Kinetics of Accumulation of Molecules into Liposomes

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In previous report (Čeh, B.; Lasic, D. D. *Langmuir* **1995**, *11*, 3356), the accumulation of external molecules into preformed liposomes by the influence of various gradients, a very important feature in recent applications of liposomes as drug carriers, was presented. The thermodynamic model of liposome loading with weak bases/acids used preset permeability coefficients that allowed or disallowed penetration of noncharged and charged species, respectively, across the vesicle membrane. Now in this article we investigate the kinetics of drug loading. Using Fick's first law, we can calculate kinetics of loading, expressing the diffusion constant as a permeability coefficient and defining an associated flow rate. Results of this theoretical approach, which in the limit of long times converge to the result of the thermodynamic model, show the filling of liposomes with particular molecules as a function of time, as well as time dependence on pH changes and concentration of any other species in the liposome internal or external environment.

Introduction

The selective transport of charged and neutral species across biological membranes of cells and some organelles, either passive or active, is one of the important phenomena in exerting and controlling biological processes. It controls the exchange of different ions, molecules, substrates, and cofactors and regulates the concentration of substances and species participating in a wide spectra of chemical reactions on both sides of the membrane.[1] In contrast to biological systems where endergonic transport of neutral and charged species and water molecules occurs also through membrane pores and channels, the passive translocation of molecules and ions across artificial phospholipid bilayers is due to transmembrane electrochemical and pressure gradients.[2,3] They represent the driving force for different diffusion processes that lead to the thermodynamic equilibration of the whole vesicular system. However, the phospholipid vesicles/liposomes and their membranes may serve as an excellent substitute for biomembranes in studying the different physicochemical phenomena and mechanisms in basic sciences like membrane permeability measurements or dynamic membrane processes.[2,4] To get insight into some of possible diffusion processes and reaction mechanisms taking place inside cytoplasm and cell organelles, different experiments with liposomes have been performed and supported by theoretical description and mathematical models.[4,5]

Since the first experiment of uptake of the cathecholamines into liposomes was undertaken more than two decades ago,[6] liposomes become widely studied and used as a drug delivery system. Effective drug loading and retention was found to be one of the major problems. In the case of weak acids and weak bases, however, remote loading methods of filling preformed liposomes with these molecules were shown to be very effective. Uptake of some drugs into preformed liposomes is forced predominantly by different concentration gradients. pH gradient, as one example, drives the neutral form of the drug from buffered exterior into the more acidic buffered vesicle interior.[7−9] On the other hand the bidirectional exchange—ammonium sulfate gradient—is an alternative way for redistribution of agents into the liposomes.[10−12]

Theory

There must be certain physicochemical complementarity between phospholipid molecules constituting the membrane, and translocated species can cross it. The hydrophobicity of bilayer phospholipid molecules as well as chemical nature of the permeating species are important factors in deciding the rate for different species to cross the membrane. A very low permeability of ions through the bilayer, for example, is due to their charge as well as their very low solubility within the hydrocarbon region. Similar is the case also for large hydrophillic molecules. In contrast to this, hydrophobic neutral molecules, owing to nonelectrostatic contributions across the bilayer, show significantly higher permeability. In general, permeation is inversely proportional to the stretching elasticity of the bilayer.[13] There is also chemical composition of the particular membrane, which can strongly affect the permeability of individual species.[2,3] Facilitated transport by carriers (protonophores and ionophores) is another way by which ions and small molecules can be translocated across phospholipid membrane, while Cl^- ions can shuttle across bilayer by complexing with lipids and consequent flip-flop. Small molecules like CO_2 and NH_3 as well some alcohols are known to be highly permeable.

There are some theories explaining the mechanisms of the transport of neutral species across lipid bilayer. By applying the absolute rate theory, for example, the process of a translocation of molecules through the lipid membrane was explained as a series of consecutive energetic jumps between a number of equilibria states within the bilayer.[14] The unusual rapid movement of small molecules across bilayers was discussed by means of so-called "mobile kink" hypotheses based on the holes proposed to exist within the hydrophobic part of the mem-

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brane.[15] The permeabilities of small molecules were measured and correlated with their partition coefficient between water and some organic solvents.[16]

Owing to the fact that membrane characteristics change with respect to the composition of both compartments, consequently, the permeability is not a constant during permeation. It depends on solute membrane interactions and transbilayer gradient.[3] However, notwithstanding the factors mentioned here, simplified models can be postulated that may serve as a powerful tool to explain transmembrane flux of species in various chemical environments. Such specific chemical environments represent the interior of the pre-filled liposomes, which interacts with the exterior solution the liposomes are placed in. Recently a general theory of loading of agents/drugs into the liposomes was published, explaining why and how efficiently the liposomes can be loaded.[17] Also, the theory was supported by experimental results explaining the efficient remote loading of two drugs, doxorubicin and ciprofloxacin into the liposomes.[12] Here, using the terminology already defined,[17−19] we shall try to explain the rate of loading of agents into the lipid vesicles, or in general, the time dependence of exchange and interchange of molecular species across the membrane.

