Synthesis of a Novel Sialic Acid Derivative (Sialylphospholipid) as an Antirotaviral Agent

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A novel sialylphospholipid (SPL) was synthesized from *N*-acetylneuraminic acid (NeuAc) and phosphatidylcholine (PC) by a chemical and enzymatic method and evaluated as an inhibitor of rotavirus. PC and 1,8-octanediol were conjugated by transesterification reaction of *Streptomyces* phospholipase D (PLD) under a water−chloroform biphasic system to afford phosphatidyloctanol, which was condensed with a protected 2-chloro-2-deoxyneuraminic acid derivative by using silver trifluoromethanesulfonate as an activator in chloroform and converted, after deprotection, to SPL. Rhesus monkey kidney cells (MA-104) were incubated with simian (SA-11 strain) and human (MO strain) rotaviruses in the presence of SPL, and the cells infected were detected indirectly with anti-rotavirus antibody. SPL showed dose dependent inhibition against both virus strains. The concentrations required for 50% inhibition (IC_{50}) against SA-11 and MO were 4.35 and 16.1 μM, respectively, corresponding to 10^3- and 10^4-fold increases in inhibition as compared to monomeric NeuAc.

Introduction

Glycoconjugates containing *N*-acetylneuraminic acid (NeuAc) have been shown to be involved in a variety of biological recognition events.[1−3] For example, sialyl glycoconjugates on the cell surface are known to play important roles as receptors for bacterial and viral infections.[4,5] Several glycoconjugates that contain NeuAc have been proposed as binding sites for animal rotaviruses in epithelial cells.[6−8] Yolken *et al.*[9] have reported that glycoproteins bind directly to rotaviruses and that the virus−glycoprotein binding is dependent largely upon sialic acid-containing oligosaccharides.

Rotaviruses are one of the most important pathogens of serious gastroenteritis in infants and children and are also a cause of traveler's diarrhea. Rotavirus has been reported to kill more than 3 million infants/year in developing countries.[10] Also it causes vomiting among 150 million children around the world every year.[11−14] Vaccines have been developed for the treatment of rotavirus infection,[15,16] but the success of vaccination trial for rotavirus has remained questionable because of the difficulties of introducing specific antibodies into the intestinal tract of the infant whose immunity has not been well developed.[17]

The role of sialic acid in cell-surface recognition has been further explored and led to the development of sialic acid derivatives as anti-inflammatory and antiviral agents.[4,5,18−20] The binding of sialic acid derivatives to their protein receptors is, however, generally weak with dissociation constants in the millimolar range, and multivalent sialic acid derivatives have to be used to enhance binding.[21−24] Both liposome-like[22] and cross-linked liposome-like[23] sialic acids have been reported to be potent inhibitors of influenza virus. These multivalent species may not be biodegradable, and whether they will be cleared after administration is not known. We envision that sialylphospholipid (SPL), like other phospholipids,[25,26] would form a liposome and be subject to enzymatic degradation by phospholipases, though this type of compound has not been prepared and evaluated as antiviral agents.[27]

Previously, we have reported the large scale preparation of sialyl oligosaccharides (NeuAc derivatives) from egg[28,29] and their inhibitory effect against rotavirus.[30] We, here, would like to report the synthesis of a novel SPL from NeuAc and phosphatidylcholine (PC) via enzymatic transphosphatidylation using phospholipase D and evaluation of SPL to inhibit the infection of rotavirus *in vitro*.

Results

Synthesis of Sialylphospholipid. PC (10 mmol) and 30 mmol of 1,8-octanediol were mixed in an water−chloroform biphasic system in the presence of *Streptomyces* phospholipase D (PLD) (370 units) to yield the phosphatidyl alcohol **1** (67.8%). The product **1** and glycosyl chloride (**2**), prepared from NeuAc in 79% yield, were condensed with silver trifluoromethanesulfonate as an activator in chloroform to give the mixture of **3** α- and β-anomers in 16% yield.[31] Thereafter, followed by deprotection including deacetylation (**4**) and debenzylation, SPL was obtained in 25% yield from **3** (Scheme 1). The SPL obtained above showed one spot on TLC, positive for both NeuAc and phospholipid chromophore reagents. The structure was elucidated by high-resonance fast atom bombardment mass spectrometry (HR-FAB-MS), 400 MHz ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR (DQFCOSY, HMQC). HRFAB-MS of SPL was mea-

