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Bulk liquid membrane transport of ferrioxamine B by neutral and ionizable carriers

Ivan Spasojević and Alvin L. Crumbliss

Department of Chemistry, Duke University, Box 90346, Durham, NC 27708–0346, USA.
E-mail: alc@chem.duke.edu

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Bulk liquid membrane (BLM) transport of ferrioxamine B ($FeHDFB^+$) across an artificial hydrophobic membrane facilitated by neutral and ionizable carriers was studied. Under controlled conditions, ferrioxamine B transport by neutral ionophores follows a simple theoretical model for a diffusion controlled process which predicts a linear dependence of flux upon aqueous-to-chloroform extraction constants. Unsubstituted 18C6 crown ether exhibits the best transport properties among the neutral carriers investigated with a flux of 1.4×10^{-10} mol s^{-1} cm^{-2}. When transport of $FeHDFB^+$, metal-free desferrioxamine B, H_4DFB^+, and $AlHDFB^+$ mediated by DC18C6 crown ether was followed, a selectivity was observed with a ratio of measured relative fluxes of 5 : 3 : 1, respectively. An ionizable carrier lasalocid {6-[7-[5-ethyl-5-(5-ethyltetrahydro-5-hydroxy-6-methyl-2*H*-pyran-2-yl)tetrahydro-3-methyl-2-furanyl]-4-hydroxy-3,5-dimethyl-6-oxonoyl]-2-hydroxy-3-methylbenzoic acid}, negatively charged at neutral pH, outperformed the crown ethers as a ferrioxamine B transporting agent. However, transport by ionizable carriers follows a different mechanism and occurs only when an electrolyte is present in the receiving phase. This requirement was exploited by designing a BLM experiment in which H_4DFB^+ acts as a counter cation at the receiving interface, whereby $FeHDFB^+$ and H_4DFB^+ were transported in opposite directions in a synergistic fashion. When the ionizable salicylic acid moiety of lasalocid is transformed into an ester, lasalocid almost entirely loses its transporting properties. This suggests a co-operative mode of binding in which electrostatic recognition of the $FeHDFB^+$ cation by lasalocid anion is a prerequisite for a subsequent encapsulation of the amine site in a host–guest fashion.

Introduction

Iron acquisition in microorganisms commonly involves molecular recognition of an iron–siderophore complex at the cell membrane and its transport through the membrane into the cell interior.[1–4] The same step is involved in strategies for iron overload therapy where both the therapeutic chelating agent and its iron complex (usually a highly hydrophilic species) are expected to penetrate the lipophilic cell membrane, each in a different direction. Desferrioxamine B (Desferal®), H_4DFB^+, is a linear trihydroxamic acid biosynthesized by *Streptomyces pilosus*.[5] Desferal is still a major drug for iron removal therapy despite great efforts to find another chelating agent with oral activity and longer body retention time.[6] Its octahedral, high-spin iron(III) complex, ferrioxamine B ($FeHDFB^+$), forms a red aqueous solution with a broad visible spectrum of maximal molar absorptivity ≈2600 M^{-1} cm^{-1} at 428 nm.[7] The stability of the trihydroxamate complex provides its dominant presence in aqueous solution over a wide range of pH (3–11) in submillimolar concentrations. A special feature of ferrioxamine B is a five-carbon pendant arm terminated by an amine that is positively charged at physiological pH (pK_a = 10.79).[8] Although its biological role may be questioned (most other naturally occurring siderophores lack such a feature), this pendant arm makes ferrioxamine B very suitable for molecular recognition studies.

A lipophilic agent, which is able to recognize and reversibly bind to ferrioxamine B and decrease its overall hydrophilicity, can increase the uptake of the siderophore complex across the cell membrane. Many natural and synthetic macrocyclic and linear molecules, including crown ethers,[9] can function as host recognition factors,[10–12] and have been used as models for cell surface receptors. We have shown that an ionophore host molecule can selectively recognize ferrioxamine B in solution through second co-ordination sphere association at the pendant arm amine site (Fig. 1).[13] The effect of various ionophore structures,[14] accompanying anions,[15] and organic phase solvents[16] on the properties of this siderophore–ionophore supramolecular assembly have been investigated in two-phase aqueous/organic extraction experiments.