According to a four-compartment model,[20] we will first derive the electroneutrality equation for a liposome interior and exterior, each of them containing an aqueous Brønsted acid/base/salt compartment and a membrane surface-binding compartment. There are three groups of species in such a Brønsted solution that must be treated separately: (i) Brønsted acid/base species, (ii) "inert" species, and (iii) H^+ and OH^- ions.[17] It is known that any of Brønsted (starting-point) species in eq 1 placed in an aqueous medium reacts with water molecules giving a series of "sister-species":

$$S_{i1}^{z_{i1}} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} S_{i2}^{z_{i2}} \underset{+H^-}{\overset{-H^+}{\rightleftharpoons}} \ldots S_{ij}^{z_{ij}} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} S_{iNi}^{z_{iNi}} \quad (S_{ij}^{z_{ij}} = HS_{ij+1}^{z_{ij+1}+1}) \qquad (1)$$

The charges of the species above decrease from z_{i1} to z_{iN_i}. The first species, $S_{i1}^{z_{i1}}$, can act only as an acid and the last one, $S_{iNi}^{z_{iNi}}$, only as a base. In the inner/outer liposomal aqueous solution, each of the species in a particular "sister-species" series has to be in protolithic equilibrium with all the other ones including the first one. So the concentration of each Brønsted sister-species designated in eq 1 above may be expressed by the concentration of the first one

$$[S_{ij+1}^{z_{ij+1}}]_c = [S_{i1}^{z_{i1}}]_c \frac{K_{i1}K_{i2}\ldots K_{ij}}{[H^+]^j} \qquad (2)$$

where K_{ij} is the acidity constant of a particular Brønsted conjugated acid/base pair $HS_{ij-1}^{z_{ij}-1}/S_{ij}^{z_{ij}}$. Additionally, for liposome interior/exterior it holds that particular species in the aqueous compartment, designated *a*, must be in chemical equilibrium with the same species bound onto the membrane surface binding domain designated *m*

$$K_{ij}^{p} = \frac{[S_{ij}^{z_{ij}}]_m}{[S_{ij}^{z_{ij}}]_a} \qquad (3)$$

where K_{ij}^{p} is a partition coefficient of species *ij* between both compartments inside/outside the liposome vesicle. Thus the total amount of a species n_{ij}, i.e., membrane-bound and remaining in the aqueous solution, in the interior/exterior is

$$n_{ij} = V_a[S_{ij}^{z_{ij}}]_a + V_m[S_{ij}^{z_{ij}}]_m = V[S_{ij}^{z_{ij}}]_c\left(1 + K_{ij}^{p}\frac{V_m}{V_a}\right) \qquad (4)$$

A total charge contribution, Q_{ij}, can also be expressed in a similar way

$$Q_{ij} = V_a z_{ij}[S_{ij}^{z_{ij}}]_a + V_m z_{ij}[S_{ij}^{z_{ij}}]_m = V_a z_{ij}[S_{ij}^{z_{ij}}]_a\left(1 + K_{ij}^{p}\frac{V_m}{V_a}\right) \qquad (5)$$

where V_a and V_m represent the volumes of an aqueous compartment and its accompanying membrane binding domain inside and outside the liposomes. Following eqs 2, and 4, the total amount of all sister-species *ij* in eq 1 can be written as

$$\sum_{j=1}^{N_i} n_{ij} = V_a[S_{i1}^{z_{i1}}]_a \sum_{j=1}^{N_i}\left(1 + K_{ij}^{p}\frac{V_m}{V_a}\right)\frac{\prod_{k=0}^{j-1} K_{ik}}{[H^+]_a^{j-1}} \qquad (6)$$

and, similarly, the total charge of the same species is obtained by combining eqs 2, and 5. By dividing eq 7 with eq 6 one obtains

$$\sum_{j=1}^{N_i} Q_{ij} = V_a[S_{i1}^{z_{i1}}]_a \sum_{j=1}^{N_i} z_{ij}\left(1 + K_{ij}^{p}\frac{V_m}{V_a}\right)\frac{\prod_{k=0}^{j-1} K_{ik}}{[H^+]_a^{j-1}} \qquad (7)$$

$$\frac{1}{V_a}\sum_{j=1}^{N_i} Q_{ij} = a_i\frac{g_i^*([H^+]_a)}{f_i^*([H^+]_a)} \qquad (8)$$

Here $c_i = \Sigma n_{ij}/V_a = n_i/V_a$ is the starting aqueous compartment concentration of a particular starting-point species designated *i*, f_i^* the "concentration function" (see eq 6)

$$f_i^*([H^+]_a) = h_{i1} + \sum_{j=1}^{N_i} h_{ij+1}\frac{\prod_{k=1}^{j} K_{ik}}{[H^+]_a^j} = \sum_{j=0}^{N_i} h_{ij+1}\frac{\prod_{k=0}^{j} K_{ik}}{[H^+]_a^j}; \quad (K_{i0} = 1) \qquad (9)$$

and g_i^* the corresponding "charge function" (see eq 7) of the sister-species series *i*

$$g_i^*([H^+]_a) = z_{i1}h_{i1} + \sum_{j=1}^{N_i} z_{ij+1}h_{ij+1}\frac{\prod_{k=1}^{j} K_{ik}}{[H^+]_a^j} = \sum_{j=0}^{N_i} z_{ij+1}h_{ij+1}\frac{\prod_{k=0}^{j} K_{ik}}{[H^+]_a^j}; \quad (K_{i0} = 1) \qquad (10)$$

where $h_{ij} = 1 + K_{ij}^{p}(V_m/V_a)$.

The appropriate charge relations may also be obtained for the second-group species present—the "inert" ions like Na^+,

Ca^{2+}, Cl^-, NO_3^-, etc. For each of them it holds

$$\frac{1}{V_c} Q_l = z_l c_l = z_l c_l^* h_l \tag{11}$$

where z_l is charge of the inert ion, c_l its initial concentration in aqueous compartment before membrane binding, c^* its concentration in either aqueous solution after (possible) membrane binding of the same ion, and $h_l = 1 + K_l^p(V_m/V_a)$, respectively. In accordance with the derivations above, for the charge of the third group of ions, those of solvent (water) acid/base species, H^+ and OH^-, one obtains

$$\frac{1}{V_a} Q([H^+]_a) = h_H[H^+]_a - \frac{K_w}{h_H[H^+]_a} \tag{12}$$

Here $[H^+]_a$ is H^+-concentration in either aqueous compartment, K_w the autoionization constant of water, and h_H has the usual form as in the equations above.

