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Synthesis and evaluation of a novel lipid–peptide conjugate for functionalized liposome

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Abstract—A novel lipid analog based on amino acids for liposome modification was developed. It consisted of three different kinds of amino acid derivatives and two fatty acids, and can react directly with the peptide synthesized first on resin by Fmoc solid-phase synthesis. In this study, lipid analog conjugated with HIV-TAT peptide (domain of human immunodeficiency virus TAT protein) was synthesized and successfully incorporated into liposome. The liposome containing the lipopeptide bearing HIV-TAT exhibited efficient cellular uptake.

Surface modification is a key step in functionalizing liposome, and actually encapsulated doxorubicin into PEG (polyethylene glycol)-modified liposome has been clinically used against kaposi-salcoma[1] for a passive targeted drug delivery system (DDS) through an enhanced permeability and retention (EPR) effect.[2] Furthermore, antibody-modified liposomes[3] are currently being evaluated in clinical trials as an active targeted DDS. Peptide-modified liposome has exhibited remarkable activity in gene delivery[4] and angiogenic vessel targeting.[5]

So far, surface modification of liposome by proteins or peptides has been carried out in reactive groups of pre-formed liposome in an aqueous environment. However, the efficiency of ligand binding to a liposome membrane is usually relatively low[6] (approximately 5%), since the reactivity and sequence specificity in various substrates depend largely on steric hindrance and the electron density of peptide residues. Accordingly, the formation of unwanted byproducts such as insufficiently reacted materials and variants of lipid–protein conjugate must be monitored, since it is difficult to pharmaceutically certify the structural reliance on quality control. A promising approach to solving this problem is to produce a lipid and protein/peptide conjugate molecule by total synthesis and then to subsequently incorporate it into liposome.

Therefore, we have designed and developed a novel lipid analog (Fig. 1) mimicking the structural features of phospholipids to avoid harmful influence on physicochemical properties of the lipid bilayer. The lipid moiety was composed of the following four domains: the anchor domain (anchoring the molecule into liposome), glycero mimic domain (structural equivalent to glycerol in phospholipids), hydrophilic domain (structural equivalent of the phosphate group in phospholipids), and

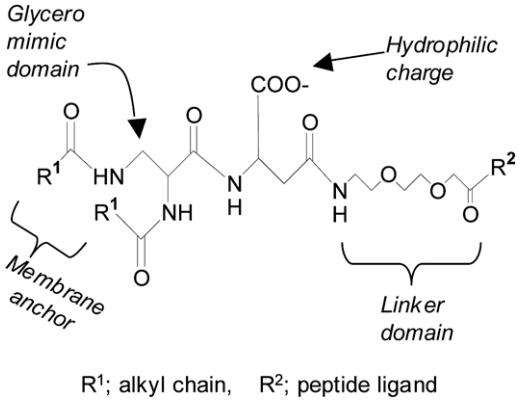

Figure 1. General structure of the novel lipid analog.

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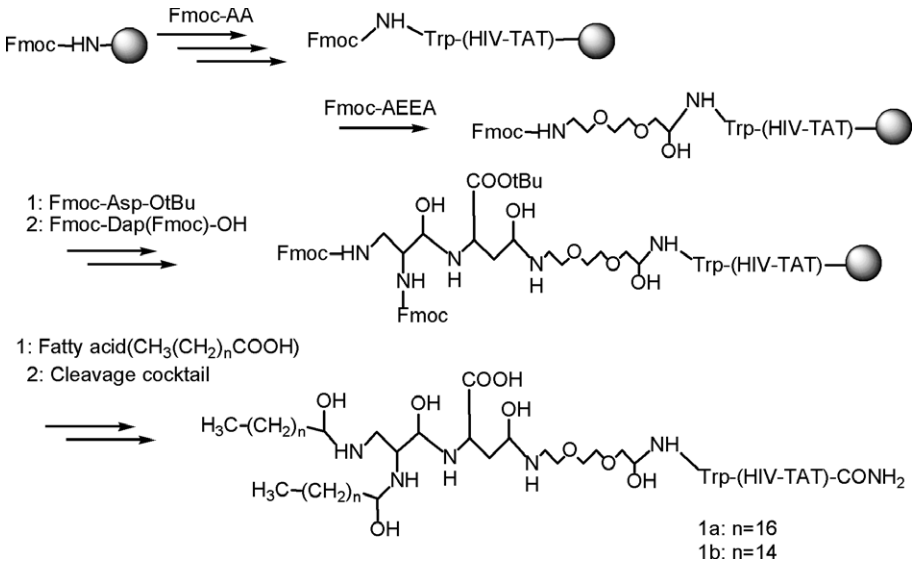
linker domain (to avoid the steric hindrance of liposome).

