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A theoretical approach to describe monolayer-liposome lipid interaction

Jordi Hernandez-Borrell [a], Francesc Mas [b] and Jaume Puy [c]

[a] *Unit of Physical Chemistry, Facultat de Farmàcia, Universitat de Barcelona, Av. Diagonal, s/n, 08028 Barcelona,* [b] *Department of Physical Chemistry, Facultat de Química, Universitat de Barcelona, C/ Martí i Franquès, 1, 08028 Barcelona and* [c] *Chemistry Section, Escola Tècnica Superior d'Enginyeries Agràries, Universitat Politècnica de Catalunya, Av. Rovira Roura, 42, 25007 LLeida, Catalonia, Spain*

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It is known from several studies on the interaction between membrane models that mechanisms such as fusion or lipid exchange can play an important role in the process of internalization by cells of lipid vesicles and also in the physical stability of liposomes. In this paper it is shown that a simple monolayer-liposome model can be used to simulate experimentally observed interactions between lipid vesicles and cell surfaces. From experimental data, a simple theoretical model is formulated to interpret the variation with time of surface pressure as a function of liposome concentration. The congruency of the physico-chemical hypothesis and its validity are studied and correlated with results from experimental systems.

1. Introduction

The concept of liposomes as a drug delivery system is well established. Several studies have shown their potential in therapeutical applications, especially in the area of cancer chemotherapy where immunological selectivity can provide specific recognition between liposomes and target cells [1]. Other mechanisms such as fusion, lipid transfer and endocytosis [2] are also involved in vesicle-cell interactions. As a consequence of such events, the permeability of liposomes and their physical stability can be seriously compromised, thus affecting the retention of entrapped substances. In order to provide a better framework for understanding the mechanisms involved in liposome-monolayer interactions, a variety of physico-chemical techniques [3] have been employed. Monolayer systems have been used to investigate the surface properties of biomembranes [4] and liposomes [5,6]. More specifically, lipid-protein [7,8] and lipid-lipid interactions [4,6] have been investigated using monolayer systems. These provide a stable interface with a composition similar to that of natural membranes.

Some reports have proposed the monolayer-bilayer system as a biophysical model for the investigation of lipid exchange and fusion between membranes [9]. Using the monolayer-bilayer system, modifications in lipid packing at the interface can be followed in a very easily monitored experimental procedure by recording the variations in surface pressure after the injection of liposomes into the aqueous subphase beneath a previously spread monolayer. Using this approach, a kinetic model for describing monolayer-bilayer interactions has been proposed [10] and a more recently proposed thermodynamic model [9] has been supported by experimental results.

This technique also offers the possibility of

Correspondence address: J. Hernandez-Borrell, Unit of Physical Chemistry, Facultat de Farmàcia, Universitat de Barcelona, Av. Diagonal, s/n, 08028 Barcelona, Catalonia, Spain.

studying the influence of chemical agents on the mechanisms involved in liposome-monolayer interactions. Specifically, calcium and other divalent cations can induce fusion of liposomes when acidic phospholipids are present in the bilayers [11–16]. It has also been demonstrated that zwitterionic phospholipids can interact with monovalent [17] and divalent cations [18–20].

In this paper a new theoretical model is proposed and its congruency tested using a liposome-monolayer system. The model is then applied to investigation of the differences between the interactions of systems where Ca^{2+} is present in the bulk phase.

2. Materials and methods

2.1. Film constituents

L-α-Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Chol) specified as 99% pure were purchased from Sigma (St. Louis, MO) and used without further purification.

2.2. Solvents

Deionized water was distilled from sodium permanganate in an all-glass apparatus and purified by reverse osmosis on a Milli-Q-System (Millipore, U.S.A.). The aqueous bulk phase was composed of Tris buffer(pH 7.4). In experiments with Ca^{2+}, $CaCl_2$ at various concentrations (5, 10, 20, 40 and 60 mM) was added to the buffer.

Organic solvents (chloroform, ethanol), obtained from Merck, were distilled twice before use to avoid the presence of surface-active impurities.

