Supplementary Material (ESI) for Chemical Communications

Towards Improved Gene Delivery: Flip of Cationic Lipids in Highly Polarized Liposomes

Richard G. Uhl II, Amalia Stevenson and Vladimir Sidorov*

Department of Chemistry, Virginia Commonwealth University, Richmond, VA 23284-2006

*Author to whom correspondence should be addressed.

Dr. Vladimir Sidorov
Assistant Professor
Department of Chemistry
Virginia Commonwealth University
1001 West Main Street
Richmond, VA 23284-2006
tel.: (804)828-7507
e-mail: vasidorov@vcu.edu

General. All fluorimetric experiments were performed on a Fluoromax 3 (Jobin-Yvon/Horriba) spectrophotometer. The dynamic light scattering experiments were carried out on a Malvern Zetasizer Nano Series Instrument. The pH of solutions was measured with a Corning 350 pH/ion analyzer using a Ag/AgCl pH-sensitive electrode (Accumet). Equivalency of osmolality of various buffer solutions was confirmed with use of VAPRO 5520 vapor pressure osmometer (Wescor). Size-exclusion chromatography (SEC) was performed with 40 -120 μm Sephadex G-75 (Aldrich) as a solid phase and various buffers as a mobile phase. High-pressure extrusion of liposomal suspensions was performed with the Avanti mini-extruder using a 0.1-μm polycarbonate membrane (Whatman Nucleopore Track-Etch Membrane). All buffers were prepared using deionized water with the resistance of 18.2 MΩ (Millipore Water purification system, Synergy series). All chemicals, except phenoxyacetamide **1** which was synthesized as described,[1] solvents and dyes were purchased from Aldrich, Sigma, Invitrogen and Fluka. Egg yolk L-α-

phosphatidylcholine (EYPC), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), and 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (Ethyl PC) were all purchased from Avanti Polar Lipids. The identity of synthesized phenoxyacetamide **1** was confirmed by ^{1}H and ^{13}C NMR on the Varian AS400 instrument operating at 400.130 MHz and 100.613 MHz respectively. The purity of phenoxyacetamide **1** was confirmed by the analytical thin layer chromatography (Kieselgel 60 F254 and Uniplate^{tm} Silica Gel GF silica-coated glass plates, visualized by UV and I_2).

Liposome Preparation for Fluorimetric Studies of Potential Buildup. Egg yolk L-α-phosphatidylcholine (EYPC ethanol solution, 60 μL, 79 μmol), was dissolved in $CHCl_3$/MeOH mixture, evaporated under reduced pressure, and dried under high vacuum for 2 hours. The lipid film was hydrated with 1.2 mL of 75 mM K_2SO_4/10 mM phosphate buffer (pH 6.4). Five freeze/thaw cycles were performed (dry ice/methanol, water bath at 40 °C). The resulting suspension was subjected to 21 high-pressure extrusions through a 0.1-μm polycarbonate membrane at room temperature. Through size-exclusion chromatography (SEC), the extra-vesicular potassium sulfate/phosphate buffer was replaced with 180 mM glucose/10 mM phosphate buffer (pH 6.4) to a final lipid concentration of 500 μM.

Fluorimetric Studies for Potential Buildup. All fluorimetric runs were performed at 15 °C. Fluorescence of potential-sensitive dye safranin O was monitored at 580 nm with excitation at 520 nm. The change in the transmembrane diffusion potential was detected through an increased fluorescence of safranin O as it partitions into the hydrophobic region of the bilayer membrane. In a typical run, 25 μL of the above liposome preparation was added under gentle stirring to a cuvette containing 1975 μL of a 100 mM LiCl or NaCl/ 10 mM phosphate buffer (pH 6.4) with 60 nM safranin O. In calibration runs, 0-130 μL of 75 mM K_2SO_4/10 mM phosphate buffer was added to achieve a desired concentration of K^+_{out}. At 90 s, 20 μL of a solution of phenoxyacetamide (31.25 μM) and / or valinomycin (3.75 μM) in DMSO was added. At 3900 s, 20 μL of 1 mM aqueous melittin solution was added. Total run time was 4000 s.

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Liposome Preparation for NBD-PE Fluorimetric Studies. Egg yolk L-α-phosphatidylcholine (EYPC ethanol solution, 60 μL, 79 μmol) and 30 μL of 1,2-Dioleoyl-sn-Glycero-3-Phospho-ethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), corresponding EYPC/NBD-PC lipid ratio of 2000:1, dissolved in $CHCl_3$/MeOH mixture, evaporated under reduced pressure, and dried under high vacuum for 2 hours. The lipid film was hydrated with 1.2 mL of 75 mmol K_2SO_4/10 mM phosphate buffer (pH 6.4). Five freeze/thaw cycles were performed (dry ice/methanol, water bath at 40 °C). The resulting suspension was subjected to 21 high-pressure extrusions through a 0.1-μm polycarbonate membrane at room temperature. Through size-exclusion chromatography (SEC), the extra-vesicular potassium sulfate/ phosphate buffer was replaced with 100 mM LiCl or NaCl/ 10 mM phosphate buffer (pH 6.4) to a final lipid concentration of 500 μM.

