Improved Preparation of an Amino Acid Type Poly(Ethylene Glycol) Derivative

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An amino acid type poly(ethylene glycol) is a useful tool for preparation of a bi- or multivalent poly(ethylene glycol) hybrid of bioactive peptides, but synthesis is problematic. The amino acid type poly(ethylene glycol) was prepared from poly(oxyethylene)diglycolic acid followed by introduction of a fluorenylmethyloxycarbonyl group. The resulting product could be purified easily by LH-20 column chromatography and HPLC.

Key words poly(ethylene glycol); hybrid; poly(ethylene glycol) hybrid; peptide hybrid; amino acid type poly(ethylene glycol)

Hybrid formation of bioactive materials with various polymers is the focus of many studies on drug delivery systems. Among various polymers, poly(ethylene glycol) (PEG) has been commonly used as a carrier system for biological proteins, since PEG has low toxicity, low immunogenicity and good solubility in both aqueous and organic solvents. However site specific pegylation (hybrid formation with PEG) of a protein is difficult and over-pegylation may result in partial or complete loss of the protein's biological activity. We were successful to synthesize PEG hybrids of small biological peptides (laminin- and fibronectin-related peptides) using an aminated PEG (aPEG) and the synthetic hybrids retained the biological activity of the parent peptides.[1,2] These results indicate that PEG offers many interesting opportunities in the development of novel drug-carrier systems, although its simple structure limits the types of hybrids that can be produced. Usually, PEG is converted to a carboxyl derivative and conjugated with a protein as a simple acyl moiety. We designed an amino acid type PEG (aaPEG) for preparation of a multifunctional PEG-peptide hybrid and were successful in developing a bivalent PEG hybrid of fibronectin- and laminin-related peptides.[3,4] However, several problems were encountered during the preparation of these bivalent PEG hybrids. A major obstacle in the synthesis of these bivalent PEG hybrids was the difficulty of preparing an aaPEG of the desired quality. Here we report improved synthesis of an aaPEG derivative, *N*-(9-fluorenylmethoxycarbonyl)-aaPEG (Fmoc-aaPEG-OH). We reported the preparation of various aaPEGs as shown in Fig. 1.

In these cases, aaPEG was prepared from a commercial diaminopropyl-PEG (apPEG) (or aPEG, which was derived from PEG according to the procedure reported by Pillai and Mutter[5]). The apPEG (or aPEG) was converted to the corresponding aaPEG by reaction with equimolar succinic anhydride. The average molecular weight of apPEG was 10000 (the only type commercially available) and reaction rate of such a large apPEG was slow. Preparation of aPEG from PEG was time-consuming, but PEGs with various molecular weight were commercially available. aaPEG was also prepared from oxidized PEG (oPEG). In this case, oPEG was prepared by oxidation of PEG with potassium permanganate according to the procedure reported by Ueyama *et al.*[6] Since purification of oPEG was problematic, it was difficult to obtain the desired quality of oPEG.

In the present study, we generated aaPEG from commercial poly(oxyethylene) diglycolic acid 3000 (carboxymethy-