2.3. Liposome preparation

Chloroform/methanol (1 : 1, v/v) solutions containing DPPC were dried in a rotary evaporator and the lipid film remaining on the walls lyophilized overnight before redispersion in buffer. After vigorous shaking, the milky solution was sonicated under N_2 for 25 min in a Labsonic 1510 B Braun sonicator at 60 kW, with the flask being immersed in cool water. Undispersed material and macroscopic aggregates were removed by centrifugation at $100000 \times g$ for 30 min, followed by chromatography on Sepharose 4B.

Eluted fractions on the descending portion of the included volume peak were pooled and characterized by negative staining and electron microscopy (Philips EM-300 at 80 kV) [21], confirmation being obtained by light scattering measurements (Fica 50) [22]. Small unilamellar vesicles (SUVs) were found to have a size of 60 $\pm$0.6 nm. Lipid concentration was determined by the method of Stewart [23].

2.4. Experimental procedure

The buffer support solution was poured into a special trough drilled in a Teflon block of 124 cm^2 surface area and 200 ml capacity. Film constituents dissolved in chloroform (10^{-3} M DPPC/Chol, 70 : 30 molar ratio) were spread over the subphase with the aid of a microsyringe (model CR-700-200, Hamilton, Reno, NE) and, subsequent to evaporation of solvent and stabilization of the lipid monolayer (3–5 min), several volumes of liposome solution (20 mM) were injected into the aqueous subphase beneath the lipid film.

Determination of the initial surface pressure of films (5, 10, 20 or 30 mN m^{-1}) and recording of the kinetics after liposome addition were made possible by use of a previously described surface barostat [24]. Briefly, interfacial tensions were evaluated by the Wilhelmy method using an electronic microbalance (Sartorius A-120-S) coupled to a chart recorder to give a continuous reading of force on the dipping plate. Throughout all assays, agitation of the subphase was maintained by two magnetic Teflon stirring bars spinning at 250 rpm.

3. Experimental results

The variation in surface pressure with time under different experimental conditions was recorded. Figs. 1–3 show plots of the surface pressure change in the presence of differing concentrations of liposomes for systems with various values of the initial surface pressure (π_0). Fig. 4 shows an

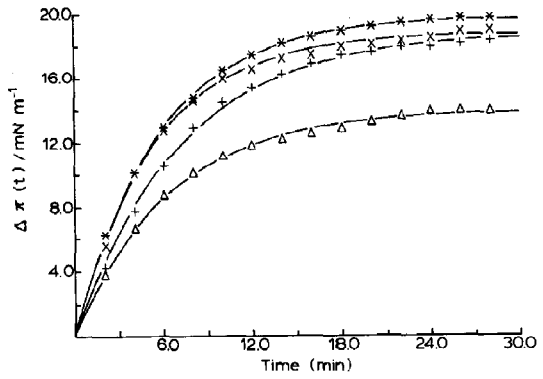

Fig. 1. Plots of surface pressure ($\Delta\pi(t) \equiv \pi(t) - \pi_0$) vs. time, with $\pi_0 = 5$ mN m^{-1}, for various volumes of liposome solution (20 mM) injected into the subphase at the initial time point ($t = 0$). V: (Δ) 0.25, (+) 0.50, (×) 0.75 and (*) 1.00 ml ($CaCl_2$ absent). Continuous lines correspond to the graphical representation of the general equation (eq. 18) with the fitted values summarized in table 1.

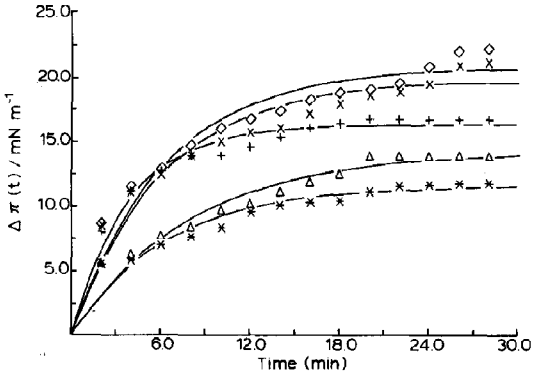

Fig. 4. As in fig. 1, in the presence of $CaCl_2$ in solution, with $\pi_0 = 10$ mN m^{-1} and 0.5 ml liposome solution (20 mM) injected into the subphase at zero time, for different concentrations of $CaCl_2$: (*) 5, (Δ) 10, (+) 20, (×) 40 and (◇) 60 mM. Continuous lines correspond to graphical representation of the general equation (eq. 18) with the fitted values summarized in table 2.