Finally, the $[H^+]_a$-dependent function, expressing the electroneutrality in the internal/external vesicular domain, including both of their subsystems, aqueous solution and membrane surface binding domain, is now

$$\sum_i c_i \frac{g_i^*([H^+]_a)}{f_i^*([H^+]_a)} + \sum_l z_l c_l + \left(h_H[H^+]_a - \frac{K_w}{h_H[H^+]_a} \right) = 0 \tag{13}$$

The designation above is based on the terminology introduced in ref 17, but the difference in the result is well-recognized by the corrective *h*-terms (see also eqs 9−12).

Following eqs 2, 6, and 9, one can express the concentration of the respective species $S_{ij}^{z_{ij}}$ in either aqueous compartment as

$$[S_{ij}^{z_{ij}}]_a = c_i \frac{1}{f_i^*([H^+]_a)} \left(\frac{\prod_{j=0} K_{ij}}{[H^+]_a^{j-1}} \right) \quad (K_{i0} = 1) \tag{14}$$

Henceforth, the (starting equilibrium) H^+ concentration in both aqueous compartments will be written as (x^{st}) x for the inner, and (y^{st}) y for the outer one. Before the permeation of any species is assumed to occur, we will describe the situation in both inner (designated I) and both outer (designated O) compartments. For the inner compartments it is valid

$$\sum_m c_{mI} \frac{g_{mI}^*(x^{st})}{f_{mI}^*(x^{st})} + u_I^*(x^{st}) = 0 \tag{15}$$

and similarly for vesicular internal domain

$$\sum_k c_{kO} \frac{g_{kO}^*(y^{st})}{f_{kO}^*(y^{st})} + u_O^*(y^{st}) = 0 \tag{16}$$

Here c_{mI}/c_{kO} are initial concentrations of starting-point species in inner/outer aqueous compartment, g_{mI}^*/g_{kO}^* and f_{mI}^*/f_{kO}^* the respective charge and concentration functions, and u_I^*/u_O^* the sum of the second and third term in eq 13. The amounts of species k/m that traverse the membrane from exterior/interior can be expressed in terms of concentrations, volumes, and permeation portions, α_k and β_m, respectively. For a species k, influxed into interior from exterior, one can write

$$V_O c_{kO} \alpha_k = V_I c_{kI} \tag{17}$$

and a similar expression is also valid for a species m that traverses the bilayer in the opposite direction

$$V_I c_{mI} \beta_k = V_O c_{mO} \tag{18}$$

Now some of the neutral molecules are allowed to cross the membrane in either direction. After the translocation of an optional number of permeable molecules, the electoneutrality function for the liposomal interior is now[17]

$$\sum_{m,I} c_{mI}(1 - B_m\beta_m) \frac{g_{mI}^*(x)}{f_{mI}^*(x)} + K_v \sum_{k,O} (A_k c_{kO} \alpha_k) \frac{g_{kO}^*(x)}{f_{kO}^*(x)} + u_I^*(x) = 0 \tag{19}$$

and for the exterior it holds

$$\sum_{k,O} c_{kO}(1 - A_k\alpha_k) \frac{g_{kO}^*(y)}{f_{kO}^*(y)} + \frac{1}{K_v} \sum_{m,I} (B_m c_{mI} \beta_m) \frac{g_{mO}^*(y)}{f_{mO}^*(y)} + u_O^*(y) = 0 \tag{20}$$

Factors A_k and B_m allow the neutral species designated k and m to permeate the bilayer (=1) or not (=0); $K_v = V_O/V_I$ is an outer-to-inner compartment volume ratio. All the functions f and g here are designated * to stress the difference to those in ref 17, where membrane-binding calculations were performed in a specific, not general, way.

It has to be pointed out that the relevance of all the expressions derived above is limited only to dilute solution where the activity coefficients in all four compartment are assumed to have values close to 1.

The transport of solute across membrane can be described by means of Fick's first law

$$j_i = -D_i \frac{dc_i}{dx} \tag{21}$$

where j_i is a molar flux of the particular species designated i crossing the bilayer, D_i is its diffusion coefficient, and dc_i/dx its concentration gradient. Assuming that the concentration gradient in the membrane of a thickness d remains linear during the permeation, one can write

$$j_i = -D_i \frac{K_i^p c_{iO}^0 - K_i^p c_{iI}^0}{d} = -\left(\frac{D_i K_i^p}{d} \right)(c_{iO}^0 - c_{iI}^0) = -P_i(c_{iO}^0 - c_{iI}^0) \tag{22}$$

Here $(c_{iO}^0 - c_{iI}^0)$ is the concentration difference of a particular neutral species across the membrane, K_i^p is the partition coefficient of the permeated species, and $P_i = K_i^p D_i/d$ is known as a permeability. In accordance with the time-dependent definition of the molar flow, the equation above, valid for a particular species permeating in either direction across the bilayer of the liposome, may be written in a differential form

$$\frac{1}{S_m} \left(\frac{V_a\, dc_i}{dt} \right) = -P_i(c_{iO}^0 - c_{iI}^0) \tag{23}$$

where S_m is the inner/outer interface bilayer surface. Applying eq 14, the concentration difference in eqs 22 and 23 can be

described in terms of a total, starting-point concentration of the particular species, c_k, and a value c_m, in the appropriate liposome aqueous compartment. For a species designated *ml* that belongs to the Brønsted acid-base series *m*, and traverses the bilayer from interior into exterior, one can write

$$K^{p}_{ml}(c^{0}_{mI} - c^{0}_{mO}) = K^{p}_{ml}c_m(1 - \beta_m)\left(\frac{\prod_{j=0} K_{mj}}{x^{l_m-1}f_{mI}^{*}(x)}\right) - K^{p}_{ml}c_{ml}\frac{1}{K_\upsilon}\beta_m\left(\frac{\prod_{j=0} K_{mj}}{y^{l_m-1}f_{mO}^{*}(y)}\right) \quad (K_{m0} = 1) \quad (24)$$