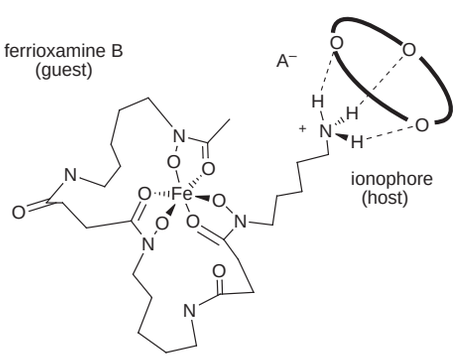
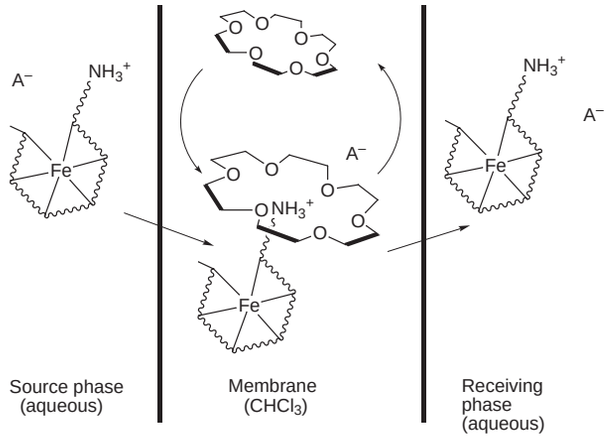
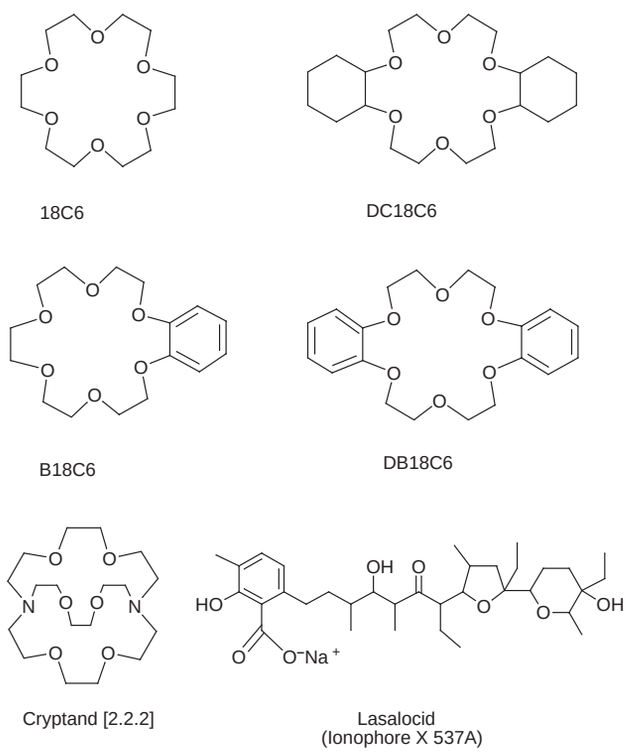


Fig. 1 Siderophore–ionophore assembly formed by second-sphere complexation of ferrioxamine B by an uncharged host.

In this work, in order more closely to approach biologically relevant membrane processes, we have extended our investigation to a three phase (aqueous/organic/aqueous) system by conducting bulk liquid membrane (BLM) transport of ferrioxamine B mediated by several synthetic and natural ionophores. The BLM is a gently stirred organic solvent placed between two aqueous phases (Scheme 1). Chloroform has been widely employed as a BLM solvent due to its immiscibility with water, low viscosity, and a relative permittivity that is similar to that of biological membranes.[17] Other three-phase systems utilizing an emulsion liquid membrane[18,19] or supported liquid membrane[20] have been investigated. However, by choosing the BLM technique and chloroform as the membrane solvent, we



Scheme 1 Representation of bulk liquid membrane (BLM) transport facilitated by recognition of the pendant ammonium site of ferrioxamine B by *neutral* crown ether carriers.



are able to utilize data accumulated from previous two-phase extraction studies and carrier facilitated BLM transport studies.[21–23] By carefully designing a small volume BLM cell equipped with a reliable stirring system (Fig. 2), we have been able to overcome two major disadvantages of the BLM technique: large membrane volume and lack of reproducibility.[24]

Investigation of metal complex–ionophore recognition and transport is important beyond considerations of the interaction of antibiotics and Desferal in the human body. An ammonium side arm may be covalently attached to many other iron chelating agents. Reversible binding of an ionophore to such species will temporarily change the overall properties of the assembly, which may open a new dimension in iron mobilization and removal studies, and in a broader sense in drug administration studies. Such investigations may be expanded to other metals and their chelating agents, and to their application in metallurgy, as well as in biological and environmental sciences.

Experimental

Materials

Ferrioxamine B ($FeHDFB^+$) was prepared[7,25] from an iron(III)

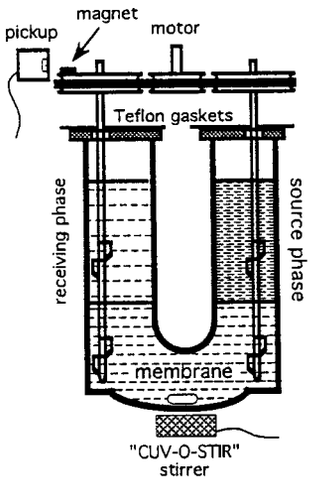


Fig. 2 "U"-shaped BLM cell (fused 1 × 1 cm glass spectrophotometric cells) used for continuous monitoring of ferrioxamine B absorbance in the receiving or membrane phase.

