Preparation and Pharmaceutical Evaluation of Liposomes Entrapping Salicylic Acid/γ-Cyclodextrin Conjugate

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To evaluate the potential use of a drug/cyclodextrin (CyD) conjugate for efficient entrapment in liposomes and prolonged residence of a drug in tissues, we synthesized a salicylic acid (SA) conjugate bound covalently with γ-cyclodextrin (SA/γ-CyD conjugate), a model drug/CyD conjugate, and then liposomes entrapping the conjugate (conjugate-in-liposome) were prepared by a freezing-thawing method. The chemical and physicochemical properties of the SA/γ-CyD conjugate in solution and solid state were investigated and then the physicochemical properties of conjugate-in-liposome, *in vitro* cellular uptake/release and *in vivo* disposition of SA/γ-CyD conjugate after intravenous administration of aqueous suspension containing conjugate-in-liposome in rats, were evaluated, comparing with those of the liposome-entrapped SA alone (SA-in-liposome) or the liposome-entrapped noncovalent SA/γ-CyD complex (complex-in-liposome). As a result, it was found that the conjugate was amorphous powder and the release of SA from the conjugate in phosphate-buffered saline (PBS) was tolerated to chemical and enzymatic degradation. Meanwhile, the particle sizes and stability of these liposomes were almost identical, and the entrapment ratio of SA/γ-CyD conjugate in liposomes was higher than those of SA alone and SA/γ-CyD complex. The cellular uptake of these liposomes was almost equivalent, but the release of SA/γ-CyD conjugate from RAW264.7 cells was markedly slower, compared with that of SA from cells following cellular uptake of the SA-in-liposome and complex-in-liposome. The disposition of SA or SA/γ-CyD conjugate following intravenous administration of aqueous suspensions containing each liposome system in rats was comparable, but the residence time of the conjugate in tissues significantly prolonged, compared with that of the SA-in-liposome and complex-in-liposome systems. These results suggest the potential use of SA/γ-CyD conjugate for efficient entrapment in liposomes as well as of liposomes containing SA/γ-CyD conjugates for prolonged residence of drugs in tissues.

Key words cyclodextrin; conjugation; salicylic acid; liposome; complex; residence time

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of 6—8 glucose units through α-1,4-glycosidic bonds and have been utilized as carriers for improvement of pharmaceutical properties such as solubility, stability and bioavailability.[1] The versatility of liposomes, such as encapsulation of hydrophilic/hydrophobic drugs, non-toxic nature, biodegradability, biocompatibility and sustained-release property, makes them attractive candidates for the delivery of anticancer drugs, proteins and genes.[2,3]

It has been widely reported that CyDs are immediately eliminated in urine following intravenous administration of their solutions in several animals and human, and CyD complexes with various drugs generally show the similar disposition behavior to CyDs themselves as well.[4—7] These pharmacokinetic properties would impair therapeutic efficacy for anti-tumor or antifungal drugs in the target cells or tissues.[8,9] On the other hand, the potential use of liposomes containing inclusion complexes of poorly water-soluble drugs with CyDs has been reported for improvement of the pharmacokinetic behavior and therapeutic efficacy of drugs. For instance, McCormack *et al.* demonstrated that liposomes are capable of entrapping both drugs and CyD while remaining stable and without disruption of liposomes.[10,11] Also, Fatouros *et al.* reported that the high entrapment of prednisolone (80% of the starting material) in phosphatidylcholine/cholesterol (CH) liposomes is achieved when the drug is used as the inclusion complex with 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD).[12] However, these still remain problems in the combination of liposomes and α- or β-CyD complexes such as rapid releases of drugs from liposomes due to the disruptive property of these CyDs against liposome membranes.[13]

Recently, many attempts have been made to improve physicochemical properties and pharmacokinetic behaviors of drugs using the CyD conjugation technique.[14,15] For example, we reported the potential use of the CyD conjugates with 4-biphenylyl acetic acid, prednisolone and *n*-butylic acid as colon targeting prodrugs.[14,16—18] In the present study, we chose salicylic acid (SA) as a model drug because it is capable of combining with γ-CyD easily. Likewise, we chose γ-CyD among CyDs because of its low degree of interaction with phospholipids and CH in liposome membranes, its high solubility in water (23.2% w/v at 25 °C) and slight hemolytic activity (the concentration to induce 50% lysis of rabbit erythrocytes is 36.7 mM), compared to α- and β-CyDs.[19,20] To evaluate the potential use of SA/CyD conjugate for efficient entrapment in liposomes and prolonged residence of SA in tissues, we prepared SA/γ-CyD conjugate (Fig. 1) and its chemical and physicochemical properties were investigated. Then, the conjugate was encapsulated in liposomes (conjugate-in-liposome) using a freezing-thawing method,[21] and the physicochemical properties of conjugate-in-liposome, *in*

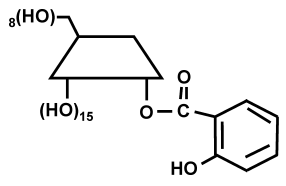

Fig. 1. Chemical Structure of SA/γ-CyD Conjugate

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vitro cellular uptake/release and *in vivo* disposition of SA/γ-CyD conjugate after intravenous administration of aqueous suspension containing conjugate-in-liposome in rats were evaluated, comparing with those of the liposome-entrapped SA alone (SA-in-liposome) or the liposome-entrapped non-covalent SA/γ-CyD complex (complex-in-liposome).

Experimental

Materials Egg yolk phosphatidylcholine (EPC), CH, carbonyldiimidazole (CDI), triethylamine (TEA) and SA were obtained from Nacalai Tesque (Kyoto, Japan). γ-CyD was donated from Nihon Shokuhin Kako (Tokyo, Japan). RPMI-1640 culture medium and fetal calf serum (FCS) were obtained from Nissui Pharmaceutical (Tokyo, Japan) and JRH Biosciences (Renexa, KS, U.S.A.), respectively. [^{14}C]SA (40—60 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Visking tube (diameter 28.6 mm) was purchased from Nihon Medical Science (Osaka, Japan). Other chemicals and solvents were of analytical reagent grade.