NBD-PE Fluorimetric Studies of lipid flop. All fluorimetric runs were made at a temperature of 15 °C. An excitation of 520 nm with an emission of 580 nm measured the transmembrane diffusion potential as detected by the increased fluorescence of safranin O as it interacts with the hydrophobic region of the liposomes. An excitation at 460 nm with an emission of 534 nm measured the decay of NBD fluorescence. In a typical run, 100 μL of the above liposome preparation was added (at 30 s) under gentle stirring to a cuvette containing 1900 μL of a 100 mM LiCl or NaCl/ 10 mM phosphate buffer (pH 6.4) with 60 nM safranin O. At 90 s, 20 μL of a solution of phenoxyacetamide **1** (0.125 mM) and valinomycin (0.015 mM) in DMSO was added. The same amount of DMSO (20 μL) with no ionophores was added to a liposomal suspension in the blank experiment. This was then followed by an incubation period at room temperature. A second scan was then initiated. At 550 s, 20 μL of 0.8 M sodium dithionate solution (in Millipore water) was added, with mixing, to the cuvette. At 1720 s, 40 μL of a 10% Triton X-100 solution (in Millipore water) was added.

Liposome Preparation for Dynamic Light Scattering Studies. These preparations were similar to the NBD-PE liposome preparation made above with the following exceptions:

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1) Instead of fluorescently labeled anionic NBD-PC, cationic Ethyl PC was used with EYPC for these preparations. 2) At higher concentrations of Ethyl PC, some vortexing was required during the freeze/thaw cycles to hydrate the lipid. 3) During SEC, extra-vesicular potassium sulfate/ phosphate buffer was replaced with 100 mM LiCl or NaCl / 10 mM phosphate buffer (pH 6.4) to a final lipid concentration of 500 μM.

Dynamic Light Scattering Studies. Preparation of samples for DLS was mostly kept consistent with fluorimetry sample preparation. Liposome preparations of 5 to 20 mol% Ethyl PC in EYPC were added (100 μL) to 1900 μL of 100 mmol NaCl or LiCl / 10 mmol phosphate buffer (pH 6.4). To some samples, 20 μL of of a solution of phenoxyacetamide (0.125 mM) and valinomycin (0.015 mM) in DMSO was added. Based on optimal results from the anionic fluorescent studies, samples were then incubated at room temperature for 12 hours. Following the incubation period, the samples were analyzed at 25 °C on Malvern Zetasizer Nano Series Instrument. Then 100 mg of human serum albumin (HSA) was added with gentle agitation. After about two minutes, DLS runs were repeated to measure resulting enlargement. With the exception of the kinetic study, which plots individual Z average values, all DLS runs consisted of 10 separate measurements of Z average obtained in about five minutes, with their average value being represented.

Ethyl PC (mol%)	External Buffer	Ionophores Present	Z-avg (nm)
12.5	NaCl	No	478±145
12.5	NaCl	Yes	315±123
12.5	LiCl	No	215±119
12.5	LiCl	Yes	127±21
15	NaCl	No	1903±522
15	NaCl	Yes	398±78
15	LiCl	No	957±507
15	LiCl	Yes	443±171
20	NaCl	No	1255±543
20	NaCl	Yes	577±240
20	LiCl	No	4263±2337
20	LiCl	Yes	710.9±321

Table S1. Results of the dynamic light scattering experiments made with liposomes containing various mol%'s of Ethyl PC in EYPC, in the presence of NaCl or LiCl phosphate buffers and HSA, with or without ionophores. All liposomes were 100 nm in size before application of HSA.

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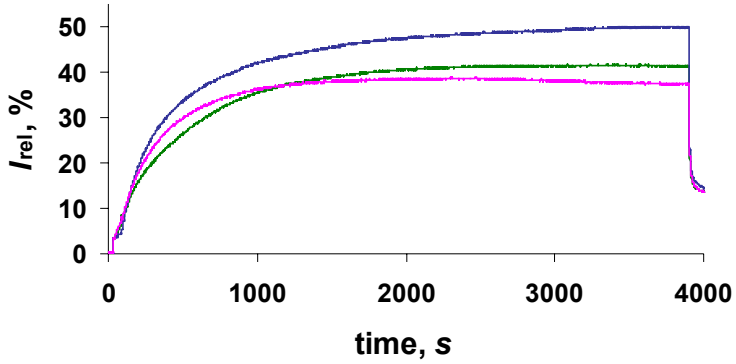

Fig S1. Monitoring of the fluorescence of safranin O (ex=520nm, em =580nm) as a function of the potential buildup upon application of 37.5 nM valinomycin (pink trace), 312.5 nM phenoxyacetamide **1** (green trace) or both (blue trace) to a suspension of EYPC (lipid concentration 125 μM) liposomes ($K_2SO_{4\ in}$/ LiCl-$K_2SO_{4\ out}$, K_{in}/K_{out} = 150). The experiments were terminated by application of 10 μM defect inducer melittin

