amicon Ultra-4 centrifugal filter unit (3K MWCO), followed by dilution with water. The resulting solution was freeze-dried to produce Exenatide-DBCO (21.4 mg, 4.05 μmol, 65% yield). ESI-TOFMS (m/z): Calcd for $C_{212}H_{308}N_{54}O_{65}S_2$ 4714.1897; found, 2358.0981 ($[M+2H]^{2+}$), 1572.4046 ($[M+3H]^{3+}$).

Preparation of Exenatide-Fc. An N_3−Fc solution (645 μL, 12.5 mg/mL, 0.300 μmol as a half-molecule) was supplemented with 0.1 M sodium phosphate buffer (pH 7.0, 5.24 mL) and 5.0 mM Exenatide-DBCO solution (299 μL, 1.50 μmol), and then shaken overnight at ambient temperature. The reaction mixture was directly purified by passing through the HiLoad 16/600 Superdex 75 pg prepacked column (GE Healthcare Life Sciences) and concentrated using an Amicon Ultra-15 centrifugal filter unit (10K MWCO) to produce an Exenatide-Fc solution in PBS (4.0 mL, 2.06 mg/mL, 87% yield). Exenatide-Fc was analyzed by ESI-TOFMS under reducing conditions. ESI-TOFMS: Calcd for $C_{1469}H_{2264}N_{378}O_{466}S_{10}$ 32996.60 (with G0F glycoform) and $C_{1475}H_{2274}N_{378}O_{471}S_{10}$ 33158.74 (with G1F glycoform); found, 32996.3 and 33157.4 (after deconvolution).

Pharmacokinetic Studies of Exenatide and Exenatide-Fc. *Materials.* Exenatide was prepared by solid-phase peptide synthesis according to the general Fmoc method. Exenatide-Fc was prepared as described above and stocked under cryogenic conditions. Exenatide (2.50 μmol/L) and Exenatide-Fc (0.313, 1.25, and 2.50 μmol/L) solutions were prepared by dissolving and then diluting the compounds with D-PBS(−) immediately before injection.

Animals. All studies were approved by the Animal Care and Use Committee of the Pharmacokinetics and Bioanalysis Center of Shin Nippon Biomedical Laboratories, Ltd. Five-week-old male C57BL/6J mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The animals had free access to food (CRF-1, Oriental Yeast Co., Ltd.) and water and were maintained under a 12:12 h light−dark cycle, 22 ± 3 °C, and 50 ± 20% humidity. Animals whose weight differed slightly from the median value after acclimatization were selected and divided into 4 groups (corresponding to doses of 25.0 nmol/kg of Exenatide and 3.13, 12.5, and 25.0 nmol/kg of Exenatide-Fc) based on body weight. After grouping, six animals were housed per cage.

Experimental Procedure. Injection solutions of Exenatide or Exenatide-Fc (10 mL/kg) were administered to 6-week-old mice intraperitoneally (n = 3 for each material, dose, and sampling time). Blood samples (ca. 0.6 mL) were collected from the posterior aorta 1, 2, 8, 24, 48, 72, 96, 120, and 168 h after administration under anesthesia by isoflurane inhalation. Aprotinin (more than 500 KIU per 1 mL of blood) was added to each blood sample, and plasma was separated by centrifugation (4 °C, 10 000 × g, 5 min, Sorvall Legend Micro 21R microcentrifuge, Thermo Fisher Scientific) and stored in a deep freezer (below −70 °C) until analysis.

Analysis. Plasma concentrations of Exenatide or Exenatide-Fc were analyzed in duplicate using the Exendin-4 EIA kit (Catalog no. S-1310, Peninsula Laboratories International, Inc.) according to the manufacturer's protocol. Briefly, the antiserum solution in EIA buffer was dispensed to each well of the immunoplate, which was then incubated at 25 °C for 1 h. Plasma samples diluted 10-fold with PBS-Tween20 or standard solutions (50 μL each) were added to each well, and the immunoplate was incubated at 25 °C for an additional 2 h. Subsequently, biotinylated tracer solution in EIA buffer (25 μL) was added to each well, and the immunoplate was then incubated at 4 °C overnight. After washing all wells with EIA buffer (300 μL, 5 times), streptavidin−horseradish peroxidase solution (100 μL) was added, and the immunoplate was incubated at 25 °C for 1 h. After washing all wells with EIA buffer (300 μL, five times), 3,3′,5,5′-tetramethylbenzidine substrate solution (100 μL) was added, and the immunoplate was incubated at 25 °C for 7.5 min. Stop solution (2 M HCl, 100 μL) was added, and the immunoplate was read at 450 nm

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

in a Multiskan Ascent plate reader (LabSystems). Standard curves of Exenatide and Exenatide-Fc were generated by 4-parameter logistic curve fitting. The quantitative range of Exenatide was 0.1580 to 79.00 nmol/L (as concentration in plasma) and that of Exenatide-Fc was 1.580 to 158.0 nmol/L. The pharmacokinetic parameters were calculated using the Phoenix WinNonlin 7.0 software (Certara).