Analytical Apparatus ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were taken on a Jeol A-500 spectrometer (Tokyo, Japan) at 25 °C. Fast atom bombardment (FAB) mass spectra were recorded on a Jeol JMS-DX 303 mass spectrometer in a negative mode using a matrix of triethanolamine/dimethyl sulfoxide (DMSO).

Preparation of SA/γ-CyD Conjugate SA/γ-CyD conjugate was prepared according to the method of McLeod *et al.*[22] SA (0.1036 g, 0.75 mmol) and CDI (0.179 g) were dissolved in 3 ml of DMSO, and the mixture was stirred at room temperature for 30 min. After adding γ-CyD (0.6485 g, 0.5 mmol) and TEA (4 ml), the mixture was stirred at room temperature for 18 h. Approximately, 200 ml of acetone was added to the reaction solution, and the precipitate was collected under reduced pressure. The precipitate was purified by ion-exchange column chromatography (Diaion™ HP-20, Mitsubishi Chemical, Tokyo, Japan) and the resulting conjugate was eluted with methanol/water with increasing methanol content. The eluents were monitored by thin layer chromatography (TLC), and the conjugate appeared in the eluents of 30—50% methanol in water. After methanol was removed under reduced pressure, the solution was lyophilized to yield 0.34 g (24%) of SA/γ-CyD conjugate. *Rf* value=0.75 (TLC: silica gel 60F_{254} (Merck, Darmstadt, Germany); ethyl acetate–2-propanol–ammonium hydroxide–water (7 : 7 : 5 : 4, v/v); indicator *p*-anisaldehyde; fast-atom bombardment (FAB)-Mass (*m*/*z*): $[M-H]^-$ was 1416; ^{1}H-NMR spectra (500 MHz, DMSO-d_6): δ (ppm) 7.93—7.91 ppm (d, 1H, SA, 6′-H), 7.59—7.54 ppm (m, 1H, SA, 4′-H), 7.02—6.99 ppm (m, 2H, SA, 3′-H, 5′-H), 4.98—4.88 ppm (m, 8H, CyD, 1-H), 3.68—3.67 ppm (m, 24H, CyD, 3-H, 5-H, 6-H), 3.65 ppm (m, 8H, CyD, 4-H), 3.40—3.37 ppm (m, 8H, CyD, 2-H), 2.50 ppm (DMSO-d_6). ^{13}C-NMR spectra (125.7 MHz, DMSO-d_6): δ (ppm) 169.05 ppm (C=O), 160.30—160.19 ppm (SA 2-C), 135.85—113.21 ppm (SA 1,3,4,5,6-C), 101.97—101.10 ppm (CyD 1-C, unsubstituted), 97.35 ppm (CyD 1′-C, substituted), 81.30—80.50 ppm (CyD 4-C), 76.51—75.89 ppm (CyD 2′-C, substituted), 73.94—72.11 ppm (CyD 2,3,5-C, unsubstituted), 71.53—71.16 ppm (CyD 3′-C, substituted), 60.04 ppm (CyD 6-C). [^{14}C]SA/γ-CyD conjugate was prepared by the similar procedure in a lower scale than described above.

Physicochemical Properties of SA/γ-CyD Conjugate The solubilities of SA and SA/γ-CyD conjugate in water at 25 °C were determined by high-performance liquid chromatography (HPLC) under the following conditions: a PU-1580 pump and a UV-970 detector (Jasco, Tokyo, Japan), a AM312 ODS column (6.0×150 mm, YMC, Kyoto, Japan), a mobile phase of 0.1 M acetic acid/methanol (50 : 50, v/v), a flow rate of 1.0 ml/min, and a detection at 303 nm. The melting and/or decomposition points of SA/γ-CyD conjugate, SA and γ-CyD were determined by a micro-melting point apparatus (Yanaco, Kyoto, Japan). The powder X-ray diffractograms of SA/γ-CyD conjugate, SA, γ-CyD and their physical mixtures (SA and γ-CyD) were measured using a Rigaku Rint-2500 diffractometer (Rigaku, Tokyo, Japan) under the following conditions: Ni-filtered Cu$K\alpha$ radiation (1.542 Å), a voltage of 40 kV, a current of 40 mA, a divergent slit of 1.74 mm (1°), a scattering slit of 0.94 mm (1°), a receiving slit of 0.15 mm, and a goniometer angular increment of 1°/min.

Preparation of SA/γ-CyD Complex The solid inclusion complex of SA/γ-CyD in a molar ratio of 1 : 1 was prepared by the kneading method[23] using water. The formation of SA/γ-CyD complex in solution and solid state was confirmed by fluorescence spectroscopy and powder X-ray analysis, respectively. For the former experiment, the concentrations of SA and γ-CyD in 10 mM Tris–HCl-buffered saline (THBS, pH 7.4) were 5 mM and 1—10 mM, respectively. The latter experiment was performed as described above.

SA Release Behavior from SA/γ-CyD Conjugate The hydrolysis of SA/γ-CyD conjugate in phosphate buffered saline (PBS, pH 7.4) or in PBS (pH 7.4) supplemented with 80% (v/v) rat plasma was performed at an initial concentration of 4.0×10^{-5} M at 37 °C. In the case of hydrolysis in PBS, the reaction solution (300 μl) was taken at appropriate intervals, an aliquot (60 μl) of which was subjected to HPLC analysis of the conjugate and SA under aforementioned conditions. In the case of hydrolysis in 80% (v/v) plasma of rats, 300 μl of the reaction solution was ultra-filtrated using a membrane filter (Amicon Kit, Millipore, Tokyo, Japan) at appropriate intervals, and an aliquot (60 μl) of the filtrate was subjected to HPLC analysis under the conditions as described above.