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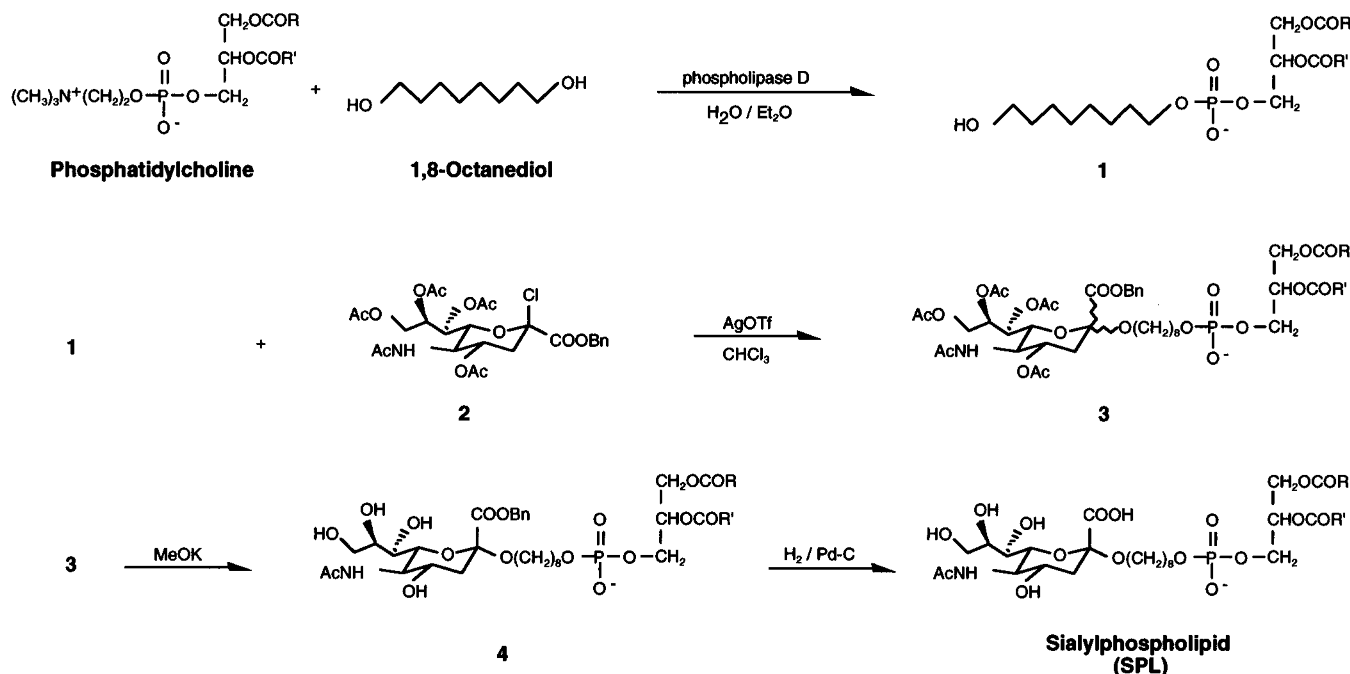
Scheme 1. Synthesis of Sialylphospholipid



sured in the negative mode. As a result, the molecular ion peak at *m/z* 1122.7451 indicates the deprotonated molecule $(M - H)^-$ of SPL (theoretical molecular weight: 1122.7433; $C_{58}H_{109}NO_{17}P$) and is consistent with the molecular formula of SPL ($C_{58}H_{110}NO_{17}P$). The NMR spectrum is also consistent with the structure of SPL, which is α-(*N*-acetylneuraminyl)phosphatidyloctanediol.[31] From these results, the structure of SPL was proposed to be that shown in Scheme 1.

