The AAPS Journal 2007; 9 (2) Article 25 (http://www.aapsj.org).

Reproducible Production of a PEGylated Dual-Acting Peptide for Diabetes

Submitted: April 11, 2007; Accepted: May 22, 2007; Published: June 22, 2007

Irene Tom,[1] Vivian Lee,[1] Michael Dumas,[1] Melanie Madanat,[1] Jun Ouyang,[1] Joanne Severs,[1] John Andersen,[1] Joanne M. Buxton,[2] James P. Whelan,[2] and Clark Q. Pan[1]

[1]Bayer HealthCare Pharmaceuticals, Biotechnology, Berkeley, CA 94701
[2]Bayer HealthCare Pharmaceuticals, Department of Diabetes Research, West Haven, CT 06516

Abstract

A PEGylated glucagon-like peptide-1 (GLP-1) agonist and glucagon antagonist hybrid peptide was engineered as a potential treatment for type 2 diabetes. To support preclinical development of this PEGylated dual-acting peptide for diabetes (DAPD), we developed a reproducible method for PEGylation, purification, and analysis. Optimal conditions for site-specific PEGylation with 22 and 43 kDa maleimide–polyethylene glycol (maleimide-PEG) polymers were identified by evaluating pH, reaction time, and reactant molar ratio parameters. A 3-step purification process was developed and successfully implemented to purify PEGylated DAPD and remove excess uncoupled PEG and free peptide. Five lots of 43 kDa PEGylated DAPD with starting peptide amounts of 100 mg were produced with overall yields of 53% to 71%. Analytical characterization by N-terminal sequencing, amino acid analysis, matrix-assisted laser desorption/ionization mass spectrometry, and GLP-1 receptor activation assay confirmed site-specific attachment of PEG at the engineered cysteine residue, expected molecular weight, correct amino acid sequence and composition, and consistent functional activity. Purity and safety analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), analytical ion-exchange chromatography, reversed-phase high-performance liquid chromatography, and limulus amebocyte lysate test showed that the final products contained <1% free peptide, <5% uncoupled PEG, and <0.2 endotoxin units per milligram of peptide. These results demonstrate that the PEGylation and purification process we developed was consistent and effective in producing PEGylated DAPD preclinical materials at the 100 mg (peptide weight basis) or 1.2 g (drug substance weight basis) scale.

Keywords: PEGylation, chromatography, GLP-1, glucagon

Introduction

Glucagon-like peptide-1 (GLP-1) and glucagon share a common precursor, preproglucagon, which upon tissue-specific processing leads to production of GLP-1 in the intestine and glucagon in the pancreas.[1] GLP-1 and glucagon and their G-protein-coupled receptors show a high degree of homology, but each is highly selective for its respective binding partner.[2-4] GLP-1 and glucagon have opposing effects on blood glucose, making them highly attractive therapeutic targets for the treatment of type 2 diabetes. GLP-1 induces glucose-dependent insulin secretion in the pancreas without provoking hypoglycemia, while glucagon stimulates glycogenolysis and gluconeogenesis in the liver, resulting in elevation of plasma glucose.[5,6] Therapies based on the individual activities of either GLP-1 or glucagon are being pursued, including a GLP-1 receptor agonist and a glucagon receptor antagonist. A dual-acting peptide for diabetes (DAPD) was engineered to activate the GLP-1 receptor yet inhibit the glucagon receptor.[7] To prolong the duration of action, DAPD was conjugated with a single polyethylene glycol (PEG) at the introduced C-terminal cysteine.

A major hurdle for clinical development of PEGylated proteins and peptides is the establishment of a consistent manufacturing-scale process with reproducible analytical techniques to characterize the final product.[8] The ability of free cysteine-containing peptides to readily form disulfides can further complicate the production process. In this article, we describe an effective and reproducible method for production of PEG-DAPD at the 100 mg scale by peptide weight or the 1.2 g scale by drug substance weight to support its development as a potential therapeutic for type 2 diabetes. A series of analytical and functional assays were developed to support PEGylation and purification development and to characterize PEGylated DAPD used in preclinical studies.

Materials and Methods

Commercial Reagents and Chemicals

DAPD (HSQGTFTSDYARYLDARRAREFIKWLVRGRC) and Cys-deleted DAPD (HSQGTFTSDYARYLDARRAREFIKWLVRGR) were synthesized by SigmaGenosys (The Woodlands, TX). Peptides were characterized by high-performance liquid chromatography (HPLC) and mass spectrometry and were >90% pure (data not shown). Methoxy polyethylene glycol (mPEG)-maleimide reagents, either a linear 22 kDa (mPEG-MAL) or a branched 43 kDa (mPEG2-MAL), were supplied by Nektar Corporation (Huntsville, AL). Chromatographic matrixes evaluated included EM FractoPrep, supplied by EM Industries (Gibbstown, NJ); CM Sepharose fast flow (FF), SP Sepharose FF, HiLoad

Corresponding Author: James P. Whelan, 400 Morgan Lane, West Haven, CT 06516. Tel: (203) 812-2131; Fax: (203) 812-6113; E-mail: james.whelan@bayer.com

16/60 Superdex 30 prep grade (pg), and HiPrep Sephadex G-25, supplied by GE Healthcare (Piscataway, NJ); and Toyopearl matrixes (Ether 650M and Phenyl 650M), supplied by TOSOH Bioscience (Montgomeryville, PA). All other chemicals used were multicompendial grade manufactured under current good manufacturing practices (cGMPs), supplied by Mallinckrodt Baker (Philipsburg, NJ). Pyrotell and Control Standard Endotoxin were supplied by Associates of Cape Cod (Falmouth, MA). Limulus amebocyte lysate (LAL) reagent water was supplied by BioWhittaker (Walkersville, MD).