Liposome Preparation Liposomes were prepared from EPC/CH (10 : 1—10 : 3, molar ratio) according to a freezing-thawing method.[21] Briefly, lipid mixtures were dissolved in chloroform, and the solvent was removed under reduced pressure by a rotary evaporator. Three milliliters of 10 mM THBS (pH 7.4) containing SA, SA–γ-CyD complex or SA–γ-CyD conjugate with a tracer amount (10 μCi) of [^{14}C]SA or [^{14}C]SA/γ-CyD conjugate were added to the resulting lipid film (28.6 μmol), and the suspensions were agitated for 5 min. Likewise, liposomes entrapping γ-CyD alone were prepared using the same procedure. Here we prepared 10 mM THBS (pH 7.4) having various concentrations of SA, SA–γ-CyD complex, SA–γ-CyD conjugate and γ-CyD to perform physicochemical, the *in-vitro* cellular uptake and *in-vivo* studies: when using in the physicochemical study and the liposomal stability study, the concentrations of SA, SA–γ-CyD complex, SA–γ-CyD conjugate and γ-CyD were 7.2 mM, 7.2 mM, 7.2 mM and 10.8 mM, when using in the *in-vitro* cellular uptake study, those were 1.1 mM, 1.1 mM, 0.15 mM and 1.6 mM, and when using the *in-vivo* study those were 18.1 mM, 18.1 mM, 2.4 mM and 27.0 mM, respectively. To prepare large unilamellar vesicles, the multilamellar vesicles were extruded by the extrusion method.[24] The vesicles were extruded through two stacked polycarbonate membranes (Nucleopore, Plesanton, U.S.A.) with pores of diameter 1 μm. The sample was subjected to 10 passes through the filter at 40 °C. The filtrates were extruded through the polycarbonate membranes (pore seize 0.4 μm) followed by extrusion through the membrane (pore size 0.1 μm) as described above. Liposome sizes were measured by a submicron particle analyzer N4 Plus (Beckman Coulter, Fullerton, CA, U.S.A.) at room temperature. After preparing liposome-entrapped SA (SA-in-liposome), non-entrapped substances were removed by the dialysis method using a visking tube. The entrapment ratios of SA or SA/γ-CyD conjugate were evaluated by determining radioactivity of [^{14}C]SA or [^{14}C]SA/γ-CyD conjugate and by calculating from the equation ($C/C_0\times100$ (%)) where C_0 and C are the concentration of SA or SA/γ-CyD conjugate before and after dialysis, respectively. Briefly, 50 μl of liposome-entrapped [^{14}C]SA or [^{14}C]SA/γ-CyD conjugate (166.7 nCi) was dissolved in 2 ml of scintillator (Hionic-Fluor™, Packard, Meriden, CT, U.S.A.) and measured radioactivity of [^{14}C]SA by an LSC-3500 liquid scintillation counter (Aloka, Tokyo, Japan). The entrapment ratio of γ-CyD was measured by anthrone-sulfuric acid method.[25] Briefly, 3 ml of anthrone reagent was added to 0.5 ml of the suspension containing liposome-entrapped γ-CyD. The tube was covered with a glass ball and was heated for 10 min in boiling water. After quenching with cold water, absorbance of the suspension was measured by a U-2000A spectrophotometer (Hitachi, Tokyo, Japan) at 620 nm.

Stability of Liposomes Stability of liposomes was monitored by determining the release of SA or SA/γ-CyD conjugate from liposomes in 10 mM THBS (pH 7.4) at 25 °C. In brief, 3 ml of the suspensions of liposomes containing SA, SA/γ-CyD complex or SA/γ-CyD conjugate prepared as described above were added to a visking tube and were incubated in 500 ml of 10 mM THBS (pH. 7.4) at 25 °C. At appropriate intervals, an aliquot of liposome suspension (60 μl) was taken from a visking tube, and then was subjected to HPLC analysis under the same conditions as described above.

Cellular Uptake and Release RAW264.7 cells, a mouse macrophage-like cell line, were used. The cells (3×10^6 cells/dish) were incubated for 3 h with 1.1 ml of RPMI-1640 culture medium supplemented with 10% FCS containing free SA, free SA/γ-CyD complex or free SA/γ-CyD conjugate or various liposomes-entrapped SA, SA/γ-CyD complex or SA/γ-CyD conjugate at the concentration of 1 μM SA or SA/γ-CyD conjugate (equivalent to [^{14}C]SA (3.3 nCi)) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. For cellular uptake experiments, after washing three times of cells with PBS (pH 2.5) to remove the cellular membrane-bound [^{14}C]SA or [^{14}C]SA/γ-CyD, the cells were lysed with 1 N NaOH, and then the amounts

of [^{14}C]SA and [^{14}C]SA/γ-CyD conjugate in the cells were determined by the liquid scintillation counter as described above. For release experiments, after washing of cells three times with PBS (pH 2.5), the cells were incubated with 1.1 ml of fresh medium supplemented with 10% FCS at 37 °C. At appropriate intervals, the amounts of [^{14}C]SA and [^{14}C]SA/γ-CyD conjugate released from the cells to the medium were determined as described above.

Pharmacokinetics of SA and Its Conjugate after Intravenous Administration in Rats Male Wistar rats (5 weeks old and weighing 140—150 g) were purchased from Kyudo (Tosu, Japan). Animal use and relevant experimental procedures were approved by the Kumamoto University Committee on Animal Care and Use of Laboratory Animals. The aqueous suspensions containing various liposomes were injected intravenously *via* the right jugular vein at a dose of 0.05 mg/kg of SA or SA/γ-CyD conjugate plus a tracer amount of [^{14}C]SA or [^{14}C]SA/γ-CyD conjugate (66.7 nCi/kg), respectively, to rats. At appropriate intervals following injection, blood was collected from the left jugular vein and various organs were also excised following phlebotomy. SA and SA/γ-CyD levels in plasma and organs were determined from their radioactivity. That is, 100 μl of plasma was dissolved in 5 ml of scintillator (Ultima Gold^{TM}, Packard, Meriden, CT, U.S.A.), whereas 100 mg of tissues was solubilized by 1 ml of tissue resolvent (Soluene^{TM}, Packard, Meriden, CT, U.S.A.) and then dissolved in 10 ml of scintillator (Hionic-Fluor^{TM}). Radioactivity of [^{14}C]SA or [^{14}C]SA/γ-CyD conjugate in these solutions was measured as described above. Pharmacokinetic parameters after intravenous administration of various liposomes were calculated by the model-independent moment analysis.[26)]

Data Analysis Data are given as the mean±S.E.M. Statistical significance of means for the studies was determined by analysis of variance followed by Scheffe's test. *p*-Values for significance were set at 0.05.