A similar expression may also be obtained for the case when neutral species *kl* traverses the bilayer in the opposite direction

$$K^{p}_{kl}(c^{0}_{kO} - c^{0}_{kI}) = K^{p}_{kl}c_k(1 - \alpha_k)\left(\frac{\prod_{j=0} K_{kj}}{y^{l_k-1}f_{kO}^{*}(y)}\right) - K^{p}_{kl}K_\upsilon c_k\alpha_k\left(\frac{\prod_{j=0} K_{kj}}{x^{l_k-1}f_{kI}^{*}(x)}\right) \quad (K_{k0} = 1) \quad (25)$$

Here l_m and l_k are the running numbers of the neutral species in a given ordered series (for example, in the ordered acid−base series BH^+/B, the neutral species, B, is in the second place; thus $l_{m/k} = 2$). Owing to the permeation of neutral molecules only, the entire amount of permeated species *m*/*k* is equal the amount of neutral molecules *ml*/*kl*, $dn_{mI} = dn^{0}_{ml}$. Thereby, for the total amount of the permeated species, we can also write

$$-d\beta_m = \frac{dn^{0}_{m}}{n_m} = \frac{d(V_1c^{0}_{m})}{V_1c_m} = \frac{dc_m}{c_m} \quad \text{and} \quad -d\alpha_k = \frac{dn^{0}_{k}}{n_k} = \frac{d(V_Oc^{0}_{k})}{V_Oc_k} = \frac{dc_k}{c_k} \quad (26)$$

Applying eqs 23−26 for a homogenous liposomal solution, a final algebraic differential equation for an outer *k*-species influxed can be obtained

$$\frac{d\alpha_k}{dt} = \frac{S_OP_k\prod_{j=0} K_{ij}}{K_\upsilon\upsilon_l}\left((1 - \alpha_k)\frac{1}{y^{l_k-1}f_{kO}^{*}(y)} - K_\upsilon\alpha_k\frac{1}{x^{l_k-1}f_{kI}^{*}(x)}\right) \quad (K_{k0} = 1) \quad (27)$$

and a similar one for an inner *m*-species effluxed

$$\frac{d\beta_m}{dt} = \frac{S_IP_m\prod_{j=0} K_{ij}}{\upsilon_l}\left((1 - \beta_m)\frac{1}{x^{l_m-1}f_{mI}^{*}(x)} - \frac{1}{K_\upsilon}\beta_m\frac{1}{y^{l_m-1}f_{mO}^{*}(y)}\right) \quad (K_{m0} = 1) \quad (28)$$

where $P_m = D_mK^{p}_{m}/d$ and $P_k = D_kK^{p}_{k}/d$ are permeability coefficients for neutral species *k* and *m* and υ_l is the volume of the internal liposome aqueous compartment. If we assume a simultaneous translocation of N_I species from the interior and N_O species from the exterior, we have $N_I + N_O$ first-order *x*- and *y*-dependent algebraic differential equations. By means of these equations, combined with two basic electroneutrality eqs 19 and 20, the calculations may be performed for an optional number, $N_I + N_O$, of neutral molecules permeating in either direction. Usually the starting conditions for each of species *k* and *m* are

$$\text{for} \quad t = 0 \Rightarrow \alpha_k = 0 \quad \text{and} \quad \beta_m = 0 \quad (29)$$

However, in such a vesicular system there are many types of processes that could affect the overall translocation rate of neutral species: protolithic acid−base reactions in both aqueous environments, diffusion of the species to/away from the membrane interface layer, the distribution of species between aqueous and membrane surface binding compartment, and traverse of molecules through the phospholipid membrane. The first three processes are assumed to be much more rapid in comparison to the fourth one. So the passive diffusion through the membrane represents the rate-determining step and justifies the equations derived above. The corrective h_{ij} terms can be easily neglected only in eq 27 because for any species the term $1 + K^{p}(V_m/K_\upsilon V_I)$ can be taken to be equal to 1 owing to relatively high values of K_υ, even when K^{p} is relatively high. But the same is not valid for the eq 28 where the simplification can be permitted only if the partition coefficients are low enough. The numerical solution of each set of differential equations, by which the vesicular system is described, has to be started with a set of α/β values: $\alpha_1 = 0$, ... $\beta_1 = 0$, ... After $x^{st} = x_0$ and $y^{st} = y_0$ are calculated from the eqs 19 and 20, the first set of values for α and β may be calculated. In any case, there may be one special case referring to the permeation of neutral Brønsted molecules through the bilayer. Namely, when the buffer capacities of both buffered aqueous vesicular compartments are high enough to keep both pH values ($-\log x$ and $-\log y$) constant during the permeation, eqs 27 and 28 can be solved analytically. For such a case, for example, eq 27 is now reduced to

$$\frac{d\alpha_k}{dt} = a - b\alpha_k \quad (30)$$

where $a = S_IP_k\prod K_{ij}/K_\upsilon\upsilon_l y^{l_k-1}f_{kO}^{*}(y)$ and $b = (S_IP_k\prod K_{ij}/K_\upsilon\upsilon_l)\cdot(x^{-l_k+1}f_{kI}^{*\,-1}(x) + K_\upsilon y^{-l_k+1}f_{kO}^{*\,-1})$

After the integration of the equation above

$$\int_0^{\alpha_k} = \frac{d\alpha_k}{a - b\alpha_k} = \int_0^{t} dt \quad (31)$$

one obtains

$$\alpha_k(t) = \frac{a}{b}(1 - e^{-bt}) \quad (32)$$

For $t \rightarrow \infty$, α_k^{eq} is obtained

$$\alpha_k^{eq} = \frac{a}{b} = \frac{f_{kI}^{*}(x)}{f_{kI}^{*}(x) + K_\upsilon\left(\frac{y}{x}\right)^{l_k-1}f_{kO}^{*}(y)} \quad (33)$$

An adequate derivation can also be performed starting from eq 28. The result of the kinetically derived expression above is

the same as that in ref 17, where the thermodynamic derivation has been performed.