Inhibitory Effect of SPL and Its Reactants against Rotaviruses (SA-11 and MO Strains). The inhibitory activity of SPL, NeuAc, and PC against simian and human rotaviruses was assayed *in vitro*. SPL was tested with concentrations ranging from 0.001 to 1 mg/mL, NeuAc was in the range from 0.1 to 100 mg/mL, and PC was in the range from 0.001 to 1 mg/mL, respectively. SPL showed a dose dependent inhibition against SA-11 with IC_{50} (50% inhibition) of 4.35 μM. The substrate (*N*-acetylneuraminic acid) used in the synthesis of SPL also inhibited SA-11 rotavirus infection with IC_{50} of 4.28 mM. The effect of SPL as an inhibitor of SA-11 was about 1000-fold better than the effect of the monomer NeuAc (4.28 mM; Figure 1). The IC_{50} of SPL against human rotavirus MO strain was 16.1 μM, compared to 142 mM when the monomer NeuAc was used. PC showed no effect against both SA-11 and MO strains (Table 1).

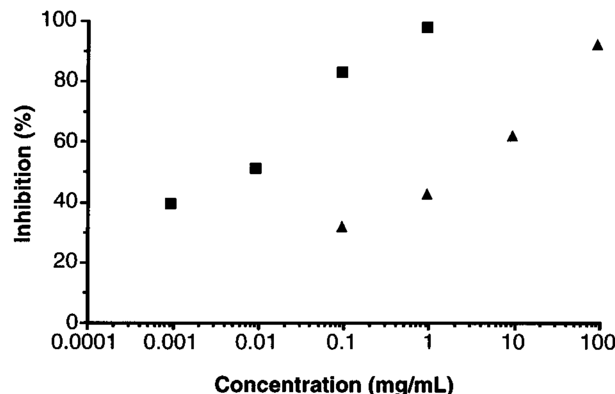


Figure 1. Effect of SPL and NeuAc on rotavirus (SA-11) inhibition: (■) sialylphospholipid (SPL) and (▲) *N*-acetylneuraminic acid (NeuAc).

Table 1. Effect of SPL and Its Reactants on Rotavirus Strains

	IC_{50} (M)	
compounds	simian rotavirus SA-11 strain	human rotavirus MO strain
SPL	4.35×10^{-6}	1.61×10^{-5}
NeuAc	4.28×10^{-3}	1.42×10^{-1}
PC	no inhibition	

Discussion

This paper describes an efficient procedure for the synthesis of SPL from PC and NeuAc. PC did not show any inhibition against the rotavirus strains described, though NeuAc showed a weak inhibitory activity against both rotaviruses (SA-11 and MO strains). SPL synthesized from NeuAc and PC enhanced the inhibitory activity by approximately 10^3- and 10^4-fold against SA-11 and MO strains, respectively, as compared with NeuAc. It is speculated that the phospholipid conjugates in aqueous solution form liposome bilayers with multivalent NeuAc (the head group) displayed on the surface, which then interacts with the virus surface receptor in a multivalent manner. Further support for the formation of liposome is revealed in the electron microscopy studies (Figure 2) which show vesicles with an average diameter of ~200 nm. Though monomeric NeuAc binds to the virus surface receptor weakly,[6,8,32] the multivalent species does enhance the binding substantially. It is believed that SPL may become a new entity as an antiviral agent.

Experimental Section

General. Reagents, solvents, and materials were purchased from Aldrich (Milwaukee, WI), from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and from Nacalai Tesque, Inc. (Kyoto, Japan). PLD was purchased from Honen Corp. (Tokyo, Japan). NeuAc and PC were prepared according

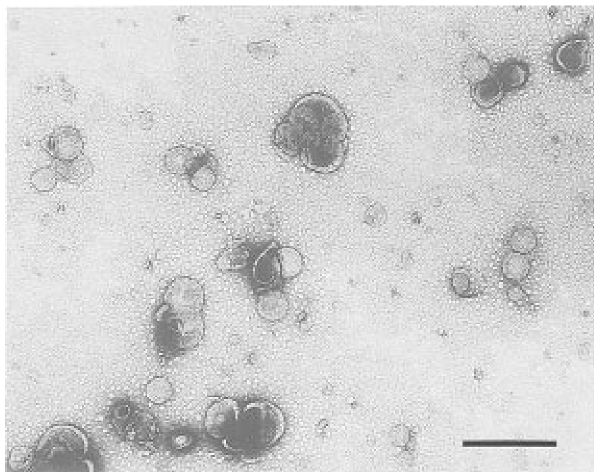


Figure 2. Electron microscope picture of SPL formed as liposome. The average diameter of a liposome is ∼200 nm; bar = 500 nm (reproduced at 50% of original size).

