Towards improved gene delivery: Flip of cationic lipids in highly polarized liposomes†

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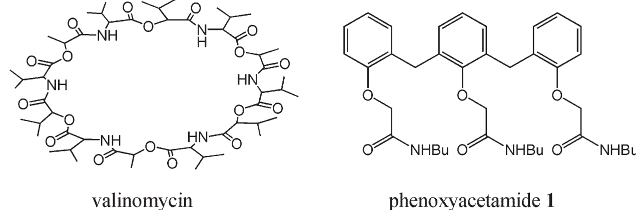
Hyperpolarization of cationic liposomes improves their stability in the presence of human serum albumin.

The lipid distribution across cell membranes is controlled by a family of enzymes known as flippases, floppases and scramblases.[1] Recent interest in the development of the synthetic molecules mimicking the function of these enzymes has emerged from their potential applicability to therapies of cancer and genetic disorders.[2] Most notably, the group of Smith demonstrated that a series of lipophilic chelators for negatively charged lipids facilitate the flip-flop of these lipids across the bilayer, ultimately equilibrating their distribution in the inner and outer leaflets.[2–4] These compounds therefore mimic the function of natural scramblases. Yet, there was no experimental evidence that the function of unidirectional transporters, such as flippase or floppase can be mimicked by a synthetic compound. Whereas the function of synthetic scramblases relies on the increased permeability of the chelated polar headgroups of lipids to the hydrophobic interior of the bilayer, the unidirectional transport of lipids, especially against the concentration gradient requires specific stimuli. In addition to mimics of flippases and floppases, unidirectional transport of synthetic cationic lipids can find a direct utility in non-viral gene delivery. Liposomes utilized as vehicles for non-viral gene delivery contain cationic lipids which compact the DNA plasmid and assist in its transport across cellular barriers.[5] However, a certain portion of cationic lipid in these lipoplexes remains in the bilayer outer leaflet, lowering the efficiency of gene delivery by lipoplexes[6] mostly due to the interactions with human serum albumin (HSA). These interactions destabilize lipoplexes, leading to their aggregation and/or premature release of DNA plasmid.[7] Although a number of technologies have been employed to overcome this difficulty, such as coating of lipoplexes with PEG-modified lipids and stabilizing them with cholesterol or DOPE, the efficiency of non-viral gene delivery systems still remains unsatisfactory.[8] Some improvement of the transfection efficiency by non-viral vectors in neural cells has been achieved through the use of synthetic cationic lipids with disulfide linkages.[9] These lipids, however, still exhibited toxicity against HEK293 cells.

If a means could be found to flip these cationic lipids to the inner bilayer leaflet, then these interactions with HSA should be reduced or eliminated. The group of Leenhouts has shown that 50–150 mV transmembrane diffusion potential ($\Delta\psi$) induced by valinomycin[10] in liposomes that are under K^+ gradient can flip the cationic octadecyl rhodamine B.[11] This single-chain amphiphile differs in structure from phospholipids comprising cell membranes or lipoplexes. Furthermore, there is an evidence that the physiological $\Delta\psi$ alone does not affect the passive translocation of phospholipids,[12] but assists in the facilitated transport of lipids across the bilayer. We hypothesized that a potential exceeding in value the physiological $\Delta\psi$ (>150 mV) can induce translocation of the charged phospholipids. The maximum value of K^+-diffusion potential in liposomes is limited by the osmotic conditions[13] and natural permeability of ions[14] to the bilayer membrane.



Recently, the group of Davis demonstrated the generation of a diffusion potential by using a Cl^--transporter phenoxyacetamide **1** in liposomes that are under outwardly directed Cl^- gradient.[15] We decided to use both ionophores simultaneously, assuming that the outward transport of K^+ and inward transport of Cl^- would result in $\Delta\psi$ higher than that achieved using valinomycin alone (Fig. 1).

Liposomes for this study were prepared from egg-yolk L-α-phosphatidylcholine (EYPC) as the major lipid component and 75 mM K_2SO_4 10 mM phosphate buffer (see ESI†). The external buffer was replaced with 100 mM NaCl or LiCl phosphate buffer. The observed potentials were more stable in liposomes suspended in LiCl buffer, probably due to the lower rate of Li^+ transport.[16]

First, we analyzed the $\Delta\psi$ buildup in 100% EYPC liposomes (K_2SO_4 $_{in}$/$LiCl_{out}$) upon application of phenoxyacetamide **1**, valinomycin, or both by monitoring the fluorescence of

Fig. 1 Schematic representation of the flippase model. Circles with "+" signs denote cationic lipids used in the formulation of lipoplexes. Induction of the transmembrane potential results in the accumulation of cationic lipids in the inner leaflet of the bilayer.

