Novel Amphiphilic Probes for [^{18}F]-Radiolabeling Preformed Liposomes and Determination of Liposomal Trafficking by Positron Emission Tomography

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Abstract: Positron-emission tomography (PET) is a noninvasive real-time functional imaging system and is expected to be useful for the development of new drug candidates in clinical trials. For its application with preformulated liposomes, we devised an optimized [^{18}F]-compound and developed a direct liposome modification method that we termed the "solid-phase transition method". We were successful in using 1-[^{18}F]fluoro-3,6-dioxatetracosane ([^{18}F]**7a**) for in vivo trafficking of liposomes. This method might be a useful tool in preclinical and clinical studies of lipidic particle-related drugs.

Liposomes can be used as multipurpose drug carriers in a wide range of applications. Liposomes have been used as one of the most ideal drug carriers for anticancer agents, antifungal antibiotics, photosensitizers, nucleic acid derivatives for gene therapy, and so on.[1–4] From the viewpoint of drug delivery systems (DDSs[a]) the pharmacokinetics, pharmacodynamics, and pharmacotoxics of such drugs have been improved through the formulation of liposomes or other kinds of lipidic particles such as lipid complexes and microspheres. To use liposomes as drug carriers, it is necessary to consider many properties of liposomal formulations, e.g., lipid composition, vesicular size, surface electrostatic potential, and functional modification, that influence independently or mutually the pharmacological characteristics of the liposomes.[5] Comprehensive evaluation of liposomes in the living body is therefore important for the development of liposomal DDS drugs and in DDS studies. For evaluation of the pharmacokinetics of liposomes in vivo, noninvasive real-time imaging of liposomes is one of the ideal techniques.

Positron emission tomography is a noninvasive technique and has been used for the clinical functional diagnosis in many applications related to oncology, neurology, cardiology, psychiatry, and so on.[6,7] Also, this technique can be applied in preclinical studies. Once a positron-labeled candidate drug is injected into animals, the distribution of the drug in the body, the tissues in which it becomes concentrated, and its eventual elimination can be monitored far more quickly and cost effectively than by the older invasive techniques of killing and dissecting the animals to obtain similar information.[8] In this study, we applied this idea to DDS drugs.

We previously reported the methodology for detecting noninvasive liposomal trafficking by PET.[9,10] In those studies, we used a water-soluble compound, [2-^{18}F]2-deoxy-2-fluoroglucose ([^{18}F]FDG), encapsulated inside the vesicles. To encapsulate the [^{18}F]FDG, we restructured the lipid bilayer by repeating freeze–thaw cycles. This method was not sophisticated in terms of efficiency of radiolabeling and prevention of occupational irradiation. Recently, Marik et al.[11] synthesized a radiolabeled amphiphilic compound for determining liposomal distribution by PET, although their method is not applicable to preformed liposomes.

In the present study, we developed not only novel [^{18}F]-positron-labeled compounds for liposomal labeling but also a new universal methodology for rapid and one-step labeling of preformed liposomes. Potent compounds were selected from a diversity of synthesized amphiphilic compounds through a series of in vitro nonradioisotope (non-RI) and RI screening studies. On the basis of the results of these studies, the structures of [^{18}F]-labeled compounds were optimized. By use of these compounds, liposomes were [^{18}F]-labeled with high efficiency and liposomal trafficking in mice was visualized by real-time analysis using a planar positron imaging system (PPIS).

To conduct the experiments for a preliminary screening of liposome-labeling compounds (vide infra), we designed novel amphiphilic compounds (**2aA**−**2cE**) and prepared them as follows: Sharma's Yb(OTf)$_3$-catalyzed etherification method[12] was applied to the coupling of the known benzyl alcohol **1a**[13] having a lipophilic *n*-octyl group to commercial diethylene glycol. The reaction proceeded gradually at 50 °C to afford **2aA**. Commercial poly(ethylene glycol)s (PEGs) with average molecular weights of 200, 285–315, 380–420, and 850–950 were used for preparation of **2aB**, **2aC**, **2aD**, and **2aE**, respectively. In these cases, the reactions proceeded at ambient temperature. Whereas **2aB** was obtained as a single compound having four PEG units after purification via SiO_2 chromatography, others were mixtures of several analogues having different lengths of the PEG chain, and the average number of its PEG units was indicated by "*m*". Similarly, **2bA**−**2cE** were synthesized by the reaction of **1a**,**b**[13] with diethylene glycol or the corresponding PEG.