Optimization of the PEGylation Reaction

DAPD at 0.5 mM concentration (peptide dry weight) was incubated with varying amounts of 22 kDa mPEG-MAL or 43 kDa mPEG2-MAL in a buffer containing 0.1 M phosphate, 0.15 M NaCl, and 0.1 M EDTA over a pH range from 5.8 to 8.0 and at ambient temperature for 0 to 4 hours. PEGylation reactions were quenched with 2 molar excess of dithiothreitol (DTT) relative to the PEG concentration. The extent of the PEGylation reaction is inversely proportional to the amount of free peptide and was a key factor for choosing the appropriate PEGylation conditions. PEGylation efficiency was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE 4% to 12% Bis-Tris gels from Invitrogen (Carlsbad, CA), performed under reducing conditions in 2-morpholinoethanesulfonic acid (MES) buffer and followed by staining with GelCode from Pierce (Rockford, IL) or a barium-iodine solution.[9] DAPD and PEGylated DAPD concentrations were determined either using amino acid analysis or by measuring the UV absorbance at 280 nm using a DAPD extinction coefficient of 2.19 mL/mg/cm (peptide mass = 3760 kDa).

Cation Exchange Chromatography

A PEGylated peptide-containing reaction mixture was applied to either a 4.5 mL (1.1 × 4.7 cm) SP Sepharose column or a 2.2 mL (1.1 × 2.3 cm) CM Sepharose column previously equilibrated with 10 mM phosphate, pH 6.5. The loading was 1.17 A_{280} peptide per milliliter of gel (or 1.17 AU/mL) and 1.6 A_{280} peptide per milliliter of gel (or 1.6 AU/mL) for the SP Sepharose and CM Sepharose column, respectively. All peptides were found to bind, and they were eluted in a ~200 to 350 mM concentration of NaCl when a salt gradient from 0 to 500 mM was applied (at pH 6.5) over 20 column volumes (CV). The PEGylation reaction mixture was also applied to 1 mL (1.1 × 1.1 cm) of either the Fracto-Prep SO,[3] the SP Sepharose, or the CM Sepharose column at pH 4.5 (25 mM Tris, 63 mM acetic acid) until the breakthrough peak started to emerge. Bound peptides were eluted in a pH gradient to 9.5 (50 mM Tris, 3 mM acetic acid), and recovery yields were determined by A_{280} measurement.

Hydrophobic Interaction Chromatography

The solubility of PEGylated DAPD solution is low in the presence of high salt. The highest concentration of ammonium sulfate allowed to completely dissolve PEG-modified DAPD was determined to be 0.75 M. Ammonium sulfate was added to a PEGylated peptide-containing reaction mixture to 0.75 M and loaded, at 1.7 A_{280} peptide per milliliter of gel, onto a 2 mL (1.1 × 2 cm) Toyopearl Phenyl column equilibrated with 0.75 M ammonium sulfate, pH 5.0, and the binding was only 1 A_{280} peptide per milliliter of gel. For elution, the ammonium sulfate concentration was decreased in a linear gradient to 0 M, and bound peptides were eluted in 0.6 to 0.2 M ammonium sulfate. When the loading ammonium sulfate concentration was reduced to 0.5 M, the reaction mixture bound to neither Toyopearl Phenyl 650M nor Toyopearl Ether 650M.

Size-Based Separation

A pool of PEG-modified DAPD from the SP and CM Sepharose columns was applied to 120 mL (1.6 × 60 cm) Superdex 30 pg, 53 mL (2.6 × 10 cm) HiPrep Sephadex G-25, or a stirred cell using a YM10 membrane. The load volume was either 17% or 8% CV for the Superdex 30 pg and 9% CV for the Sephadex G-25 column. The linear flow rate was 28 to 30 cm/hr.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed on an Applied Biosystems Voyager RP instrument (Foster City, CA) in positive, linear mode. A calibration mixture, composed of bovine insulin (5733 Da), thioredoxin (11 673 Da), and apomyoglobin (16 951 Da) (Applied Biosystems), was used to calibrate the *m/z* range. Samples were mixed with the matrix, composed of saturated sinapinic acid (Sigma Aldrich, St Louis, MO) in 70% trifluoroacetic acid (TFA)/30% acetonitrile, then applied to the MALDI target plate and allowed to crystallize. A laser intensity of 2000 V was applied to the target spots, and an accelerating voltage of 25 kV was applied to the ions. Spectra provided were an accumulation of 80 laser shots.

N-Terminal Sequencing

N-terminal sequencing was conducted on an Applied Biosystems (Foster City, CA) Procise 494 sequencer using the standard operation procedure recommended by the manufacturer. Relevant sequencing chemicals and reagents were obtained from Applied Biosystems. Using a calculated mass of 3750 for 43 kDa PEG-DAPD (based on peptide weight) and DAPD

with a concentration of ~0.8 mg/mL, each sample was diluted 1:10 into 0.1% TFA (5 μL + 45 μL diluent). Exactly 9.3 μL (200 pmol) of sample was then transferred onto a Biobrene (Applied Biosystems, Foster City, CA) treated glass fiber filter. The sample cartridge was then assembled and placed onto the instrument. The sequencer was programmed to collect 33 cycles of amino acids for each sample analyzed. The repetitive yield of the β-lactoglobulin control was 96.5%, meeting the instrument specifications of ≥94%. Data analysis was conducted using the Model 610A, version 2.1, software package provided by the manufacturer.