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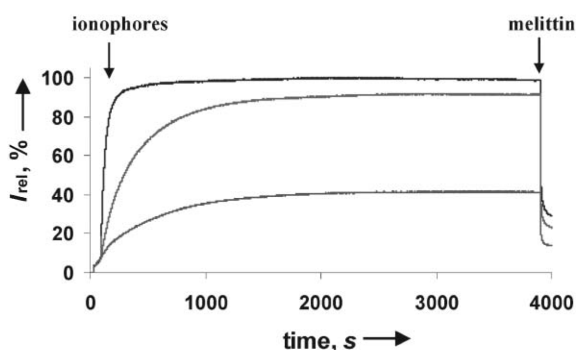

Fig. 2 Monitoring of the fluorescence of safranin O (λ_{ex} = 520 nm, λ_{em} = 580 nm) as a function of the potential buildup upon application of 312.5 nM phenoxyacetamide **1** (bottom trace), 37.5 nM valinomycin (middle trace), or both (upper trace) to a suspension of EYPC (lipid concentration 125 μM) liposomes (K_2SO_4 in/$LiCl_{out}$). The experiments were terminated by application of 10 μM defect inducer melittin.

exogenously added potential-sensitive dye safranin O (Fig. 3).[17] The results for the optimized system are shown in Fig. 2. Phenoxyacetamide **1** induced a substantially lower $\Delta\psi$ than valinomycin. This is probably due to the high permeability of Cl^- to the bilayer membrane and some potential offset by co-transport of H^+.[15] The $\Delta\psi$ induced by valinomycin into the liposomes suspended in K^+-free buffer can be estimated only by extrapolation of fluorimetric data (see Fig. S2, ESI†), which gives a value in excess of 300 mV. The simultaneous application of both transporters resulted in a much faster $\Delta\psi$ buildup, which was stable for at least 3 h. A somewhat higher fluorescence of safranin O observed for the latter case indicates the higher potential, and extrapolation suggests a value in excess of 350 mV. This extrapolation can be used only with care as the fluorimetric response of safranin O does not change linearly with $\Delta\psi$ (Fig. S2, ESI†).[18] Although the fluorimetric response of safranin O was not additive in both K^+-free and 1 mM K^+ external buffers (Fig. S1, ESI†), the combined application of ionophores always gave higher $\Delta\psi$ than the application of one of these ionophores alone.

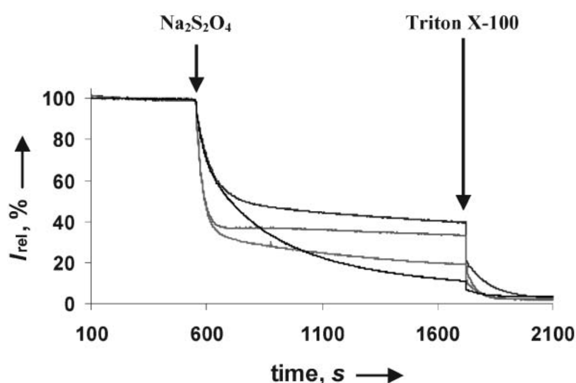

Fig. 3 Monitoring of NBD-PE fluorescence in liposomes under $\Delta\psi$ for various incubation periods at RT. Time events: 600 s: $Na_2S_2O_4$ injected; 1800 s: lysis of liposomes with 40 μL of 2.5% Triton X-100. Traces from top to the bottom at 1600 s correspond to (1) blank experiment (no ionophores added); (2)–(4) liposomes preincubated with ionophores for 5 min (trace 2), 2 h (trace 3) and 12 h (trace 4).

Next, we determined, using fluorimetric technique of Armstrong,[19] whether this high $\Delta\psi$ could translocate a charged lipid. We used the anionic, fluorescently labelled lipid *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) in the liposome preparation (2000 : 1 EYPC/NBD-PE). After addition of the ionophores, the liposomes were incubated for 5 min, 2 and 12 h. A blank experiment was also performed where only DMSO without ionophores was added. After the incubation period, 20 μL of an aqueous 0.8 M $Na_2S_2O_4$ (membrane-impermeable NBD fluorescence quencher) solution was added. In symmetrical liposomes, the NBD-PE should be equally distributed between the outer and inner bilayer leaflets. Since the dithionite solution can only act on the portion of NBD-PE residing in the outer leaflet, a 50% quench of total fluorescence was expected, which was confirmed in the blank experiment. However, incubation of liposomes with the transporters resulted in higher than 50% quench of fluorescence consistent with the flop of anionic lipid towards the positive pole of $\Delta\psi$. The extent of quench was proportional to the incubation times, culminating at 90% after 12 h of incubation. Different kinetics of fluorescence quench observed after 12 h of incubation indicates that other processes, such as diffusion of dithionite through the bilayer of aged liposomes may take place in addition to the lipid flop. Safranin O, included in the extravesicular buffer, indicated that the $\Delta\psi$ did not dissipate even after 12 h. No significant deviations from 50% quenching was observed when the liposomes were incubated only with valinomycin or phenoxyacetamide **1** alone. All experiments were terminated with 0.2% Triton X-100, which lyzed the liposomes and caused a complete quenching of fluorescence.