The toluene sulfonates (**3bA**−**3bC** and **3cA**) were obtained by reactions of the sodium alkoxides derived from the corresponding **2** with TsCl.[14] Purification of crude products via SiO_2 chromatography afforded **3bA**−**3bC** and **3cA**, each as a single compound. In the same manner, the toluene sulfonates (**6a** and **6b**) without the aromatic group were prepared from commercially available **5a**,**b**. The fluorinated compounds (**4bA**−**4bC**, **4cA**, **7a**, and **7b**) were prepared as references for the [^{18}F]-labeled compounds ([^{18}F]**4** and [^{18}F]**7**) by the direct fluorination of the corresponding alcohols (**2** and **5**) with (diethylamino)sulfur trifluoride (DAST, Scheme 1).[15] The preparation of the [^{18}F]-labeled compounds ([^{18}F]**4bA**−**4bC**, [^{18}F]**4cA**, [^{18}F]**7a**, and [^{18}F]**7b**) was effectively achieved via nucleophilic substitution of the corresponding toluene sulfonates (**3** and **6**) with [^{18}F]KF/K[2.2.2] obtained by the previously reported method[16] with minor modifications (Scheme 2).

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[a]Abbreviations: PET, positron emission tomography; DDS, drug delivery systems; FDG, 2-deoxy-2-fluoroglucose; RI, radioisotope; PEG, poly(ethylene glycol); DAST, (diethylamino)sulfur trifluoride; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum; HDL, high-density lipoprotein; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; PPIS, planar positron imaging system.

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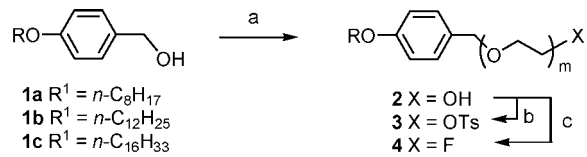
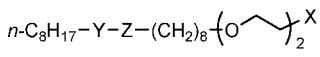
Scheme 1[a]



2aA R = n-C_8H_{17}, m = 2, X = OH
2aB R = n-C_8H_{17}, m = 4, X = OH
2aC R = n-C_8H_{17}, m = 7, X = OH
2aD R = n-C_8H_{17}, m = 9, X = OH
2aE R = n-C_8H_{17}, m = 20, X = OH
2bA R = n-$C_{12}H_{25}$, m = 2, X = OH
2bB R = n-$C_{12}H_{25}$, m = 4, X = OH
2bC R = n-$C_{12}H_{25}$, m = 7, X = OH
2bD R = n-$C_{12}H_{25}$, m = 9, X = OH
2bE R = n-$C_{12}H_{25}$, m = 20, X = OH
2cA R = n-$C_{16}H_{33}$, m = 2, X = OH
2cB R = n-$C_{16}H_{33}$, m = 4, X = OH
2cC R = n-$C_{16}H_{33}$, m = 7, X = OH
2cD R = n-$C_{16}H_{33}$, m = 9, X = OH
2cE R = n-$C_{16}H_{33}$, m = 20, X = OH

3bA R = n-$C_{12}H_{25}$, m = 2, X = OTs
3bB R = n-$C_{12}H_{25}$, m = 4, X = OTs
3bC R = n-$C_{12}H_{25}$, m = 7, X = OTs
3cA R = n-$C_{16}H_{33}$, m = 2, X = OTs
4bA R = n-$C_{12}H_{25}$, m = 2, X = F
4bB R = n-$C_{12}H_{25}$, m = 4, X = F
4bC R = n-$C_{12}H_{25}$, m = 7, X = F
4cA R = n-$C_{16}H_{33}$, m = 2, X = F

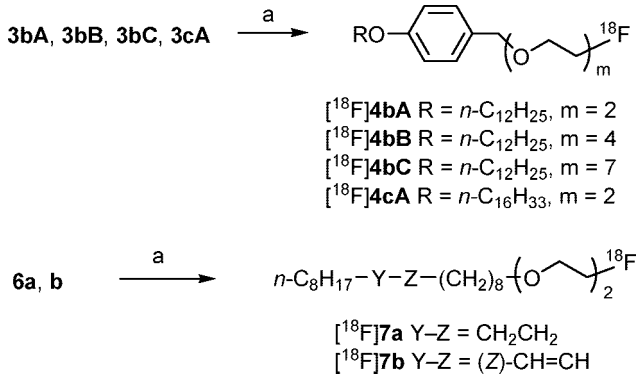


5a Y–Z = CH_2CH_2, X = OH
6a Y–Z = CH_2CH_2, X = OTs
7a Y–Z = CH_2CH_2, X = F
5b Y–Z = (*Z*)-CH=CH, X = OH
6b Y–Z = (*Z*)-CH=CH, X = OTs
7b Y–Z = (*Z*)-CH=CH, X = F

