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BIPHASIC KINETICS AND FLIP-FLOP BEHAVIOUR OF VESICLES OF FLUOROCARBON AMPHIPHILES WITH A 1,3-DISUBSTITUTED GLYCEROL STRUCTURE

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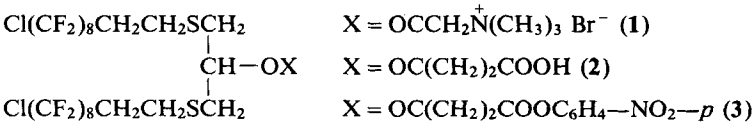
Fluorocarbon amphiphiles with a 1,3-disubstituted thioglycerol structure form stable unilamellar vesicles. The fluorocarbon bilayer of an amphiphile which has an ammonium salt as head group is an 'insulator' for the permeation of hydroxide anions. In the presence or absence of a fluorocarbon nucleophile, hydrolysis of a probe amphiphile carrying a cleavable *p*-nitrophenyl ester group in the vesicle under alkaline conditions shows a pattern of biphasic kinetics, in which the fast and slow reaction can be attributed to the hydrolysis of probe molecules at outer and inner surfaces of vesicles, respectively. The fact that the slow rate constant always remains constant at 0·25 min^{-1} at 25 °C, independent of the pH of the system and of concentration of the nucleophile, indicates that the slow process is an outward flip-flop process of probe molecules within the fluorocarbon domain.

INTRODUCTION

The movement of lipophilic molecules in or between cells, which includes diffusion in the membrane plane, spontaneous movement between membrane surfaces and migration across a membrane (flip-flop), has important effects on life processes. Some workers have studied the transverse diffusion and exchange movement and flip-flop behaviour in phospholipid liposomes.[1–4] The results showed that the lateral diffusion of membrane lipids is strongly dependent on the fluidity and composition of the host membrane, but bears little relationship to the chemical composition of diffusing species. However, the exchange between membranes depends strongly on the length and composition of the hydrophobic chains of phospholipids (i.e. hydrophobicity). Although some work on the flip-flop of phospholipids in liposomes has been reported, the flip-flop behaviour in fluorocarbon vesicle systems has not been investigated. Fluorocarbon amphiphiles possess surfactant behaviours very different from those of corresponding hydrocarbon amphiphiles.[5–8]

Recent work in this laboratory has been directed towards fluorocarbon amphiphiles with a 1,3-disubstituted thioglycerol structure and their vesicles. The amphiphiles have the structures shown in Scheme 1. Vesicles formed from **1** and **2** have good stability owing to the strong hydrophobic interaction between



thionucleophile: $Cl(CF_2)_8CH_2CH_2SH$ (**4**)

Scheme 1

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fluorocarbon chains. In this work, the permeability of vesicles **1** and **2** for hydroxide ion was studied. The fluorocarbon amphiphile **3** has a cleavable head group. The different location of **3** in the outer or inner monolayer of vesicle **1** affects their reactivities under attack of hydroxide ion or nucleophiles and it may provide an alternative means of studying the flip-flop process of amphiphiles under vesicular conditions.

RESULTS AND DISCUSSION

The characterization of vesicles of **1** and **2** has been reported elsewhere.[9] Vesicles of **1** and **2**, prepared by sonication as also mentioned previously,[9] have diameters in the ranges 60–100 and 100–300 nm, respectively, with a membrane thickness of 40–50 Å corresponding to a monolamella structure. The vesicles are very stable and they can survive at room temperature for several weeks without change. The phase transition temperature for the vesicle of **1** is 60 °C and that for the vesicle of **2** is 71·8 °C.

The permeability of hydrocarbon vesicles and liposome bilayers for hydroxide and other ions has been investigated using a variety of methods.[10–14] Thymol blue, a pH-sensitive probe, was chosen for measuring the permeability of our systems. Thymol blue-encapsulated vesicles were prepared by sonication of the amphiphiles in thymol blue solution and then removal of the dye in solution by gel filtration with Sephadex G-50. In the presence of potassium hydroxide, the absorbance change of thymol blue encapsulated in the vesicles (monitored at 590 nm, λ_{max} for the salt form) is given in Figure 1. No observable absorbance change for vesicle **1** was found up to 300 min.