In this study, we employed the 11-mer peptide sequence (abbreviated as HIV-TAT: Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) of the TAT sequence[7] found in HIV-1 as a functional ligand connected to a lipid analog. The HIV-TAT peptide is well documented as a protein transduction domain, and it enables delivery of such entities as macromolecules, nucleic acids, and liposome into living cells.[8–10] Therefore, we have examined here the suitability of the present lipopeptide for preparation of functionalized liposomes.

Lipopeptide (**1a** and **1b**) conjugated with HIV-TAT peptide was synthesized on TGS RAM resin (15.0 μmol, Shimadzu Co.) by the Fmoc solid-phase synthesis method using an automatic peptide synthesizer (Shimadzu PSSM-8 Peptide Synthesizer Simultaneous Multiple) (Scheme 1). Tryptophan residue was added at the N-terminus of HIV-TAT peptide as a fluorescence probe. Then, Fmoc-AEEA (9-fluorenylmethoxycarbonyl-8-amino-3,6-dioxaoctanoic acid, linker domain),[11] Fmoc-Asp-*O*-*t*-Bu (hydrophilic domain), and Fmoc-Dap(Fmoc)-OH (glycero mimic domain) were coupled sequentially. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), *N*-hydroxybenzotriazole (HOBt), and *N*-methylmorpholine (NMM) were used, respectively, for the peptide coupling reaction with 1.0, 1.0, and 1.5 equivalents based on amino acids. Fmoc amino acid and alkyl chain were used in 7 and 5 excess equivalents, respectively. The coupling reactions were carried out for 30 min. Since the last condensation reaction with stearic or palmitic acid (anchor domain) did not proceed satisfactorily on a machine due to their insolubility in DMF, the reaction was carried out in a manual mode with checking of the reaction progress by a ninhydrin test. De-protection and cleavage of resin were accomplished with a cleavage cocktail (10 mg/mL of 2-methylindole containing TFA (trifluoroacetic acid)/H_2O/thioanisole/1,2-ethanedithiol/ethylmethyl sulfide/phenol = 82.5/5/3/2/3) for 16 h at room temperature, and **1** was then precipitated by adding a large amount of ether (Scheme 1).

In the case of stearic acid, CN-HPLC (Cyano-column used for high performance liquid chromatography)[12] analysis showed the main peak (retention time at 10.0 min) accompanied by the existence of some impurities (2.1 and 8.4 min, Supplemental Fig. 1b). These peaks were fractionated by HPLC, and analyzed by MALDI-TOFMS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry, ABI Voyager-DETM STR), giving an exact mass of *m*/*z* 2091, 2357 (Supplemantal Fig. 2a and 2b), and 2623 (Supplemental Fig. 1a),[13] which correspond to diamine, mono-acylated product, and the desired products **1a**, respectively (Supplemental Figs. 1 and 2). In contrast, the synthesis of conjugated lipopeptide attached with palmitoyl chain (**1b**) as anchor domain proceeded very smoothly.[14] HPLC analysis showed almost one peak, and MALDI-TOFMS showed an exact mass of *m*/*z* 2568 $M+H^+$ (Fig. 2a and b). In addition, ^1H NMR analysis (JEOL JMN-AL400) also showed the structure of **1b**; for example, the molar ratio of the TAT-peptide and palmitoyl moiety showed the correct proton ratio of the ortho position on the tyrosine residue (2H as a characteristic signal of the peptide at 6.62 ppm) to the methyl signal at 0.84 ppm in the alkyl chain end (6H as characteristic signal of lipid) using an integration value of ^1H NMR spectra (Fig. 2c). The overall yield of **1b** was greater than 80% based on the molar ratio of the amino group on TGS-RAM resin.

The liposome containing **1b** was then prepared. Compound **1b** was mixed with egg phosphatidylcholine (11:89 molar ratios, 13 mmol) in chloroform, and lipid thin film was formed by evaporation with recovery flask and then dried in vacuo. Calcium- and magnesium-free phosphate-buffered saline was added to lipid thin film at a lipid concentration of 5 mg/mL, and suspended by



Scheme 1. Synthesis of the HIV-TAT peptide conjugated lipid analog.