Results

There is a wide range of liposomal systems possible where a number of neutral Brønsted acid/base species can permeate the bilayer simultaneously. The kinetics of such systems may be simulated usually resulting in a form of numerically calculated time-dependent functions/graphs like x (=pH_i), y (=pH_o), as well as in a series of permeation portions, α_k's and β_m's. Here, only a few of the simplest liposome solutions are presented in the form of diagrams, all of them consisting of families of curves.

Until now there are two ways known to trap particular agents into the liposomes, both of them theoretically described elsewhere.[17,19] The first way, known as a pH-gradient loading, takes advantage of relatively large pH difference between the acidic liposomal interior (lower pH) and less acidic exterior. Such a pH gradient established drives, for example, the neutral molecules of a base down the pH gradient, from less acidic to more acidic liposome aqueous compartment. If the buffer concentration in both solutions is high enough to ensure an insignificant drop of the pH gradient during the "pumping" of one or (simultaneously) more kinds of neutral base/acid species into the interior, the drug molecules, after crossing the lipid membrane, are protonated/deprotonated immediately, and a concentration gradient is reestablished constantly.[21] In such a case any of the α_i's can be calculated analytically by means of eq 32. In general, during the ΔpH-driven loading of the drug(s) the pH gradient changes. The kinetics of the permeation of only one species down the pH gradient is described by three equations written in Appendix A (including eq A4, which describes boundary conditions). Shown in Figure 1 is the time-dependent portion of the loaded drug (1A), and pH_i/pH_o changes during the loading (1B). The course of the particular curves and their mutual positions is straightforward to explain. The rate of both the drug loading (loading efficacy in 1A) as well as pH changes is found to be proportional to the permeability of the molecules. However, the thermodynamic equilibrium state does not depend on the kinetics. Thus, if the time taken for calculations is long enough "to allow" the complete saturation of also the most slowly loading liposome system (curve 1), both the loading efficacy as well as pH-gradient curves fall into the same straight line. Owing to the fact that in this case the simulated outer buffer concentration is 10 times higher than that of the drug, only a very slight acidification in the exterior is observed after a complete drug encapsulation (ca. 60%). In contrast to this, the interior buffer capacity is substantially reduced (almost two-thirds of the inner buffer is neutralized) resulting in a reduction of the inner acidity for little more than 0.6 pH units. The curves in Figure 2 describe the kinetics of the liposome systems closely related to those previously shown in Figure 1. The calculations are performed for different liposome concentrations (or outer-to-inner volume ratios, K_v). The loading efficiencies at higher K_v values are lower, but the concentrations of the drug uptaken, c_u, are higher ($c_u = K_v c_d \alpha$). Higher amounts of drug influxed reduce the inner buffer capacity more effectively, which implies higher increase/decrease of the inner/outer pH values at higher K_v values (Figure 2B). It can be easily deciphered from Figure 2A that, for this particular case, the saturation times within a relatively wide liposome concentration range are almost the same. For more dilute liposomal solutions (higher K_v values; curves 3 and 4) the saturation times are even slightly lower in comparison with those at lower K_v values.

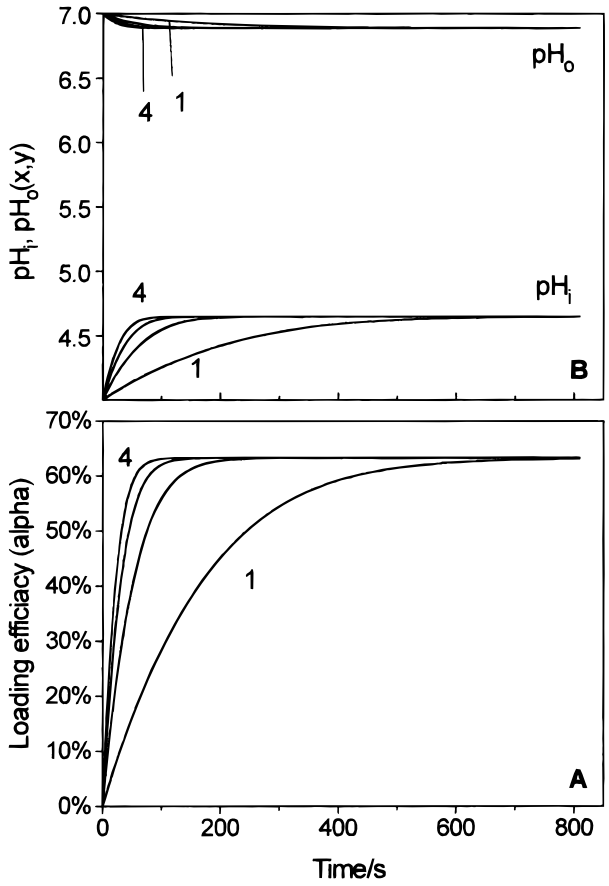

Figure 1. Effect of the permeability on the kinetics in the case of the pH-gradient loading. Curves are calculated on the basis of the following parameters: inner buffer (b_i), $c_{bi} = [HB_i] + [B_i^-] = 0.200$ M, $[HB_i]/[B_i^-] = 1{:}1$, $pK_{bi} = 4.00$; outer buffer (b_o), $c_{bo} = [HB_o] + [B_o^-] = 0.0100$ M, $[HB_o]/[B_o^-] = 1{:}1$, $pK_{bo} = 7.0$; drug (d), $c_d(DH^+Cl^-) = 1.00$ mM, $pK_d = 9.00$; $r = 1000$ Å; $d = 70$ Å; $K_v = 100$; $10^4 \cdot P_d$(cm s^{-1}) = (1) 1.00, (2) 3.25, (3) 5.50, (4) 7.75.