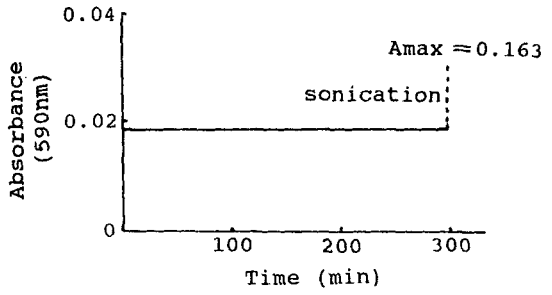
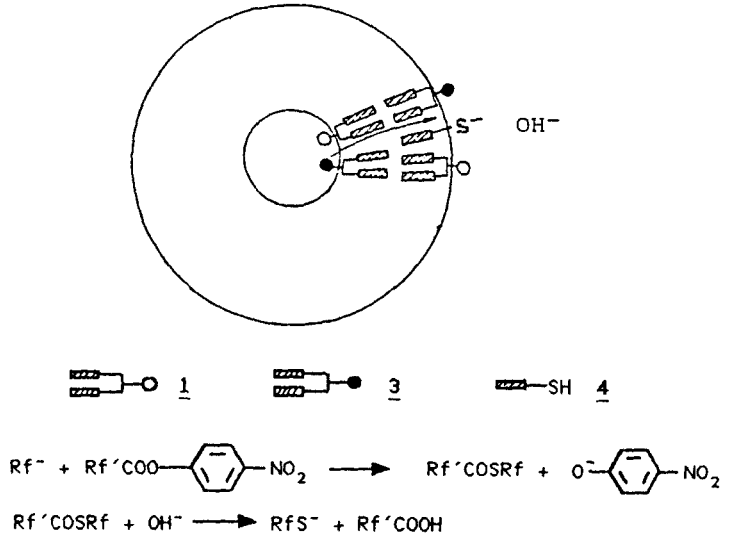


Figure 1. Measurement of permeability of vesicles of **1** for hydroxide ion. Temperature, 25 °C; concentration of KOH, 5×10^{-3} M

Generally, the permeation for proton or hydroxide could be explained by either 'hydrogen-bond band' ('water wire') or a 'weak acid' hypothesis. It is considered that water is slightly soluble in phospholipid bilayers. Therefore, protons and hydroxide ions can be transferred rapidly through bilayers by hydrogen-bond rearrangement. Fendler and Tundo[14] reported a fast permeation rate of 10^{-4} cm s^{-1} of proton and hydroxide anions through liposome bilayers. Fluorocarbon compounds are more hydrophobic than their hydrocarbon analogues, which limits the water content in their bilayers and hence rules out the existence of a 'hydrogen-bond band' in the fluorocarbon vesicle bilayer of **1**. The result for vesicle **1** indicates that the bilayer of **1** is an 'insulator' for hydroxide ion.



Scheme 2. Thiolate nucleophile-catalysed hydrolysis of **3** in inner monolayer of vesicle of **1** through the flip-flop process

According to the weak acid hypothesis, the vesicle formed by **2**, a weak acid, should be permeable to hydroxide anion to some extent, which was confirmed by the present results.

When a covesicle of **1** and **3** is formed, the probe molecules **3** should be homogeneously distributed in the outer and inner layers as illustrated in Scheme 2. ω-Chloro-1*H*,1*H*,2*H*,2*H*-perfluorododecanethiol(**4**) acted as a nucleophile to catalyse the hydrolysis of **3** under basic conditions (the pK_a of heptanethiol in vesicles of dioctadecylmethylammonium chloride is 9·5.[15] If we use the same value for our nucleophile, **4** exists mainly as the thiolate conjugate base within the pH range 9·25–10·35 under which our kinetic experiments were conducted). As mentioned previously, the bilayer of **1** is an 'insulator' for hydroxide ion, hence the only way for probe **3** located in the inner monolayer to be hydrolysed is by flip-flop into outer monolayer (Scheme 2). Therefore, so-called biphasic kinetics appear.[16] As shown in Figure 2, a fast reaction which is complete within a few minutes is followed by a slower reaction which requires 30 min for completion. The fast reaction apparently obeys first-order kinetics, with a rate constant $k_f = 7.9\ \text{min}^{-1}$.