Results and Discussion

Characterization of SA/γ-CyD Conjugate SA/γ-CyD conjugate was prepared according to the method of McLeod *et al.*,[22)] *i.e.* the carboxyl group of SA was reacted with CDI and the resulting active ester was directly conjugated to the hydroxyl group of γ-CyD in the presence of TEA. The conjugate gave a molecular ion of 1416 in the FAB mass spectrum, which was identical to the molecular ion $[M-H]^-$ of the 1 : 1 (molar ratio) SA/γ-CyD conjugate linked though an ester bond between the carboxyl group of SA and the hydroxyl group of γ-CyD. We assumed that SA is introduced at one of the secondary hydroxyl groups of γ-CyD, because the ^{13}C-NMR chemical shift of the C6 primary hydroxyl groups of γ-CyD was not significantly changed by the conjugation, *i.e.* δ=60.00 and 60.04 ppm in parent γ-CyD alone and SA/γ-CyD conjugate, respectively. On the other hand, the chemical shifts of the C2 and C3 carbons of γ-CyD were significantly changed by the conjugation, *i.e.* δ=72.57 and 72.93 ppm for the C2 and C3 carbons of parent γ-CyD and 76.51—75.89 and 71.53—71.16 ppm for those of SA/γ-CyD conjugate. It was hard to determine in which positions (C2 or C3) SA is introduced, because ester groups are known to be subject to fast acyl migration between the C2 and C3 hydroxyl groups of CyDs.[18)]

Table 1 depicts some physicochemical properties of SA, γ-CyD and SA/γ-CyD conjugate. The aqueous solubility of SA/γ-CyD conjugate in water was about 2 times and 20 times higher than that of γ-CyD and SA, respectively. In general, the CyD conjugate having a substituent with an appropriate size at the primary hydroxyl side has a low aqueous solubility due to the formation of a stable columnar packing structure in the crystalline state, whereas the introduction of the substituent to the secondary hydroxyl group of CyDs gives an amorphous conjugate with high aqueous solubility.[18)] As expected, the present SA/γ-CyD conjugate was actually in an amorphous state, as mentioned below, leading to the high aqueous solubility.

Table 1. Some Physicochemical Properties of SA, γ-CyD and SA Conjugate

	SA	γ-CyD	SA conjugate
Molecular weight	138	1297	1417
Melting point (°C)	160	275[b)]	>300[b)]
Description	White needle	White crystal	White powder
Solubility (M)[a)]	1.58×10^{-2}	1.77×10^{-1}	3.32×10^{-1}

a) In water at 25 °C. *b*) Decomposition.

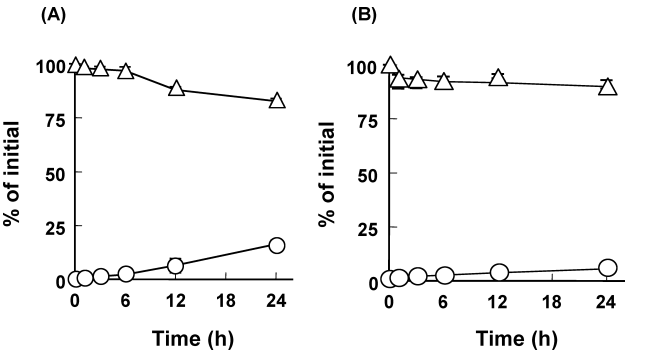

Fig. 2. *In Vitro* SA Release Behavior from SA/γ-CyD Conjugate

The hydrolysis of SA/γ-CyD conjugate in (A) PBS (pH 7.4) or (B) rat plasma-PBS (80%v/v, pH 7.4) was performed at an initial concentration of 4.0×10^{-5} M at 37 °C. SA (open circle) and SA/γ-CyD conjugate (open triangle) were determined using HPLC. Each point represents the mean±S.E.M. of 3—4 experiments.

The release of SA/γ-CyD conjugate in PBS (pH 7.4) and in 80% rat serum in PBS (pH 7.4) was investigated to evaluate chemical and enzymatic stabilities of SA/γ-CyD conjugate. As shown in Fig. 2, the release of SA from the conjugate was extremely slow under both conditions. In fact, the release rate constants for SA in PBS and 80% serum were $3.4\times10^{-3}\pm0.3\times10^{-3}$ (h^{-1}) and $3.0\times10^{-4}\pm1.2\times10^{-4}$ (h^{-1}), respectively. These results indicate that the conjugate is tolerated to chemical and enzymatic degradations. The reason for the slower release rate constant of SA from the conjugate in serum than in PBS still remains unclear, but it might be due to the stabilizing effect of serum on the chemical degradation of the ester bond between SA and γ-CyD, possibly through the interaction of some serum components with SA/γ-CyD conjugate. Further investigation should focus on this mechanism.

Various Properties of SA–γ-CyD Complex The interaction of SA with γ-CyD in aqueous solution and solid state was investigated by fluorescence spectroscopy and powder X-ray diffractometry. The fluorescence spectroscopic study demonstrated that the stability constant of SA complex with γ-CyD is 233 M^{-1} at 25 °C, suggesting the moderate interaction between SA and γ-CyD.[23)] Figure 3 shows powder X-ray diffraction patterns of the SA/γ-CyD system. SA and γ-CyD (Figs. 3A, B) showed the crystalline patterns, and the physical mixture of SA and γ-CyD (Fig. 3C) gave a superimposed diffraction pattern of each component. On the other hand, the diffraction pattern of SA/γ-CyD complex (Fig. 3D) was different from that of the physical mixture, indicating that SA forms a solid complex with γ-CyD. The SA/γ-CyD conjugate (Fig. 3E) gave a halo-pattern, indicating that it is in amorphous state.