b, c

[a] Reagents and conditions: (a) $H(OCH_2CH_2)_mOH$, $Yb(OTf)_3$, $ClCH_2$-CH_2Cl, 50 °C (for **2A**), room temp (for **2B**–**E**), 10–50% yields; (b) NaH, THF, 0 °C to room temp, then TsCl, room temp, 40–70% yields; (c) DAST, CH_2Cl_2, 0 °C to room temp, 10–35% yields.

Scheme 2[a]



[a] Reagents and conditions: (a) [^{18}F]KF/K[2,2,2], MeCN, reflux, 10 min.

In this study, we developed a new method of liposomal labeling named the "solid-phase transition method" and determined the incorporation efficiency of the diverse nonradiolabeled compounds (**2aA**−**2cE**). At first, the amphiphilic compounds (**2aA**−**2cE**) were dried to make a thin film. Liposomes were added to the solvent-free compound (100:1 as the molar ratio of lipids to compounds) and incubated at 65 °C for 15 min. Then the liposomal solutions were transferred to centrifuge tubes, and the compounds not incorporated were removed by an ultracentrifugation. Compounds in the liposomal fraction (pellet), supernatant, and residue were quantified by HPLC. Figure 1 shows the incorporation efficiency of the various compounds. In the case of some compounds such as **2aD** and **2aE** with a shorter aliphatic hydrocarbon chain and a longer PEG chain, the incorporation efficiency was decreased. The

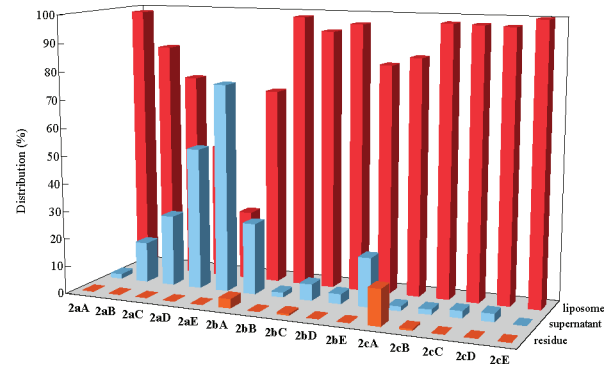


Figure 1. Efficiency of incorporation of novel amphiphilic compounds into liposomes. Incorporation experiments were performed by the solid-phase transition method, and the amount of compound in each fraction (liposome, supernatant, and residue) was determined by HPLC.

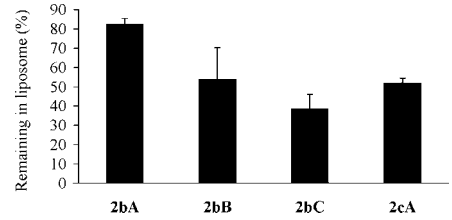


Figure 2. Stability of modified liposomes. Liposomes modified with nonradiolabeled amphiphilic compounds were incubated for 1 h in the presence of FBS, and the amounts of compounds in the liposome were determined. Data represent the mean ± standard deviation (n = 4).

analysis of particle size in water (data not shown) led us to consider that these compounds had formed micelles.

On the basis of the above-mentioned experiments, we selected four compounds (**2bA**–**2bC**, and **2cA**) that had relatively high incorporation efficiency and different physicochemical properties and examined the stability of incorporation of these compounds in the presence of serum. Liposomes were incubated at 37 °C for 1 h in the presence of 50% fetal bovine serum (FBS). It is well-known that amphiphilic compounds associated with the lipid bilayer of liposomes via weak hydrophobic interactions are transferred from liposome to serum, and that phenomenon is due mainly to high-density lipoprotein (HDL) in the serum. Liposomes and other components including serum lipoproteins were fractionated by gel filtration chromatography, and the amount of compounds that remained in the liposomal fraction was determined. Strikingly, there were significant differences among the compounds in terms of stability in serum (Figure 2), and a structure–activity relationship was indicated.

