pH gradients and membrane transport in liposomal systems

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Among many possible applications, liposomes show particular promise as drug delivery vehicles, with demonstrated advantages over other approaches in the therapy of cancer and infectious disease[1]. The advantages of liposomes for drug-delivery are primarily the reduced toxic side effects exhibited by liposomal formulations of anticancer and other drugs as compared to the free drug, while maintaining or increasing efficacy against the disease state. Until recently, development of this potential has been frustrated by a variety of problems, including the inability to load liposomes efficiently in order to achieve stable systems with high internal concentrations of drug. Recent work indicates that transmembrane pH gradients in liposomal systems can facilitate the encapsulation of many commonly employed drugs, certain ions and peptides.

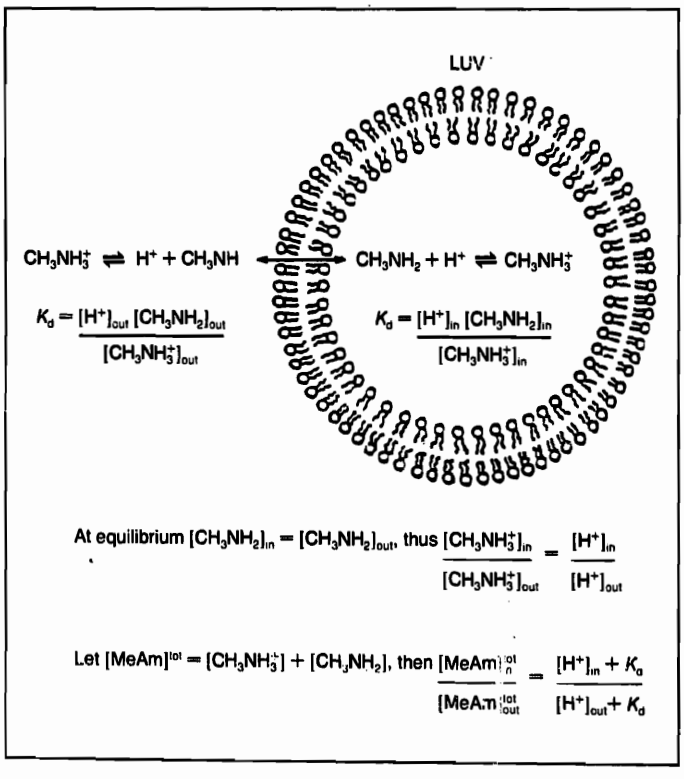

Figure 1

Influence of a pH gradient on the transbilayer distribution of methylamine in a large unilamellar vesicle (LUV). K_d is the dissociation constant. $[MeAm]^{tot}$ represents the total concentration of methylamine, both the charged ($CH_3NH_3^+$), and uncharged (CH_3NH_2) forms.

Transmembrane gradients

Research into liposomes as drug-delivery vehicles stems from their use in modeling biological membranes in studies of transmembrane pH and electrical potential gradients, and lipid mobility. Transmembrane electrical potentials ($\Delta\psi$) and pH gradients (ΔpH) in biological systems can be conveniently monitored by employing certain lipophilic cations or anions as probes, as well as observations that organelles such as chromaffin granules[2] and mitochondria[3] can accumulate weak bases or acids, respectively, in response to endogenous pH gradients. By way of example, in order to measure transmembrane pH gradients, weak bases such as methylamine are often used. These probe molecules are taken up into membrane-bound systems by a mechanism that involves the transmembrane diffusion of the neutral form of the molecule, which is highly membrane permeable, whereas the charged (protonated) form is not membrane permeable. In the case of methylamine, for example, the probe can adopt a charged or uncharged form according to the equilibrium:

$$CH_3NH_3^+ \rightleftharpoons CH_3NH_2 + H^+$$

with a dissociation constant:

$$K_d = \frac{[CH_3NH_2][H^+)}{[CH_3NH_3^+]}$$

Thus, if methylamine is introduced into a medium containing closed, vesicular membrane systems exhibiting a transmembrane ΔpH, equilibrium will be established when the neutral (membrane permeable) form occurs at equal concentrations on both sides of the membrane. As indicated in Fig. 1, this corresponds to a situation where $[MeAm]^{tot}_{in}/[MeAm]^{tot}_{out} = [H^+]_{in}/[H^+]_{out}$, for situations where $K_d << [H^+]_{in}, [H^+]_{out}$. Since the p$K$ of methylamine is 10.6, this condition is easily satisfied. This can result in substantial concentration gradients of the probe across the membrane. For example, for a three-unit pH gradient (ΔpH = 3) (inside acidic), this corresponds to an equilibrium concentration of probe inside the vesicle system which is 1000 times

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higher than outside. Determination of inside: outside ratios of such a probe using, for example, a radiolabelled species, can provide a convenient measure of ΔpH.