Characterization of Liposomes Entrapping SA, SA

Complex or SA/γ-CyD Conjugate Physicochemical properties of liposomes, such as particle size and entrapment ratio of a drug in liposomes, strikingly affect cellular uptake and *in vivo* pharmacokinetic behavior. Therefore, we examined the effects of lipid composition on the entrapment ratio of SA, γ-CyD and SA/γ-CyD conjugate in liposomes. Here we used four liposomes at the molar ratio of EPC/CH of 10 : 0—10 : 3 (molar ratio). At all molar ratios used here, the entrapment ratio of SA/γ-CyD conjugate in liposomes (Fig. 4C) was much higher than those of SA in the SA-in-liposome (Fig. 4A) and complex-in-liposome (Fig. 4B) systems. The higher entrapment ratios of SA/γ-CyD conjugate could be ascribed to its higher hydrophilic property, compared with SA. In addition, the lower entrapment ratio of SA in the complex-in-liposome system compared to the conjugate-in-liposome system could be attributed to the dissociation of SA from the complex during the preparation of liposomes. Of the various molar ratios, the entrapment ratios of SA and SA/γ-CyD conjugate were highest at the molar ratio of 10 : 1 (EPC : CH) in all systems (Fig. 4). The fact that the entrapment ratios of SA and SA/γ-CyD conjugate decreased in liposomes with higher CH contents is consistent with the finding reported by Ganapathi *et al.*[27] Although the precise mechanism for the existence of the optimal molar ratio still remains unknown, we used liposomes composed of EPC : CH = 10 : 1 (molar ratio) in the subsequent studies. Additionally, as described in the materials and methods section, we used 10 mM THBS including SA and SA–γ-CyD conjugate having the various initial concentrations to prepare liposomes with suitable SA and its conjugate concentrations for each experiment. As a result, the entrapment ratios of SA in the SA-in-liposome and complex-in-liposome and those of SA/γ-CyD conjugate in the conjugate-in-liposome were confirmed to be equivalent in the range of the various initial concentrations used in this study (data not shown).

To reveal the entrapment ratio of γ-CyD in liposomes, the amounts of γ-CyD entrapped in liposomes were determined by anthrone-sulfate method.[25] As shown in Table 2, the entrapment ratios of γ-CyD were 11.9% and 12.0% in the γ-CyD-in-liposome and the complex-in-liposome, respectively. These results indicate that the entrapment of γ-CyD in the γ-CyD-in-liposome is almost identical to that in the com-

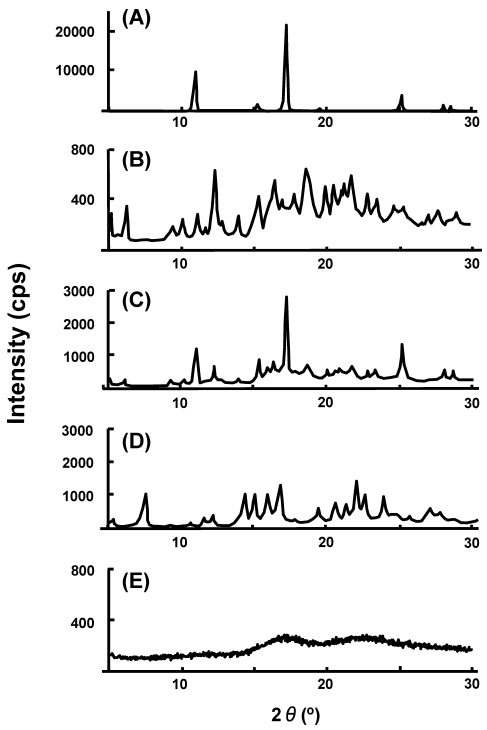

Fig. 3. Powder X-Ray Diffraction Patterns of SA/γ-CyD System

(A) SA; (B) γ-CyD; (C) physical mixture of SA and γ-CyD in a molar ratio of 1 : 1; (D) SA/γ-CyD complex in a molar ratio of 1 : 1; (E) SA/γ-CyD conjugate.

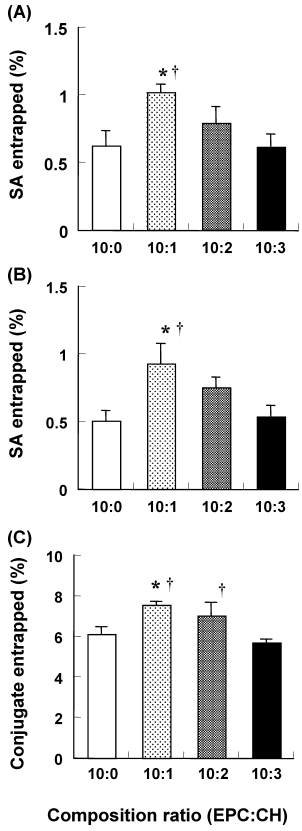

Fig. 4. Entrapment Ratios of ^{14}C-SA or ^{14}C-SA/γ-CyD Conjugate into Liposomes

(A) SA-in-liposome; (B) complex-in-liposome, (C) conjugate-in-liposome. The entrapment ratios were evaluated by using the formula ($C/C_0 \times 100$ (%)). C_0 and C show the concentration of SA or SA/γ-CyD conjugate before and after dialysis. Each value represents the mean±S.E.M. of 4—6 experiments. $*p<0.05$ *versus* liposome (10 : 0). $\dagger p<0.05$ *versus* liposome (10 : 3).

