Squeezing a Synthetic Ionophore and Mechanistic Insight Out of a Lipid Bilayer

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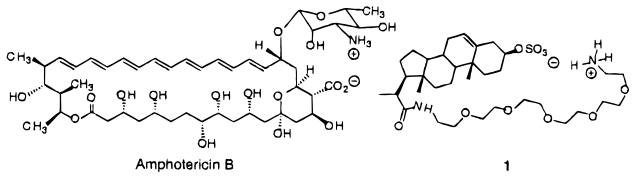
In this paper we report the first example of a phase-induced expulsion (i.e., "squeezing out") of a synthetic ionophore from a phospholipid bilayer.[1] Specifically, we show how a synthetic ionophore, derived from 23,24-bisnor-5-cholenic acid and 1,17-diamino-3,6,9,12,15-pentaoxaheptadecane (i.e., **1**), is expelled from vesicles made from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) when the temperature of the dispersion falls below the membrane's liquid-crystalline phase transition temperature (T_c = 41.4 °C). We also show how such expulsion can be used to provide detailed mechanistic insight into the nature of the transport-active species.

We have previously reported the design and synthesis of **1**, which was patterned after the macrolide antibiotic, Amphotericin B.[2] Specifically, a long and rigid hydrophobic unit (sterol nucleus), a flexible hydrophilic chain (polyether), and a pendant polar headgroup (ammonium sulfate) were used to mimic the heptaene, polyol, and carboxyl/mycosamine components of Amphotericin B, respectively. In very recent studies, we have shown that **1** promotes Na^+ transport across fluid bilayers derived from 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (C14:1) PC, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (C16:1) PC, and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (C18:1) PC.[3] On the basis of transport rates that exhibited a second-order dependency on the mol % of **1**, and also a strong activity-dependence on bilayer thickness, a model was proposed in which **1** produces *membrane-spanning, transport-active dimers*.[3]



During the course of related studies, it occurred to us that further support for this membrane-spanning model should be obtainable by measuring the activity of **1** in gel- versus fluid-phase membranes. In particular, it is well-known that a comparison of ionophoric activity above and below the phase transition temperature of a host membrane can be used to distinguish between an ion carrier mechanism and a membrane-spanning pathway.[4] Thus, ion transport that occurs via a carrier mechanism is expected to exhibit significantly lower rates in the gel phase as compared with those in the fluid phase, because the ionophore is "frozen" in each monolayer, i.e., transbilayer movement is greatly reduced. In contrast, ion transport rates are expected to be almost independent of the fluidity of a bilayer when a membrane-spanning mechanism is operative. To our chagrin, the results of gel- versus fluid-phase experiments in DPPC bilayers appeared to favor a carrier mechanism (vide infra). Careful examination of this system, however, revealed that **1** was being expelled from the bilayer as the membrane entered the gel phase.[5] More importantly, we have found that such expulsion can be used to gain mechanistic insight into the nature of the transport-active species.

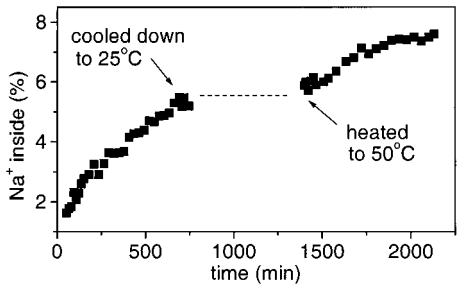


Figure 1. Entry of Na^+ into DPPC vesicles containing 0.4 mol % of **1** as a function of time at 50 °C (■), before and after an interruption period (- -) in which the temperature of the dispersion was reduced to 25 °C.[6]

Using experimental protocols similar to those previously described, **1** was incorporated into both leaflets (double-sided addition) of 200 nm unilamellar DPPC vesicles that were prepared in LiCl solution.[3] After dilution with an aqueous NaCl solution containing a shift reagent ($DyCl_3$), the rate of entry of Na^+ into the vesicles was monitored using $^{23}Na^+$-NMR spectroscopy. While significant ionophoric activity was observed at 50 °C, no Na^+ transport could be detected at 25 °C (Figure 1).

To determine whether **1** remained associated with the DPPC membranes while in the gel state, a dispersion was filtered (Sephadex G-25 M) at 25 °C and the vesicles analyzed for ionophore content using a fluorescamine assay.[7] With an initial loading of 1.5 mol % of **1**, we found that 53 ± 5% of the conjugate was removed after filtration. In sharp contrast, gel filtration of a similar dispersion that was prepared and filtered at 50 °C resulted in a 94 ± 5% recovery of **1** in the vesicles. These results strongly suggested that in the gel phase half of the ionophore was being expelled into the bulk aqueous phase and half into the aqueous interior of the vesicles where it cannot be removed by gel filtration.