Probes of ΔpH, such as methylamine, are of great use in their own right to determine transbilayer pH gradients in biological systems. However, their behaviour has much more general implications. This was first suggested by Nichols and Deamer[4], whose early studies showed that the presence of a ΔpH (inside acidic) can result in the rapid sequestration of catecholamines (the weak bases dopamine, epinephrine and norepinephrine) into liposomal systems. These authors suggested that these observations were related to the mechanism of accumulation and storage of catecholamines in secretory organelles. A more general implication is that any weak base which exhibits suitably lipophilic character will be accumulated into membrane-bound systems with an acidic interior. Alternatively, lipophilic weak acids should be accumulated into membrane systems exhibiting a basic interior. Studies employing liposomal systems which support these conclusions are discussed below. First, however, we summarize techniques available for generating a ΔpH in liposomal systems which provide convenient experimental systems for these investigations.

Constructing liposomal systems with pH gradients

Studies aimed at characterizing the influence of ΔpH on membrane transport in liposomes require an appropriate model system. The specifications of the type of liposome are quite rigorous: they should be of a uniform size and unilamellar for ease of data interpretation, and large enough that sufficient buffer can be entrapped to generate stable electrochemical potentials (see below). Large unilamellar vesicles (LUVs), with a diameter of 100 nm or larger are preferred. Until recently, techniques for generating such LUVs have been relatively tedious and often employ organic solvents or detergents which can influence permeability properties. These include protocols involving diluting lipids dissolved in organic solvent or detergent into aqueous media, or reverse-phase evaporation procedures[5]. We have found a medium-pressure extrusion procedure[6] provides a major technical advantage. This protocol involves extruding preformed multilamellar vesicles through filters of defined pore size and gives rise to a relatively homogeneously sized LUV population within minutes. A freeze-fracture micrograph of 100 nm LUVs generated by this technique is shown in Fig. 2.

Techniques for generating ΔpH in the LUV systems are straightforward. A proton gradient can be generated by forming the LUVs in a well-buffered medium of defined pH and subsequently adjusting the exterior pH to achieve the desired

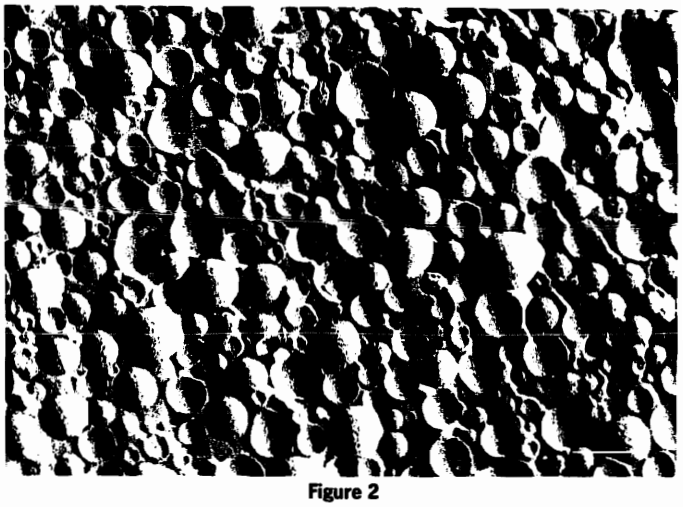

Figure 2
Freeze-fracture electron micrograph of LUVs composed of egg phosphatidylcholine (400 mg ml^{-1}) after extrusion (10 times) through two stacked Nuclepore filters (pore size 100 nm). The bar corresponds to 100 nm. For further details, see Ref. 6.

ΔpH. In these systems, there are two important points to note.