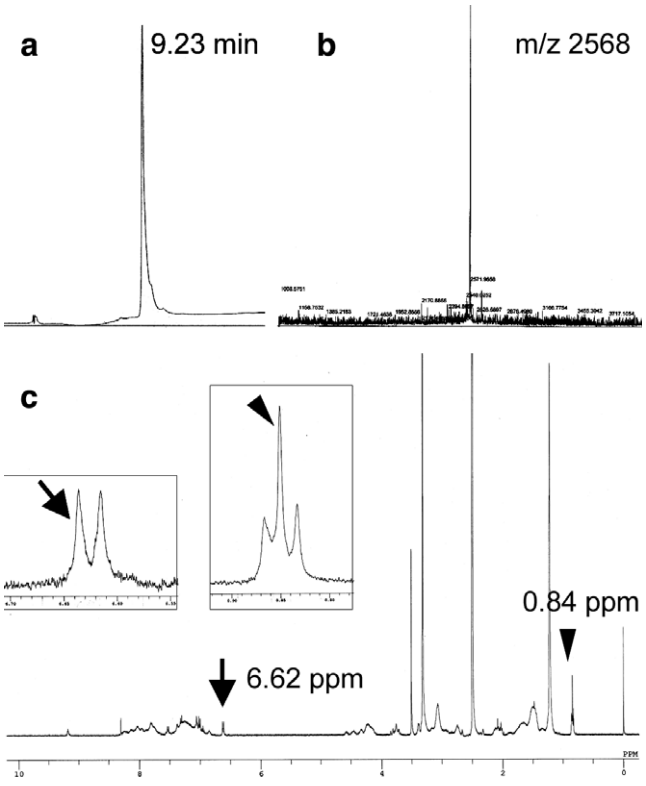


Figure 2. Identification of HIV-TAT peptide conjugated lipid analog **1b**. (a) MALDI-TOFMS, (b) CN-HPLC, (c) 1H NMR, arrowhead and arrow indicate methyl signal in alkyl chain and ortho position on tyrosine, respectively.

a vortex mixer. The lipid suspension was extruded through polycarbonate membrane (100 nm pore, Track-Etch membrane, Nuleopore) 21 times using an extruder (LiposoFast-basic, Avestin, Inc.), yielding the peptide-modified liposome. The liposome showed 107 nm diameter with monodispersion (polydispersity index = 0.07) and +15.8 mV of zeta-potential by dynamic light scattering measurement (Zetasizer Nano ZS, Malvern Instruments Ltd). The positive charge of the peptide-modified liposome indicated the presence of basic peptide HIV-TAT on the liposome surface.

Cellular uptake of peptide-modified liposome was examined. African green monkey kidney fibroblast-like cell line COS-7 (American type culture collection) was used, and the liposome was labeled with 3 mol% of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE). The liposome was dispersed in DMEM containing 10% FCS at 8.66 nmol/mL lipid concentration and then added to cultured COS-7 cells. After incubation for 1 h at 37 °C, the cells were inspected by confocal microscopy. Only the cells incubated with HIV-TAT peptide-modified liposome exhibited remarkable fluorescence, and neither control liposome nor untreated COS-7gave a positive indication (Fig. 3).

It has previously been reported that liposomes modified with basic oligomer peptide are incorporated into living cells through endocytosis and/or macropinocytosis.[4] In the present study, fluorescence was scattered in COS-7 cells, suggesting that liposomes are incorporated into membranous organelles by basic peptide HIV-TAT mediated cellular uptake.

In conclusion, a feasible and efficient method of novel lipid analogs has been developed for functionalized liposomes. Actually, these conjugated molecules with peptide and lipid are easily incorporated into a lipid bilayer