to the procedure previously reported.[33,34] The following solvents and reagents have been abbreviated: sodium acetate (NaOAc), calcium acetate ($Ca(OAc)_2$), ethyl acetate (EtOAc), chloroform ($CHCl_3$), methanol (MeOH), phosphate-buffered saline (PBS), and fluorescein isothiocyanate (FITC). The SA-11 and MO strains of rotavirus and MA-104 cells (an established cell line derived from rhesus monkey kidney) were obtained from the Department of Bacteriology, Tohoku University School of Medicine. TLC was performed on silica gel 60 F_{254} (art. 5717, E. Merck, Darmstadt, Germany) and stained by resorcinol[35] and Dittmer's reagent[36] for sialic acid and phospholipid, respectively. High-resonance fast atom bombardment mass spectrometry (HRFAB-MS) of SPL was measured on a JEOL JMS-HX-110 mass spectrometer (JEOL Co., Ltd., Tokyo, Japan) in the negative mode using triethanolamine as matrix. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100.64 MHz), DQFCOSY, and HMQC were recorded on a JEOL-GSX-400 spectrometer, operating in the pulsed Fourier transform mode. Tetramethylsilane (TMS) was used as an internal standard.

Phosphatidyloctanediol (1; Scheme 1). To a solution of 1,8-octanediol (4.39 g, 30 mmol) and distearoylphosphatidylcholine (PC; 7.90 g, 10 mmol) in 200 mL of chloroform were added 30 mL of NaOAc buffer (pH 5.6) and 3 mL of 0.4 M aqueous $Ca(OAc)_2$. PLD from *Streptomyces* species (Honen Corp.) (370 units in 3 mL of 0.4 M NaOAc buffer) was then added in portions, and the mixture was stirred at 35 °C for 50 h. The reaction mixture was evaporated to dryness and then purified by silica gel chromatography (EtOAc, $CHCl_3$, $CHCl_3$/MeOH = 3/1, v/v, successively) to afford the desired product **1** (5.65 g, 67.8%) as a white powder: mp 73−75 °C dec; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.88 (t, 6H, J = 6.6 Hz), 1.26 (m, 62H), 1.58 (m, 6H), 2.02 (m, 4H), 2.29 (dd, 4H, J = 8.3, 17.0 Hz), 3.62 (t, 2H, J = 5.5 Hz), 3.84 (m, 2H), 3.97 (m, 2H), 4.18 (dd, 1H, J = 6.6, 11.6 Hz), 4.41 (d, 1H, J = 10.5 Hz), 5.26 (m, 1H); ^{13}C-NMR (100.64 MHz, $CDCl_3$) δ 14.0, 22.6, 24.9, 25.0, 25.2, 25.7, 27.3, 28.5, 28.6, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 30.1, 31.5, 31.9, 32.2, 34.2, 34.3, 62.5, 63.0, 66.1, 70.9, 173.5, 173.7; MS calcd for $C_{47}H_{93}O_9P$ 832, found 831 (negative mode).