First, an adequate pool of interior ions is essential to achieve a stable ΔpH. As an example, it is useful to consider a 100 nm diameter LUV exhibiting a ΔpH of 3 (inside acidic, e.g. an exterior pH of 7 and an interior pH of 4) in a Na^+ buffer. Due to the high intrinsic permeability of protons through bilayers[7], H^+ ions will permeate out. This efflux is self-limiting, however, as the outward movement of H^+ ions sets up an electrical potential (Δψ, positive outside) which acts to inhibit further H^+ efflux. At 'electrochemical equilibrium', the electrical potential thus generated will satisfy the Nernst equation:

$$\Delta\psi = -59 \log \frac{[H^+]_{in}}{[H^+]_{out}}$$

Assuming that the ΔpH of three units remains constant, this results in a Δψ of −177 mV. Any further efflux of protons will be coupled to the inward movement of Na^+ ions, which is a much slower process. Assuming a membrane capacitance (C) of ~0.5 μF cm^{-2}, approximately 150 H^+ ions will diffuse out to set up this potential according to the capacitance relationship Q = CV, where Q is the charge and V is the transmembrane voltage Δψ. Thus, the number of trapped proton equivalents (i.e. singly charged, positive ions), must be considerably greater than 150 to allow a stable ΔpH (and Δψ) to be maintained. This is readily achieved by employing high concentrations of entrapped buffer. A 300 mM citrate buffer in a 100 nm diameter LUV, for example, corresponds to a requirement for approximately 10^5 proton equivalents to titrate between pH 4 and pH 7.

The second point – one that is central to the usefulness of LUV systems exhibiting a Δψ and

ΔpH – is that once electrochemical equilibrium is achieved, the systems are highly stable. This is due to the relatively low permeability of ions other than H^+ ions through membranes. For an egg-phosphatidylcholine–cholesterol (EPC-chol) LUV system, for example, $\Delta\psi$ values in excess of 100 mV, and ΔpH values in excess of two units can be readily maintained for days or weeks under convenient experimental conditions.

Uptake of drugs into LUVs in response to ΔpH

A large proportion of commonly employed pharmaceuticals are relatively lipophilic molecules containing primary, secondary or tertiary amines. Indeed, the generality of this observation argues that such characteristics are important for function – probably related to the ability of such molecules to traverse cell membranes to gain access to intracellular sites of action. As for methylamine, the presence of an ionizable amino function allows the compound to adopt a net neutral form which may be expected to be considerably more membrane-permeable than its charged (protonated) counterpart. In any event, the fact that a large proportion of drugs are lipophilic amines has two important consequences with regard to their response to liposomal systems exhibiting a pH gradient.

First, by analogy with the response of methylamine, it would be expected that incubation of a lipophilic drug containing an ionizable amino function with LUVs with an acidic interior would result in drug accumulation. Thus, depicting a drug containing an amino function as $R\text{-}NH_3^+$, the equilibrium transbilayer distribution would be expected to follow the proton gradient according to $[R\text{-}NH_3^+]_{in}/[R\text{-}NH_3^+]_{out} = [H^+]_{in}/[H^+]_{out}$. This, of course, assumes that $K_d << [H^+]_{out}$, $[H^+]_{in}$, and again ignores effects due to drug partitioning into the bilayer. High drug–membrane partition coefficients lead to even higher levels of internalized drug.

Second, in addition to high interior:exterior drug concentration ratios, high interior concentrations of drug would be expected if the interior contains high concentrations of buffer. The requirement for a high interior buffering capacity arises from the re-protonation of the drug in the LUV interior following transport of the neutral form. This depletes the interior proton pool and can collapse the transmembrane pH gradient if the interior buffering capacity is insufficient.

These predictions regarding the uptake of lipophilic, amino function-containing drugs into LUVs with an acidic interior have received substantial experimental confirmation, best illustrated by recent studies using 100 nm egg-phosphatidylcholine LUVs exhibiting a ΔpH of 3.5 (exterior pH 7.5, interior pH 4.0) containing 300 mM citrate as internal buffer[8]. As shown in Table 1, a variety of antineoplastic agents, antiarrhythmic agents, antimalarials and other drug classes can be accumulated in LUVs to high interior concentrations corresponding to 130 mM or higher, for an initial exterior drug concentration of 0.2 mM. It may also be noted that such accumulation can be highly efficient, as interior drug concentrations of 120 mM correspond to entrapment of greater than 90% of the total drug under these experimental conditions.

