Molecular recognition-controlled membrane disruption by a bis(crown ether) bola-amphiphile

Thomas M. Fyles*† and Binqi Zeng

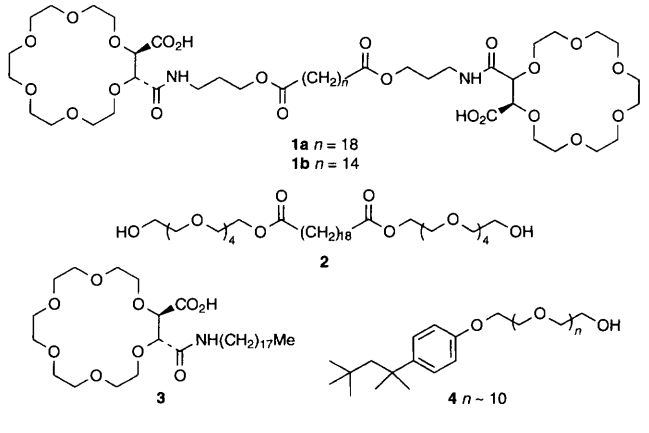
Department of Chemistry, University of Victoria, Victoria, B.C., Canada V8W 3V6

Release of liposome-entrapped carboxyfluorescein is switched 'on' *via* molecular recognition of barium ion by a bis(crown ether carboxylate) bola-amphiphile.

Drug delivery using liposomes[1] relies on cell uptake of the liposome by fusion or phagocytosis.[2] Unfortunately, stabilized liposomes capable of specific cell targeting *in vivo* are resistant to these uptake mechanisms.[3] A solution to this impasse would couple molecular recognition of the target to the release of liposome contents. This is one example of a general problem in signal transduction; specifically, how can a molecular binding event be amplified to a supramolecular membrane disrupting event? Consider the series of reports by Regen and co-workers[4] on membrane disrupting systems based on bis(oligoethyleneoxide) bola-amphiphiles[4a] and related polymeric derivatives.[4b,c] There is considerable evidence that membrane disruption occurs *via* a U-shaped bola-amphiphile conformation.[4] Perhaps molecular recognition-based control of membrane disruption might be achieved *via* U-shaped bis(crown ether) complexes[5] in which formation of a 'sandwich' complex would create the required U-shaped conformation in the linking chain.

Here we report the behaviour of a bis(crown ether) bola-amphiphile system (**1a,b**) in comparison with **2**, an analogue of the compounds described by Regen,[4a] and with **3**, a previously reported surfactant crown ether carboxylate.[6] We expected **1a,b** to be less effective membrane disrupting agents than **2** due to repulsion of the charged head groups. However, the binding of a divalent cation in one of the crown ethers would favour approach of the head groups, thereby stabilizing a U-shaped conformation.

The synthesis of compounds **1a,b** follows from the structures. Dicarboxylic acids were converted *via* the acid chlorides to the bis(esters) of Z-protected 3-aminopropanol. Catalytic transfer hydrogenolysis of the protecting groups in formic acid[7] afforded the stable bis(formate) salts. In basic solution, the free amine could be intercepted by the anhydride of (*R*,*R*)1,4,7,10,13,16-hexaoxacyclooctadecane-2,3-dicarboxylic acid[6] to give the target compounds in 35–45% yield over the three steps. Compound **2** was prepared following the published procedure[4a] starting from pentaethylene glycol in place of hexaethylene glycol.‡



Membrane disruption was assessed by monitoring the release of liposome-entrapped 5[6]-carboxyfluorescein (CF) following procedures described previously.[4] Liposomes were formed in 0.1 M Na^+CF or K^+CF by a sonication–freeze–thaw method[8] using 8:1:1 egg phosphatidylcholine–phosphatidic acid–cholesterol. The crude liposomes were sized though 0.45 μm Nucleopore filters, and external CF was removed by gel filtration on Sephadex G-25 in a NaCl- or KCl-containing tris–HCl buffer. Aliquots of liposomes were incubated with varying amounts of **1–3** for 30 min, diluted with buffer, and the fluorescence emission intensity was recorded. The extent of release of entrapped CF was calculated from eqn. (1),

$$\text{CF release}(\%) = (I_{obs} - I_{blank})/(I_{max} - I_{blank}) \times 100 \quad (1)$$

where I is fluorescence intensity at 515 nm, and the subscripts denote the observed value for the sample (obs), the value for a blank sample (blank) and the maximum emission (max) for release of CF in excess Triton X-100 **4**. The extent of CF release as a function of the concentration of amphiphile is given in Fig. 1.