If the slow process shown in Figure 2 reflects the hydrolysis of **3** in the inner monolayer through an outward flip-flop mechanism, the kinetic process should obey the following equation:

$$A_i \underset{k_i}{\overset{k_o}{\rightleftharpoons}} A_o \xrightarrow{k} P \qquad (1)$$

where A_i and A_o are the moieties of *p*-nitrophenyl ester **3** in the inner monolayer and outer monolayer, respectively, P is the hydrolytic products of **3** and k_o, k_i and k are the rate constants of outward and inward flip-flop and esterolysis of **3**, respectively.

As the fast and slow reactions are both independent processes, they can be treated separately:

Fast reaction:

$$A_o^f \xrightarrow{k} P \qquad (2)$$

$$\ln[A_o^f] = -kt + \ln[A_o^f]_o \qquad (3)$$

Slow reaction:

$$A_i^s \xrightarrow{k_o} A_o^s \underset{k_i}{\overset{k}{\rightarrow}} P \qquad (4)$$

where A_i^s represents **3** in the inner monolayer and A_o^s is **3** in outer monolayer produced by the outward flip-flop. For $k \gg k_o \gg k_i$ and $[A_i^s] \gg [A_o^s]$, equation (4) can be simplified to

$$A_i^s \xrightarrow{k_o} A_o^s \xrightarrow{k} P \qquad (5)$$

and with an approximate treatment, we have the equation

$$[P] = [A_i^s]_o[1 - \exp(-k_{ot})] \qquad (6)$$

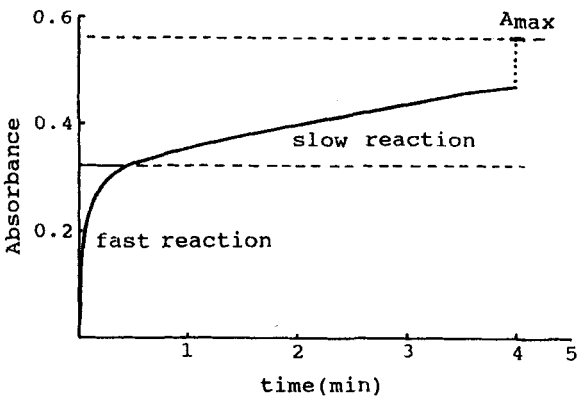


Figure 2. Catalytic hydrolysis of **3** in vesicle of **1** by thiolate nucleophile **4** under basic conditions (pH 9·80). Temperature, 25·0 °C. Concentrations: **1**, $8{\cdot}4 \times 10^{-3}$ M; **3**, $1{\cdot}4 \times 10^{-3}$ M; nucleophile **4**, $1{\cdot}9 \times 10^{-4}$ M

where $[A_i^s]_o$ is the concentration of **3** in inner monolayer before reaction. Therefore, the appearance of the *p*-nitrophenolate ion from *p*-nitrophenyl ester **3** in the inner monolayer also obeys first-order kinetics. The rate constant k_o (i.e. k_s) corresponding to the flip-flop rate of **3** was calculated to be $0{\cdot}25\ \text{min}^{-1}$. It can be seen that the flip-flop of double-fluorocarbon chains is a slow process.

Because the rate constants of fast reactions in the outer monolayer are much higher than those of the slow reactions in the inner monolayer, it is reasonable simply to take the ratio of A^f_{max} and A^s_{max}, the maximum absorbances of the fast and slow reaction, respectively, as the equilibrium constant of **3** between two monolayers:

$$K = k_o/k_i = A^f_{max}/A^s_{max} = 1{\cdot}28$$

The molar ratio, 1.28:1, of molecule **3** in the outer monolayer to that in inner monolayer can be calculated from the respective A_{max} values of the fast and slow reactions shown in Figure 2, which is very close to the area ratio, 1·20:1, of the outer to the inner wall for vesicles of **1** calculated from the vesicle diameter and thickness.

