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A synthetic method for peptide-PEG-lipid conjugates: Application of Octreotide-PEG-DSPE synthesis

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ABSTRACT

A solid phase synthesis method was established for the synthesis of peptide-poly(ethylene glycol)-lipid (peptide-PEG-lipid) conjugates. Octreotide-PEG_{2000}-DSPE (OPD_{2000}) was used as an example to demonstrate the synthetic approach. The OPD_{2000} obtained had confirmed structure, activity, and purity providing a targeting molecule for preparation of well-defined drug delivery systems, such as targeted liposomes, for further studies.

Ligand-PEG-lipid is a type of conjugate that is widely used as targeting molecules in drug delivery systems. An example was found in ligand-based targeted liposomes[1,2] for cancer drug delivery. A receptor-specific ligand-PEG-lipid in liposomes can bind to its counter receptor in particular tumor cells. Through the binding of the ligand to its receptor and the endocytosis of cell membranes, the therapeutic agent containing liposomes can be transported into the tumor cells. Several types of molecules have been used as ligands in ligand-PEG-lipid conjugates, such as antibodies,[3] proteins,[4] small molecules,[5] and peptides.[6] Among these molecules, peptides exhibit the greatest potential for applying as a ligand, because many tumors were found to have certain over-expressed[7] receptors, and several peptides[7] were known to have specific affinity to these receptors. Hence, the peptide-PEG-lipid conjugates for these peptides are currently being developed.

Although peptide-PEG-lipid conjugates are required for preparing targeted liposomes, the availability of conjugates is still limited. Moreover, a peptide-PEG-lipid conjugate with a confirmed structure, activity, and purity is difficult to be found. The difficulty in the synthesis of conjugates is due to the complex nature of the peptide-PEG-lipid. Specifically, in peptide-PEG-lipid conjugates, the chemical properties of the peptidyl side chains are diverse, the molecular mass of the PEG is big and heterogeneous, and the characteristic of lipids is amphiphilic. These properties cause difficultly in the synthetic processes, such as side chain protection, purification, and conjugation. Currently, the synthesis of peptide-PEG-lipid is commonly performed via direct conjugating peptide ligand with end-group activated PEG-lipid.[6,8,9] In this synthesis method, if the peptide ligand has more than one conjugation site, the obtained product was often a mixture, containing several non-site-specific conjugates of the peptide-PEG-lipid and non-reacted PEG-lipid. The components in the mixture with heterogeneous mass and amphiphilic property were difficult to separate and purify. Thus, it was generally difficult to obtain a structurally well-defined peptide-PEG-lipid. Additionally, it is also difficult to characterize the biological properties of the obtained peptide-PEG-lipid, such as binding affinity. Then if un-natural linkers were used among the peptide, PEG, and lipid in the conjugates, they might produce undesired immuno-responses in biological systems.[10] In this study, we anticipated the development of a synthetic method that is capable of synthesizing a broad range of peptide-PEG-lipid conjugates. From the synthesized products, a structurally well-defined peptide-PEG-lipid can be obtained.

An example of a peptide-PEG-lipid, Octreotide-poly(ethylene glycol)$_{2000}$-distearyl phosphatidylethanolamine (Octreotide-PEG_{2000}-DSPE; or OPD_{2000}) (see Fig. 1), was designed and synthesized in this study. The OPD_{2000} is composed of three molecular parts including eight amino acids consisting of the cyclic peptide (Octreotide), PEG_{2000}-diacid, and distearyl phosphatidylethanolamine (DSPE). The linkage between each molecular part was an amide bond. Hence, the OPD_{2000} could be formed through

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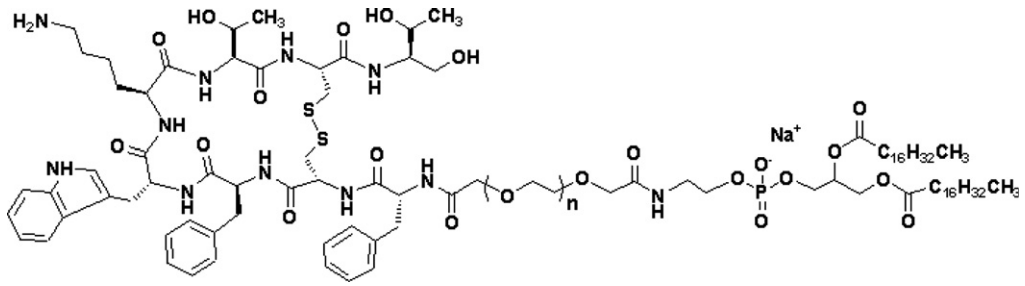


Figure 1. Chemical structure of the Octreotide-PEG-DSPE.