The behaviour of compounds **2** and **4** is qualitatively and quantitatively as expected.[4] Both amphiphiles achieve full disruption at high concentration in either sodium- or potassium-containing CF solutions (0.14 M NaCl or KCl), and are unaffected by added calcium, strontium or barium chlorides (up to 0.01 M). Conversely, compound **1a** is notably inactive in a sodium-containing buffer system, shows a small tendency to disrupt in a potassium-containing system, but shows a marked

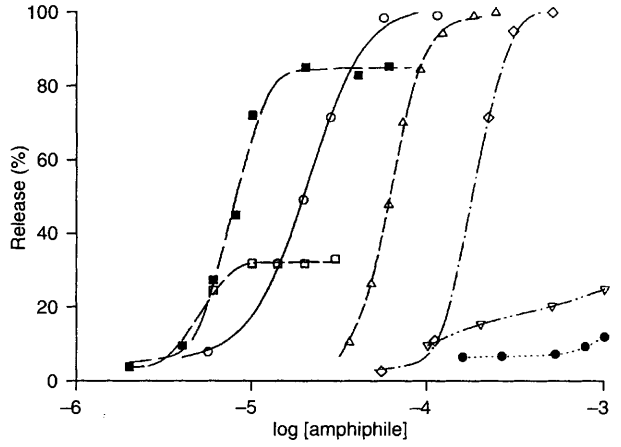


Fig. 1 Extent of carboxyfluorescein release as a function of amphiphile concentration after 30 min incubation of K^+CF containing liposomes in 0.14 M KCl–tris–HCl buffer (pH 7.5) for **1a** (□), **1a** including additional 1 mM $BaCl_2$ (■), **1a** with NaCl in place of KCl (●), **1b** (▽), **2** (○) **3** (△) and **4** (◇). Experimental uncertainty in percentage release = ±5%. Curves for **2**–**4** in 0.14 M NaCl–Tris–HCl buffer, or buffers containing up to 0.01 M Ca, Sr or Ba chlorides are identical to those shown within experimental error (points omitted for clarity). Curves are calculated using non-linear fits to a 4 parameter logistic function.

ability to induce CF release in the presence of 1 mM barium added to either the sodium or potassium containing the buffer systems. The extent of disruption induced by Ba^{2+} never reaches the full extent of entrapped CF accessible to **2** or **4** (see below). Addition of 1 mM Ca^{2+} or Sr^{2+} also induces membrane rupture but the effect is less than with Ba^{2+} (Ca^{2+} 35%, Sr^{2+} 55% at log [**1a**] = −3.6). Compound **1b** is substantially less active than **1a**, as expected from Regen's results.[4a] Comparison of **1a** and **3** reveals that cation selectivity is not an inherent property of crown ether surfactants; disruption by **3** is insensitive to Na^+, K^+ or additional alkaline earth salts.

The following features have been established. (i) The extent of release by **1a** is related to the fraction of unilamellar liposomes in the mixture. The uni-/multi-lamellar ratio was determined by a melittin assay.[9] The liposomes for Fig. 1 were 88 ± 4% unilamellar. The **1a**:Ba^{2+} curve appears to approach this value as an asymptotic limit. To test this hypothesis, liposomes were prepared with a smaller unilamellar proportion (60 ± 3%); **1a** released 61 ± 5% of the entrapped CF in the presence of Ba^{2+}. Similarly, an alternate liposome preparation containing 80 ± 4% unilamellar liposomes released 79 ± 5% of the entrapped CF.§ (ii) The cation selectivity is a kinetic effect. In the Ba^{2+}:**1a** system, >75% of the release eventually achieved occurs in the first 10 min, and the process is >95% complete within the 30 min incubation period. Without the Ba^{2+}, 55% release requires 6 h, and >95% release requires >40 h. This slower release is still a factor of seven faster than a control without any added **1a**. (iii) The induced membrane disruption is related to molecular recognition of Ba^{2+} by the crown ether of **1a**. In addition to the cation selectivity, the extent of CF release as a function of the Ba^{2+}:**1a** molar ratio appears as a hyperbolic curve. Analysis as a 1:1 binding isotherm yields an apparent binding constant of log K = 3.6 ± 0.3. This value is reasonable when compared to log β_{110} = 5.5 ± 0.2 in 90:10 methanol–water solution for the butyl homologue of **3** plus Ba^{2+},[10] or to an apparent interfacial association constant of log K_i = 3.0 ± 0.4 determined for Sr^{2+}:**3** at a $CHCl_3$–H_2O interface.[11]