Table 1. Accumulation of drugs into LUVs in response to a pH gradient (pH_{out} = 7.5, pH_{in} = 4.0)[a]

Drug	Maximum uptake levels (mM)
Antineoplastic	
Mitoxanthrone	132
Epirubicin	133
Daunorubicin	134
Doxorubicin	134
Vincristine	119
Vinblastine	117
Local anaesthetics	
Lidocaine	58
Chlorpromazine	65
Dibucaine	129
Adrenergic antagonists	
Propranolol	132
Timolol	65
Antiarrhythmic agents	
Quinidine	134
Biogenic amines	
Dopamine	127
Serotonin	53
Antidepressant	
Imipramine	125
Antihistamine	
Diphenhydramine	117
Antimalarial	
Quinine	97
Chloroquine	69
Antiprotozoan	
Quinacrine	49

[a] Egg phosphatidylcholine (EPC) large unilamellar vesicles (LUVs) (100 nm diameter) were prepared with an interior buffer of 300 mM citrate (pH 4.0) and an exterior buffer of 300 mM NaCl, 20 mM HEPES (pH 7.5). LUVs (1 mM phospholipid) were then incubated with drug at an initial exterior concentration of 0.2 mM and uptake monitored at 25°C (Ref. 8). The internal concentration of drug is calculated assuming an internal trapped volume of 1.5 μl per μmol phospholipid.

These observations have important implications for loading liposomal systems for drug-delivery applications. A prime example is doxorubicin, a major anticancer drug active against a variety of ascitic and solid neoplasms. It is well established (in animal models), that liposomal encapsulation buffers the acute toxicity (LD_{50}),

and cardiotoxicity of this drug without reducing anticancer potency[9]. However, methods for stably and efficiently entrapping the drug at high drug : lipid ratios have not been available. As shown in Fig. 3, however, doxorubicin can be rapidly entrapped in response to a ΔpH (inside acidic) to achieve interior drug concentrations of 300 mM or higher. As indicated elsewhere[10], trapping efficiencies of greater than 98% can also be readily achieved. This technology has resulted in liposomal doxorubicin preparations which exhibit superior toxicity and efficacy properties[10], and which are currently in clinical trials, and has also been extended to liposomal preparations of the anticancer drug vincristine[11]. A particular advantage of the ΔpH loading procedure is that encapsulation can be performed immediately prior to administration, thus eliminating the possibility of drug leakage during extended storage.

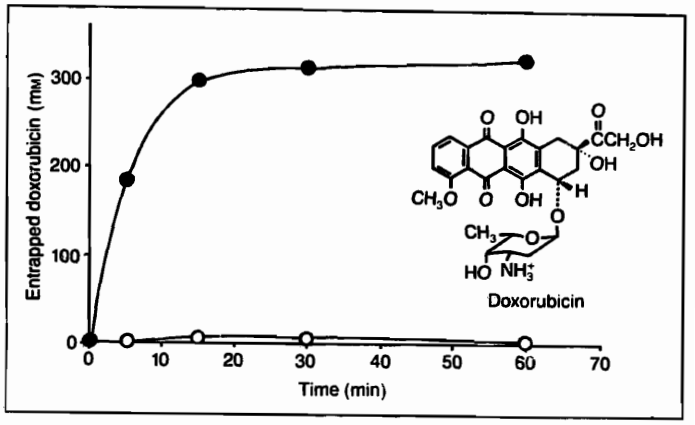

Figure 3

Accumulation of doxorubicin into egg-phosphatidylcholine–cholesterol (55:45, mol:mol) LUVs (sized through filter with 200 nm pore size) in the presence of a transmembrane pH gradient (pH_{out} = 7.4, pH_{in} = 4.0) (●), and in the absence of a pH gradient (pH_{out} = pH_{in} = 7.4) (○). The experiment was conducted at 37°C and the internal concentration of doxorubicin calculated assuming an internal trapped volume of 2.8 μl per μmol phospholipid.

