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PROTON/HYDROXIDE PERMEATION ACROSS AMMONIUM ION BILAYER VESICLES DETECTED BY SELF-INDICATING HEAD GROUPS

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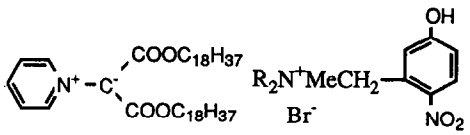
Summary. p-Nitrophenol-functionalized vesicular surfactants (2) are sensitive, reversible and continuous reporters of local pH conditions at endovesicular and exovesicular surfaces

The decay of pH gradients across lipid bilayers is governed by their permeability toward proton equivalents.[1] When permeability is sufficiently low, the imposition of an exovesicular/endovesicular pH gradient can hydrolytically differentiate susceptible surfactant head groups on the outer and inner surfaces of synthetic bilayer vesicles or liposomes.[2] In this methodology, which is broadly applicable,[3] decay of the pH gradient can be rate limiting for endovesicular hydrolyses. Accordingly, it is useful to have a direct probe of the decay of pH gradients across vesicles composed of surfactants that are closely related to those of chemical interest.[2]

Pertinent previous studies include Kunitake's report that riboflavin (pk_a ~ 10), entrapped within dioctadecyldimethylammonium bromide (DODAB) vesicles, resisted deprotonation when subjected to an endo/exo pH gradient of 9.0/11.4 ($\tau_{1/2}$ ~ 10 min); deprotonation was very rapid, however, when the gradient was 9.0/12.3.[4] Similarly, the endovesicular hydrolysis of 5,5'-dithiobis(2-nitrobenzoic acid) in DODAC(hloride) vesicles was ~90 times slower than the exovesicular hydrolysis, and appeared to be rate limited by OH^-/H^+ permeation when driven by an endo/exo pH gradient of 5.3/10.9.[5] Recently, self-indicating, vesicle-forming, pyridinium malonate diester ylides (e.g., 1) were used as probes of pH gradients across dipalmitoylphosphatidyl choline-cholesterol liposomes.[6] The internal pH of liposomes doped with 1 equilibrated within "minutes" upon exovesicular acidification, but equilibration required "hours upon basification."[6]

Now we describe surfactants 2 as self-indicating pH probes for use with the ubiquitous[1b] dialkyldimethylammonium ion vesicles constructed from surfactants 3. The fast p-nitrophenol ⇌ p-nitrophenoxide prototropic equilibration that obtains at the head groups of 2a - 2c makes these molecules spectroscopic reporters of the dynamics of pH gradient decay near pH 4-5.

Surfactants 2a (mp 76-78°C), 2b (mp 80-85°C), and 2c (mp 85-90°C) were prepared by hydrolysis (aq. ethanolic NaOH/$CHCl_3$ at 25°) of their corresponding benzoate esters,[7-9] followed by aq. HBr neutralization of the resulting $CHCl_3$ solution of the surfactant p-nitrophenoxides. Drying, removal of solvent, and several recrystallizations from EtOAc afforded 2a - 2c, which were characterized by nmr spectroscopy and microanalysis.



1

2a, R = n-$C_{16}H_{33}$
2b, R = n-$C_{18}H_{37}$
2c, R = n-$C_{20}H_{41}$

$R_2N^+Me_2$, Br^-

3a-c
(as in 2)

$(n\text{-}C_{18}H_{37})_2N^+MeCH_2CH_2OCOOC_6H_4NO_2$ Cl^-

4

Dialkyldimethylammonium bromide surfactant 3c (mp 135-140°C) was prepared from eicosyl bromide and dimethylamine, following the procedure described for 3a.[10] Surfactants 3a (mp 164-169°C)[10] and 3b (Sigma, mp 156-159°C) were available.