Amino Acid Analysis

Glass hydrolysis tubes were obtained from Waters Corporation (Milford, MA). Hydrochloric acid, 6N sequanal grade and the protein standard bovine serum albumin (BSA) at a concentration of 2.0 mg/mL were purchased from Pierce (Rockford, IL). Amino acid calibration standards, internal standards, and the derivatizing reagents o-phthalaldehyde (OPA) and 9-fluorenylmethyl-chloroformate (Fmoc) were purchased from Agilent Technologies (Wilmington, DE). Hydrolysis of the polypeptide bonds was conducted using 6 N HCl at 110°C for 22 to 24 hours under vacuum. The free amino acids were reacted with OPA (in the presence of 3-mercaptopropionic acid) for primary amino acids and Fmoc for secondary amino acids. The derivatives were separated by reversed-phase HPLC (Agilent 1090 HPLC system) and quantified by comparison to amino acid calibration standards. Internal standards were included in all analyses.

Analytical Ion-Exchange Chromatography

Analytical ion exchange was performed using the ÄKTAexplorer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) system. The sample was applied to a Mono S HR 10/10 column (GE Healthcare), equilibrated with 10 mM phosphate, 20 mM NaCl, pH 6.5, at a linear velocity of 153 cm/hr (2 cc/min). Following loading, the column was washed with 5 CV of the equilibration buffer. After washing, the salt concentration was increased in a linear gradient (from 2% to 100%) to 1 M over 20 CV for elution. The amount of residual unreacted mPEG2-MAL in purified PEGylated DAPD was estimated by comparing the mPEG2-MAL peak area (in the flow-through fraction) to peak areas determined for an mPEG2-MAL standard curve generated by the same method.

Reversed-Phase HPLC

Reversed-phase HPLC was used to estimate the amount of free DAPD in the PEG-DAPD sample. PEG-DAPD was run on a C18 HPLC (Beckman System Gold), using a 2 mL loop and flow rate of 1 mL/min with the following program: 5 minutes of Buffer A (0.1% TFA/H_20) followed by 30 minutes of gradient to 100% Buffer B (0.1% TFA/acetonitrile [ACN]).

LAL Test

LAL is an aqueous extract of blood cells (amebocytes) from the horseshoe crab, *Limulus polyphemus*. A series of 2-fold dilutions was made for PEGylated DAPD. The LAL test was performed by adding 0.1 mL Pyrotell, containing an aqueous extract of amebocyte of *L polyphemus,* to 0.1 mL of the test sample PEGylated DAPD in a 10 × 75 mm depyrogenated, flint (soda lime) glass reaction tube. The reaction solution was mixed thoroughly and placed immediately in a noncirculating water bath at 37°C ± 1°C for 60 ± 2 minutes. At the end of the incubation, the tube was removed from the water bath and inverted. If a gel formed and remained intact in the bottom of the tube after inversion of 180°, the test was positive. The endotoxin concentration of the sample was determined by using a standard curve of control standard endotoxin (*Escherichia coli*).

Measurement of Peptide Signaling Through GLP-1 Receptor

Rat insulinoma (RINm5F) cells were maintained in RPMI 1640 medium containing 5% fetal bovine serum (JRH Biosciences, a division of Sigma-Aldrich Corp., St Louis, MO) and 1% antibiotic-antimycotic solution (Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Seeded in 96 well plates and grown overnight were 1.5×10^5 RINm5F cells per well. The cells were washed twice with phosphate-buffered saline (PBS). The cells were incubated with peptide in HEPES-PBS containing 1% BSA and 100 μM 3-isobutylmethylxanthine (IBMX) for 15 minutes at 37°C. The cells were lysed, and intracellular cAMP was determined using the cAMP Scintillation Proximity Assay direct screening assay system (GE Healthcare).

Results and Discussion

Optimization of the PEGylation Reaction

In an earlier publication, we described the engineering of a long-acting dual GLP-1 agonist and glucagon antagonist peptide as a potential therapeutic for type 2 diabetes.[7] The C-terminus of this DAPD was determined to be the optimal position to introduce a cysteine residue to enable site-specific attachment of long-chained PEG. To scale up production of PEGylated DAPD for clinical studies, we first optimized PEGylation conditions with the 22 kDa mPEG-MAL. The pH optimization experiment performed at ambient temperature for 30 minutes indicated that the PEGylation reaction was highly efficient for pHs ranging from 5.8 to 8, as confirmed by the disappearance of the free peptide band

at 4 kDa and the appearance of the PEGylated band at ~40 kDa (Figure 1A). The 7 kDa band corresponds to the oxidized dimer peptide because when the same samples were run on reducing gels, DAPD migrated as a single band at 4 kDa and the dimer band disappeared (data not shown). Mass spectrometry revealed that the dimer had a mass of 7514 Da (data not shown), confirming the disulfide band formation. To maintain the equilibrium between disulfide formation and dissociation, the dimer converted to the monomer peptide as more of the PEG-peptide conjugate formed over time. The apparent molecular weight of PEG-DAPD on the gel is much greater than the theoretical molecular weight because of heavy hydration of the PEG moiety. To minimize potential side reactions with amines at higher pH, pH 6.0 was chosen for the next set of optimization studies.