The second way to trap agents into liposomes, known as an ammonium sulfate or an exchange-gradient loading, drives the molecules across lipid bilayer on the basis of the concentration gradient of one or more neutral species.[11,12,17] The gradients force the species to cross the membrane in the opposite directions. After deprotonation of a particular protonated base and its translocation, the protons remaining in either liposome compartment reestablish the concentration gradients by protonation of translocated molecules. The uptake of two or more species is going on until the last concentration gradient disappears; i.e., the system is completely equilibrated. The kinetics of the most simple liposome system of this kind, driven by exchange gradient of only two (protonated) amines, i.e., the simultaneously permeation of two species in the opposite directions across the bilayer, is described by four equations written in Appendix B (including eq B5, which describes boundary conditions). The simulation performed on this system, and consequently its behavior, is represented in Figure 3A−C. It can be proved that for such a system, when loading is finished, the amount ratio of exchanged molecules is always close to 1 (to be published). Consequently, the course of the curves for portions of the influxed/effluxed drug is the same, but at any time the ratio $K_v c_o \alpha / c_i \beta$ is equal 1. For curve 1, the permeability of both the inner and outer neutral base is taken to be in the proportion equal to K_v. For each of the curves 2 and 3, the permeability of the influxed molecules is higher than that of curve 1. Namely, if the ratio of two permeabilities is high, the species with the lower permeability value almost completely

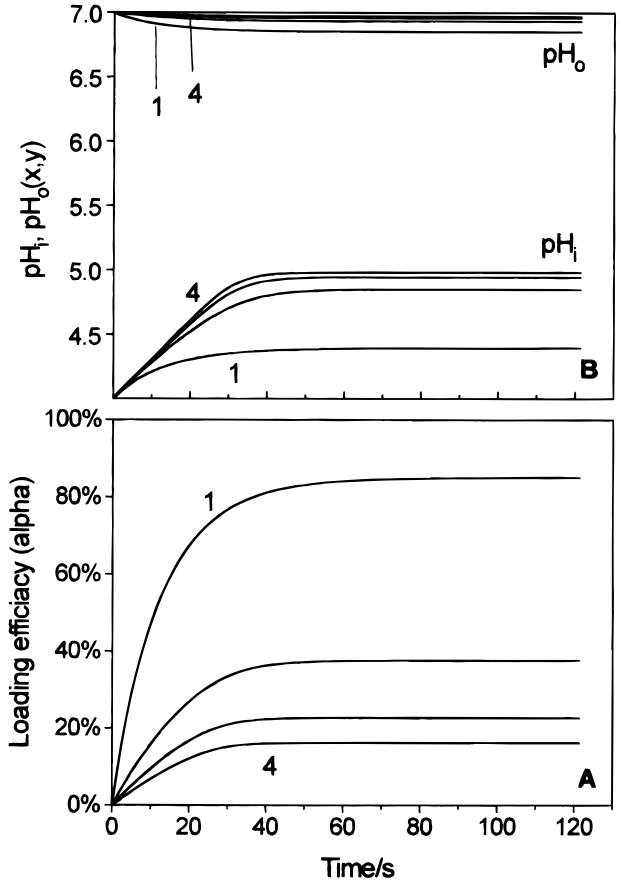

Figure 2. Effect of the drug concentration on the kinetics in case of the pH-gradient loading. The calculations are performed as follows: inner buffer (b_i), $c_{bi} = [HB_i] + [B_i^-] = 0.200$ M, $[HB_i]/[B_i^-] = 1{:}1$, $pK_{bi} = 4.00$; outer buffer (b_o), $c_{bo} = [HB_o] + [B_o^-] = 0.0100$ M, $[HB_o]/[B_o^-] = 1{:}1$, $pK_{bo} = 7.00$; drug (d), $c_d(DH^+Cl^-) = 1.00$ mM, $pK_d = 9.00$; $r = 1000$ Å; $d = 70.0$ Å; $P_d = 1.00 \times 10^{-3}$ cm s^{-1}; $K_\nu = (1)$ 50.0, (2) 200, (3) 350, (4) 500.