In the absence of a nucleophile, the results of the pH dependence of the simple alkaline hydrolysis of **3** in vesicles of **1** were as shown in Figure 3. At pH < 9·8, the hydrolysis is a monophasic process, which indicates that the rate of hydrolysis of **3** in the outer layer is slow enough to be comparable to that of the outward flip-flop of **3** and so only one process can be observed. However at pH > 10·06, the hydrolysis becomes a biphasic process and the rate constant of hydrolysis of **3** in the outer layer is several times faster than that of the flip-flop of **3**, so that it is possible to differentiate kinetically the fast from the slow process. The pH

dependence of the fast reaction shows a typical pattern of specific alkaline hydrolysis, but the slow reaction is independent of the pH of the system with a constant rate constant of 0·25 min^{-1} at 25 °C.

Figure 4 shows clearly that the variation in nucleophile concentration only influences the fast and not the slow reaction of the biphasic process. The fluorocarbon nucleophile **4** can be solubilized into the fluorocarbon vesicle bilayer and catalyse the esterolysis of **3** by its conjugate base under basic conditions. For the fast reaction, the dependence of the rate constant, k_f, on the concentration of the nucleophile shows a typical pattern of a micellar solubilization system,[17] i.e. it first shows certain binding characteristics and then the gradual increase in nucleophile concentration results in first-order kinetics. It is noteworthy that the change in nucleophile concentration does not influence the rate constant of the slow reaction, remaining constant at 0·25 min^{-1} at 25 °C, which is the same as those of simple alkaline hydrolyses. The fact that the slow rate constant is not influenced by pH and the presence of the thiolate nucleophile confirms that the rate-determining step for the slow reaction is the flip-flop process of probe **3**.

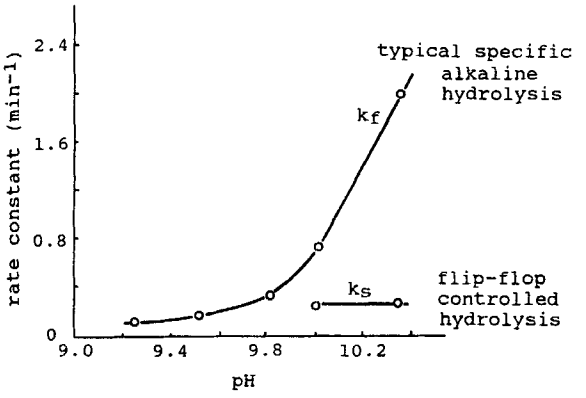


Figure 3. pH dependence of the hydrolysis of **3** in vesicle of **1**. Temperature, 25·0 °C. Concentrations: **1**, $8{\cdot}4 \times 10^{-4}$ M; **3**, $1{\cdot}4 \times 10^{-3}$ M

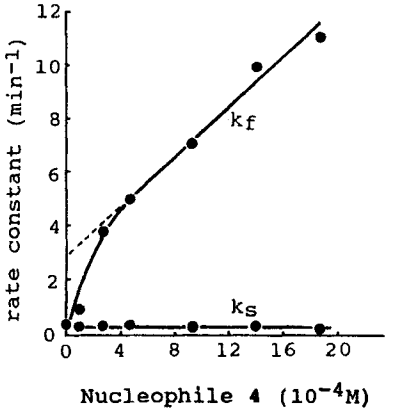


Figure 4. Influence of concentration of nucleophile **4** on the biphasic kinetics of hydrolysis of **3** in vesicle of **1** at pH 9.80. Temperature, 25·0 °C. Concentrations: **1**, $8{\cdot}4 \times 10^{-3}$ M; **3**, $1{\cdot}4 \times 10^{-3}$ M