Influence of ΔpH on the transbilayer distributions of lipids

It is well established that biological membranes exhibit asymmetric transbilayer distributions of lipids[12]. However, the mechanisms whereby this asymmetry is generated and maintained are not understood. By analogy with the behaviour of drugs which are weak bases, however, it may be expected that lipids which are weak bases (and weak acids) should respond to transbilayer pH gradients via similar mechanisms. This idea has received considerable experimental support. In the case of the weak base stearylamine, for example, the presence of a pH gradient (interior acidic) results in rapid migration of stearylamine to the inner monolayer[13]. The behaviour of naturally occurring lipids which are weak acids is of particular interest. Such compounds assume neutral or negative charge according to the equation:

$$H^+ + A^- \rightleftharpoons HA$$

Assuming that the neutral (HA) form is membrane permeable, it is straightforward to show that at equilibrium $[A^-]_{in}/[A^-]_{out} = [H^+]_{out}/[H^+]_{in}$, where $[H^+]_{out}$ and $[H^+]_{in}$ are the proton concentrations at the exterior and interior membrane–water interfaces, respectively. Thus, a net accumulation of lipids which are weak acids into the inner monolayer would be expected for LUVs with a basic interior. This has been observed for fatty acids[13], where a relatively complete and rapid transfer of oleic acid from the outer to the inner monolayer is observed in LUVs experiencing a ΔpH of three units (exterior pH 7, interior pH 10). This work has been extended to certain phospholipids which are weak acids, namely phosphatidylglycerol (PG) and phosphatidic acid (PA)[14,15]. Three conclusions arise from this work. First, both PG and PA migrate to the inner monolayer of LUVs with a basic interior. Second, a kinetic analysis of the PG transport strongly indicates that such migration proceeds as a first order process where the neutral (protonated) species are traversing the membrane. Finally, the rate of transbilayer movement of the neutral species can be extremely rapid, exhibiting half-times for transbilayer movement of PG on the order of seconds at 45°C.

The ability to modulate the transbilayer distribution of lipids in liposomes is potentially important in drug delivery since the clearance of liposomes from the circulation and their ability to fuse with target cells is very sensitive to the types of lipids found in the outer monolayer. In addition, this is important technology for modifying the surface charge of liposomes.

These observations also have biological implications concerning the flow of fatty acids *in vivo* and the transbilayer distributions of PG, PA and other lipid species which are weak acids. In the case of fatty acids, an outward flow from organelles and cells with acidic interiors would be expected, for example. Similarly, lipids such as PG would be expected to be present in the inner monolayer of prokaryotes such as *Clostridium butyricum*, which grow in acidic environments[16]. Alternatively, in chloroplast membranes, which can exhibit a highly acidic interior[17], minority acidic lipids such as PG would be expected to reside predominantly in the outer monolayer.

Influence of ΔpH on the transbilayer movements of cations and peptides

Transmembrane pH gradients can also strongly influence membrane transport and accumulation of divalent cations and certain peptides into

LUVs. In the case of cations such as Ca^{2+}, accumulation is accomplished employing ionophores such as A23187, which transport Ca^{2+} ions in exchange for protons. Employing the 100 nm LUV system containing entrapped citrate at pH 4.0, it has been shown that interior Ca^{2+} concentrations in excess of 200 mM can be readily achieved for initial exterior Ca^{2+} concentrations of 0.5 mM[18]. Potential applications for cation accumulation include developing highly radioactive liposomes for cancer therapy, heavy-metal chelation *in vivo* and electron-dense liposomes for imaging *in vivo* and *in vitro*.

The development of small peptides as therapeutics is likely to generate increased interest in liposomes as a means for transporting and targeting the peptides to their site of action. In the case of peptides, it has been shown that peptides which are lipophilic amines can be accumulated into LUVs in response to $\Delta\psi$ (negative interior)[19]. Such observations are intriguing, given the present lack of understanding regarding the forces driving transbilayer movement of signal peptides during protein insertion and translocation – signal peptides usually contain one or more basic residues and have distinctly lipophilic character[20].

Concluding remarks

The results summarized here illustrate the remarkable and general transport abilities of liposomal systems exhibiting transmembrane pH gradients. These capacities considerably expand the utility of liposome technology and have direct implications for biological systems. With regard to application, for example, in addition to loading liposome systems with drugs, ions and peptides, there are clear potential applications for purification of weak acids and bases from complex media or for performing novel microchemistry within the liposome interior. Alternatively, these observations provide new insight into mechanisms regulating the transmembrane distribution of lipids, drugs and peptides *in vivo*.

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