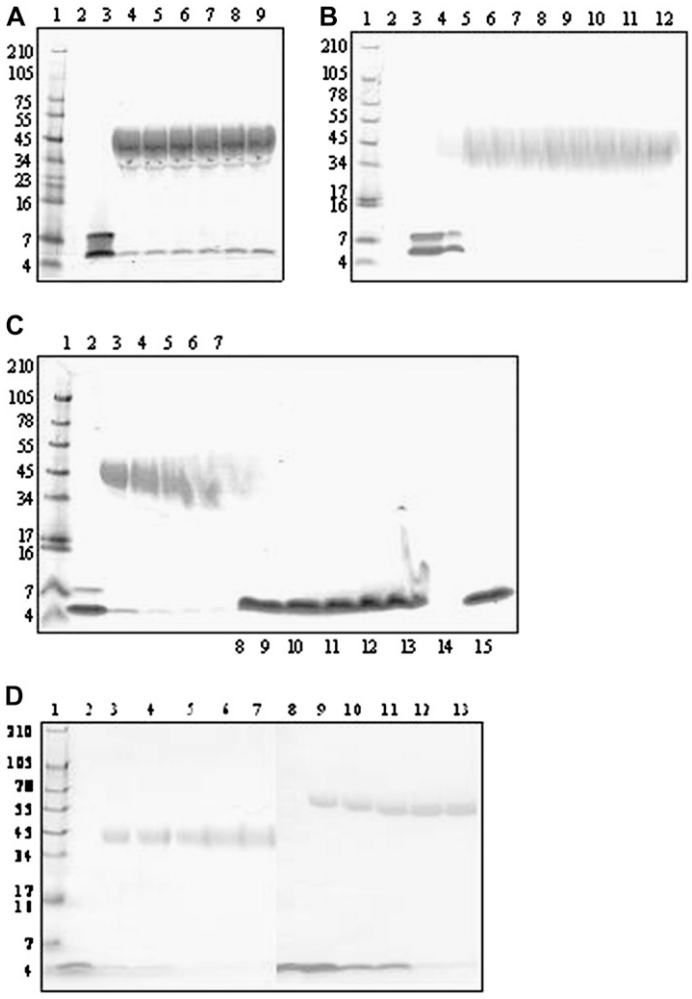

Figure 1. PEGylation condition optimization. DAPD at 0.5 mM was reacted with 1 mM 22 kDa mPEG-MAL at pH 5.8, 6.0, 6.6, 7.0, 7.6, or 8.0 (lanes 4-9 of A) for 30 minutes or at pH 6.0 for 0.2, 5, 10, 20, 40, 60, 120, 180, or 240 minutes (lanes 4-12 of B) at room temperature. DAPD and mPEG-MAL were loaded in lanes 2 and 3, respectively. (C) DAPD (lanes 3-7) or Cys-deleted DAPD (lanes 9-13) at 0.5 mM was reacted with 1, 2.5, 5, 10, or 25 mM 22 kDa mPEG-MAL at pH 6.0 and room temperature for 30 minutes. DAPD was loaded in lane 2 and Cys-deleted DAPD in lanes 8 and 15. Molecular weight marker was loaded in lane 1. (D) DAPD (lanes 2 and 8) at 0.5 mM was reacted with 0.15, 0.3, 0.6, 0.7, and 0.8 mM 22 kDa mPEG-MAL (lanes 3-7) or 43 kDa mPEG-MAL (lanes 9-13) at pH 6.0 and room temperature for 30 minutes. DAPD indicates dual-acting peptide for diabetes; mPEG, methoxy polyethylene glycol; MAL, maleimide.

To optimize reaction time, the PEGylation reaction was performed at pH 6.0 from 0.2 minutes to 4 hours. The result showed that the reaction went to completion in 5 minutes or less (Figure 1B). A reaction time of 30 minutes was chosen for the next set of optimization studies to ensure a complete site-directed PEGylation reaction without the formation of any unwanted side reactions.

To determine the optimal PEG-to-peptide ratio for the PEGylation reaction, 22 kDa mPEG-MAL with a concentration ranging from 2- to 50-fold molar excess over the concentration of DAPD was reacted with DAPD at pH 6.0 for 30 minutes. The concentration of peptide was kept at 0.5 mM to minimize dimerization and maintain peptide solubility. The minimum molar PEG-to-peptide ratio to achieve maximum PEGylation was determined to be ~1:1 for both 22 kDa and 43 kDa PEG reactions (Figures 1C and 1D). No reaction was detected with the Cys-deleted DAPD, confirming the high specificity of PEGylation at the C-terminal cysteine of DAPD.

The optimized reaction parameters described above were further adjusted specifically for DAPD modification with the 43 kDa mPEG2-MAL. DAPD was not always completely soluble when resuspended to 1.88 mg/mL (or 0.5 mM) in a reaction buffer consisting of 100 mM phosphate, 150 mM NaCl, and 100 mM EDTA, pH 6.0. When the phosphate buffer concentration was reduced from 100 mM to 10 mM, the solubility of the peptide improved. EDTA was initially added to prevent the reoxidation of disulfides that might form intermolecularly through the cysteine located at the C-terminus of DAPD. Nonetheless, following the PEGylation of DAPD, only PEGylated peptide was observed—not PEGylated dimer species (see below). Thus, EDTA was unnecessary for the reaction. Salt was initially included to prevent possible nonspecific interactions, but these interactions did not interfere with PEGylation, as the removal of salt did not appear to affect PEGylation (data not shown). The lower salt level has the added advantage of allowing the PEGylation reaction to be directly applied to a cation exchange column without further dilution, thereby streamlining the process. DTT was replaced by L-cysteine to quench the reaction because of DTT's possible in vivo toxicity. The final condition for PEGylation was 0.5 mM DAPD reacted with 1.5-fold molar excess of the 43 kDa mPEG2-MAL in 10 mM phosphate, pH 6.5, at room temperature for 0.5 hours.