EXPERIMENTAL

Synthesis of **4**. ω-Chloro-1*H*,1*H*,2*H*,2*H*-perfluorododecanyl iodide (17·7 g, 30 mmol) was placed in a 250 ml round-bottomed flask and 6·0 g (79 mmol) of thiourea and 130 ml of anhydrous ethanol were added. The mixture was heated under reflux for 40 h. After the solution had been concentrated to a small volume, a solution of potassium hydroxide (9·0 g) in water (50 ml) was added. The reaction mixture became dark brown with an unpleasant odour. The mixture was refluxed for 4 h, then ethanol was evaporated and the residue was neutralized with 10% H_2SO_4. Steam distillation of the reaction mixture gave **4** (10·9 g, 73%) as a white solid. The product showed one spot on TLC (1:4 diethyl ether–light petroleum). M.p. 30 °C. ^{1}H NMR ($CDCl_3$): δ 2·68 (2H, m, CH_2S), 2·40 (2H, m, CF_2CH_2), 1·53 (1H, t, SH). ^{19}F NMR (CF_3COOH as external standard): δ 9·2 (2F, s, ClF_2), −37·0 (2F, s, CH_2CF_2), −44·0 [12F, t, $(CF_2)_6$].

Synthesis of **1**. To a solution of **4** (4·97 g, 10 mmol) in benzene (20 ml) was added sodium hydride (0·24 g, 10 mmol). The mixture was stirred for 3 h at 35 °C, then a solution of 2,3-epoxypropyl chloride (0·463 g, 5 mmol) in benzene (10 ml) was added dropwise. The resulting mixture was stirred for 5 h at 35 °C, poured into saturated ammonium chloride (10 ml), and extracted with diethyl ether. The organic layer was washed with water, dried (Na_2SO_4), filtered and concentrated. The solid residue was crystallized from benzene to give 1,3-di-*S*-(ω-chloro-1*H*,1*H*,2*H*,2*H*-perfluorododecyl)-1,3-dithioglycerol (3·57 g, 68%) as a white solid, m.p. 101–103 °C. ^{1}H NMR ($CDCl_3$): δ 3·88 (1H, m, CHO), 2·78 [8H, m, $(CH_2SCH_2)_2$], 2·45 [4H, m, $(CF_2CH_2)_2$]. ^{19}F NMR (CF_3COOH as the external standard): δ 9·3 [4F, s, $(CF_2Cl)_2$], −36·5 [4F, s, $(CF_2CH_2)_2$], −43·8 [24F, t, $(CF_2)_{12}$). IR (KBr, cm^{-1}): 3600–3100. Analysis: calculated for $C_{23}H_{14}OF_{32}S_2Cl_2$, C 26·33, H 1·34, S 6·10; found, C 26·36, H 1·06, S, 6·17%.

To a solution of above 1,3-disubstituted glycerol (1·05 g, 1·0 mmol) in THF (10 ml) containing pyridine (0·17 g, 2·10 mmol) was added dropwise a solution of bromoacetyl bromide (0·40 g, 2·0 mmol) in THF (5 ml); a white solid appeared immediately. After 4 h,

TLC showed the complete reaction of starting material. The usual work-up gave a yellow solid which was crystallized from light petroleum (b.p. 60–90 °C) to afford a pale yellow solid (0·82 g, 72%). ^{1}H NMR ($CHCl_3$): δ 5·11 (1H, t, CHO), 3·85 (2H, s, CH_2Br), 2·83–3·04 [8H, m, $(CH_2CH_2S)_2$], 2·44 [4H, m, $(CH_2CF_2)_2$]. IR (KBr, cm^{-1}): 1740.

The above pale yellow solid (0·5 g) was dissolved in anhydrous acetone (5 ml) and transferred into a 10 ml sealed tube, then cooled with an acetone–dry-ice bath. After trimethylamine (3 ml) had been added, the tube was sealed and the mixture was allowed to react for 18 h at room temperature. The reaction mixture was filtered and the solid was washed with cold acetone, then crystallized from acetone to give **1** (0·25 g, 48%) as white crystals, m.p. 178–180 °C. ^{1}H NMR ($CDCl_3$): δ 5·16 (3H, m, $CHOOCCH_2N^+$), 3·70 (9H, s, N^+Me_3), 2·88 [8H, m, $(CH_2CH_2S)_2$], 2·46 [4H, m, $(CH_2F_2)_2$]. IR (KBr, cm^{-1}): 1745. Analysis: calculated for $C_{28}H_{24}O_2NF_{32}S_2Cl_2$, C 27·35, H 1·97, N 1·14; found, C 27·25, H 1·85, N 1·14%.