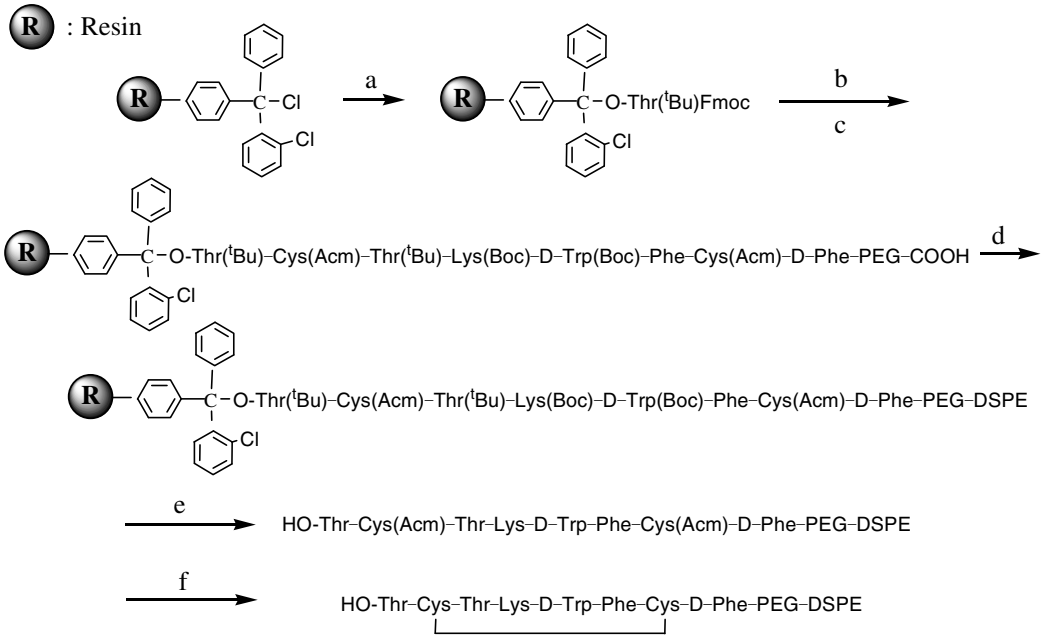
connecting the N-terminal of the peptide and the amino group of the DSPE to each end of the PEG_{2000}-diacid. The selection of an amide bond as the linkage functional group was based on its simplicity, stability, and non-immunogenicity. To date, the OPD_{2000} and Octreotide-related peptide-PEG-DSPE have not been synthesized.

In consideration of the pharmaceutical setting, we selected the Octreotide as the ligand because it has high binding affinity to somatostatin receptors (SSTRs).[11] SSTRs have been recognized as tumor markers in several cancers,[12] and they have been used as the targets for tumor diagnoses[13] and therapeutic treatments.[14] Therefore, if the OPD_{2000} could be synthesized with desired biological activities, it can be potentially applied as a targeting molecule in drug delivery systems for somatostatin receptor-positive tumors.

A solid phase synthetic method was developed to synthesize the OPD_{2000} in this study (Scheme 1). This method was considered to have several advantages in the synthesis of the OPD_{2000}. Specifically, the synthesis was simplified by conjugating commercially available amino acid derivatives, PEG-diacid, and then DSPE onto the resins to form the octapeptide-PEG-DSPE. The conjugating reactions were all amide bond formations, which avoided the task of inducing other types of linkages and functional groups. More importantly, during the conjugating steps, the amino acid side chains were all protected. Therefore, only an amino group and an acid group were allowed for conjugation in each coupling step. Through this, the obtained octapeptide-PEG-DSPE had a unified structure without non-site-specific conjugates. In addition, the solid phase synthetic method was known to simplify the purification procedures after each conjugating step.

The purification of octapeptide-PEG-DSPE was also important in this method. In this synthesis, the crude final conjugates cleaved from the resins were expected to contain three major components including octapepetide-PEG-DSPE, octapepetide-PEG-acid, and octapepetide. The coupling reaction of the big PEG-diacid with resin-peptide is expected to be incomplete. In addition, it was difficult for the coupling reaction of amphiphilic DSPE with resin-peptide-PEG-acid to reach a high yield. Therefore, the uncompleted conjugates, octapepetide-PEG-acid, and octapepetide should be found in the crude product. However, the uncompleted conjugates did not contain the amphiphilic DSPE. Hence, they should have distinct properties from octapepetide-PEG-DSPE, and could be conveniently removed. This was observed in the actual synthesis of the OPD_{2000} (Fig. 2). The purified octapepetide-PEG-DSPE was then performed to cyclize and form the structural unified OPD_{2000}.