On the basis of the data that are shown in Figure 1, we conclude that the return of gel-phase vesicles to their fluid state results in complete re-incorporation of the ionophore into the bilayer. In principle, such reversible expulsion should allow one to determine whether movement of the ionophore between the adjoining monolayers (flip-flop) is significant on the time scale of these experiments. We were particularly interested in testing for flip-flop because it bears directly on the question of whether **1** functions as an ion carrier, i.e., negligible flip-flop would argue against a carrier mechanism. As illustrated in Scheme 1, if flip-

(1) For reviews of synthetic ionophores, see: (a) Gokel, G. W.; Murillo, O. *Acc. Chem. Res*. **1996**, *29*, 425. (b) Koert, U. *Chem. Unserer Z.* **1997**, *31*, 20. For recent examples, see: (c) Fyles, T. M.; Loock, D.; Zhou, X. *J. Am. Chem. Soc*. **1998**, *120*, 2997. (d) Clark, T. A.; Buehler, L. K.; Ghadiri, M. R. *J. Am. Chem. Soc*. **1998**, *120*, 651. (e) Jin, T.; Kinjo, M.; Kobayashi, Y.; Hirata, H. *J. Chem. Soc., Faraday Trans*. **1998**, *94*, 3135. (f) Murray, C. L.; Gokel, G. W. *Chem. Commun*., **1998**, 2477. (g) Fritz, M. G.; Walde, P.; Seebach, D. *Macromol*. **1999**, *32*, 574. (h) Tedesco, M.; Ghebremariam, B.; Sakai, N.; Matile, S. *Angew. Chem., Int. Ed. Engl*. **1999**, *38*, 540. (i) Sakai, N.; Majumdar, N.; Matile, S. *J. Am. Chem. Soc.* **1999**, *121*, 4294.

(2) Sadownik, A.; Deng, G.; Janout, V.; Regen, S. L. *J. Am. Chem. Soc*. **1995**, *117*, 6138.

(3) Otto, S.; Osifchin, M.; Regen, S. L. *J. Am. Chem. Soc*. **1999**, *121*, 7276.

(4) (a) Krasne, S.; Eisenman, G.; Szabo, G. *Science* **1971**, *174*, 413. (b) Boheim, G.; Hanke, W.; Eibl, H. *Proc. Natl. Acad. Sci., U.S.A.* **1980**, *77*, 3403.

(5) A recent report of an amphiphilic, fluorescent pyrrolopyrimidine (U-104067) has shown that the molecule exhibits full solubility in the liquid disordered phase of DPPC membranes, and complete insolubility in the solid ordered phase, while remaining in the bilayer: Epps, D. E.; Wilson, C. L.; Vosters, A. F.; Kezdy, F. J. *Chem. Phys. Lipids* **1997**, *86*, 121.

(6) When higher concentrations of **1** were used, a cooling/reheating cycle caused extensive leakage, allowing even the shift reagent to enter the vesicles.

(7) (a) Udenfriend, S.; Stein, S.; Bohlen, P.; Dairman, W. *Science* **1972**, *178*, 871. (b) Imai, K.; Watanabe, Y. *Anal. Chim. Acta* **1981**, *130*, 37. The experimental procedure was similar to that reported in ref 3.

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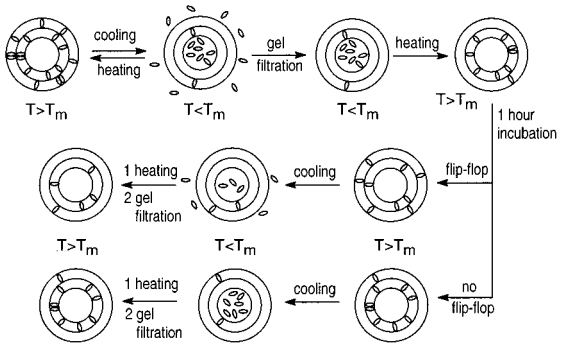
Scheme 1



flop were rapid, then one would expect that cooling and gel-filtration, followed by reheating, incubation, cooling, and a second gel filtration should result in the removal of additional ionophore. If flip-flop were slow, however, then no further removal of ionophore should be observed. Experimentally, we have found that after a 1-h incubation period at 50 °C and a second gel filtration at 25 °C, half (49 ± 5%) of the ionophore was still retained by the vesicles. These results imply that flip-flop and ion carrier processes are negligible.