perchlorate stock solution whose concentration of 0.1004 M was determined spectrophotometrically[26] and the acidity of which was ≈0.1 M $HClO_4$. The ligand, desferrioxamine B (H_4DFB^+), was dissolved in a small volume of water and added to the $Fe(ClO_4)_3$ solution in 5% excess to ensure that no Fe^{III} existed in forms other than its hexadentate complex. While vigorously stirred, the solution was neutralized by slow addition of solid $Mg(OH)_2$ up to pH 3.2. The ferrioxamine B concentration, typically 0.02 M, was checked spectrophotometrically ($\varepsilon = 2600\ M^{-1}\ cm^{-1}$ at 428 nm).[7] The solution was then filtered and stored at ≈0 °C. Crown ethers and the sodium salt of lasalocid were purchased from Aldrich (Kriptofix (Cryptand) [2.2.2] from Merck) and used as received. Lasalocid acid was prepared as described previously.[27] Lasalocid ester was prepared from lasalocid acid by reaction with diazomethane[28] that was generated from Diazald® purchased from Aldrich.[29] Purity of the lasalocid ester was confirmed by mass spectrometry, the parent ion peak (expected value) at *m*/*z* 605.4 (604.8), and by ^1H NMR spectra that matched that of lasalocid acid,[30] with an additional peak at δ 3.96 consistent with the spectrum of the methyl ester of salicylic acid.[31]

Instrumentation

Bulk liquid membrane transport experiments were carried out in a specially designed U-shaped, 1 cm pathlength spectrophotometer cell that was equipped with a stirring mechanism (Fig. 2). Special care was taken to provide reproducible stirring and geometrical consistency throughout the multiple experiments. Stirring was powered by a continuous-duty synchronous a.c. motor which ran impellers with no observable deviation from 960 rpm. When experiments with different stirring rates were performed, a variable speed a.c. motor was used instead. After setting the speed of the motor, the stirring rate was measured by an oscilloscope acquiring an amplified signal from the pick-up coil that was sensing the rotation of a small magnet placed on one of the pulleys directly connected to the stirring shafts. The cell assembly was placed in a Beckman Acta (III) double beam UV/VIS spectrophotometer and the absorbance in the receiving or membrane phase was continuously monitored. A personal computer equipped with a data acquisition board and software (OLIS) was interfaced to the spectrophotometer for data storage, manipulation and graphical presentation.

Methods

The three phase BLM transport experiments were typically

performed as follows. An appropriate amount of ionophore (carrier) was dissolved in chloroform and 4 ml of this solution were added to the bottom of the U-cell. This volume of "membrane" solution was needed in order to divide the U-cell into two compartments. To both sidearm compartments 2 ml of "source" aqueous solution and 2 ml of "receiving" aqueous solution were simultaneously added. The lower stirring paddles were wetted with chloroform (so that they would not bring a part of the aqueous solution down into the membrane) and the stirring shafts were then immersed so that the chloroform/aqueous interface was exactly between the lower and upper stirring paddles (Fig. 2). The whole assembly was then carefully placed into a spectrophotometer and the light beam aligned to pass through either the source, membrane or receiving phase solution. After 3–6 h, the slope of the absorbance *vs.* time trace (excluding the non-linear portion recorded during the "lag-time" period, *i.e.* the initial 15–30 min) was calculated as a flux (mol cm^{-2} s^{-1}). The chloroform/aqueous phase interface surface area was taken as 1 cm^2 and it was not corrected for the surface deformation due to the presence of 1 mm^2 shafts and "meniscus" formed due to the water/glass wall interaction. The molar absorptivity of ferrioxamine B was taken as 2600 M^{-1} cm^{-1} at 428 nm.[7] Two phase experiments were performed as described previously.[14a]

Results and discussion

Background

The overall equilibrium for the *two-phase* batch extraction of a hydrophilic ion pair (*e.g.* $FeHDFB^+$, A^-) into an organic phase, facilitated by a neutral ionophore, *e.g.* crown ether (CE), may be expressed as in eqn. (1). This process may be conceived as proceed-

$$FeHDFB^+(aq) + A^-(aq) + CE(org) \overset{K_{ex}}{\rightleftharpoons} FeHDFB^+\cdot CE\cdot A^-(org) \quad (1)$$

ing through an ion pairing and *distribution*, eqn. (2), followed

$$FeHDFB^+(aq) + A^-(aq) \overset{K_d}{\rightleftharpoons} FeHDFB^+\cdot A^-(org) \quad (2)$$

by the *association* of the ion pair with the ionophore, eqn. (3).

$$FeHDFB^+\cdot A^-(org) + CE(org) \overset{K_a}{\rightleftharpoons} FeHDFB^+\cdot CE\cdot A^-(org) \quad (3)$$

Consequently, the overall extraction constant, K_{ex}, may be expressed as in eqn. (4).[13]

$$K_{ex} = K_d K_a \quad (4)$$

Carrier facilitated transport in a *three phase* (aqueous/organic/aqueous) bulk liquid membrane (BLM) study consists of an extraction process (K_{ex}) at the source aqueous phase/membrane interface, and a subsequent re-extraction process (K_{ex}^{-1}) at the membrane/receiving phase interface (Scheme 1). The process may be visualized to consist of the following stages:[32] (1) diffusion of the ions through the unstirred layer of the aqueous source phase driven by the concentration difference between the aqueous bulk and the immediate vicinity of the membrane; (2) transition of ions across the aqueous/organic interface and ion pairing [eqn. (2)]; (3) diffusion of the ion pair through the membrane until it is complexed by the ionophore [eqn. (3)]; and (4) diffusion of the cation–ionophore–anion assembly through the rest of the unstirred membrane layer. At the membrane/receiving phase interface re-extraction goes on through the same stages in reverse order. The driving force for the overall flux is the difference in substrate concentration between source and receiving aqueous phases; the transport will cease once the concentrations become equal. The role of the carrier (ionophore) is to act as a solubilizing agent, *i.e.* to make the membrane more permeable to the hydrophilic species and thus to increase the transport rate.