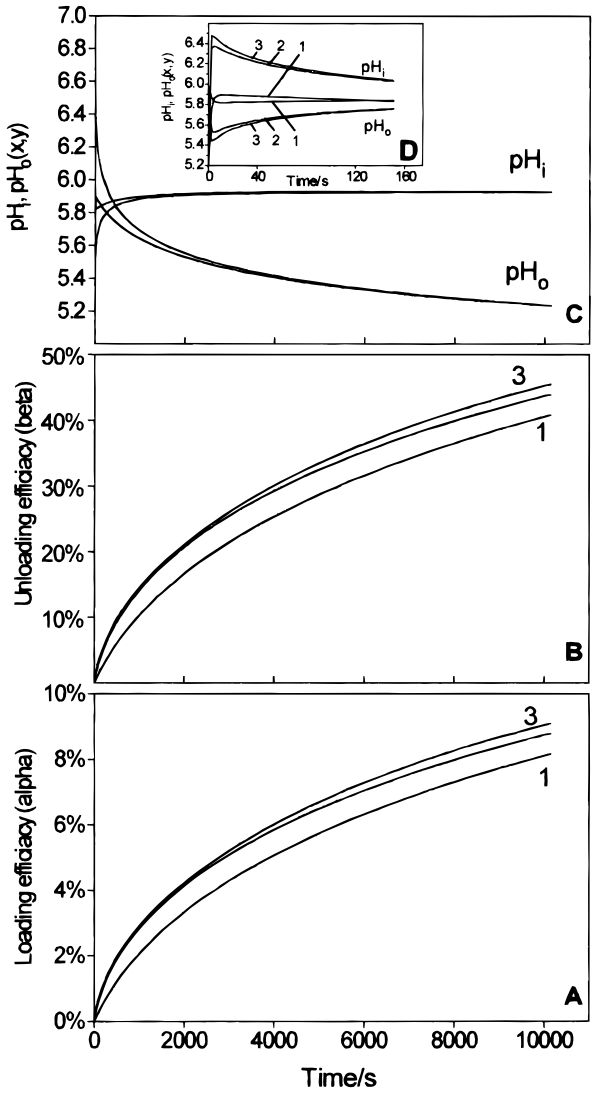

Figure 3. Effect of the permeability of the outer drug on the kinetics in case of the ammonium sulfate (exchange-gradient) loading. The parameters describing this systems are inner drug (i), $c_i = 0.100$ M, $pK_i = 10.0$; outer drug (o), $c_o = 0.010$ M, $pK_o = 10.0$; $r = 1000$ Å; $d = 70$ Å; $P_i = 5.00 \times 10^{-7}$ cm s^{-1}, $K_\nu = 50.0$, $10^7 \cdot P_o$ (cm s^{-1}) = (1) 1.00, (2) 3.00, (3) 5.00.

determines the kinetics of loading. This can be explained easily: if the amount of much more permeable molecules L traversed in the opposite compartment is much higher in comparison with that crossing the membrane in the opposite direction, M, the protonation of the molecules L greatly reduces the proton reservoir in the compartment of less permeable species, M. This causes an increase of the concentration of the neutral species L in the opposite compartment and, consequently, a rapid drop of its concentration gradient. Thus, the flow of molecules L is diminished. Owing to proton-reservoir reduction, the gradient of species M rises simultaneously, so the process is self-adjusting. There is another detail that deserves to be mentioned here. In the Figure 3C,D, a sudden drop/jump of the outer/inner pH is observed in the very first few seconds of the encapsulation and also the exchange ratio of permeated molecules in this region is higher than 1. Soon after the maxima/minima are reached, the exchange ratio drops down to the value 1 (not shown here). All these facts confirm the explanation given above.

Discussion

We present here the first general model that allows the calculation of the kinetics of liposome (lipid vesicle) loading. Applying Fick's first law for a rate-determining transmembrane movement of only neutral species and taking into account all the possible time-dependent equilibria states within all four compartments (within inner and outer Brønsted aqueous solution and partition of all species between either aqueous compartment and inner/outer membrane binding domain), a series of equations can be obtained, which, together with the boundary conditions, completely describe the time-dependent behavior of any particular liposome solution during loading. The first two (electroneutrality) nonlinear equations are solved by Newton−Raphson method. The values found here (x and y) are inserted simultaneously into the series of the differential equations. Keeping the time step small enough, and repeating the calculation several (ten) thousand times, the correct solution can be found within the whole time range taken. This kinetic model is complementary to the thermodynamic model,[17] and indeed, the calculated values for $t \rightarrow \infty$ are found to be the same.

The simulations performed with this kinetic model can help to get insight into the influence of different parameters on the time course of loading of different liposomal systems. The loading parameters can vary either within a wide range of values of particular variables or within their mutual proportions. Sometimes the results of such simulations can also help us to find a reasonable interpretation especially concerning particular

details (for example, the course of the curves in Figure 3C,D) in the calculations performed.

Certainly, there are also some weaknesses of this model that have to be mentioned. First there is a possible formation of an electrical double layer, which can generate a surface potential at the membrane interface that could change during the loading and thus affects the particular equilibrium constants as well as permeability of particular molecules. However, the surface potential can be reduced by the higher ionic strength. Second, in the case of very high permeabilities, the rates of restoration of particular equilibria can be comparable with those of translocation of molecules through the bilayer. Nevertheless, a mathematical and physicochemical construction of this model seems to be appropriate to take into account also the processes mentioned above.

Glossary

A_k = factor that "allows" the neutral species designated *kl* to load into the interior of the vesicles (=1) or not (=0)

B_m = factor that "allows" the species *ml* to release from the inner liposome domain (=1) or not (=0)

c_i = total internal/external aqueous concentration of all sister-species of the corresponding starting-point species *ik*

c_{kI}^0, c_{kO}^0, c_{mI}^0, c_{mO}^0 = inner/outer aqueous compartment concentration of neutral molecules designated *kl*/*ml* during the permeation

c_{kO}, c_{mI} = total starting-point concentration of the species *k*/*m* in both aqueous compartments before loading

c_l, c_l* = concentration of the "inert" species *l* (which does not react with water molecules) before/after (*) membrane binding

d = thickness of the liposome bilayer

D_i = diffusion coefficient of the species *i*

f_i*([H$^+$]$_a$) = [H$^+$]$_a$-dependent concentration function

f_{kI}*, f_{kO}*, f_{mI}*, f_{mO}* = concentration function of the species *k*/*m* during loading/unloading

g_i*([H$^+$]$_a$) = [H$^+$]$_a$-dependent charge function

g_{kI}*, g_{kO}*, g_{mI}*, g_{mO}* = charge function of the species *k*/*m* in the interior/exterior during loading/release

h_{ij}, h_H, h_l = corrective term for the Brønsted species *ij*, H$^+$, and "inert" species *l*

[H$^+$]$_a$ = H$^+$ concentration in a corresponding aqueous compartment

j_i = molar flux of the neutral species *i*

K_{ij}, K_{ik} = acidity constant connecting the species *i* and *j*/*k*

K_{ij}^p = partition coefficient of the species *ij* between the membrane surface binding domain and the aqueous compartment

K_k^p, K_m^p, K_l^p = partition coefficient of the neutral species *k*, *m*, and "inert" species *l*