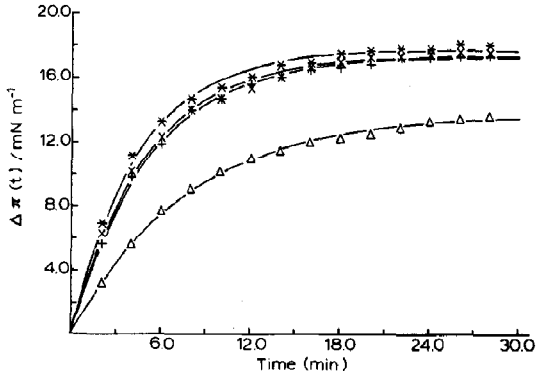

Fig. 2. As in fig. 1, with $\pi_0 = 10$ mN m^{-1}.

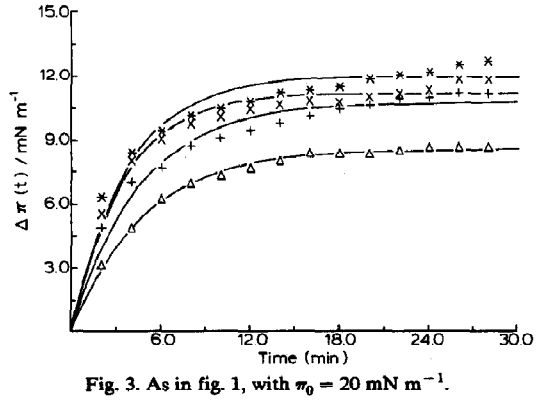

Fig. 3. As in fig. 1, with $\pi_0 = 20$ mN m^{-1}.

example of the results from similar systems to which various concentrations of Ca^{2+} were added.

On inspection of figs. 1–4, one can observe a qualitative difference in behaviour between the systems according to whether or not Ca^{2+} was present, since in its presence, an inflection point exists that does not occur when Ca^{2+} is omitted. In both cases, a constant value of the relative surface pressure is found at later time points.

In contrast, figs. 1–3 show a correlation between the amount of liposomes injected at the initial time point and the relative values of the surface pressure ($\Delta\pi \equiv \pi - \pi_0$). Furthermore, in the case of equal amounts of liposomes injected, the value of the initial surface pressure is correlated with that of the relative surface pressure when Ca^{2+} is present. These observations indicate that a correlation may exist between the initial surface pressure, initial concentration of liposomes injected, Ca^{2+} concentration (if present), and the relative surface pressure.

In this paper, a mathematical model is formulated in order to describe quantitatively the above-stated correlation, together with its inadequacies when applied to systems where Ca^{2+} is present and possible extensions.

4. Theoretical treatment

In order to formulate a mathematical model to describe the experimental results, it is necessary to put forward several physico-chemical hypotheses to explain the following phenomena.

4.1. Mass transport

In the experiments, predetermined amounts of liposomes are injected into the bulk phase which is stirred during the procedure. Thus, it can be presumed that the lipid concentration in the bulk phase (constituted by liposomes) is homogeneous:

$$c_L(x,t) = c_L(x=0,t) = c_L^* \quad \forall t \in (0,\infty) \tag{1}$$

where $x = 0$ represents the limit surface of the bulk phase around the monolayer. It can also be assumed that c_L^* is independent of time because the amount of lipids exchanged between the monolayer and bulk phase represents only a small proportion of the total injected in the form of liposomes.