Finally, we analyzed the effects of transmembrane potential on the stability of liposomes containing cationic phospholipid used in formulations of lipoplexes (Ethyl PC) in the presence of HSA. It is known that the interactions of HSA with cationic liposomes result in a formation of large aggregates. We monitored the size of particles in the liposome suspensions in the presence of HSA by the dynamic light scattering (DLS) techniques and used this size as a measure of the relative stability of liposomes. Several populations of liposomes (K_2SO_4 in/LiCl or $NaCl_{out}$) containing varying mol percentages of cationic Ethyl PC (0–20 mol%) in EYPC were prepared. Pure EYPC liposomes were used as negative control. Liposome preparations were added to NaCl or LiCl phosphate buffer with or without ionophores. After a 12 h incubation period at room temperature, a DLS experiment was performed to verify that the *Z*-average size was still on the order of 100 nm. Then the physiological amount of HSA was added to the suspension of liposomes, and the DLS experiment was repeated. Once HSA was added, measurement to measurement variability of *Z*-average values occurred. Therefore, each Z_{avg} value, Table S1, ESI,† represents the average of 10 separate Z_{avg} measurements, spaced 30 s apart. We found that 100% EYPC liposomes actually experienced a decrease in Z_{avg} values due to smaller HSA particulates contributing to the average. However, preparations of 12.5, 15 and 20 mol% Ethyl PC always demonstrated an increase in Z_{avg} values attributed to aggregation with HSA. In each of these instances, the size of the particles formed upon addition of HSA was smaller for the liposomes preincubated with ionophores, compared to liposomes preincubated with pure DMSO, which is consistent with higher stability of ionophore-treated liposomes. Although NaCl buffer is more representative of

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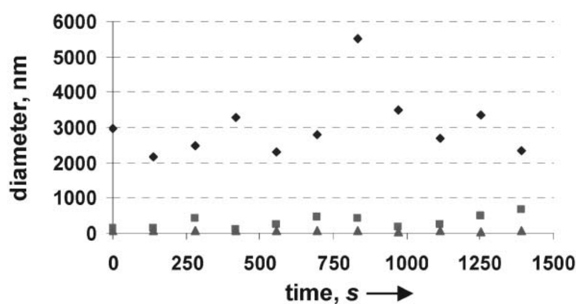

Fig. 4 Kinetic DLS measurements performed on 15 mol% Ethyl PC/EYPC ($K_2SO_{4\ in}$, $LiCl_{out}$) liposomes preincubated with (squares) or without (diamonds) valinomycin and phenoxyacetamide **1**, suspended with HSA. Triangles correspond to the negative control experiment performed on 100% EYPC liposomes suspended in the identical HSA solution.

biological conditions, we also experimented with LiCl[20] buffer due to concerns of valinomycin-facilitated antiport of Na^+[21] that causes some negation of potential stability. However, no significant variation in the relative size increase of ionophore-treated liposomes was observed between NaCl and LiCl buffers.

With ten DLS measurements taken only 30 s apart, the tabulated runs were completed in the span of only about 5 min each. We were interested in whether reduced Z_{avg} values would continue for a longer period of time, more consistent with the needs of gene therapy regimens. We performed extended kinetic runs, Fig. 4, using a 15 mol% Ethyl PC liposome preparation in LiCl phosophate buffer. After a 12 h incubation with or without the ionophores, 100 mg of HSA was added to 2 mL of liposome suspensions and a kinetic DLS experiment was performed. Individual Z_{avg} measurements were spaced about 2 min apart. When compared to ionophore-free preparation, it was found that the ionophore-containing preparation could maintain a significantly lower Z_{avg} value for at least 40 min. As expected, a control performed on a 100% EYPC preparation did not demonstrate any elevation of Z_{avg} values over the same time period. As the fluorescence quenching experiments suggested that other than lipid flip processes may take place after 12 h of incubation, we also performed DLS kinetic experiments with liposomes preincubated with ionophores for only 2 h. In this case, the Z_{avg} values increased to 900 nm in only 6 min (see Fig. S3 in ESI†). Clearly, longer incubation times for sufficient stabilization of cationic liposomes are required.

In conclusion, we demonstrated that the simultaneous application of K^+-specific carrier valinomycin and Cl^--specific transporter phenoxyacetamide **1** into a suspension of lipsomes that are under inwardly directed Cl^- gradient and outwardly directed K^+ gradient results in a formation of strong transmembrane potential. The potential buildup was rapid and this potential was stable over extended period of time. We demonstrated that this potential can flop the fluorescently-labelled anionic lipids towards the positive pole, resulting in the accumulation of this lipid in the bilayer outer leaflet. We also demonstrated that the cationic liposomes that are under K^+ and Cl^- gradients exhibit higher stability in HSA solution when preincubated with K^+- and Cl^- -specific ionophores. This improved stability is consistent with the $\Delta\psi$-induced flip of cationic lipids into the inner bilayer leaflet. Still, some aggregation of pretreated cationic liposomes was evidenced. Z_{avg} values did not decrease upon the addition of HSA as they did for the neutral liposomes, but showed small, compared to ionophore-free liposomes, increase in the size. Further optimization is required to increase the effectiveness of this procedure. One area currently under investigation is the development of scramblases specific to cationic lipids.[2,3] If an optimized process for the flipping of cationic lipids in liposomes is established, then the next step would be testing its effectiveness on lipoplexes, DNA plasmid-containing liposomes. Success could bring about an improved non-viral delivery system for genetic therapies.

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