In Vitro Evaluation of Biological Activities of Exenatide and Exenatide-Fc. *Cell-Based Human GLP-1 Receptor/cAMP Assay.* *X. laevis* melanophore cells expressing the human GLP-1 receptor were seeded in a 96-well plate at a concentration of 0.8×10^5 cells/well and cultured in Leibovitz's L-15 culture medium (Thermo Fisher Scientific) containing 0.2% BSA for 48 h. After precultivation, the culture medium was removed. Melatonin (Sigma-Aldrich Corp.) solution (2 nM) was added, and after cultivation at 25 °C for 1 h, the absorbance of each well at 620 nm [$A620(a)$] was measured. Subsequently, Exenatide or Exenatide-Fc was added to the culture medium, and after cultivation at 25 °C for 1 h, absorbance [$A620(b)$] was measured again. GLP-1 activity was calculated using the following equation:

$$\text{GLP-1 activity (a.u.)} = [A620(b) - A620(a)]/A620(a)$$

Pharmacodynamic Studies of Exenatide and Exenatide-Fc. *Animals.* All studies were approved by the Animal Care and Use Committee of Ajinomoto Co., Inc. Five-week-old male BKS.Cg-*Dock*^{7m}+/+*Lepr*^{db}/J mice (*db*/*db* mice) were purchased from Charles River Laboratories Japan, Inc. One animal was housed per cage and maintained under a 12:12 h light−dark cycle, 22 ± 1 °C, and 55 ± 5% humidity. The animals had free access to food (CRF-1, Oriental Yeast Co., Ltd.) and water throughout the acclimatization and experimental period. After acclimatization for 1 week, body weight and blood glucose levels (via the tail vein) were measured 1 day before dosing. The animals were divided into five groups (corresponding to doses of 25.0 nmol/kg of Exenatide, 3.13, 12.5, and 25.0 nmol/kg of Exenatide-Fc, and placebo) based on equality of body weight and blood glucose level.

Experimental Procedure. Exenatide (2.50 μmol/L) and Exenatide-Fc (0.313, 1.25, and 2.50 μmol/L) solutions were prepared by dissolving and then diluting the compounds with D-PBS(−) immediately before injection. Injection solutions of Exenatide, Exenatide-Fc, or PBS (10 mL/kg) were administered to six-week-old mice intraperitoneally (n = 5 for each material and dose). Blood samples were collected via the tail vein 1, 2, 8, 24, 48, 72, and 168 h after administration, and blood glucose levels were measured using the Accu-Chek blood glucose meter (Roche Diabetes Care).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.9b00235.

Additional data include an overview of known methods for the N-terminal conjugation of proteins, detailed experimental procedures and data on the development of the semisynthetic method, supplemental pharmacokinetic parameters, surface plasmon resonance analysis with human neonatal Fc receptor, and the sequence of Lys-Fc (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: shigeo_hirasawa@ajinomoto.com.
*E-mail: taki@pc.uec.ac.jp.

ORCID

Shigeo Hirasawa: 0000-0003-3077-8887
Masumi Taki: 0000-0002-1811-8029

Author Contributions

S.H. conceived the idea of the bioconjugation approach for Fc-fusion synthesis, supervised the study, and synthesized the compounds. Y.K., Y.O., and T.F. designed the biological activity evaluation methodology and conducted the experiments. Y.K., A.N., S.U., C.I., and F.F. planned the pharmacokinetic study. S.U. supervised the pharmacokinetic study. K.N. conducted the physicochemical experiments and contributed to the discussion on the physicochemical properties of the compounds. M.T. contributed to the discussion on the establishment of the semisynthetic methodology and provided expertise and materials for the NEXT-A reaction. S.H. and M.T wrote the manuscript. All authors have read and approved the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): S.H., Y.K., Y.O., T.F., A.N., S.U., C.I., F.F., and K.N. were employed by Ajinomoto Co., Inc. at the time of this study.

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■ ABBREVIATIONS

DBCO, dibenzocyclooctyne; FcRn, neonatal Fc receptor; GLP-1, glucagon-like peptide-1; Lys-Fc, Fc protein with N-terminal Lys; NEXT-A, N-terminal extension using leucyl/phenylalanyl-tRNA-protein transferase and aminoacyl-tRNA synthetase; SPAAC, strain-promoted azide−alkyne cycloaddition

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Bioconjugate Chem. XXXX, XXX, XXX−XXX