Purification Process Development

PEGylated DAPD generated according to the optimized PEGylation procedure was used as a starting material for purification process development. Our initial purification strategy attempted to use a single column; specifically, cation exchange chromatography was used to resolve uncoupled PEG, free peptide, and PEGylated peptide in a single-unit operation based on the fact that both free and PEG-modified peptides carry positive charges at the operating pH, but not PEG. Additionally, cation exchange chromatography might also separate free peptide from PEGylated peptide because the PEG-modified peptide theoretically would bind less tightly to the cation exchange resin than the free peptide, because of steric interference from the attached PEG. However, SDS-PAGE analysis showed that a small amount of free peptide coeluted with the PEGylated peptide in most of the fractions collected from both SP Sepharose and CM Sepharose runs (data not shown). Hydrophobic interaction chromatography (HIC) was also found ineffective in separating the 2 forms of DAPD, as SDS-PAGE analysis showed that all fractions, column bound or unbound, contained both the free and the PEG-modified peptides (data not shown). Thus, an additional separation method was needed to remove the small amount of free peptide impurity.

Cation exchange chromatography was selected over HIC as the initial capture step for purifying PEGylated DAPD because of its low solubility in ammonium sulfate and lower capacity in HIC binding. To choose a cation exchange matrix, the binding capacity of 3 matrixes was compared. The binding for EM FractoPrep SO,[3] SP Sepharose, and CM Sepharose was 1.4, 1.5, and 1.3 AU per mL of gel which corresponds to 0.64, 0.71, and 0.58 mg per mL of gel on the peptide weight basis. SP Sepharose was chosen because of its slightly higher binding capacity and easy accessibility.

To completely eliminate the unreacted DAPD, which may interfere with the functional assay, as the free peptide is more potent than the 43 kDa PEGylated DAPD (see below), a second purification step was needed to separate DAPD and PEG-DAPD based on the large size difference. HiLoad 16/60 Superdex 30 pg, HiPrep Sephadex G-25, and stirred cell equipped with a YM10 (10 000 molecular weight cut-off) membrane were compared in their ability to completely remove free peptide. The matrix of Superdex 30 pg is highly cross-linked porous agarose, covalently bound with dextran, and is suitable for either fractionation when a good resolution is desired or group separation that allows for a bigger sample application. HiPrep Sephadex G-25 is also ideal for group separation. The molecular weight for group separation with Superdex 30 pg and HiPrep Sephadex G-25 is <10 000 and <5000, respectively. The absorbance-based yield analysis indicated that greater than 90% of the PEG-modified DAPD applied was recovered. SDS-PAGE analysis showed that additional removal of free peptide by either size exclusion columns or a membrane-based filtration device was achieved and that all methods showed comparable results (data not shown). Both the membrane-based filtration step and size exclusion chromatography were included in the final purification process scheme to ensure maximum removal of the free peptide.

Preparation of PEGylated DAPD for Preclinical Studies

To produce the 5 batches of PEGylated DAPD used in preclinical studies, 0.5 mM DAPD was reacted with 1.5-fold molar excess of the 43 kDa mPEG2-MAL in 10 mM phosphate, pH 6.5, at room temperature for 0.5 hours and quenched by 2 molar excess of cysteine over the mPEG2-MAL. A 3-step purification process consisting of cation exchange chromatography on SP Sepharose, membrane filtration on stirred cell, and size exclusion chromatography on Superdex 30 pg was used to purify PEGylated DAPD (Figure 2). Average ± SD yield from 5 separate preparations for the cation exchange step was 84% ± 5%; for the membrane filtration step, 84 ± 12%; and for the size exclusion step, 87% ± 9%. The overall yield was 63% ± 7%.

Analytical Characterization of 43 kDa PEG-DAPD

The 43 kDa PEG-DAPD preclinical batches were characterized by a series of analytical methods to confirm product

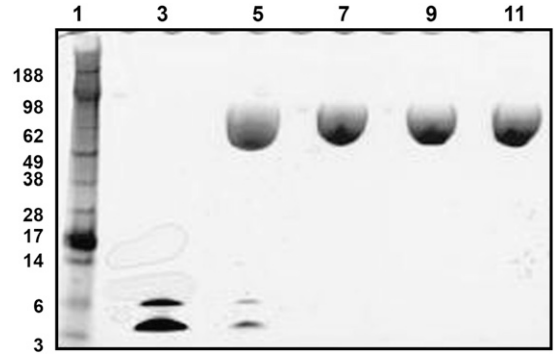

Figure 2. SDS-PAGE analysis of DAPD PEGylation and purification. The samples, 2 µg by peptide weight, are DAPD (lane 3), DAPD reacted with 43 kDa mPEG2-MAL followed by DTT quenching (lane 5), SP Sepharose eluate (lane 7), concentrated SP eluate via stirred cell with a membrane of 10 kDa MWCO (lane 9), and Superdex 30 size exclusion pool (lane 11) of the PEGylated DAPD. Molecular weight marker was loaded in lane 1. SDS-PAGE indicates sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DAPD, dual-acting peptide for diabetes; mPEG2, 2 methoxy polyethylene glycol chains; MAL, maleimide; DTT, dithiothreitol; MWCO, molecular weight cut off.

safety, identity, strength, purity, and potency. SDS-PAGE followed by protein and PEG staining was used to confirm that the purified PEG-DAPD contained both PEG and the peptide (Figure 3). Peptide composition and concentration was confirmed by amino acid analysis (data not shown). The amount of peptide impurity was found to be ~1% by reversed-phase HPLC based on the integration of the PEG-DAPD peak vs the putative DAPD peak on the left (Figure 4A). The purified 43 kDa PEG-DAPD was also loaded onto SDS-PAGE at increasing amounts to determine peptide impurity levels (Figure 4B). The amount of free peptide impurity was estimated to be <1% based on the lowest detection limit at 0.03 nmol (Lane 5) for the unmodified peptide and that no free peptide was detected at the highest level of 43 kDa PEG-DAPD loaded (2.7 nmol, Lane 7).