Since the bulk of the organic phase is being stirred, only the unstirred layers next to the aqueous phases mimic a lipophilic membrane. However, this "membrane" is still relatively thick (50–300 μm)[32,33] and, providing the ion pairing and association equilibria [eqns. (2) and (3)] are established fast enough, the diffusion of the $FeHDFB^+\cdot CE\cdot A^-$(mem) species in the unstirred portion of the membrane will control the overall flux.

The conditions in a typical BLM transport experiment are usually such that the source phase concentration of the substrate is high so that it remains essentially constant. When the extraction constant K_{ex} and the concentration of all species are low enough, and when the diffusion of ions and ion pairs relative to the diffusion of ion pair–ionophore is negligible (a reasonable assumption), the conditions for a steady-state flux of ions through the membrane are met as described by Reusch and Cussler;[21] eqn. (5). Equation (5) relates the flux (mol cm^{-2} s^{-1}), measured in a BLM experiment, to the extraction constant K_{ex} [eqn. (4)], which can be obtained in a separate two-phase batch extraction experiment and may be independently confirmed by microcalorimetry.[34] Here D_{mem} is the diffusion coefficient of the $FeHDFB^+\cdot CE\cdot A^-$(mem) assembly in the membrane solvent, l is the diffusion layer thickness at the membrane side (chloroform) and $[FeHDFB^+_{src}]$ and $[A^-_{src}]$ are the ferrioxamine B and anion concentrations in the aqueous source phase, respectively.

$$\text{flux} = K_{ex}\frac{D_{mem}}{l}[CE_{mem}][FeHDFB^+_{src}][A^-_{src}] \quad (5)$$

More elaborate mathematical models have also been developed[23,32] which include fewer constraints and account for the fact that for a high extraction constant, K_{ex}, the carrier will become saturated and the re-extraction processes at the receiving phase/membrane interface will become limiting. Thus the theory predicts, and it has been confirmed experimentally,[32] that for a broad range of extraction constants the flux will increase to a maximum value that corresponds to an "optimal" value of the extraction constant K_{ex}, and then fall, giving rise to a bell-shaped dependence of flux on log K_{ex}.[23,32]

Neutral carriers, crown ethers and cryptand [2.2.2], in the membrane

Crown ether derivatives with an 18C6 cavity, together with cryptand [2.2.2], recognize and transport ferrioxamine B in our BLM experiments (Table 1). The most efficient carrier is unsubstituted 18C6, as implied by its extraction constant. Both cyclohexano and especially benzo substituents diminish the observed flux. It is believed that electron withdrawing benzene rings lower the polarity of the oxygen atoms in the crown ether ring. Also, the rigidity, introduced into the crown ether ring by the benzo groups, may limit the flexibility of the host ring to adapt its size and shape to the cation guest. The negative effect of the cyclohexane rings is most likely due to a steric hindrance to the approach of the cation guest to the crown ether cavity, as well as to a loss in ring flexibility.† [36]

A linear dependence of flux on K_{ex} is obtained (Fig. 3) as predicted by eqn. (5). The quotient D_{mem}/l can be calculated from the slope, and by setting the thickness of the membrane diffusion layer as l = 50–300 μm,[32,33] we obtain a reasonable range for the diffusion coefficient $D_{mem} = 2 \times 10^{-6}$–$1.2 \times 10^{-5}$ cm^2 s^{-1}.

† See discussion in ref. 14 and refs. therein.

Table 1 BLM Transport data for ferrioxamine B with various ionophore carriers

Carrier	Flux[a]/10^{-10} mol s^{-1} cm^{-2}				
	Deionized water[b]	$Mg(ClO_4)_2$[b]	$KClO_4$[b]	K_{ex}[c]/M^{-2}	log K_{ex}
None	0.012				
DB18C6	0.023	0.026	0.028	0.07	−1.18
B18C6	0.126	0.128	0.122	0.49	−0.31
DC18C6	0.891	0.847	0.693	5.51	0.74
Cryptand [2.2.2]	1.190	1.061	0.728	7.43[d]	0.87
18C6	1.396	1.246	0.923	8.53	0.93
Na^+lasalocid$^-$	0.102	5.448	13.04	2140[e]	3.33

[a] Values reported are an average of 2–4 measurements with a maximum observed deviation of 5%. Room temperature (23 ± 0.5 °C). Source phase: pH 3.2 0.02 M $FeHDFB^+$ $MeSO_3^-$, 0.04 M Mg^{2+}, 0.08 M ClO_4^-. Membrane: 0.02 M carrier in $CHCl_3$. [b] Receiving phase: deionized water or 0.04 M Mg^{2+}, 0.08 M ClO_4^- or 0.08 M K^+, 0.08 M ClO_4^-; room temperature (23 ± 0.5 °C). [c] From ref. 14(*a*). [d] From ref. 14(*b*). [e] Experiment performed under identical conditions as in the case of neutral carriers; K_{ex} calculated as described in ref. 35.