4.2. Exchange of lipids at the interphase

It is assumed that the exchange process between lipids in the monolayer and those in the bulk phase:

$$c_L(x=0,t) \underset{k_b}{\overset{k_f}{\Leftrightarrow}} \Gamma(t) \tag{2}$$

can be described by the following differential equation [25,26]:

$$\frac{d\Gamma(t)}{dt} = k_f c_L^* \Gamma_m [1 - \Theta(t)] - k_b \Gamma(t) \tag{3}$$

where $\Gamma(t)$ and $\Theta(t)$ (defined as $\Gamma(t)/\Gamma_m$) denote the surface concentration of lipids and coverage of the monolayer, respectively. Γ_m represents the maximum surface concentration of lipids and is inversely proportional to the molecular area, σ, occupied by a lipid molecule in the monolayer:

$$\Gamma_m = \frac{1}{\sigma N_A} \tag{4}$$

where N_A is Avogadro's number.

4.3. State equation of the monolayer

The monolayer can be studied thermodynamically by applying the Gibbs model for interphases [25,27]. For ideal, dilute solutions the variation in surface tension with solute concentration is linear in behaviour, thus allowing integration of the Gibbs adsorption isotherm, for a binary system, and derivation of the state equation [27]:

$$\pi \equiv \gamma_0 - \gamma = \Gamma RT \tag{5}$$

where π is the surface pressure of the monolayer. Eq. 5 shows that in dilute solutions the film of adsorbed solute obeys the equation of state for a two-dimensional ideal gas.

Since it has been assumed that the reorganization of lipids in the monolayer occurs much more rapidly than the exchange process, the state equation, eq. 5, remains valid at all time points:

$$\pi(t) = \Gamma(t) RT \quad \forall t \in (0,\infty) \tag{6}$$

5. Mathematical solution

In experiments, as a response function of the system, we record the variation in surface pressure with time, $\pi(t)$. From the theoretical treatment described above, we can obtain $\pi(t)$ through solving the system of differential equations (eqs. 3 and 6) with the initial conditions

$$\left.\begin{aligned} \pi(t=0) &= \pi_0 \\ \Gamma(t=0) &= \Gamma_0 \end{aligned}\right\} \tag{7}$$

This is detailed in the appendix and, if an isothermal process (T = constant) is considered, the solution obtained is:

$$\Delta\pi(t) \equiv \pi(t) - \pi_0 = \delta(1 - e^{-\alpha t}) \tag{8}$$

where π_0 designates the initial surface pressure of the monolayer before injection of liposomes (initial thermodynamic equilibrium for the monolayer is assumed). Eq. 8 comprises two variables, α and δ, defined by (eq. A6):

$$\left.\begin{aligned} \alpha &= k_b + k_f c_L^* \\ \delta + \pi_0 &= \frac{k_f c_L^*}{k_b + k_f c_L^*} \Gamma_m RT \end{aligned}\right\} \tag{9}$$

6. Results and discussion

In this section, we deal with analyses aimed at ascertaining whether eq. 8 can reproduce the experimental results in order to verify the physico-chemical hypothesis considered. This can be performed by minimizing the sum of squares of the differences between experimental data, and theoretical results obtained from eq. 8 (nonlinear regression fit). To do so, a modified Gauss-Newton algorithm for the determination of an unconstrained minimum of a sum of squares of a set of nonlinear functions has been used [28], obtaining as a final result the best parameters of eq. 8. Two cases are considered below.

6.1. Absence of Ca^{2+} in solution

The continuous lines in figs. 1–3 correspond to a graphical representation of the general equation (eq. 8) with the fitted values summarized in table 1. These figures demonstrate a good agreement between experimental values and fitted curves, which is also evident in table 1 from inspection of the values of the minimized sum of squares.