Next, the practically useful compounds 1-[^{18}F]fluoro-3,6-dioxatetracosane ([^{18}F]**7a**) and (*Z*)-1-[^{18}F]fluoro-3,6-dioxatetracos-15-ene ([^{18}F]**7b**) were prepared, and the efficiency of liposomal labeling using these [^{18}F]-labeled compounds was determined. Since the [^{18}F]-labeled compounds possessed very high radioactivity, only a very small amount of compound was needed for liposomal labeling in comparison with the amount of nonradiolabeled compounds used in the non-RI experiment mentioned above. Therefore, it is possible to conclude a difference in labeling efficiency between a trace amount of radiolabeled compounds and UV detectable non-RI compounds by use of the "solid-phase transition method". Figure 3 indicates the labeling efficiency of [^{18}F]-labeled compounds (**4bA**−**4bC**, **4cA**, **7a**, and **7b**). There was no significant difference between it and that of the corresponding nonradiolabeled compounds.

We then examined the stability of [^{18}F]-labeled compounds. Figure 4 indicates that there was no significant difference in

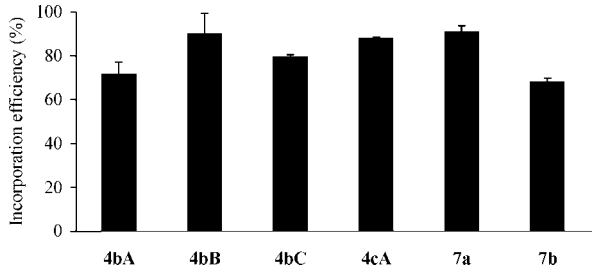


Figure 3. Incorporation efficiency of [^{18}F]-radiolabeled amphiphilic compound. Incorporation experiment with [^{18}F]-radiolabeled probe was performed by the solid-phase transition method. The incorporation efficiency was determined by measuring γ-ray radioactivity. The data represent the mean ± standard deviation ($n = 4$).

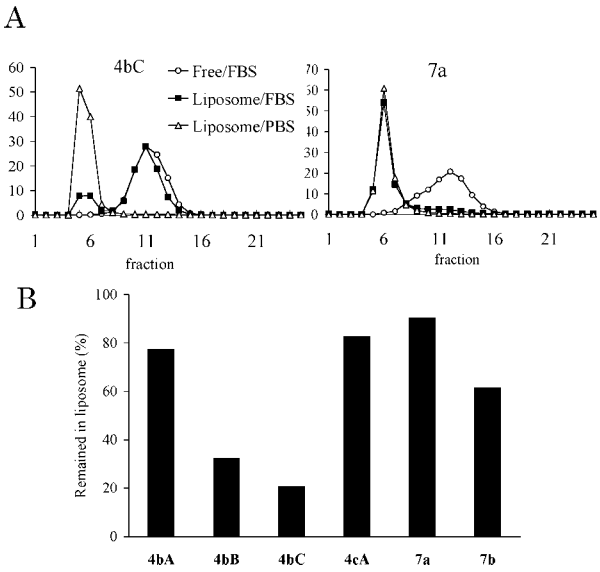


Figure 4. Stability in the serum of liposomes modified with [^{18}F]-radiolabeled amphiphilic compounds. Liposomes modified with [^{18}F]-radiolabeled amphiphilic compounds were incubated in the presence or absence of FBS for 30 min. The reactants were fractionated by gel filtration chromatography, and the radioactivity of each fraction was measured. (A) Typical elution patterns are shown: liposome fractions 5–9; serum lipoprotein fractions 9–16. (B) The radioactivity in liposome fractions is shown.

stability in liposomes between non-RI and RI compounds. These results proved the feasibility of screening with nonradiolabeled compounds to obtain appropriate radiolabeled compounds. Interestingly enough, although there is only one difference in the structures between [^{18}F]**7a** and [^{18}F]**7b**, which is the presence of an unsaturated bond in the aliphatic hydrocarbon chain of the latter, the stability of [^{18}F]**7a** in serum was much better. Therefore, we selected [^{18}F]**7a** as the probe with the most potential for in vivo imaging of liposomes.