In an effort to obtain more detailed insight into the nature of the transport-active species, we examined the extent of Na^+ entry into fluid-phase DPPC vesicles (50 °C) as a function of time and ionophore content, *before and after removal of* **1** *from the outer monolayer leaflet*. In contrast to our previous results for (C16:1) PC bilayers, the observed pseudo-first-order rate constants (k_{obsd}) were found to have a *fourth*-order dependency on the mol % of **1** (Figure 2).[3] As noted previously, a nonlinear dependence of transport activity on ionophore concentration supports a model in which only a small fraction of the ionophore is assembled into transport-active aggregates. More specifically, it can be readily shown that:

$$k_{obsd} = k_o + k_2[\text{monomer}]^n/K \qquad (1)$$

where K is the equilibrium constant for dissociation of an assembly of n ionophore molecules into monomers, k_o is the rate constant for ion transport in the absence of ionophore, and k_2 is an intrinsic rate constant.[3] Thus, a fourth-order dependency of rate on the mol % of **1** supports the existence of transport-active *tetramers*. A similar series of experiments that were carried out, in which the vesicles were gel-filtered at 25 °C, prior to dilution with aqueous NaCl and shift reagent, showed a *second*-order dependency on the mol % of **1** indicating the presence of transport-active dimers in the inner leaflet. The picture that emerges from these results, therefore, is that the transport-active tetramers that exist within these membranes consist of dimers in one monolayer leaflet that are aligned with dimers in the adjoining leaflet (Figure 3a).[8] It is noteworthy that such a structure bears a resemblance to the "barrel stave" model that has been proposed for Amphotericin B in (C22:1) PC bilayers under double-sided conditions.[9]

One final mechanistic issue that we sought to clarify in this study was the apparent shift from transport-active dimers in fluid phase membranes composed of (C16:1) PC (35 °C), as reported previously, to transport-active tetramers in fluid DPPC bilayers (50 °C).[3] Since the only differences in these two systems are temperature and degree of unsaturation, we examined the rate of Na^+ transport in (C16:1) PC as a function of ionophore concentration at 50 °C. The fact that a second-order dependency on the mol % of **1** was observed indicates that the shift to the tetramer-active aggregates in DPPC results from the removal of unsaturation within the alkyl chains (Figure 2).

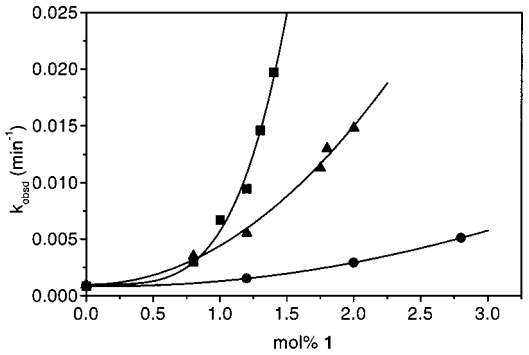


Figure 2. Plot of k_{obsd} versus mol % **1** in DPPC before (■) and after (●) removal of **1** via gel filtration at 25 °C; the solid lines represent the best-fit curves according to eq 1, where $n = 4.2 \pm 0.7$ and 2.2 ± 0.7, respectively. For comparison, a plot of k_{obsd} versus mol % of **1** in (C16:1) PC (50 °C) is also given (▲); here, a best-fit curve was obtained with $n = 2.0 \pm 0.3$.

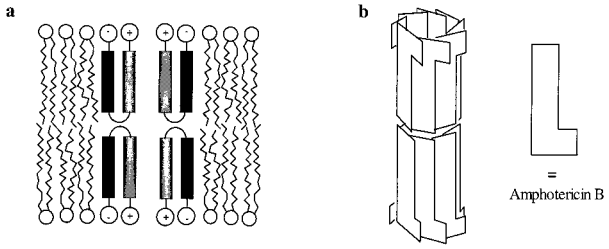


Figure 3. (a) Hypothetical geometry of the transport-active tetramer of **1** in fluid-phase DPPC vesicles. Darkened and lightly shaded rectangles represent sterol and polyether units, respectively; **1** is represented in its zwitterionic form (b) "Barrel-stave" model for Amphotericin B when present in both leaflets of (C22:1) PC vesicles.[8]

In summary, we have presented the first example of a phase-induced expulsion of a synthetic ionophore from a phospholipid membrane. We have also shown that this behavior can be used to obtain insight into the structure of a transport-active aggregate. Given the similarity between tetramers of **1** and the proposed active form of Amphotericin B in (C22:1) PC membranes (Figure 3), it is tempting to speculate that **1** (like Amphotericin B) may be capable of exhibiting selectivity toward lipid membranes based on their sterol content, e.g., membranes containing ergosterol versus cholesterol.[9a] Studies aimed at exploring such a possibility, with a view toward the creation of improved antifungal agents, are in progress.

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Supporting Information Available: Experimental procedures for vesicle formation and ionophore analysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) We do not presently understand the nature of the transport-active dimers, which remain after gel filtration.

(9) (a) Bolard, J. *Biochim. Biophys. Acta* **1986**, *864*, 257. (b) Van Hoogevest, P.; DeKruijff, B. *Biochim. Biophys. Acta*. **1978**, *511*, 397.