A mechanism consistent with the results would involve partition of **1a** to the liposome and insertion into the outer leaflet. In the presence of K^+ alone, the two crown ether head groups repel one another both sterically and electrostatically. This inhibits the formation of U-shaped loops in the connecting chain. The addition of Ba^{2+} allows formation of neutral 1:1 complexes in which the connecting chain is in a tight U-shaped loop leading to rapid membrane disruption. We are exploring other systems based on the same mechanistic principle to establish the generality of this type of signal transduction.

The authors thank Rose Yen for the preparation of **2**. The ongoing support of the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged. B. Z. thanks the University of Victoria for Fellowship support.

Footnotes

† E-mail:tmf@uvaix.uvic.ca

‡ All new compounds were characterized by ^1H and ^{13}C NMR, IR and electrospray mass spectra. Compound purity was established by analytical gel permeation chromatography and elemental analysis.

§ Extent of release is unrelated to size distribution: 88% release from a bimodal population distribution of liposomes [140 nm diameter (12%); 470 nm diameter (88%)], 61% release from a similar population distribution [170 nm diameter (15%); 540 nm diameter (85%)], but 79% release from a significantly different population distribution [140 nm diameter (30%); 400 nm diameter (70%)]. Size distributions were determined using a Nicomp 370 particle size analyser.

References

1 *Liposomes as Drug Carriers—Recent Trends and Progress*, ed. G. Gregoriadis, Wiley, New York, 1988.
2 J. H. Senior, *Crit. Rev. Ther. Drug Carrier Syst.*, 1987, **3**, 123.
3 D. Papahadjopoulos, *J. Liposome Res.*, 1992, **3**, 3; D. Papahadjopoulos and A. A. Gabizon, in *Liposomes as Tools in Basic Research and Industry*, ed. J. R. Philipott and F. Schuber, C.R.C. Press, Boca Raton, 1995.
4 (*a*) N. Jayasuriya, S. Bosak and S. L. Regen, *J. Am. Chem. Soc.*, 1990, **112**, 5844; (*b*) S. L. Regen, N. Jayasuriya and W. Fabionowski, *Biochem. Biophys. Res. Commun.*, 1989, **159**, 566; (*c*) N. Jayasuriya, S. Bosak and S. L. Regen, *J. Am. Chem. Soc.*, 1990, **112**, 5851.
5 K. Kimura and T. Shono, in *Cation Binding by Macrocycles*, ed. Y. Inoue and G. W. Gokel, Marcel Dekker, New York, 1990.
6 L. A. Frederick, T. M. Fyles, N. P. Gurprasad and D. M. Whitfield, *Can. J. Chem.*, 1981, **59**, 1724.
7 B. El Amin, G. M. Anantharamaiah, G. P. Royer and G. P. Means, *J. Org. Chem.*, 1979, **44**, 3442.
8 *Liposomes—a practical approach*, R. R. C. New, IRL Press, Oxford, 1989.
9 M. Bhakoo, T. H. Birkbeck and J. H. Freer, *Can. J. Biochem. Cell Biol.*, 1985, **63**, 1; T. M. Fyles, T. D. James and K. C. Kaye, *J. Am. Chem. Soc.*, 1993, **115**, 12315.
10 T. M. Fyles and D. M. Whitfield, *Can. J. Chem.*, 1984, **62**, 507.
11 T. M. Fyles, *J. Chem. Soc., Faraday Trans. 1*, 1986, **82**, 617.

Received, 11th June 1996; Com. 6/04097G