It is well-known that one of the most important elements in trafficking of liposomes in vivo is the diameter of the particles. Larger-sized liposomes are quickly trapped by the reticuloendothelial system (RES) in the liver and spleen. To demonstrate the usefulness of [^{18}F]**7a**, we examined the typical in vivo behavior of differently sized liposomes. We prepared ^{18}F-labeled liposomes with three different diameters, namely, 90, 170, and 570 nm, as well as the liposome-free compound dispersed in 1% DMSO/PBS, and injected them into mice via a tail vein. Whole-body imaging of [^{18}F]**7a** in normal mice was obtained by using PPIS. Real-time imaging from PPIS showed that the larger the liposomes, the more the liposomes were trapped and accumulated in the spleen (Figure 5A). These imaging results

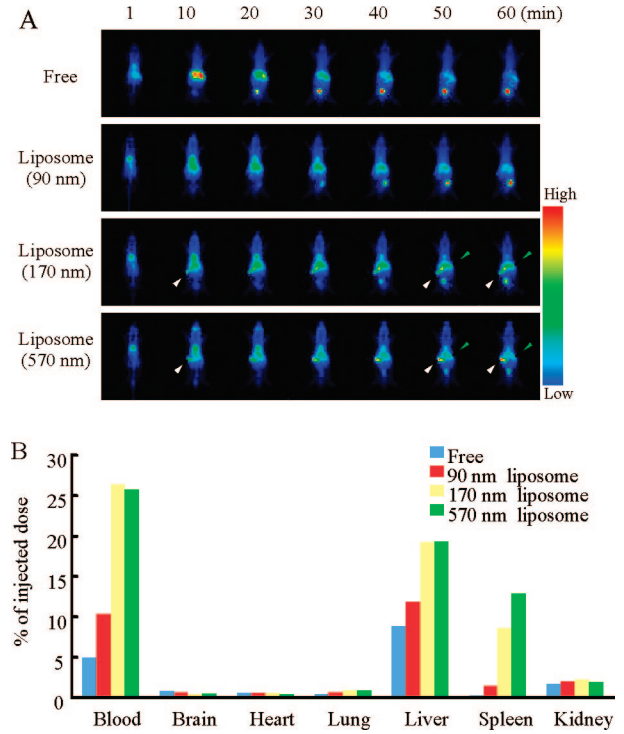


Figure 5. Whole-body imaging of [^{18}F]**7a** and liposome labeled with [^{18}F]**7a** in BALB/c mice by use of PPIS. Each radiolabeled sample at a dose of 2.5 MBq was injected via a tail vein. (A) Data were acquired with a 1 min frame at 1, 10, 20, 30, 40, 50, and 60 min after injection. Liposomes with diameters of 170 and 570 nm were accumulated in spleen (white arrowheads) and liver (green arrowheads). (B) Shown is the biodistribution in BALB/c mice after intravenous injection of [^{18}F]**7a** or liposomes labeled with [^{18}F]**7a**.

were also supported by the data from radioactivity at autopsy (Figure 5B). In contrast, the free compound accumulated in the kidneys immediately after injection and was then excreted in the urine. This result reflected the nature of liposomes. These results suggest that this novel methodology of noninvasive real-time whole-body imaging enables us to visualize the behavior of various kinds of liposomes or lipidic particles such as lipid complexes and lipid microspheres. Also, this technique may be applied to larger animal models and three-dimensional imaging provided by PET. In the future, it may be possible to use this technique for the detection of liposomal behavior in preclinical and clinical studies. Moreover, when lipidic particles specifically targeted to disease sites are developed, these particles will be useful not only for drug delivery but also for diagnostic imaging of the sites by use of the present technology.

In summary, here we introduced novel [^{18}F]-labeled amphiphilic compounds for liposomal modification that were highly incorporated into liposomes and stable in serum. This universal method of liposomal modification, i.e., the solid-phase transition method, can be used for various kinds of liposomes and lipidic particles. The compounds are useful as PET probes for imaging liposomal trafficking in the living body. An in vivo study with PPIS revealed the feasibility of the developed ^{18}F-probe. These findings suggest that [^{18}F]**7a** is a promising PET probe for imaging of liposomal behavior. Furthermore, only one or a few kinds of ^{18}F-probe can be applied to various kinds of liposomes and lipidic particles. Further study should clarify the usefulness and utility of the current probes and methodology in preclinical and clinical studies on lipidic particle-based DDS drugs.

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Supporting Information Available: Experimental procedures and characterization data for all new compounds; details of incorporation experiments, in vitro stability assays using non-RI and RI compounds, and mice positron imaging studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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