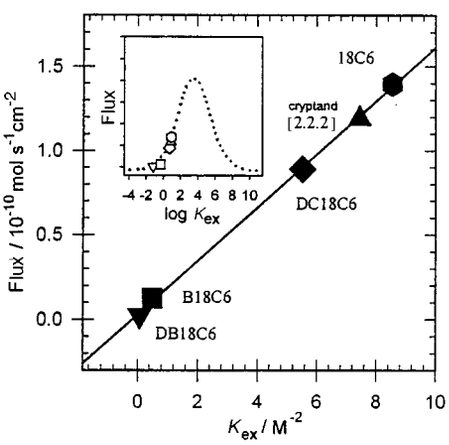


Fig. 3 Ferrioxamine B BLM transport by *neutral carriers*; flux plotted against water/chloroform extraction constant, K_{ex}, and log K_{ex} (inset). Slope of the line is 1.6×10^{-11} mol cm^{-2} s^{-1} M^2 with $r^2 = 0.999$. Data and experimental conditions are given in Table 1.

The extraction constants for the various ionophore carriers are relatively small. Therefore, when the flux obtained from transport experiments is plotted against the logarithm of the extraction constant only the left portion of the predicted[23,32] bell-shaped curve is observed (Fig. 3, inset). An arbitrary function with a maximum value at an optimum extraction constant, log $K_{ex} = 4$, was drawn through the experimental data to illustrate the efficiency of the carriers used.[23]

When an ion pair is transported by a neutral ionophore theory predicts that the flux will increase with the second power of the substrate concentration because twice as many species (cation and anion) must be transported [eqn. (5)]. Since our source phase contains non-stoichiometric amounts of ferrioxamine B and perchlorate, their influence on flux has been tested separately.‡ The flux was found to vary linearly with the initial ferrioxamine B concentration in the source phase over the range 2.5–20 mM. Also, a ten-fold variation in perchlorate concentration increased the flux by an order of magnitude. Both experiments confirm the validity of [eqn. (5)] and suggest that the simple model for carrier facilitated transport of an ion pair is operative in the case of bulk liquid membrane transport of ferrioxamine B by neutral carriers, crown ethers and cryptand [2.2.2].

The effect of the receiving phase composition on flux was investigated by using either deionized water, 0.04 M $Mg(ClO_4)_2$ or 0.08 M $KClO_4$ (Table 1). In the experiments with magnesium ion the salt concentration (0.04 M) was selected to be the same as it was in source phase ferrioxamine B solution throughout the study. Potassium ion was introduced because of its known high affinity for the 18C6 family of crown ethers[37] and the concentration of its salt (0.08 M) provided the same ClO_4^- concentration as was present in the experiments with $Mg(ClO_4)_2$. The presence of electrolyte in the receiving phase resulted in the same or decreased flux relative to a pure aqueous receiving phase (Table 1). This is ascribed to a decrease in effective free carrier concentration in the membrane due to a competition for the carrier between magnesium or potassium ion on one side and ferrioxamine B on the other.

‡ Our source phase contains an "innocent" cation Mg^{2+} which has an extraction constant *ca.* 1/100 that of ferrioxamine B[14] and a "innocent" anion, $MeSO_3^-$, which makes ferrioxamine B three times less extractable into chloroform than in the case of perchlorate.

Proof of diffusion regime

Estimation of the critical rate of complexation. As outlined above, the host–guest complexation/decomplexation processes are in most cases fast enough that physical diffusion through the unstirred boundary layers is the rate limiting step. The second Damköhler number, a semiempirical relationship involving critical parameters (diffusion coefficient D_{mem}, diffusion layer thickness l, and the complexation rate constant k), should be large for a diffusion controlled process, *i.e.* $kl^2/D_{mem} \gg 1$.[38] Crown ether–cation decomplexation rates are lower than complexation rates, and thus can be critical for the transport regime.[39] The closest comparable reaction with available kinetic data is the unimolecular dissociation of Na^+ from its complex with 18C6 with an estimated rate constant 3.8×10^3 s^{-1}.[40] The only available data for the ammonium cation are from Chock,[41] who found essentially identical complexation and decomplexation rates for Na^+ and NH_4^+ with 30C10. Using $l = 50$–300 μm and $D_m = 2 \times 10^{-6}$–1.2×10^{-5} cm^2 s^{-1} (from the discussion above), we calculate the second Damköhler numbers to be in the range 3.8×10^4–2.4×10^6. This strongly suggests that the transport of ferrioxamine B by the 18C6 family of crown ethers and cryptand [2.2.2] is inside a diffusion controlled regime. From the equilibrium constant for the association of 18C6 with ferrioxamine B, log $K_a = 4.38$,[14a] and the unimolecular dissociation rate constant for Na^+–18C6 (3.8×10^3 s^{-1}),[40] we can estimate the rate constant for the bimolecular association of 18C6 and ferrioxamine B to be $\approx 9 \times 10^7$ M^{-1} s^{-1}, a value that is in agreement with data previously published for polyether–cation interactions.[39]