(1-Benzyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminyl)-phosphatidyloctanediol (3; Scheme 1). NeuAc was converted to 1-benzyl-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid (**2**) according to the literature procedure.[37,38] 1-Benzyl-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid (2.34 g, 4.0 mmol) and phosphatidyloctanediol (3.33 g, 4.0 mmol) were dissolved in 200 mL of chloroform, and to the solution were added powdered anhydrous disodium hydrogen phosphate (1.14 g, 8.0 mmol) and molecular sieves (4A, 2.34 g). Then, the mixture was cooled at −50 °C, silver trifluoromethanesulfonate (1.03 g, 4.0 mmol) in 15 mL of toluene was added slowly, and the mixture was stirred at 30 °C for 1 h. The reaction mixture was filtered through Celite (Celite Co., CA), evaporated to dryness, and then purified by silica gel chromatography ($CHCl_3$/MeOH = 3/1, v/v) to afford the mixture of **3** α- and β-anomers (900 mg, 16.0%) as a pale yellow powder: mp 101−103 °C dec; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.88 (t, 6H, J = 7.1 Hz), 1.25 (m, 62H), 1.43 (m, 1H), 1.57 (m, 6H), 1.87 (d, 2H, J = 2.7 Hz), 2.04 (m, 6H), 2.13 (d, 2H, J = 2.7 Hz), 2.30 (m, 8H), 2.45 (dd, 1H, J = 4.9, 12.6 Hz), 2.62 (dd, 1H, J = 4.9, 12.6 Hz), 3.12 (m, 1H), 3.27 (m, 1H), 3.45 (m, 1H), 3.70 (m, 2H), 3.81 (m, 2H), 3.95 (m, 4H), 4.13 (m, 4H), 4.31 (d, 1H, J = 9.9 Hz), 4.43 (d, 1H, J = 11.5 Hz), 4.75 (d, 1H, J = 9.9 Hz), 4.83 (m, 1H), 5.21 (m, 2H), 5.35 (m, 3H), 7.26 (s, 1H), 7.36 (d, 4H, J = 3.8 Hz); ^{13}C-NMR (100.64 MHz, $CDCl_3$) δ 14.1, 20.8, 22.7, 24.9, 25.8, 29.3, 29.4, 29.5, 29.7, 29.8, 30.3, 31.9, 34.2, 34.3, 38.1, 67.3, 67.6, 98.6, 98.8, 128.3, 128.4, 128.6, 128.7, 135.0, 135.1, 168.0, 170.0, 170.2, 170.5, 170.8, 170.9, 173.8, 173.9; MS calcd for $C_{73}H_{123}NO_{21}P$ 1380, found 1380.

(1-Benzyl-*N*-acetylneuraminyl)phosphatidyloctanediol (4; Scheme 1). To a solution of (1-benzyl-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminyl)phosphatidyloctanediol (234 mg, 0.17 mmol) in 70 mL of methanol was added potassium methoxide (142 mg, 0.60 mmol) in 30% methanol at 0 °C, and the reaction was monitored by silica gel TLC ($CHCl_3$/MeOH/water = 70/30/5). The reaction mixture was neutralized with DOWEX-50 resin (H^+ form) after completion of reaction and filtered through Celite. The filtrate was evaporated to dryness and purified by silica gel chromatography ($CHCl_3$/MeOH = 2/1, v/v) to afford the desired **4** α-anomer (111 mg, 54.0%) as a pale yellow powder: mp 95−98 °C; ^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD = 1/1, v/v) δ 0.89 (t, 6H, J = 6.6 Hz), 1.28 (m, 62H), 1.61 (m, 6H), 2.03 (s, 3H), 2.34 (m, 4H), 2.77 (dd, 1H, J = 4.9, 12.6 Hz), 3.21 (dd, 1H, J = 6.1, 15.4 Hz), 3.56 (m, 3H), 3.74 (m, 3H), 3.86 (m, 6H), 3.97 (m, 3H), 4.06 (t, 1H, J = 6.6 Hz), 4.17 (m, 1H), 4.42 (dd, 1H, J = 3.3, 12.1 Hz), 5.24 (m, 1H), 5.29 (d, 1H, J = 3.3 Hz), 7.39 (m, 4H), 7.59 (s, 1H); MS calcd for $C_{65}H_{115}NO_{17}P$ 1212, found 1212.