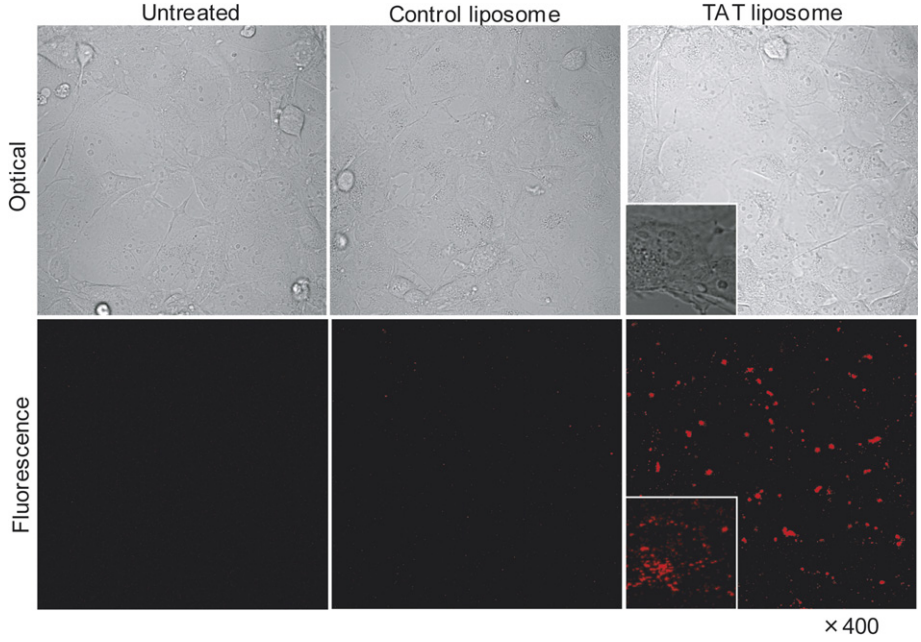


Figure 3. Microscopic images of cellular uptake of Rh-PE labeled TAT-modified liposome; 8.66 nmol/mL lipid concentration and 1 h incubation. Upper panel, optical image; lower panel, fluorescence image. Left, Untreated COS-7 cells; middle, incubated with Rh-labeled non-modified liposome; right, Rh-labeled HIV-TAT-modified liposome. Cells were cultured on thin glass. Liposome treated cells washed and directly observed in PBS using Carl ZEISS LSM510 MATE confocal laser microscopy equipped with 543 nm laser.

by simple mixing with phospholipids, and they robustly deliver liposomes into living cell. This method enables us to generate functionalized liposomes with arbitrary numbers and arbitrary sequences of peptide. Attitude optimization of the peptide moiety will regulate liposome function in a time- and environment-dependent manner. We consider that this method could be applicable to gene delivery, a tool of molecular biology, and targeted DDS.

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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.2007.02.001.

References and notes

1. Krown, S.; Northfelt, D.; Osoba, D.; Stewart, J. *Semin. oncol.* **2004**, *31*, 36.
2. Iyer, A. K.; Khaled, G.; Fang, J.; Maeda, H. *Drug Discovery Today* **2006**, *11*, 812.
3. Allen, T. M. *Nat. Rev. Cancer* **2002**, *2*, 750.
4. Khalil, I. A.; Kogure, K.; Futaki, S.; Harashima, H. *J. Biol. Chem.* **2006**, *281*, 3544.
5. Oku, N.; Asai, T.; Watanabe, K.; Kuromi, K.; Nagatsuka, M.; Kurohane, K.; Kikkawa, H.; Ogino, K.; Tanaka, M.; Ishikawa, D.; Tsukada, H.; Momose, M.; Nakayama, J.; Taki, T. *Oncogene* **2002**, *21*, 2662.
6. Gregoriadis, G.; Florence, A.; Patel, H. *Liposome in Drug Delivery*; Harwood Academic Publishers: NJ, 1993, pp 227–238.
7. Green, M.; Loewenstein, P. M. *Cell* **1998**, *55*, 1179.
8. Torchilin, V. P. *Ann. Rev. Biomed. Eng.* **2006**, *8*, 343.
9. Torchilin, V. P.; Levchenko, T. S.; Rammohan, R.; Volodina, N.; Papahadjopoulous-Sternberg, B.; D'Souza, G. G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1972.
10. Fretz, M. M.; Koning, G. A.; Mastrobattista, E.; Jiskoot, W.; Storm, G. *Biochim. Biophys. Acta* **2004**, *1665*, 48.
11. Koskinen, A. M. P.; Valo, T.; Vihavainen, S.; Hakala, J. M. L. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 573.
12. HPLC coditions; Colum, CAPCELL PAK UG CN (SHISEIDO) 4.6×150 mm; Gradient, 0.1%TFA H_2O/0.1%TFA CH_3CN from 100:0 to 0:100; Detection, 280 nm; Flow rate, 1.0 mL/min.
13. MALDI-TOFMS of **1** was performed in linear mode at 20k acceleration voltage using sinapic acid as matrix. For ionization, a nitrogen laser (337 nm, 0.5 ns pulse width, 3 Hz, 25 times/spectrum) was used. All spectra were measured using external calibration.
14. The undesired side products were resulted with C18 simply due to their solubility differences in DMF during the condensation reaction.