Influence of stirring rate on flux. For a diffusion controlled BLM process it is expected that the transport rate will increase linearly with stirring rate as long as the hydrodynamic profile of the stirring is unchanged; *i.e.* the unstirred diffusion layer thickness, l [eqn. (5)], is linearly diminished as the rotation speed of the paddles increases, providing there is no disturbance of the water/chloroform interface. All transport experiments presented in this work were done at 960 rpm of the paddle shafts driven by a fixed-speed continuous duty a.c. synchronous motor. A variable-speed a.c. motor was used only for

Table 2 Selectivity in BLM transport of desferrioxamine B (H_4DFB^+) and its iron(III) and aluminium(III) complexes when neutral carrier DC18C6 and ionizable lasalocid are used as carriers

	Flux[a]/10^{-11} mol s^{-1} cm^{-2}		
Carrier	H_4DFB^+	$AlHDFB^+$	$FeHDFB^+$
DC18C6	9.80	3.44	16.5
Na^+lasalocid$^-$	20.4	19.0	30.0

[a] Values reported from single measurements. Source phase: 0.02 M $MHDFB^+$ $MeSO_3^-$, 0.04 M Mg^{2+}, 0.08 M ClO_4^-. Membrane: 0.05 M DC18C6 or 1 mM Na^+lasalocid$^-$ in $CHCl_3$. Receiving phase: 0.1 M $Fe(ClO_4)_3$, 0.1 M $HClO_4$; room temperature (23 ± 0.5 °C).

the purpose of determining the flux dependence on the stirring rate. The ferrioxamine B flux mediated by DC18C6 was found to vary linearly with stirring rates from 300 to 1680 rpm. This indicates that the diffusion layer thickness is the rate limiting resistance for the overall flux.

Transition metal selectivity

In addition to ferrioxamine B, $FeHDFB^+$, we investigated the BLM transport of its aluminium analog, $AlHDFB^+$, as well as transport of the "free" ligand desferrioxamine B, H_4DFB^+. Table 2 illustrates the selectivity observed in the BLM transport of these species, facilitated by DC18C6 crown ether.§ The importance of this result rests in the fact that, in addition to the $>10^6$ selectivity in metal–ligand complexation ($\beta_{FeHDFB^+}/\beta_{AlHDFB^+} = 4 \times 10^6$),[8] the transport selectivity of Fe over Al is fivefold increased through a selective binding of the metal chelate to an ionophore. The data also show that an ionophore will prefer the iron complex over the "free" ligand, but on the other hand will prefer "free" ligand over the aluminium complex, a property that can be very important under certain biologically relevant conditions.

We have already observed such behavior in two phase batch extractions of desferrioxamine B complexes of the trivalent metals In, Fe, Ga and Al by crown ethers.[13] Namely, the change in ionic radius of the metals, which corresponds to the change in "charge density" and thus potentially to the "thickness" of the water solvation shell "wrapped" around the metal–LNH_3^+ complex, is directly correlated to the stability of the [(metal–LNH_3^+)–crown ether] supramolecular assembly.[13]

Since the volume of the metal chelate water solvation shell is thought to be responsible for the metal selectivity observed, it is anticipated that shortening the amine side-arm of the metal–ligand complex may further enhance the extraction and thus BLM transport selectivity as well.

Ionizable carrier lasalocid in the membrane

In our search for a more efficient carrier, lasalocid has been utilized, since this natural ionophore is known to mediate the transport of many cations,[27,42,43] as well as metal amine complexes and alkylammonium cations of the type $[RNH_3]^+$.[44] Lasalocid is a more effective extraction agent for ferrioxamine B, $FeHDFB^+$, than the most efficient crown ether by more than two orders of magnitude (Table 1; see also ref. 45). As a result, only micromolar concentrations of lasalocid are needed to transport ferrioxamine B at a very high flux (Fig. 4). The crown ethers and cryptand [2.2.2] are neutral species and in order to carry the $FeHDFB^+$ across the membrane their assemblies must include an accompanying anion (*e.g.* ClO_4^-). Lasalocid, on the other hand, due to the presence of the salicylic acid group ($pK_a = 3.7$),[46] can act as a hydrophobic counter anion as well as an ionophore when complexed with ferrioxamine B. We have previously demonstrated that an anionic arm covalently attached to a crown ether, *e.g.* a carboxylate lariat ether, will greatly increase the stability of its interaction with ferrioxamine B and the extractability of the entire assembly into an organic phase.[35]

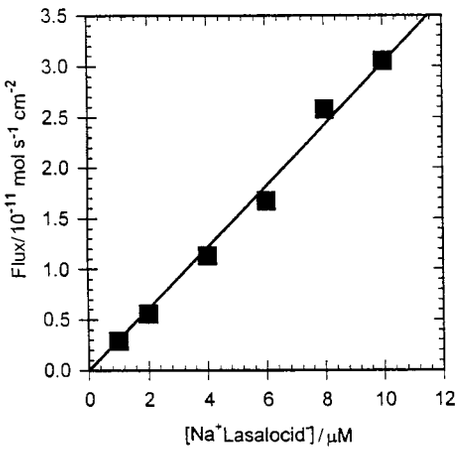


Fig. 4 Flux of ferrioxamine B as a function of membrane phase sodium lasalocid concentration. Source phase: 0.02 M $FeHDFB^+$ $MeSO_3^-$, 0.04 M Mg^{2+}, 0.08 M ClO_4^-, pH 9. Membrane phase: 1–10 μM Na^+lasalocid$^-$ in $CHCl_3$. Receiving phase: 0.1 M $Na^+ClO_4^-$, pH 9.