Table 2. Mean Diameters of Liposomes and Entrapment Ratios of SA, γ-CyD and SA Conjugate

	SA-in-liposome	γ-CyD-in-liposome	Complex-in-liposome	Conjugate-in-liposome
Mean diameter (nm)[a]	116.3±3.4	128.0±1.8	122.3±4.5	132.8±7.2
Entrapment ratio of SA (%)	1.09±0.12	—[b]	0.97±0.20	7.46±0.08*,†
Entrapment ratio of γ-CyD (%)	—[b]	11.92±0.40	12.0±2.31	—[b]

a) Liposome size was determined by dynamic light scattering at 25 °C. *b*) Not determined. Each value represents the mean±S.E. of 3—6 experiments. $*p<0.05$ *versus* SA alone. $\dagger p<0.05$ *versus* SA/γ-CyD complex.

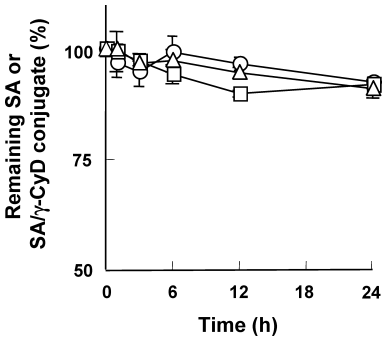

Fig. 5. Release of SA or SA/γ-CyD Conjugate from Various Liposomes

Liposomes containing SA, SA/γ-CyD complex or SA/γ-CyD conjugate were incubated in 10 mM THBS (pH. 7.4) at 25 °C. The remaining extent of SA and SA/γ-CyD conjugate in liposomes was determined with HPLC apparatus. Open circle, SA-in-liposome; open square, complex-in-liposome; open triangle, conjugate-in-liposome. Each point represents the mean±S.E.M. of 3 experiments.

plex-in-liposome, indicating that the influence of SA on entrapment ratio of γ-CyD is only very slight.

Next, we determined particle sizes of various liposomes using a submicron particle analyzer. As shown in Table 2, the mean diameters of the complex-in-liposome and the conjugate-in-liposome were slightly larger than that of the SA-in-liposome, but no significant difference in liposome size was observed under these experimental conditions. To reveal the stability of liposomes, we determined the remaining extent of SA or SA/γ-CyD conjugate in various liposomes after incubation in THBS (pH 7.4). The release of SA or SA/γ-CyD conjugate was found to be markedly slow, and these release rates were statistically insignificant (Fig. 5). Thus, it is evident that γ-CyD and SA/γ-CyD conjugate are unlikely to affect the liposome size and liposome stability, which are consistent with our assumption that γ-CyD interacts with liposome membranes only slightly.

Cellular Uptake and Release of SA and SA/γ-CyD Conjugate It is well known that CyDs fail to enter cells owing to their hydrophilic property and higher molecular weight.[28] To improve the low membrane permeable property, encapsulation of CyDs into liposomes was envisaged to be a very useful technique because liposomes are capable not only of entrapping hydrophilic and hydrophobic compounds but also of permeating through cellular membranes.[29] Moreover, we proposed that if CyD forms the complex or conjugate with a certain drug, the release rate of the drug from cells should decrease, resulting in higher retention of the drug in cells. Hence, we examined cellular uptake of liposomes into RAW264.7 cells, and the release of SA or SA/γ-CyD conjugate from cells after cellular uptake. The reason why we used RAW264.7 cells here is the phagocytotic property, assuming the uptake into reticuloendothelial system (RES) *in vivo*. When free SA, SA/γ-CyD complex and SA/γ-CyD conjugate was incubated with RAW264.7 cells for 3 h, the extent of free SA and SA/γ-CyD conjugate entered cells was found to be 1.59±0.24% (SA), 1.54±0.22% (SA) and 0.37±0.14% (conjugate), respectively (data not shown), suggesting the only slight cellular uptake of free SA and its conjugate. Meanwhile, the extent of cellular uptake of SA or SA/γ-CyD conjugate in their liposome systems was higher than that in their free form systems, but there was no significant difference in the cellular uptake of SA or SA/γ-CyD

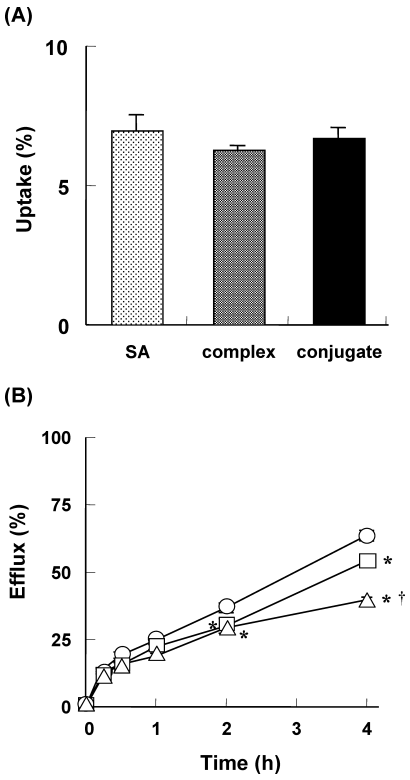

Fig. 6. Cellular Uptake (A) and Release (B) of ^{14}C-SA or ^{14}C-SA/γ-CyD Conjugate after Treating of RAW264.7 Cells with Various Liposomes

(A) RAW264.7 cells (3×10^6 cells) were incubated for 3 h with various ^{14}C-SA-in-liposomes (1 μM) at 37 °C. After washing three times with PBS (pH 2.5), the amount of ^{14}C-SA in the cells was determined by an Aloka LSC-3500 liquid scintillation counter after lysed in 1 N NaOH. Each point represents the mean±S.E.M. of 5—6 experiments. (B) After uptake of various liposomes for 3 h, the cells were washed three times with PBS (pH 2.5), and then fresh medium supplemented with 10% FCS was added to the dish and incubated at 37 °C. At appropriate intervals, the release amount of ^{14}C-SA or ^{14}C-SA/γ-CyD conjugate from the cells in the medium was determined by an Aloka LSC-3500 liquid scintillation counter. Open circle, SA-in-liposome; open square, complex-in-liposome; open triangle, conjugate-in-liposome. Each point represents the mean±S.E.M. of 5—6 experiments. * $p<0.05$ *versus* SA-in-liposome. † $p<0.05$ *versus* complex-in-liposome.