Synthesis of 2. A 0·5 g (0·42 mmol) amount of the 1,3-disubstituted glycerol was dissolved in 5 ml of dry pyridine containing 10 mg of 4-dimethylaminopyridine. To the solution was added 0·20 g (2 mmol) of succinic anhydride, then the mixture was heated in a sealed tube at 120 °C for 3 h and cooled. Pyridine was removed under reduced pressure. The solid residue was dissolved in diethyl ether, washed with 1% H_2SO_4 and water, dried (Na_2SO_4), filtered and concentrated. The residue was recrystallized from benzene to give **2** (0·48 g, 88%) as white crystals, m.p. 78–79 °C. ^{1}H NMR ($CDCl_3$): δ 5·12 (1H, t, CHO), 2·87 (8H, m, $(CH_2SCH_2)_2$], 2·68 (4H, m, CH_2CH_2), 2·42 [4H, m, $(CF_2CH_2)_2$]. IR (KBr, cm^{-1}): 3600–3000, 1740, 1715.

Synthesis of 3. To 10 ml of ethyl acetate containing 3·4 g (2·96 mmol) of **2** in a 25 ml round-bottomed flask was added 0·42 g (3·0 mmol) of *p*-nitrophenol. After the solid had completely dissolved, 0·62 g (3·0 mmol) of (dicyclohexylcarbodiimide was added. The mixture was placed in a refrigerator for 0·5 h and then kept at room temperature overnight. The precipitate was filtered and washed three times with ethyl acetate. The combined filtrate was washed several times with water and dried over anhydrous sodium sulphate. The solvent was stripped to give a solid which was then recrystallized twice from absolute ethanol to afford 2·1 g (57%) of **3**, m.p. 59–61 °C. ^{1}H NMR ($CDCl_3$): δ 7·34–8·08 (4H, m, ArH), 5·14(1H, t, CHO), 2·80–3·00[12H, m, $(CH_2CH_2SCH_2)_2$], 2·41 (4H, m, $OCCH_2CH_2CO$). IR (KBr, cm^{-1}): 1730(s), 1770(s), 1525(s). Analysis: calculated for $C_{33}H_{21}O_6NF_{32}S_2Cl_2$, C 31·39, H 1·67, N 1·10; found, C 30·90, H 1·44, N 1·36%.

Generation of vesicles. Typically, covesicles of an amphiphile and a probe compound were prepared by sonication of their mixture for several minutes with the microprobe of a JC-Chu Li Ji sonicator at 120 W, pH 7·0 and 80 °C.

The thymol blue-encapsulated vesicle was prepared by sonicating the amphiphile in thymol blue solution for 2 min, then the vesicle solution was eluted through a Sephadex G-50 column (65 cm × 1·5 cm i.d.) at 1 ml min^{-1} using doubly distilled water for the vesicle of **2** and 0·001 M potassium chloride solution for the vesicle of **1**. Fractions were collected at intervals of 3 ml, and their visible absorption was measured at 430 nm.

Kinetic study. The esterolytic reaction was normally initiated by mixing the vesicle solution with aqueous sodium hydrogen carbonate–sodium carbonate buffer at 25 °C, and it was followed at 410 nm on a Perkin-Elmer Lambda 5 UV–visible spectrophotometer. The temperature was controlled by a digital controller.

The reaction of thymol blue encapsulated in vesicles was initiated by mixing 3 ml of vesicle solution with 0·05 ml of 0·300 M potassium hydroxide solution. The reaction was followed at 590 nm on a Perkin-Elmer Lambda 5 UV–visible spectrophotometer. When the measurements were finished, absorption spectra were taken immediately: the solution was sonicated at room temperature for 2 min at 120 W to destroy the vesicular structure and its absorption spectrum was recorded.

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