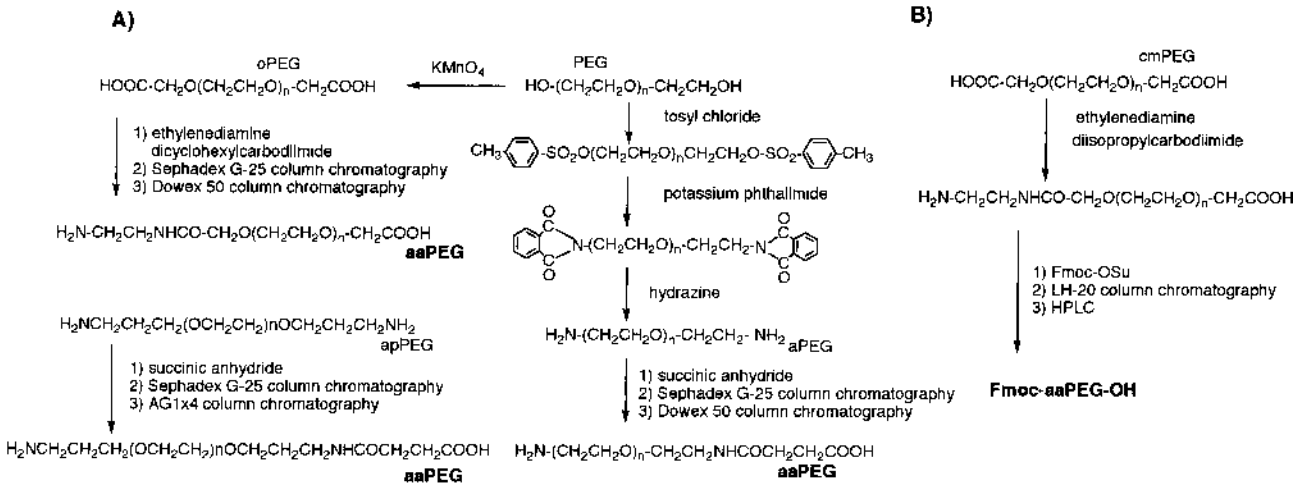

Fig. 1. Synthetic Scheme for Preparation of aaPEG and Fmoc-aaPEG-OH

A) A previously reported scheme for preparation of aaPEG.[3,4] B) New synthetic scheme for Fmoc-aaPEG-OH.

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lated PEG, cmPEG, MW 2400-3500, Wako Chemical Ind. Ltd.). Both oPEG and cmPEG have 2 carboxymethyl groups at each terminal of PEG. Prior to use in subsequent reactions, commercial cmPEG required additional purification by extraction and precipitation procedures, since, for our purposes, it was not suitable as a reactant. The commercial cmPEG had a strong pungent odor and had the appearance of melted wax. It did not react without purification. The cmPEG was purified by extraction and precipitation procedures. The resulting cmPEG was white powder and was odorless. In a previous report,[4] aaPEG was prepared from oPEG and ethylenediamine using dicyclohexylcarbodiimide (DCC)[7] as a coupling reagent. The limited solubility of a reaction product, dicyclohexylurea, was problematic, when the reaction mixture was purified by a column chromatography. Since diisopropylcarbodiimide (DIC)[8] became commercially available recently, it was used instead of DCC in the present study. The enhanced solubility of the resulting product, diisopropylurea, did not give any problems during column chromatography. In a previous report,[3,4] Sephadex G-25 and ion-exchange column chromatography were used successively for purification of the product. Since aaPEG does not have a well-defined absorption peak suitable for selective detection by spectrophotometric procedures, purification of aaPEG by chromatography necessitated the use of ninhydrin testing.[9] Ion-exchange column chromatography and intermittent ninhydrin testing was unacceptably time-consuming. In the present study, crude aaPEG was acylated with the 9-fluorenylmethoxycarbonyl (Fmoc) group[10] and the resulting Fmoc-aaPEG-OH was readily detected by a UV spectrometer. cmPEG and ethylenediamine (1 to 2 molar ratio) were reacted with DIC (at equimolar concentration to cmPEG) to give aaPEG. The crude aaPEG was washed with ethyl acetate to remove diisopropylurea and converted to Fmoc-aaPEG-OH by the reaction with *N*-(9-fluorenylmethoxycarbonyl)succinimide. Then the material was purified by Sephadex LH-20 column chromatography and HPLC successively. As shown in Fig. 2, two major peaks appeared in HPLC and their eluents were collected. A by-product, Fmoc-aPEG-Fmoc (Fmoc-$NHCH_2CH_2NHCOCH_2$-PEG-$CH_2CONHCH_2CH_2NH$-Fmoc), was found in the eluate of peak II and was identified by mass spectroscopy. Materials contained in peak I and peak II (Fig. 2) exhibited an average molecular weight of 3433 and 3696, respectively (Fig. 3) which corresponded to molecular weight of Fmoc-aaPEG-OH and Fmoc-aPEG-Fmoc.

PEG exhibits a comb-shaped peak by MS, because it is a mixture consisting of a relatively uniform distribution of molecular weights within a fixed range. The number of aromatic protons and methylene protons next to fluorene in NMR spectra of a sample from peak I was half of that of peak II. The number of aliphatic protons of oxyethylene portion in peak I material and peak II material were same This result also indicates that peak II material was Fmoc-aPEG-Fmoc. Comparing the peak area of peak II with that of peak I in HPLC, formation of the by-product appeared significant, but the intensity of the absorbance of the by-product (peak II, 2 Fmoc groups) is double of the desired material (peak I, 1 Fmoc group). Yields of the desired material and the by-product was 45% and 10%, respectively. We found that formation of the by-product depends on the concentration of the reactants when aaPEG was formed. Reactions of cmPEG and ethylenediamine were examined at various concentrations and the results are summarized in Fig. 4.