The first 20 amino acids of DAPD were confirmed by N-terminal sequencing. However, a significant drop in amino acid yield in cycle 4 followed by a steady drop in recovery thereafter suggested significant washout of sample, most likely because of the presence of the PEG, as the drop was not as significant in the sequencing of the non-PEGylated peptide. This, together with the fact that no reaction was detected with the Cys-deleted DAPD, suggests that PEGylation is specific for the C-terminal cysteine of DAPD.

MALDI-MS confirmed the predicted mass of 43 kDa PEG-DAPD. Uncoupled 43 kDa PEG yielded a broad spectral peak at ~40 to 50 kDa and centered at 43 to 44 kDa (Figure 5A). The broad spectrum is due to the heterogeneous population of the PEG polymer species.[10,11] The 43 kDa PEG-DAPD gave a broad spectral peak centered between 47 and 48 kDa, as expected (Figure 5B), confirming that a single PEG was reacted with a single peptide. The peak observed at ~24 kDa represents the doubly charged ion. A small rise in the baseline was observed at 94 kDa because of the MALDI-induced dimer rather than a second PEG chain bound to the peptide. The presence of free PEG in a sample containing 43 kDa PEG-DAPD would not be detected by this method because of the heterogeneity of the polymeric samples and the lower efficiency of ionization of free PEG. Although a relatively low laser power was applied, the MALDI process causes cleavage to a low percentage of the PEG-peptide bonds, leading to free peptide and PEG. Thus, this method cannot be used to accurately quantify the amount of free DAPD.

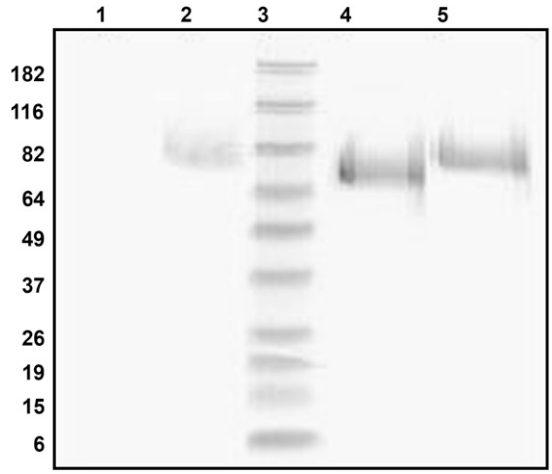

Figure 3. Confirmation of PEGylation by iodine staining. Exactly 100 pmol of 43 kDa mPEG2-MAL (lanes 1 and 4) or purified 43 kDa PEG-DAPD (lanes 2 and 5) along with the molecular weight markers (lane 3) were run on a single gel. The gel was cut in between lanes 3 and 4 and stained with GelCode blue (lanes 1 and 3) or iodine (lanes 4 and 5). mPEG2 indicates 2 methoxy polyethylene glycol chains; MAL, maleimide; PEG, polyethylene glycol; DAPD, dual-acting peptide for diabetes.

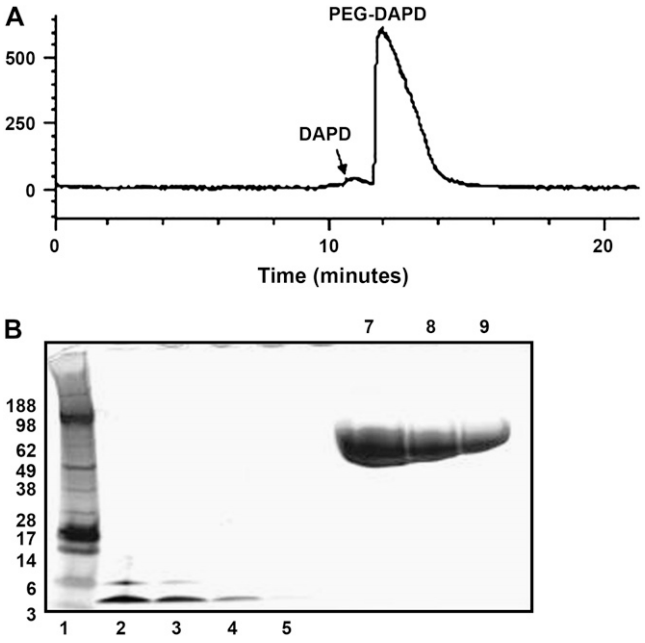

Figure 4. Estimation of free peptide impurity level in 43 kDa PEG-DAPD. (A) Analytical reversed-phase HPLC analysis. DAPD peak was identified by a previous run of DAPD alone. (B) SDS-PAGE analysis. Lane 1 is molecular weight marker; lanes 2 to 5 are DAPD at 1, 0.5, 0.3, and 0.125 μg; and lanes 7 to 9 are PEG-DAPD at 10, 5, and 2.5 μg by peptide weight. PEG indicates polyethylene glycol; DAPD, dual-acting peptide for diabetes; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electorphoresis.