conjugate into cells after incubation with their liposome systems for 3 h, probably because of invariable physicochemical properties of their liposomes (Fig. 6A). However, the release of SA and its conjugate from the cells after the cellular uptake decreased in the order of the systems of SA>SA/γ-CyD complex>SA/γ-CyD conjugate (Fig. 6B). These obvious differences in the cellular release rate may be due to the intrinsic properties of SA, SA/γ-CyD complex and SA/γ-CyD conjugate such as hydrophilicity and molecular size, since there was no substantial difference in the extent of cellular uptake. Indeed, after cellular uptake *via* endocytosis, liposomes are believed to transfer to endosomes and lysosomes, and some of them translocate to cytoplasm. In cytoplasm, SA/γ-CyD complex should partially dissociate to each component, depending on the magnitude of the stability constant of the complex, and the resulting free SA is eliminated in the same way as SA itself. However, intact SA/γ-CyD conjugate appears to reside in cells, because of its high tolerance to chemical and enzymatic degradation (Fig. 2), as well as its large molecular size and hydrophilic nature (Table 1). Therefore, the *in-vivo* residence time of SA/γ-CyD conjugate in tissues after intravenous administration of the conjugate-in-liposome can be expected to be prolonged, compared with

those of the SA-in-liposome and the complex-in-liposome.

***In Vivo* Study** It is well known that the pharmacokinetic behavior of a drug after intravenous administration of the solution containing drug/CyD complex is almost the same as the drug alone owing to dilution and competitive inclusion phenomena.[4—6] In marked contrast, pharmacokinetic behavior of a drug/CyD conjugate is known to be totally different from that of the drug alone: the conjugate tends to disappear rapidly from blood and to eliminate in urine, which is similar to that of CyD alone.[1,30] In fact, we confirmed the very short half life ($t_{1/2}$=1.2±0.1 h) of SA/γ-CyD conjugate in plasma after intravenous administration of THBS containing the conjugate to rats, compared with SA ($t_{1/2}$=5.8±1.5 h) and SA/γ-CyD complex ($t_{1/2}$=5.4±1.4 h). In fact, the SA/γ-CyD conjugate level in kidney was markedly higher than that in the other tissues such as liver, spleen, lung and heart 2 h after intravenous administration to rats. These results indicate that SA/γ-CyD conjugate maintained an intact form and very rapidly eliminated from rat's plasma (data not shown). On the other hand, liposomes have been known to distribute to the RES following intravenous administration.[31,32] However, the fate of SA/γ-CyD complex or SA/γ-CyD conjugate still remains unclear. To test *in vivo* pharmacokinetic behaviors of the SA-in-liposome, the complex-in-liposome and the conjugate-in-liposome, we determined SA and SA/γ-CyD conjugate levels in plasma and tissues after intravenous administration of various liposome preparations in rats. Here, the doses of SA and SA/γ-CyD conjugate were set at 0.05 mg/kg (equivalent to SA) with a tracer amount of [^{14}C]SA or [^{14}C]SA/γ-CyD, respectively. As shown in Fig. 7, plasma SA levels in the SA-in-liposome system were similar to those in the complex-in-liposome system. In addition, the SA/γ-CyD conjugate levels in the conjugate-in-liposome system were slightly lower than those in the other two liposome systems, but there was no statistical significance (Fig. 7). These results suggest that the disappearance of SA or SA/γ-CyD conjugate from blood may be dependent on pharmacokinetic property of liposome themselves. Table 3 summarizes the pharmacokinetic parameters. The half-life ($t_{1/2}$), mean residence time (*MRT*) and area under the curve (*AUC*) of the plasma levels of SA/γ-CyD conjugate in the conjugate-in-liposome tended to be somewhat lower than those of SA in the SA-in-liposome and complex-in-liposome systems. On the other hand, total clearance (CL_{tot}) of SA/γ-CyD conjugate in the conjugate-in-liposome tended to be higher than those of SA in the other systems, but the difference was not statistically significant either. Considering the *in vitro* slow release profile and *in vivo* rapid elimination property of SA/γ-CyD conjugate from rat's plasma as mentioned above, it seems that bioconversion of SA/γ-CyD conjugate to SA hardly occurs *in vivo*. Thus, SA/γ-CyD conjugate may be pharmacologically inactive under this experimental condition, thereafter the rational design of drug/CyD conjugate as a potent prodrug is necessary for evaluation of the therapeutic effect *in vivo*. Therefore, the present data suggest that pharmacokinetic behavior of SA and SA/γ-CyD conjugate after intravenous administration of these liposome systems was almost the same, probably because it is dependent on the physicochemical properties of the liposome itself.

To study the tissue distribution of SA and SA/γ-CyD conjugate, we determined radioactivity of [^{14}C]SA and [^{14}C]SA/γ-CyD conjugate in various tissues at the designated times after administration. The SA levels in liver 12 h after intravenous administration of the complex-in-liposome were much higher than those of the SA-in-liposome (Fig. 8A), but not in the other tissues (Figs. 8B—E). Meanwhile, the SA level in liver and spleen 24 h after administration of the conjugate-in-liposome was much higher than those in the SA-in-liposome and the complex-in-liposome (Figs. 8A, B). The high retention of the conjugate in these tissues may be ascribed to the resistance to chemical and enzymatic hydrolyses of the conjugate, as described above (Fig. 2), suggesting that higher retention of SA/γ-CyD conjugate in various tissues can be achieved by utilizing the conjugate-in-liposome system. As expected, higher retention of SA/γ-CyD conjugate was observed in RES such as liver, spleen and lung as well as heart at various times (Figs. 8D, E). However, it remains unknown whether SA/γ-CyD conjugate exists in interstitial space of tissues or in cells in the present study. Further elaborate studies are required to clarify this pivotal issue.