The synthesis of the peptidyl-resin was performed according to Arano and co-workers' method.[15] 2-Chlorotrytylchloride resin with Fmoc peptide synthesis method was applied in this process. The octapeptide was assembled according to the repeated cycle consisting of the following steps: (1) removing the Fmoc protecting group with 20% piperidine, in *N*,*N*-dimethylformamide (DMF) for 30 min, and (2) coupling of the Fmoc amino acid derivative



Scheme 1. Synthesis of Octreotide-PEG_{2000}-DSPE. Reagents and conditions: (a) Fmoc-Thr(tBu)-ol, pyridine; (b) elongation of peptide; (c) PEG_{2000}-diacid, DIPCDI, HOBt, DMF, 2 h; (d) NHS, EDC, DMF, 4 h; DSPE, $CHCl_3$, 50 °C, overnight; (e) TFA/TFE/CH_2Cl_2; (f) I_2, 1 h.

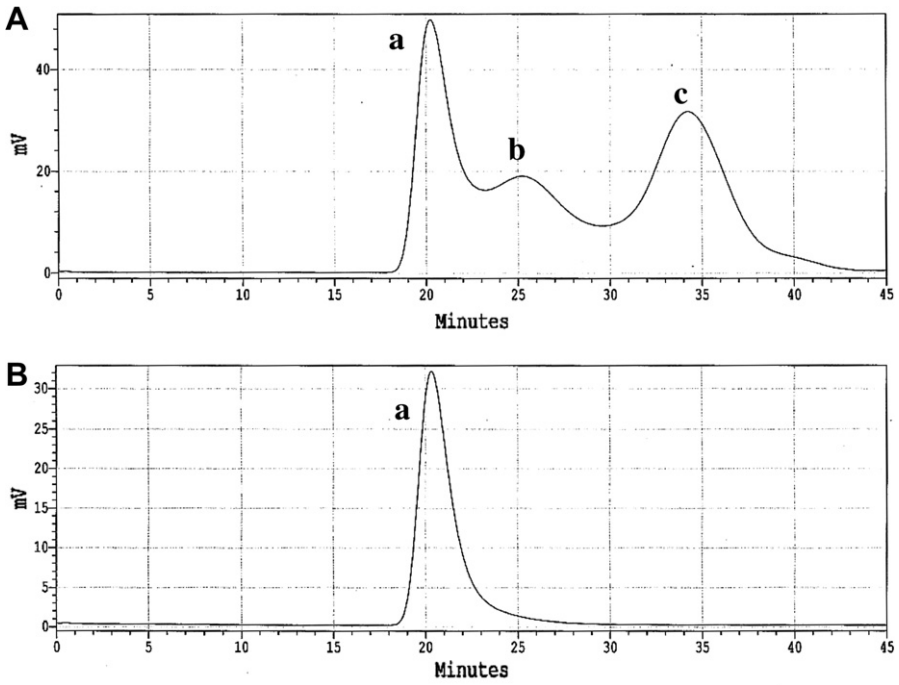


Figure 2. Chromatographies of the synthesized crude products. (A) Crude product contained the following: a—octapepetide-PEG-DSPE; b—octapepetide-PEG-acid; and c—octapepetide; (B) purified octapepetide-PEG-DSPE.

(2 equiv) with 1,3-diisopropylcarbodiimide (DIPCDI) (2 equiv) and *N*-hydroxybenzotriazole (HOBt) (2 equiv) in DMF for 2 h. The completeness of the coupling reaction was monitored via Kaiser test.[16] A requirement of 99% completeness in each amino acid coupling step was essential in this synthesis. After all amino acids were assembled, a small amount of peptidyl-resin was cleaved to ensure that the peptide was correct. A mixture consisting of trifluoroacetic acid (TFA), chloroform, thioanisole, ethanedithiol (EDT), and anisole was used to cleave the peptide. The obtained peptide was then cyclized by I_2, which is expected to form Octreotide. A mass spectrum of the cyclic peptide was then measured and confirmed to be Octreotide (calculated M.W. 1019.24, measured M.W. 1019.4).

Conjugation of PEG_{2000}-diacid to the octapeptide-resin was reacted in DMF with DIPCDI (2 equiv) and HOBt (2 equiv) for 2 h. After the coupling reaction, a small portion of the reacted resins was cleaved. The product was measured by ^1H NMR, which showed an intense peak of poly(ethylene glycol) at 3.5 ppm and protons of octapeptide. The estimated yield of the octapeptide-PEG-acid was about 80% based on the amount of octapeptide in the resins.