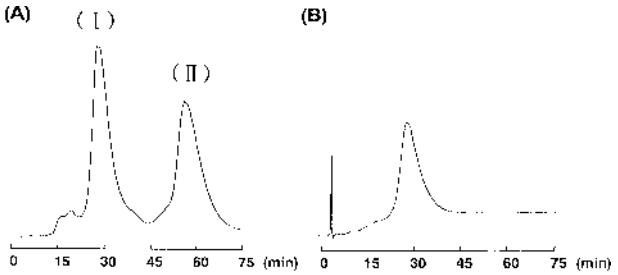

Fig. 2. HPLC Profile of Crude (A) and Purified Fmoc-aaPEG-OH (B)

Peaks I and II of (A) contained Fmoc-aaPEG-OH and Fmoc-aPEG-Fmoc respectively. Column: DAISOPAK-SP-120-ODS-B (4.6×250 mm). Flow rate, 1 ml/min. Gradient: CH_3CN/H_2O containing 0.05% TFA; 40/60→50/50→50/50 (0→40→100 min). OD at 220 nm.

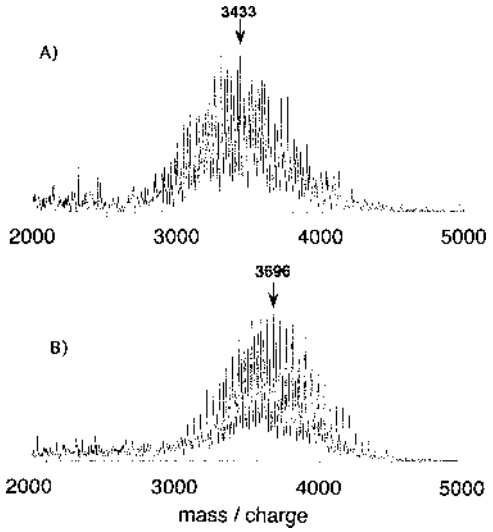

Fig. 3. TOF-MS of Fmoc-aaPEG-OH and Fmoc-aPEG-Fmoc

A) TOF-MS of Fmoc-aaPEG-OH obtained from peak I eluate in HPLC. B) TOF-MS of Fmoc-aPEG-Fmoc obtained from peak II eluate in HPLC.

The vertical axis represents the ratio of peak area (Fmoc-aPEG-Fmoc/Fmoc-aaPEG-OH) and reaction yield of Fmoc-aaPEG-OH. The horizontal axis represents the amount of solvent used for the coupling reaction of cmPEG and ethylenediamine. Figure 4 indicates that a reduced concentration of reactants surpresses the formation of the by-product (ethylenediamine-cmPEG-ethylenediamine, amino-PEG, aPEG). Yield of the desired material (Fmoc-aaPEG-OH) was better in diluted reaction conditions (solvent volume 250 ml, 10 mmol/l concentration of cmPEG), but with extensive dilution (solvent volume 450 ml, 7.4 mmol/l concentration of cmPEG) the yield of the desired material became low. This phenomenon may be explained by the possibility of the formation of a by-product (ethylenediamine-cmPEG-ethylenediamine). Formation of the by-product under dilute reaction conditions may be less than at higher concentrations. To obtain these results, subsequent reactions with cmPEG were performed at 10 mmol/l concentration. The quality of Fmoc-aaPEG-OH obtained in the present study is better than that for the previously reported aaPEG.[3,4] It is important to recognize that aaPEG and its derivatives are useful tools for preparation of multivalent PEG-peptide hybrids, but it is dif-