Covesicles of 2a/3a - 2c/3c (2:3 = 1:10) were generated by sonication of $CHCl_3$-evaporated surfactant films in 0.01 N aqueous HCl-0.01 M KCl solutions.[11] Dynamic light scattering[2a] of 2 (1×10^{-4} M)/3 (1×10^{-3} M) covesicles at pH 2 afforded hydrodynamic diameters of 42, 35 and 22 nm, respectively, in the C_{16}, C_{18}, and C_{20} systems. A similar trend was observed with holovesicles of 3a-c (36, 29, and 26 nm, respectively). Tighter packing and smaller size with increasing chain length appears evident.

The gel to liquid crystalline phase transition temperatures (T_c) of these covesicles (conditions as above) were determined from discontinuities in the fluorescence polarization of (4×10^{-5} M) covesicallized 1,6-diphenyl-1,3,5-hexatriene as a function of temperature.[2a,12] We observed T_c values of 20, 36, and 45°C, respectively, for the C_{16}, C_{18}, and C_{20} covesicles. Therefore, under kinetic conditions at 25°C, both the C_{18} and C_{20} covesicles will initially be in their more rigid gel phases, but the C_{16} covesicles will be in the relatively fluid liquid crystalline phase.

Kinetics. The pK_a of 2 in 1:10 covesicular 2/3 is between 4 and 5 (uv spectroscopy), and is sensitive to minor variations in vesicular conditions. Accordingly, solutions of covesicular 2 (5×10^{-5} M) and 3 (5×10^{-4} M) were prepared by sonication[11] in 0.01 N HCl (pH 2-2.2), 0.01 M KCl, where the p-nitrophenol headgroups of 2 were very largely protonated (UV, 310 nm). Endo/exo 2/8 pH gradients were then imposed on the 2/3 covesicles by the addition of 0.02 M aqueous Tris (0.01 M in KCl), leading to a final bulk aqueous pH of 8.0-8.1. The ensuing conversion of 2 (p-nitrophenol) to 2 (p-nitrophenoxide) was monitored at 400 nm by UV spectroscopy, using stopped-flow methods for the 2a/3a case.

For each covesicular system, we observed "instantaneous" (<1 msec) deprotonation of 50-60% of the p-nitrophenol (PNPOH) head groups to their p-nitrophenoxide (PNPO) forms, followed by kinetically-discrete, "slow" deprotonation of the remaining PNPOH moieties. The latter reactions were system (i.e., chain length) dependent. Moreover, all reactions were reversible upon acidification. We take the very rapid PNPOH → PNPO conversions to represent exovesicular deprotonation in immediate response to the imposition of the pH gradient. The slower, subsequent deprotonation is then attributable to the decay of the gradient, mediated by H^+/OH^- permeation across the bilayers.[1] The apparently pseudo-first-order rate constants associated with these processes are: 2a/3a ~1.0; 2b/3b, 0.019 ± 0.002; 2c/3c, 7.1×10^{-4} s^{-1}.[13]

The high H^+/OH^- permeability and rapid gradient decay of the **2a/3a** (C_{16}) covesicles can be associated in part with their low T_c (20°C) and corresponding fluidity at 25°C. (However, even at 15°C, pH 2/8 gradients across these covesicles decay with $\tau_{1/2}$ ~ 3-4 s). The slower gradient decays observed with the C_{18} and C_{20} covesicles, where T_c > 25°C, reflect their more rigid, gel phase construction, increasingly tighter packing, and (consequently) greater resistance to permeation.[14]

Further details of the kinetic processes were explored with the **2b/3b** (C_{16}) covesicles. We found that the presence of Tris buffer had little effect[5] on the rate of gradient decay; using 0.5 N NaOH solution to impose a pH 2.2/8.1 gradient gave $\underline{k}$ = 0.015 s^{-1} ($\tau_{1/2}$ ~ 46 s), compared to $\underline{k}$ = 0.019 s^{-1} (τ ~ 36 s) in the presence of Tris. The size of the initial pH gradient also had minimal effect on the rate of gradient decay. Thus, $\underline{k}$ = 0.017 or 0.018 s^{-1} for gradient decay with initial pH gradients of 3.9/8.1 or 2.2/9.8, respectively, compared to $\underline{k}$ = 0.019 s^{-1} in the pH 2/8 gradient experiment.