Lasalocid does not appreciably extract ferrioxamine B when the salicylic acid moiety is converted into an *ester*. However, although the extraction of ferrioxamine B by lasalocid ester in a two-phase batch extraction experiment was not spectrophotometrically observable, we have been able quantitatively to detect the flux of a very small amount of ferrioxamine B transported by lasalocid ester due to the cumulative nature of the BLM experiment. A 500-fold decrease in ferrioxamine B flux was observed for lasalocid ester as a carrier relative to a lasalocid salt (Fig. 5). This result suggests that lasalocid exhibits very modest ionophore properties and thus binds to and transports ferrioxamine B cation solely as an accompanying hydrophobic anion.

However, if we compare lasalocid with carboxylate lariat ethers, which also have ionophore properties and an ionizable side chain, we see that their ability to extract ferrioxamine B in a batch experiment is not even close to the extraction ability of lasalocid.[35,45] Simple hydrophobic anions (picrate, octanoate, and decanoate) also proved to be much less efficient when compared to lasalocid in qualitative two-phase extractions of ferrioxamine B under identical conditions. These results clearly rule out any possibility that lasalocid acts only as a hydrophobic anion.

Therefore, the observed behavior suggests a *co-operative* action of two binding modes, electrostatic and ionophoric, in which an electrostatic interaction between ferrioxamine B and lasalocid is a requirement for the wrapping of the lasalocid backbone around the ferrioxamine B ammonium site. This leads to a remarkably strong overall binding that makes lasalocid, in its anionic form, the best transporting agent for ferrioxamine B tested to date.[45]

At pH values below its pK_a, lasalocid is protonated and its transporting ability falls somewhere between lasalocid salt and lasalocid ester (Fig. 5). However, the relatively high acidity of its salicylic group ($pK_a = 3.7$)[46] enables lasalocid to be an efficient ionophore, over a broad range of pH conditions.

§ The BLM transport selectivity experiment was complicated by the fact that the desferrioxamine B ligand and its aluminium complex are colorless. Thus, a highly acidic (0.1 M H^+) solution of high Fe concentration (0.1 M Fe^{3+}) was used as the receiving phase so that any incoming ligand, aluminium or iron complex was transformed into a stable iron *mono* complex $Fe(H_3DFB)(H_2O)_4^{3+}$ as the only iron-containing species in solution. The absorbance of the receiving phase was followed at 500 nm where the $Fe(H_3DFB)(H_2O)_4^{3+}$complex has a molar absorptivity $\varepsilon \approx 1000$ M^{-1} cm^{-1}.[7]

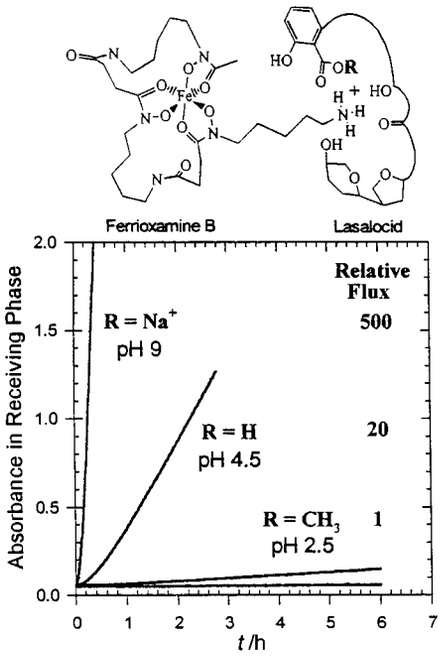


Fig. 5 Traces of ferrioxamine B absorbance in the receiving phase as a function of time with Na^+lasalocid$^-$ (R = Na^+), H^+lasalocid$^-$ (R = H), or lasalocid methyl ester (R = CH_3), as carriers. Bottom trace: no carrier present in membrane. Source phase: 0.02 M $FeHDFB^+$ $MeSO_3^-$, 0.04 M Mg^{2+}, 0.08 M ClO_4^-. Membrane phase: 0.01 M R-lasalocid in $CHCl_3$; receiving phase: 0.1 M $Na^+ClO_4^-$. Relative flux values obtained from relative slopes of the traces for R = Na^+ (500), H (20) or CH_3 (1).

Synergistic transport of ferrioxamine B and desferrioxamine B. BLM Transport studies presented in this work reveal some specific aspects associated with ionizable carriers. The principle of the BLM experiment calls for a hydrophobic carrier to be confined to the membrane phase. This means, in the case of an ionizable carrier, that the anion, being a part of the carrier, is confined to the membrane as well. Hence, the release of a cationic substrate from the membrane into a receiving aqueous phase will take place only through a hydrophobic/hydrophilic phase ion exchange process. As a consequence, lasalocid will not release the ferrioxamine B from the membrane phase when deionized water is used as the receiving phase. Any electrolyte in the receiving phase, however, dramatically increases the flux, since this facilitates the ion exchange process.