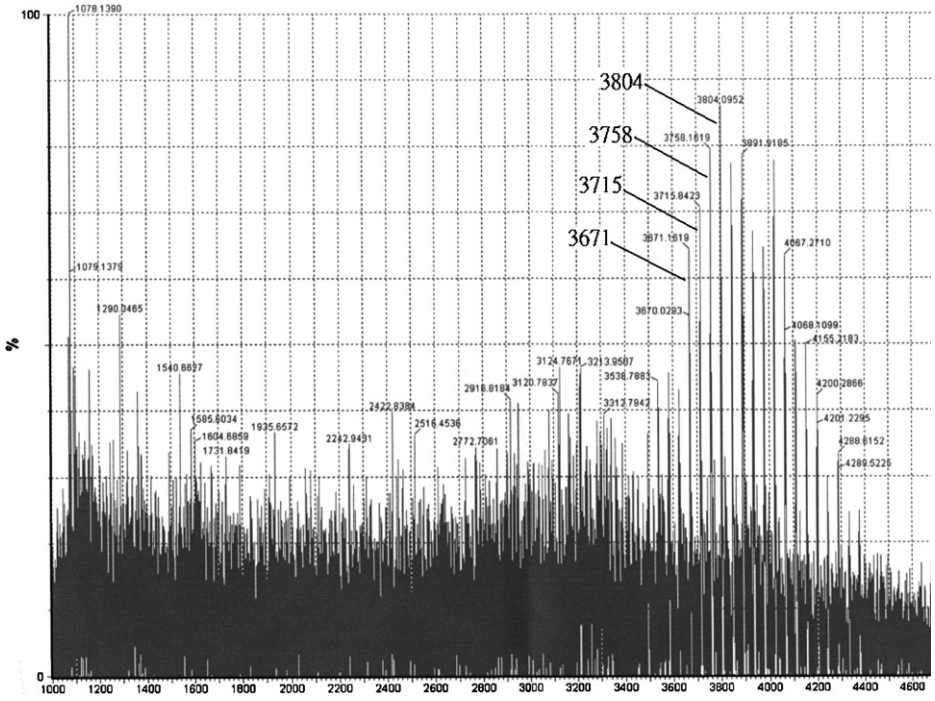


Figure 3. MALDI-MS of Octreotide-PEG_{2000}-DSPE.

In the coupling reaction of DSPE with the resin-octapeptide-PEG-acid, the resins were treated with *N*-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethyl-carbodimide in DMF for 4 h to activate the acid group. Subsequently, the activated resins were reacted with DSPE in chloroform at 50 °C overnight. Afterwards, the resins were washed with chloroform to remove uncoupled free DSPE, and then were subjected to cleavage through a mixture containing trifluoroacetic acid/$CHCl_3$/trifluoroethanol at 60:35:5 volume ratio. To precipitate the crude conjugates, the mixture was cooled in an ice bath, and then cold ether was added. The precipitate was spun and washed with cold ether for three times. The crude product was purified by liquid chromatography with C8 silica column (1 × 25 cm, LC-8 Supelcosil, Supelco) and eluted with a methanol gradient (0–90% v/v) in water (10% increments every 5 ml). After removing the solvent, a light yellow octapeptide-PEG_{2000}-DSPE was obtained.

The purified octapeptide-PEG_{2000}-DSPE was cyclized with 10 equivalents of I_2 at room temperature for 1 hour. The resulting solution was transferred into a dialysis tube (Spectra/Por™, MWCO 2000), and then was dialyzed against water at 4 °C (3 × 1000 ml, 8–16 h per period) to remove excess I_2. The completeness of the disulfide formation was further verified using Ellman's reagents (Pierce, Rockford, US). Free thiol group was not detected in the product, which showed complete disulfide formation. The conjugate was then dried and measured by ^{1}H NMR[17] and MALDI-MS (Fig. 3). The product was confirmed to be OPD_{2000}. The purity of the OPD_{2000} was then determined by measuring the mole ratio of phosphorus[18] in the product, and it obtained a purity level of more than 96%. The final yield obtained was 280 mg in per gram of resin. The binding affinity of OPD_{2000} to SSTR2A was measured according to the method described by Liapakis et al.[19] We obtained K_i at 1.6 nM, which was comparable to the free Octreotide (K_i 2.1 nM).[20]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.027.