Most importantly, protonation-deprotonation changes with the **2b/3b** covesicles were reversible, occurred at comparable rates, and could be cycled. Thus, vesicles prepared at pH 2.2 first deprotonated with $\underline{k}$ = 0.020 s^{-1} when the external pH was adjusted to 8.0 - 8.1 with Tris, and then <u>reprotonated</u> with $\underline{k}$ = 0.019 s^{-1} upon addition of HCl (to pH 2.1 - 2.2).[15] A second readjustment with 0.5 N NaOH (to pH 8.0 - 8.1) gave deprotonation with $\underline{k}$ = 0.021 s^{-1}. Covesicles prepared at pH 8 in Tris buffer gave $\underline{k}$ = 0.022 s^{-1} for protonation with HCl (pH 2.1-2.2).

Comparisons of the rate constants for pH gradient decay of covesicular **2b/3b** with the rate constants for endovesicular esterolyses (with initial pH 4/8 gradients) of covesicular 4/3b by OH^-[2a] or of **2b-benzoate/3b** by glutathione/OH^-[2a,12a] indicate that the pH gradient decays are ~10 times faster than the endovesicular esterolyses. This could be consistent with rate-limiting H^+/OH^- permeation if the establishment of endovesicular pH 8 required more time than endovesicular pH 6.[13] Alternatively, pH gradient decay and the concommitant increase in endovesicular [OH^-] may not be the sole rate limiting factors governing the endovesicular esterolyses. Lower reagent activity or lower substrate reactivity might obtain at the endovesicular membrane surfaces.

Covesicles constructed of surfactants 2 and 3 are sensitive, reversible, and continuous reporters of "microscopic" pH at the exovesicular and endovesicular surfaces. The dynamics of pH gradient decay across these bilayers are particularly apparent, and exhibit clear structural dependencies on chain length and packing. These and related reporter surfactants are likely to be valuable probes in continuing studies of vesicular reactions, and the chemical differentiation of vesicular surfaces.

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(7) The benzoate esters of 2a[8] and 2b[2a] have been described. The benzoate of 2c (mp 85-95°C) was similarly[2a] prepared in 43% yield by quaternization of N,N-dieicosyl-N-methylamine[9] with 4-nitro-3-(bromomethyl)phenyl benzoate[2a] ($CHCl_3$, 25°, 48 h). It was purified by recrystallization from EtOAc, and characterized by nmr and microanalysis.

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(9) This tertiary amine (mp 42-43°C) was prepared in 76% yield from 10 mmol of methylamine, 20 mmol of eicosyl bromide, 24 mmol of NaOH, 33 mg of KI (catalyst), and 1.5 ml of water (140°C, sealed tube, 7 days, recryst. EtOAc).

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(11) Sonication conditions: Braunsonic Model 2000 sonicator, 108 x 19 mm immersion probe, 55-65 W, 6 min. For series a and b, the sonication temperature rose to 45-50°C; for series c, it was raised from 45-65° C during sonication. Sonicated solutions were cooled slowly and filtered through 0.8 μM Millex (Millipore) filters before use.

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(13) The corresponding approximate half-lives are 0.7, 36, and 976 seconds, respectively. Note that because the pKa's of covesicular 2a-c are 4-5, the apparent rate constants and half-lives most closely report the dynamics of gradient decay in the pH 3-6 interval.

(14) We do not explicitly consider surfactant flip-flop[2a] here, which (on the basis of T_c) should be important with 2a, unimportant with 2c, and of some importance with 2b. Note that until the pH gradient decays, an exo-endo flip of 2 will convert a PNPO group to its PNPOH form, whereas an endo → exo flip will have the opposite effect. We do not know the relative rates of these flips. If comparable, their effects on the overall rate of PNPOH → PNPO conversion would cancel, leaving permeation as the only important contributor to pH gradient decay.

(15) The cited rate constants are for endovesicular protonation or deprotonation. In each case, about 50% of the overall change was too fast to follow, and is ascribed to exovesicular prototropy.

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