This observation was exploited by using the metal-free ligand desferrioxamine B (H_4DFB^+) as an exchangeable cation in the receiving phase, eqn. (6). This experimental set-up represents a

$$FeHDFB^+\cdot lasalocid^-(mem) + H_4DFB^+(rec)\cdot A^-(rec) \rightleftharpoons FeHDFB^+(rec)\cdot A^-(rec) + H_4DFB^+\cdot lasalocid^-(mem) \quad (6)$$

self contained and complete (in a simplified form) model for iron chelation and transport across a biological membrane (Scheme 2). Theory predicts that a system with two substrates, ferrioxamine B and desferrioxamine B, transported at similar rates (Table 2), is optimal for BLM transport by ionizable carriers.[23] Fig. 6 illustrates the dramatic increase in the flux of ferrioxamine B from the aqueous *source* phase caused by introducing more of the "free" ligand, desferrioxamine B, in the aqueous *receiving* phase (which mimics a cell interior). It can also be seen that the flux approaches its maximum value (thick-solid line) as the carrier becomes less "loaded" (thin-solid line). Optimum conditions for maximum flux in both directions are expected when the carrier is 50% saturated[23] (25% with ferrioxamine B and 25% with desferrioxamine B).

The system illustrated in Scheme 2 and Fig. 6 represents the *synergistic* transport of ferrioxamine B in one direction across a bulk liquid membrane with concomitant transport of desferrioxamine B across the membrane in the opposite direction.

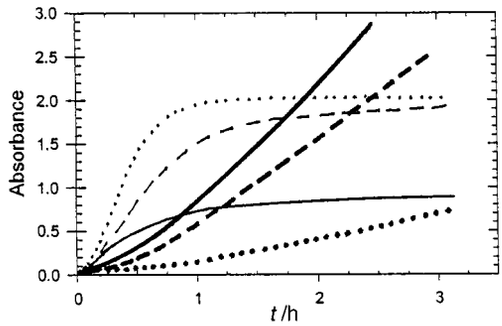
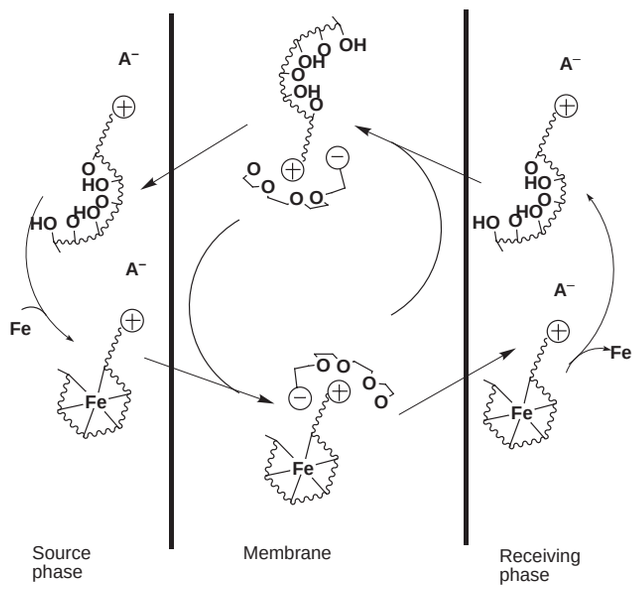


Fig. 6 Traces of ferrioxamine B absorbance as a function of time, in the membrane phase (thin lines) and receiving phase (thick lines) for BLM transport of ferrioxamine B by sodium lasalocid for different concentrations of desferrioxamine B as an exchangeable cation in the receiving phase (1 mM, dotted line; 10 mM, dashed line; and 100 mM, solid line). Source phase: 0.02 M $FeHDFB^+$ $MeSO_3^-$, 0.04 M Mg^{2+}, 0.08 M ClO_4^-, pH 3.2. Membrane phase: 1 mM Na^+lasalocid$^-$. Receiving phase: 1–100 mM H_4DFB^+ $MeSO_2Ph^-$ only.



Scheme 2 Synergistic BLM transport of ferrioxamine B (from left to right) and desferrioxamine B (from right to left) by the *ionizable* carrier lasalocid.

Conclusions

Bulk liquid membrane transport experiments demonstrate that ferrioxamine B can be efficiently transported across a hydrophobic artificial (chloroform) membrane by neutral crown ethers, cryptand, or the ionizable ionophore lasalocid. The transport flux is diffusion controlled and influenced by the structure of the neutral ionophore carrier in a predictable fashion according to K_{ex}. A search for more efficient neutral carriers should be continued since fluxes obtained with 18C6 cavity crown ethers are still far from the maximum obtainable values predicted by theory. The ability of crown ethers selectively to recognize and transport uncomplexed desferrioxamine B ligand and its iron(III) and aluminium(III) complexes is of biological and environmental importance. Although less selective, lasalocid proved to be an outstanding carrier for ferrioxamine B due to a dual mode of binding that was clearly revealed through BLM transport experiments with lasalocid salt, lasalocid acid, and lasalocid ester. The use of lasalocid anion as an ionophore carrier has enabled us to demonstrate

the synergistic transport of ferrioxamine B and desferrioxamine B across a BLM in opposite directions.

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