Analytical cation exchange chromatography was used for monitoring the amount of unreacted PEG (Figure 6). A small amount of the unreacted PEG was found in the flow-through fraction, as detected by A_{215} nm absorbance. PEG does not absorb at A_{280} nm, so that this A_{215} nm peak in flow-through could also be due to the smaller A_{280} nm peak in flow-through as a result of a small portion of PEGylated peptide not binding to the column. A_{215} nm standard curves generated with 43 kDa PEG (quenched with cysteine)

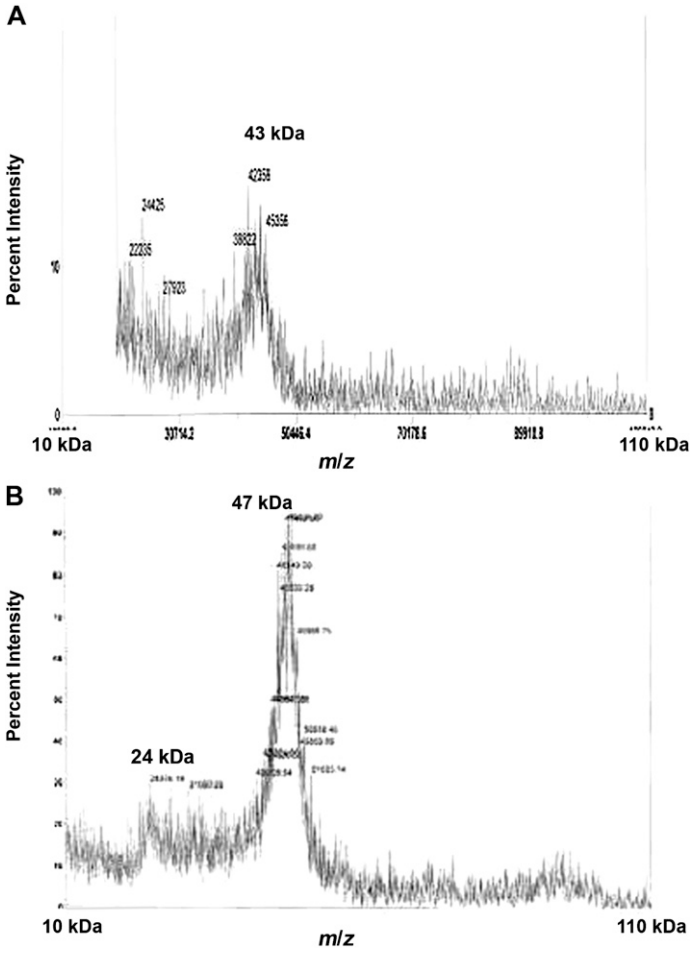

Figure 5. MALDI-MS spectrum of 43 kDa mPEG2-MAL (A) and 43 kDa PEG-DAPD (B). MALDI-MS indicates matrix-assisted laser desorption/ionization mass spectrometry; mPEG2, 2-methoxy polyethylene glycol chains; MAL, maleimide; PEG, polyethylene glycol; DAPD, dual-acting peptide for diabetes.

allowed for an approximation of the amount of 43 kDa PEG impurity in the 43 kDa PEG-DAPD sample: <5%.

To facilitate in vitro and in vivo functional testing of 43 kDa PEG-DAPD, all lots were also tested for endotoxin levels. The LAL test showed that all lots had endotoxin levels less than the detection limit of 0.2 endotoxin units (EU)/mg.

GLP-1 receptor activity was assessed for DAPD, 22 kDa PEG-DAPD, and 43 kDa PEG-DAPD against the benchmark GLP-1 peptide (Figure 7). The different lots of 43 kDa PEG-DAPD showed reproducible GLP-1 receptor activation, although to a lesser extent than 22 kDa PEG-DAPD, DAPD, and GLP-1, as expected following modification with a higher PEG molecular weight.[7]

Process Scale-Up Feasibility

The high overall purification yield, the low SD for the yield at each step of purification, and the extensive analytical characterization detailed above demonstrate that PEGylated DAPD can be reproducibly synthesized and purified. The PEGylation

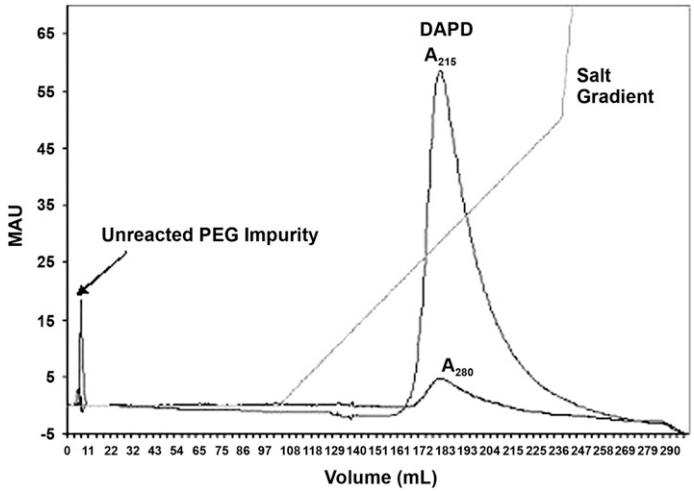

Figure 6. Estimation of mPEG2-MAL impurity level in 43 kDa PEG-DAPD by cation exchange chromatography. mPEG2 indicates 2-methoxy polyethylene glycol chains; MAL, maleimide; PEG, polyethylene glycol; DAPD, dual-acting peptide for diabetes.

reaction can be performed under simple and well-known conditions. The separation techniques are straightforward and familiar. PEGylation and purification can readily be performed at the 100 g (peptide weight basis) or 1.2 kg (drug substance basis) scale, involving vessels and chromatography columns of less than 250 L. The projection represents the most conservative possible case. Improvements in process performance can be expected even if their magnitude cannot be predicted. These include increasing reactant concentrations, reducing the number of purification steps, increasing the yield of each unit operation, reducing the reaction excess of mPEG2-MAL, and increasing the loading of chromatography columns.