***(N*-Acetylneuraminyl)phosphatidyloctanediol (Sialylphospholipid; Scheme 1).** **4** (100 mg, 0.08 mmol) was dissolved in 30 mL of ethanol and 10 mL of chloroform, and 10% palladium on activated carbon (20 mg) and 600 μL of 0.01 N HCl were added. The mixture was stirred vigorously under hydrogen (1 atm) at 35 °C for 24 h. The reaction mixture was filtered through Celite and evaporated to dryness, and then the product was purified by preparative silica gel thin layer chromatography ($CHCl_3$/MeOH/water = 70/30/5, v/v/v) to afford the desired sialylphospholipid (43.2 mg, 46.0%) as a white powder: mp 143 °C; ^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD = 1/1) δ 0.90 (t, 6H, J = 7.3 Hz), 1.26 (m, 62H), 1.54 (m, 1H), 1.60 (m, 6H), 2.00 (s, 3H), 2.32 (m, 4H), 2.82 (dd, J = 3.9, 12.2 Hz, 1H), 3.44 (ddd, J = 6.8, 6.8, 8.8 Hz, 1H), 3.49 (dd, J = 1.9, 9.3 Hz, 1H), 3.57 (dd, J = 1.9, 10.3 Hz, 1H), 3.61 (dd, J = 5.4, 11.2 Hz, 1H), 3.66 (m, 2H), 3.75 (ddd, J = 6.8, 6.8, 8.8 Hz, 1H), 3.82 (m, 2H), 3.83 (dd, J = 2.5, 11.2 Hz, 1H), 3.86 (ddd, J = 2.9, 5.9, 9.3 Hz, 1H), 3.96 (t, J = 5.7 Hz, 2H), 4.18 (dd, J = 6.3, 12.2 Hz, 1H), 4.45 (dd, J = 3.4, 12.2 Hz, 1H), 5.21 (m, 1H); ^{13}C-NMR (100.64 MHz, $CDCl_3$/CD_3OD = 1/1) δ 14.5, 22.6, 23.1, 26.1, 27.1, 27.3, 30.3, 30.5, 30.6, 30.7, 30.8, 31.1, 31.9, 32.0, 33.1, 35.0, 35.2, 42.8, 54.2, 63.7, 64.4, 64.6, 65.3, 66.8, 69.6, 70.4, 72.1, 73.0, 74.3, 101.8, 113.8, 174.5, 174.6, 175.0, 175.6; HRFAB-MS calcd for $C_{58}H_{109}NO_{17}P$ 1122.7433, found 1122.7451.

Rotavirus Infectivity Assay *in Vitro*. The inhibition activity of each compound against rotavirus infection was measured as previously reported.[7,30] Rhesus monkey kidney cells (MA-104) were grown to confluence in 96-well tissue culture microplates (Nunc microwell plate 96F with lid) in the presence of Eagle's minimum essential medium (EMEM) and 10% fetal bovine serum at 37 °C in 5% CO_2 environment. The cells were washed with serum-free media before use. Aliquots of SPL, NeuAc, and PC were diluted with EMEM, sonicated, and then mixed with an equivalent volume of rotavirus (SA-11, 1×10^5−10^6 FCFU/mL; MO strain, 1×10^5−10^6 FCFU/mL), and incubated at 37 °C for 1 h. This mixture was absorbed onto the cells. After incubation at 37 °C for 1 h, the inoculum was decanted and the cells were incubated in EMEM at 37 °C for 17 h. After incubation, EMEM was decanted and the cells were air-dried and fixed in cold MeOH (−80 °C) for 1 h. After MeOH was removed and cells were air-dried, guinea

pig antiserum against rotavirus was added and the mixture was incubated at 37 °C for 30 min. After cells were washed with PBS, FITC-labeled goat IgG against guinea pig IgG was added and the mixture was incubated at 37 °C for 30 min. Cells were washed and air-dried. The number of positively fluorescent cell focus forming units (FCFU) was counted under a fluorescence microscope.

Preparation of SPL Liposome.[39,40] SPL (3.0 mg, 2.67 nmol), D-α-phosphatidylcholine dipalmitoyl (15.5 mg, 21.1 nmol), and cholesterol (6.5 mg, 16.8 nmol) were dissolved in a mixture of MeOH (1 mL) and $CHCl_3$ (1 mL). After the solution was thoroughly evaporated *in vacuo*, the residue was lyophilized to dryness. The resultant dry powder was hydrated with 2 mL of 50 mmol of potassium phosphate buffer (pH 7.0) and mechanically vortexed. The obtained suspension was sonicated using a probe sonicator (B. Braun-2000U) until the turbidity dissipated to form small unilamellar liposomes. After incubation at room temperature for 2 h, the suspension of liposomes was centrifuged at 23000*g* for 10 min, and the liposome on the top layer was collected by a pipet.

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