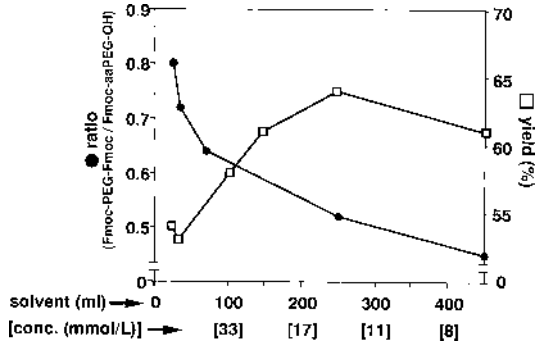

Fig. 4. Relationship between Yield, By-product Formation and Solvent Volume [Concentration of cmPEG] Used for the Synthetic Reaction of Fmoc-aaPEG-OH

cmPEG (10 g, 3.33 mmol), diisopropylcarbodiimide (0.52 ml, 3.33 mmol) and ethylenediamine (0.44 ml, 6.66 mmol) were reacted at room temperature in dichloromethane for 24 h. After the reaction, the product was fluorenylmethoxycarbonylated and examined by HPLC. The vertical axis for ● indicates ratio of peak area of the desired material (Fmoc-aaPEG-OH) and the by-product (Fmoc-aPEG-Fmoc). The vertical axis for □ indicates reaction yield of Fmoc-aaPEG-OH. The horizontal axis indicates volume of the solvent (dichloromethane) used for the reaction. [Square brackets] under the volume indicate the concentration of cmPEG at each solvent volume.

ficult to obtain a pure aaPEG, since commercial PEG is a mixture. We have improved the synthetic procedure and obtained Fmoc-aaPEG-OH of high quality. A problem with our synthetic procedure is based upon HPLC for purification of Fmoc-aaPEG-OH. Laboratory scale HPLC limits preparation of a large quantity of aaPEG. We will continue to investigate alternative strategies for synthesis of aaPEG of high quality and greater quantities.

Experimental

RP-HPLC was performed using a Waters 600 with a DAISOPAK column and gradient systems of CH_3CN/water containing 0.05% TFA. TOF-mass spectra were obtained from a SHIMADZU/KRATOS KOMPACT MALDI IV spectrometer. ^{1}H-NMR specra were obtained on a Varian 400 (400 MHz) spectrometer.

Fmoc-aaPEG-OH Commercial poly(oxyethylene)diglycolic acid (cmPEG, 80 g) was dissolved in chloroform and the solution was washed 3 times with saturated NaCl solution (200 ml). The chloroform layer was dried with Na_2SO_4 and evaporated. The residue was precipitated from ethanol/ether to give white powder. Yield 65 g. The purified cmPEG (average molecular weight 3000, 10 g, 3.33 mmol) and DIC (0.52 ml, 3.33 mmol) in dichloromethane (DCM, 330 ml) was stirred for 2 h at 0 °C and ethylenediamine (0.44 ml, 6.6 mmol) was added to the solution. The reaction mixture was stirred overnight at room temperature and the solvent was removed by evaporation. The residue was dissolved in water and washed with ethyl acetate 3 times. The water layer was evaporated and the residue was precipitated from ethanol/ether. *N*-(9-Fluorenylmethyloxycarbonyl)succinimide (2.2 g, 6.6 mmol) was added to a solution of the crude aaPEG and pyridine (2.67 ml, 33 mmol) in DCM (100 ml) and the mixture was stirred for 24 h at room temperature. The solvent was removed by evaporation and the residue was precipitated from ethanol/ether. The crude Fmoc-aaPEG-OH was first purified by Sephadex LH-20 column chromatography using methanol as an eluent and eluate was checked by absorbance at 265 nm. One major peak was observed in the eluate and 9.1 g of a material was obtained from the peak eluent. This material was purified by HPLC using a DAISOPAK SP-120-5-ODS-B column as shown in Fig. 2. The crude material (200 mg) was applied to the column (4.6×250 mm) and 90 mg of Fmoc-aaPEG-OH from peak I was obtained. Yield 45%. TOF-MS *m*/*z*: 3433 (average). ^{1}H-NMR (D_2O) δ: 7.81 (2H, d, J=7.4 Hz), 7.65 (2H, d, J=7.4 Hz), 7.40 (2H, d, J=7.4 Hz), 7.32 (2H, d, J=7.4 Hz), 4.38 (2H, d, J=6.8 Hz), 3.69 (296H, s). Fmoc-aPEG-Fmoc (20 mg, yield 10%) was obtained from peak II. TOF-MS *m*/*z*: 3696 (average). ^{1}H-NMR (D_2O) δ: 7.81 (4H, d, J=7.4 Hz), 7.65 (4H, d, J=7.4 Hz), 7.40 (4H, d, J=7.4 Hz), 7.32 (4H, d, J=7.4 Hz), 4.38 (4H, d, J=6.8 Hz), 3.69 (296H, s).

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