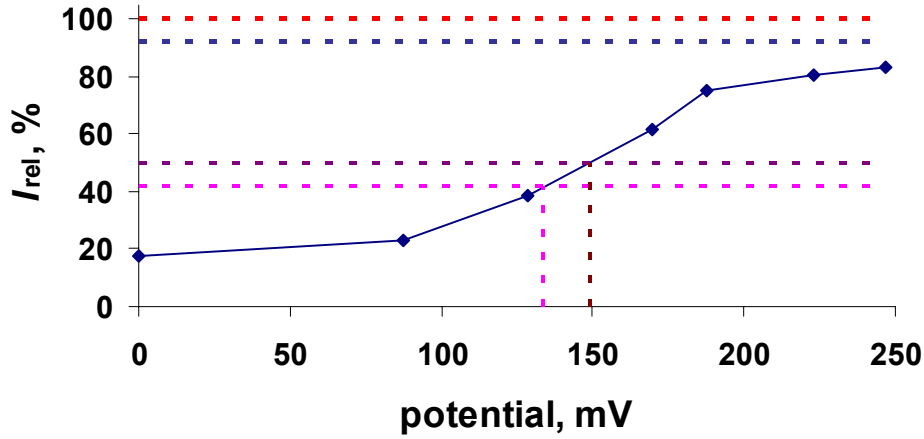

Fig S2. Calibration plot for the K^+ diffusion potential induced by valinomycin in liposomes that are under various K_{in}/K_{out} gradients. The potential values were calculated according to the Nernst equation: $\Delta\Psi$ (mV) = 59.1log(K_{in}/K_{out}). Dashed lines represent fluorescence responses of safranin O in liposomes that are under K^+ and Cl^- gradients. From top to bottom: red line: K^+_{in} = 150 mM, K^+_{out} = 0 mM; Cl^-_{in} = 0 mM, Cl^-_{out} = 100 mM, valinomycin and phenoxyacetamide **1** applied; blue line: K^+_{in} = 150 mM, K^+_{out} = 0 mM; Cl^-_{in} = 0 mM, Cl^-_{out} = 100 mM, valinomycin only applied; brown line: K^+_{in} = 150 mM, K^+_{out} = 1 mM; Cl^-_{in} = 0 mM, Cl^-_{out} = 100 mM, valinomycin and phenoxyacetamide **1** applied; magenta line: K^+_{in} = 150 mM, K^+_{out} = 1mM; Cl^-_{in} = 0mM, Cl^-_{out} = 100 mM, valinomycin only applied.

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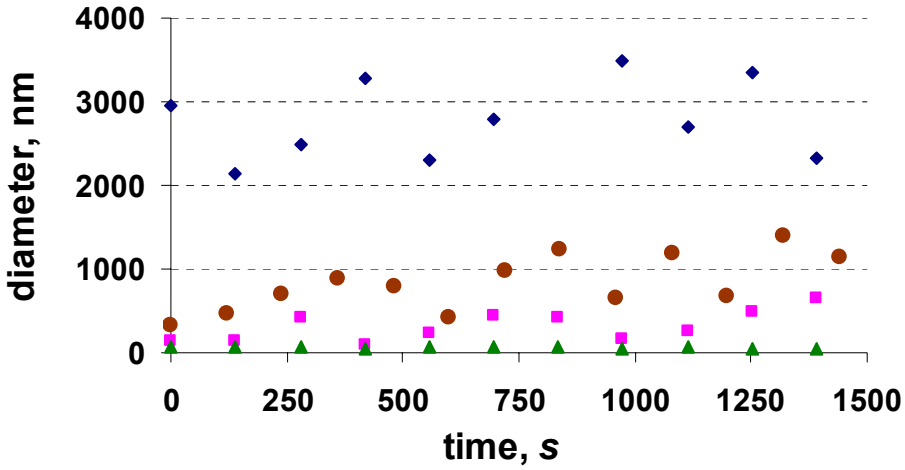

Fig. S.3. Kinetic DLS measurements performed on 15 mol%EthylPC/EYPC (K_2SO_4 $_{in}$, LiCl $_{out}$) liposomes preincubated for 12 hours with (squares) or without (diamonds) valinomycin and phenoxyacetamide **1**, suspended in a solution of HSA. Note, that these data are the same as in Fig. 4 of the main text. Circles correspond to the experiment performed on liposomes of the same preparation, incubated for 2 hours with ionophores. Triangles correspond to the negative control experiment performed on 100% EYPC liposomes suspended in the identical HSA solution.

(1) Sidorov, V.; Kotch, F. W.; Kuebler, J. L.; Lam, Y. F.; Davis, J. T. Chloride transport across lipid bilayers and transmembrane potential induction by an oligophenoxyacetamide. *J. Am. Chem. Soc.* **2003**, *125*, 2840-2841.