References and notes

1. Sapra, P.; Tyagi, P.; Allen, T. M. *Curr. Drug Deliv.* **2005**, *2*, 369.
2. Noble, C. O.; Kirpotin, D. B.; Hayes, M. E.; Mamot, C.; Hong, K.; Park, J. W.; Benz, C. C.; Marks, J. D.; Drummond, D. C. *Expert Opin. Ther. Targets* **2004**, *8*, 335.
3. Sofou, S.; Sgouros, G. *Expert Opin. Drug Deliv.* **2008**, *5*, 189.
4. Wu, J.; Lu, Y.; Lee, A.; Pan, X.; Yang, X.; Zhao, X.; Lee, R. J. *J. Pharm. Pharm. Sci.* **2007**, *10*, 350.
5. Wu, J.; Liu, Q.; Lee, R. J. *Int. J. Pharm.* **2006**, *316*, 148.
6. Zalipsky, S.; Mullah, N.; Harding, J. A.; Gittelman, J.; Guo, L.; DeFrees, S. A. *Bioconjug. Chem.* **1997**, *8*, 111.
7. Reubi, J. C. *Endocr. Rev.* **2003**, *24*, 389.
8. Zalipsky, S. *Bioconjug. Chem.* **1993**, *4*, 296.
9. Lee, T.; Lin, C.; Kuo, S.; Chang, D.; Wu, H. *Cancer Res.* **2007**, *67*, 10958.
10. Boeckler, C.; Frisch, B.; Muller, S.; Schuber, F. *J. Immunol. Methods* **1996**, *191*, 1.
11. Patel, Y. C.; Srikant, C. B. *Endocrinology* **1994**, *135*, 2814.
12. Florio, T. *Front. Biosci.* **2008**, *13*, 822.
13. Kwekkeboom, D. J.; Krenning, E. P. *Eur. Radiol.* **1997**, *7*, 1103.
14. Delaunoit, T.; Rubin, J.; Neczyporenko, F.; Erlichman, C.; Hobday, T. J. *Mayo. Clin. Proc.* **2005**, *80*, 502.
15. Arano, Y.; Akizawa, H.; Uezono, T.; Akaji, K.; Ono, M.; Funakoshi, S.; Koizumi, M.; Yokoyama, A.; Kiso, Y.; Saji, H. *Bioconjug. Chem.* **1997**, *8*, 442.
16. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.
17. The NMR data for OPD_{2000}. ^{1}H NMR (500 MHz, CD_3OD) δ 0.80 (6H, t, J = 7.05 Hz, phospholipid–CH_3), 1.10 (3H, d, J = 6.36 Hz, threonine–CH_3), 1.16 (3H, d, J = 6.38 Hz, threonine–CH_3), 1.19 (58H, br s, phospholipid–$(CH_2)_{14}$–, lysine γCH_2), 1.50 (5H, br s, phospholipid βCH_2, lysine βH), 1.58 (1H, br s, lysine βH), 1.68 (1H, br s, lysine δH), 1.92 (1H, br s, lysine δH), 2.10 (1H, t), 2.21 (4H, t, J = 7.05 Hz, phospholipid-βCH_2), 2.59 (1H, m), 2.70–3.20 (≈8H, overlapping peaks, tryptophan, phenylalainine βCH_2, lysine εCH_2), 3.39 (2H, overlapping peaks), 3.51 (≈180H, s, PEG), 3.60–4.02 (≈10H, overlapping peaks), 4.10 (1H, m, phospholipid*sn1*-PO_4-CH_2), 4.14 (1H, m phospholipid *sn1*-PO_4-CH_2), 4.23 (1H, m, amino acid αH), 4.33 (2H, m, amino acid αH), 4.45 (1H, m, amino acid αH), 4.54 (1H, m, amino acid αH), 4.54 (2H, overlapping peaks, amino acid αH), 5.25 (1H, m, phospholipid *sn2*-CH), 6.87 (1H, s, tryptophan *H2*), 6.94 (1H, dd, J = 7.70, 7.33 Hz, tryptophan *H5*), 7.03 (1H, dd, J = 7.75, 7.33 Hz, tryptophan *H6*), 7.10–7.23 (10H, overlapping peaks, phenyl *H*), 7.27 (1H, d, J = 7.75 Hz, tryptophan *H7*), 7.38 (1H, d, J = 7.70 Hz, tryptophan *H4*).
18. Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*, 446.
19. Liapakis, G.; Fitzpatrick, D.; Hoeger, C.; Rivier, J.; Vandlen, R.; Reisine, T. *J. Biol. Chem.* **1996**, *271*, 20331.
20. Patel, Y. C.; Greenwood, M. T.; Panettb, R.; Demchyshyn, L.; Niznik, H.; Srikant, C. B. *Life Sci.* **1995**, *57*, 1249.