CONCLUSION

Although protein PEGylation has been well established, few examples of successful PEGylation of peptides have been reported to date. This is most likely due to the higher

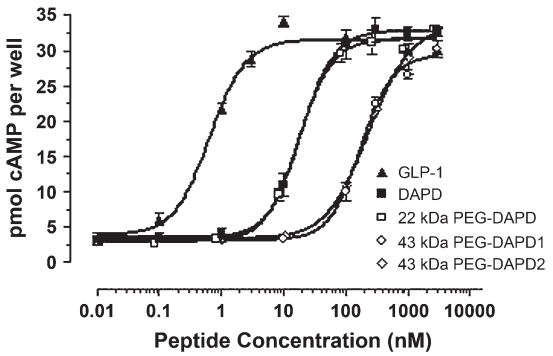

Figure 7. Reproducible cAMP activation by different lots of PEG-DAPD in rat insulinoma cell line. Representative of at least 3 trials. PEG indicates polyethylene glycol; DAPD, dual-acting peptide for diabetes; GLP-1, glucagon-like peptide-1.

likelihood of a reduction in activity upon PEGylation of many peptides. The small size difference between the PEGylated peptide and PEG, the heterogeneity of PEG, and the ability of PEG to shield the peptide charges have made it extremely challenging to purify PEGylated DAPD in a consistent manner and with a high degree of purity.

We have developed a highly efficient and reproducible PEGylation procedure for site-specific PEGylation of a 32-mer dual-acting peptide for type 2 diabetes. A nearly complete reaction was achieved at ambient temperature with a wide range of pH from 5.8 to 8, within 5 minutes, and a slight molar excess of PEG over the peptide. A purification process was developed to purify PEG-modified DAPD that included cation exchange chromatography to remove the unreacted PEG, membrane filtration to separate the uncoupled peptide and to reduce the pool volume before loading onto the size exclusion column, and size exclusion chromatography to resolve the trace amounts of free peptide and to formulate the product in phosphate-buffered saline (pH 7.4). The average overall yield of four 43 kDa PEG-DAPD lots was 63%. Analyses of 43 kDa PEG-DAPD preparations showed consistent product safety, identity, strength, purity, and potency and that the preparations were mono-PEGylated; possessed the expected sequence and amino acid composition; contained <1% of free peptide, <5% uncoupled PEG, and <0.2 EU/mg endotoxin; and had consistent GLP-1 receptor activation. The PEGylation and purification development studies described here demonstrate that 43 kDa PEG-DAPD can be synthesized and purified reproducibly and reliably at the 100 mg scale based on peptide weight. Moreover, the series of analytical methods developed to fully characterize the PEGylated product establishes a blueprint for future qualification of PEGylated peptides.

As far as we are aware, PEGylated DAPD is the only PEGylated peptide that can be produced on a scale sufficient to support extensive preclinical studies. PEGylated DAPD has dramatically prolonged pharmacological activity in vivo when compared with nonmodified GLP-1, which has a half-life of less than 5 minutes in vivo. PEGylated DAPD has demonstrated glucose-lowering activity in a glucose tolerance test for up to 65 hours following subcutaneous injection in rats.[12] This prolonged activity confirms the improved stability and reduced clearance of the PEGylated peptide.

Acknowledgments

The authors would like to thank N. He, S. Yung, S. Roczniak, J. Greve, J. Wang, W. Wang, T. Cui, and W. Froland for helpful suggestions.

References

1. Mayo KE, Miller LJ, Bataille D, et al. International Union of Pharmacology, XXXV: the glucagon receptor family. *Pharmacol Rev*. 2003;55:167-194.

2. Jelinek LJ, Lok S, Rosenberg GB, et al. Expression cloning and signaling properties of the rat glucagon receptor. *Science*. 1993;259:1614-1616.

3. Thorens B. Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *Proc Natl Acad Sci USA*. 1992;89:8641-8645.

4. Hjorth SA, Adelhorst K, Pedersen BB, Kirk O, Schwartz TW. Glucagon and glucagon-like peptide 1: selective receptor recognition via distinct peptide epitopes. *J Biol Chem*. 1994;269:30121-30124.

5. Roges OA, Baron M, Philis-Tsimikas A. The incretin effect and its potentiation by glucagon-like peptide 1-based therapies: a revolution in diabetes management. *Expert Opin Investig Drugs*. 2005;14:705-727.

6. Jiang G, Zhang BB. Glucagon and regulation of glucose metabolism. *Am J Physiol Endocrinol Metab*. 2003;284:E671-E678.

7. Pan CQ, Buxton JM, Yung SL, et al. Design of a long acting peptide functioning as both a glucagon-like peptide-1 receptor agonist and a glucagon receptor antagonist. *J Biol Chem*. 2006;281:12506-12515.

8. Veronese FM, Pasut G. PEGylation, successful approach to drug delivery. *Drug Discov Today*. 2005;10:1451-1458.

9. Lee KC, Tak KK, Park MO, et al. Preparation and characterization of polyethylene-glycol-modified salmon calcitonins. *Pharm Dev Technol*. 1999;4:269-275.

10. Chowdhury SK, Doleman M, Johnston D. Fingerprinting proteins coupled with polymers by mass spectrometry: investigation of polyethylene glycol-conjugated superoxide dismutase. *J Am Soc Mass Spectrom*. 1995;6:478-487.

11. Watson E, Shah B, DePrince R, Hendren RW, Nelson R. Matrix-assisted laser desorption mass spectrometric analysis of a PEGylated recombinant protein. *Biotechniques*. 1994;16:278-280.

12. Claus TH, Pan CQ, Buxton JM, et al. Dual-acting peptide with prolonged glucagon-like peptide-1 receptor agonist and glucagon receptor antagonist activity for the treatment of type 2 diabetes. *J Endocrinol*. 2007;192:371-380.