From the viewpoint of drug-in-liposome strategy, covalently-bound SA/CyD conjugate is more likely to be of advantage than noncovalent SA complexes, because 1) the release rate of SA from SA/γ-CyD conjugate is extremely slow (Fig. 2) in contrast to CyD complex which is in fast equilibrium with free components in solution, 2) entrapment ratio of SA/γ-CyD conjugate was higher than that of drug alone and

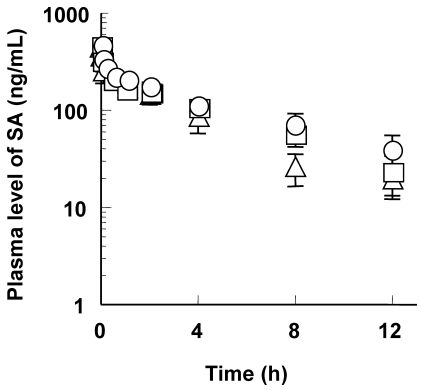

Fig. 7. Plasma Levels of SA after Intravenous Administrations of Various Liposomes in Rats

Various SA preparations were injected intravenously *via* the right jugular vein at a dose of 0.05 mg SA plus ^{14}C-SA/kg or 0.05 mg/kg SA/γ-CyD conjugate plus ^{14}C-SA/γ-CyD conjugate to male Wistar rats. At appropriate intervals, blood was collected from the left jugular vein. The plasma levels of SA or SA/γ-CyD conjugate were measured by radiation activity of ^{14}C-SA and ^{14}C-SA/γ-CyD conjugate. Plasma was dissolved in scintillator and measured radiation activity of ^{14}C-SA or ^{14}C-SA/γ-CyD conjugate by an Aloka LSC-3500 liquid scintillation counter. Open circle, SA-in-liposome; open square, complex-in-liposome; open triangle, conjugate-in-liposome. Each point represents the mean±S.E.M. of 4—6 experiments.

Table 3. Pharmacokinetic Parameters of SA after Intravenous Administrations of Aqueous Suspensions containing Various Liposomes to Rats

	SA-in-liposome	Complex-in-liposome	Conjugate-in-liposome
$t_{1/2}$ (h)	3.3±0.9	3.5±0.5	2.9±0.8
MRT (h)	4.7±1.2	5.0±0.7	3.9±1.0
CL_{tot} (ml/h/kg)	44.2±6.8	47.8±6.1	66.0±24.6
AUC (μg h/ml)	1.3±0.2	1.1±0.1	0.9±0.2
V_d (ml/kg)	180±17.5	227±11.7	195±16.0

Each value represents the mean±S.E. of 4—6 experiments.

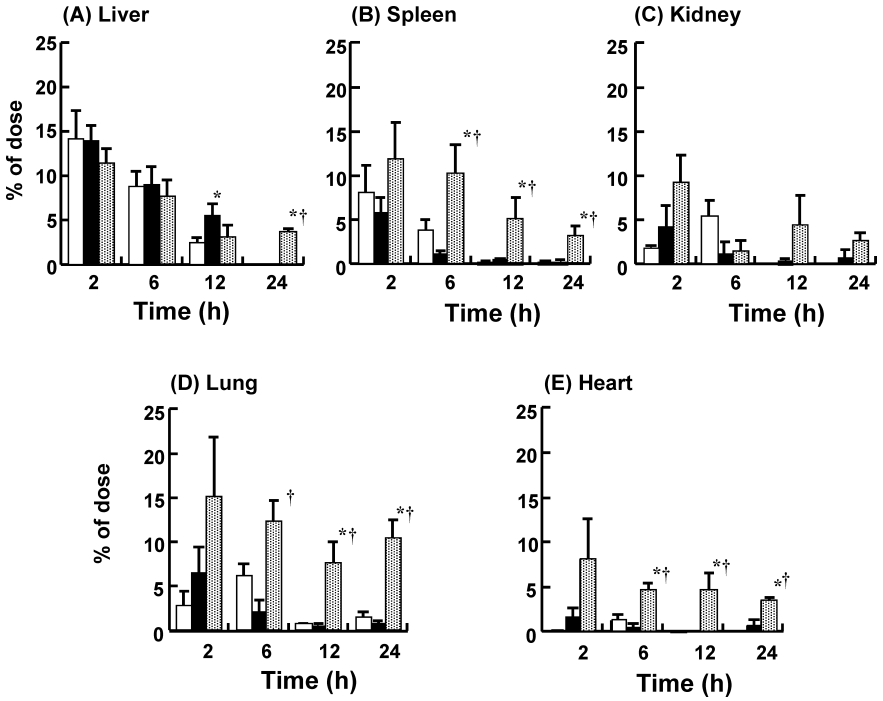

Fig. 8. Organ Levels of SA after Intravenous Administrations of Various Liposomes in Rats

Various SA preparations were injected intravenously *via* the right jugular vein at a dose of 0.05 mg/kg SA plus ^{14}C-SA or 0.05 mg/kg SA/γ-CyD conjugate plus ^{14}C-SA/γ-CyD conjugate to male Wistar rats. At appropriate intervals following injection, organs (liver, spleen, kidney, lung, heart) were excised. The organ levels of SA were determined from radioactivity of ^{14}C-SA or ^{14}C-SA/γ-CyD conjugate using a liquid scintillation counter. Open column, SA-in-liposome; closed column, complex-in-liposome; hatched column, conjugate-in-liposome. Each point represents the mean±S.E.M. of 3—10 rats. $*p<0.05$ *versus* SA-in-liposome. $\dagger p<0.05$ *versus* complex-in-liposome.

its CyD complex, 3) release of the SA/γ-CyD conjugate through liposome membrane may be slower than that of the drug alone due to its large molecular size and hydrophilicity. However, prolonged residence of drugs through the conjugate-in-liposome is likely to have a deleterious effect. Hence further study regarding its safety would be necessary *in vivo*.

In conclusion, these results suggest the potential use of SA/γ-CyD conjugate for efficient entrapment in liposomes as well as of liposomes containing SA/γ-CyD conjugates for prolonged residence of drugs in tissues.

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