K_υ = outer-to-inner aqueous compartment volume ratio

$k_\upsilon = K_\upsilon^{-1}$

K_w = autoionization constant of water

l_k, l_m = running number of the neutral species *k*/*m* in a given ordered series (e.g., in the series NH_4^+/NH_3, NH_3 is in the second place; therefore for this two-member series, $l_k = 2$)

n_{ij} = amount of the Brønsted species *ij*

N_i = number of Brønsted sister-species in a particular sister-species series

P_i = permeability of the species *i*

Q_{ij}, Q_l = charge of the Brønsted species *i*, *j*, and "inert" species *l*

r = inner liposome radius

S_I, S_O = inner/outer bilayer surface

υ_l = vesicle volume

V_a, V_m = aqueous compartment volume and membrane surface binding domain volume

V_I, V_O = inner/outer aqueous compartment volume of the liposome system

u_I*, u_O* = collective charge of the "inert" species and H$^+$/OH$^-$ ions in the inner/outer compartment

x, y = [H$^+$]$_a$ in the inner/outer compartment

x^{st}, y^{st} = initial [H$^+$]$_a$ in the inner/outer aqueous compartment before loading

z_{ij}, z_l = charge of the Brønsted species *ij* and "inert" species *l*

α_k, β_m = percentage of the entrapped/released species *k*/*m*

Appendix A

The simulation of time-dependent unidirectional (pH gradient) loading of one species into the liposomes:

(1) inner solution: inner buffer M$^+$B$_i^-$/HB$_i$, with concentration c_{bi} and concentration ratio [B$_i^-$]/[HB$_i$] 1:1, and K_a(HB$_i$) = K_{bi};

(2) outer solution: protonated amine (drug), DH$^+$X$^-$, with concentration c_d, and K_a(DH$^+$) = K_d; outer buffer M$^+$B$_o^-$/HB$_o$, with concentration c_{bo} and concentration ratio [B$_o^-$]/[HB$_o$] = 1:1, and K_a(HB$_o$) = K_{bo};

(3) vesicles with bilayer of thickness d and inner vesicles radius r; $K_\upsilon = V_O/V_I$; P_d is permeability of neutral molecules B; α is portion of influxed neutral drug's species, B$_d$, respectively. The corresponding corrective h_{ij} terms are taken to be negligible. The vesicles are taken to be homogenous and spherical-symmetrical. All three functions, x, y, and α, are time-dependent. The appropriate equations are

$$K_\upsilon c_d \alpha \frac{1}{1 + K_d/x} + c_{bi} \frac{-K_{bi}/x}{1 + K_{bi}/x} + \frac{1}{2} c_{bi} + \left(x - \frac{K_w}{x}\right) = 0 \quad \text{(A1)}$$

$$c_d(1 - \alpha) \frac{1}{1 + K_d/y} + c_{bo} \frac{-K_{bo}/y}{1 + K_{bo}/y} + \left(\frac{1}{2} c_{bo} - c_d\right) + \left(y - \frac{K_w}{y}\right) = 0 \quad \text{(A2)}$$

$$\frac{d\alpha}{dt} = \frac{3}{r}\left(1 + \frac{2d}{r} + \frac{d^2}{r^2}\right) P_d K_d \left(\frac{1}{K_\upsilon}(1 - \alpha)\frac{1}{K_d - y} - \alpha \frac{1}{K_d + x}\right) \quad \text{(A3)}$$

$$\text{for} \quad t = 0 \Rightarrow \alpha = 0 \quad \text{(A4)}$$

$$\frac{3}{r} = \frac{S_I}{\upsilon_l} = \frac{4\pi r^2}{4\pi r^3/3} \quad \text{and}$$

$$\frac{3}{r}\left(1 + \frac{2d}{r} + \frac{d^2}{r^2}\right) = \frac{S_O}{K_\upsilon \upsilon_l} = \frac{4\pi(r + d)^2}{4\pi r^3/3} \quad \text{(A5)}$$

Appendix B

The simulation of time-dependent bidirectional exchange of two species:

(1) inner solution: protonated amine, D$_i$H$^+$X$^-$, with concentration c_i, and K_a(D$_i$H$^+$) = K_i;

(2) outer solution: protonated amine, D$_o$H$^+$X$^-$, with concentration c_o, and K_a(D$_o$H$^+$) = K_o;

(3) vesicles with bilayer of thickness d and inner vesicles radius r; $K_\upsilon = V_O/V_I$, P_i and P_o are permeabilities of neutral molecules B$_i$ and B$_o$; α and β are (time-dependent) portions of influxed and effluxed neutral species, B$_o$ and B$_i$, respectively.

The corresponding corrective h_{ij} terms are taken to be negligible. All four functions, x, y, α, and β, are time-dependent. The corresponding equations are

$$c_i(1-\beta)\frac{1}{1+K_i/x}+K_v c_o \alpha \frac{1}{1+K_o/x}-c_i+\left(x-\frac{K_w}{x}\right)=0 \tag{B1}$$

$$c_o(1-\alpha)\frac{1}{1+K_o/y}+\frac{1}{K_v}c_i\beta\frac{1}{1+K_i/y}-c_o+\left(y-\frac{K_w}{y}\right)=0 \tag{B2}$$

$$\frac{d\beta}{dt}=\frac{3}{r}P_i K_i\left((1-\beta)\frac{1}{K_i+x}-\frac{1}{K_v}\beta\frac{1}{K_i+y}\right) \tag{B3}$$

$$\frac{d\alpha}{dt}=\frac{3}{r}\left(1+\frac{2d}{r}+\frac{d^2}{r^2}\right)P_o K_o\left(\frac{1}{K_v}(1-\alpha)\frac{1}{K_o+y}-\alpha\frac{1}{K_o+x}\right) \tag{B4}$$

$$\text{for}\quad t=0 \Rightarrow \alpha=0 \quad \text{and} \quad \beta=0 \tag{B5}$$

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