The question arising from the fitted values of the parameters of the model concerns whether the physico-chemical parameters of the experimental system can be obtained. To answer this question the congruency of the model must be analyzed. In order to do so, we can operate with the definitions of both mathematical parameters, α and δ, to establish the relationship between them. From eqs. 9, defining the parameters α and δ, the following relationship is obtained:

$$\delta + \pi_0 = \Gamma_m RT - k_b \Gamma_m RT \frac{1}{\alpha} \tag{10}$$

A linear regression test [29] performed between $\delta + \pi_0$ and $1/\alpha$ (fig. 5) yields the following values:

$$\left.\begin{aligned} &\text{intercept at the origin} = (39.0 \pm 3.0)\ \text{mN m}^{-1} \\ &\text{slope} = -(2.3 \pm 0.5)\ \text{mN m}^{-1}\ \text{min}^{-1} \\ &\text{correlation coefficient} = -0.8037 \end{aligned}\right\} \tag{11}$$

These values indicate agreement between the model and the experimental results. Moreover, only two physico-chemical parameters of the model, Γ_m and k_b, can be obtained from eq. 10:

$$\left.\begin{aligned} k_b &= (5.9 \pm 1.4) \times 10^{-2}\ \text{min}^{-1} \\ \Gamma_m &= (1.6 \pm 0.1) \times 10^{-5}\ \text{mol m}^{-2} \end{aligned}\right\} \tag{12}$$

when the temperature is kept constant at 25 °C:

According to the relationship between Γ_m and σ (eq. 4), the molecular area of a lipid in the monolayer would amount to $\sigma = (10.5 \pm 0.6)$ Å^2. This fitted value for σ is quantitatively lower than that determined experimentally, which lies within

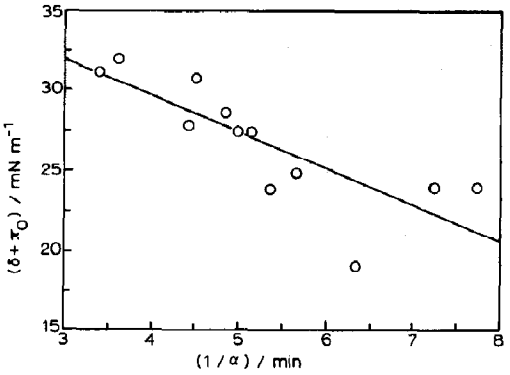

Fig. 5. Plot of linear regression fit of $(\delta + \pi_0)$ vs. $1/\alpha$ from table 1 (in the absence of $CaCl_2$).

Table 1

Fitted parameters of eq. 18 (in the absence of Ca^{2+})

π_0 (mN m^{-1})	V [a] (ml)	α (min^{-1})	δ (mN m^{-1})	Sum of squares
5	0.25	0.1574	14.03	0.4399
	0.50	0.1381	18.92	0.7293
	0.75	0.1865	18.84	0.9216
	1.00	0.1767	19.82	0.3159
10	0.25	0.1294	13.90	0.3405
	0.50	0.1937	17.45	0.5062
	0.75	0.2001	17.53	1.3859
	1.00	0.2247	17.85	2.1110
20	0.25	0.2059	8.659	0.2694
	0.50	0.2218	10.82	0.3449
	0.75	0.2926	11.23	2.7420
	1.00	0.2750	12.01	6.4340

[a] Volume of liposome solution (20 mM) injected into subphase.

the range 30–40 Å^2 [30], but is of the same order of magnitude. This could be due to the simplifications employed for the model, and to the fact that, initially, the monolayer is a mixture of DPPC and Chol, which could reduce this value. Therefore, this value only indicates that the model used is congruent with several experiments performed, but does not allow prediction of the precise experimental value of σ.

At this point, only k_f remains undetermined. From eq. 9:

$$\frac{\delta + \pi_0}{\Gamma_m RT} = \frac{k_f c_L^*}{k_b + k_f c_L^*} \tag{13}$$

The value of $\delta + \pi_0$, from eqs. 6 and 8, has the following meaning:

$$\delta + \pi_0 = \lim_{t \to \infty} \pi(t) = \lim_{t \to \infty} \Gamma(t) RT \tag{14}$$

Eq. 14 then becomes:

$$\frac{\delta + \pi_0}{\Gamma_m RT} = \lim_{t \to \infty} \frac{\Gamma(t)}{\Gamma_m} \equiv \theta_\infty = \frac{k_f c_L^*}{k_b + k_f c_L^*} \tag{15}$$

or, if the term $k_f c_L^*$ is isolated:

$$K c_L^* = \frac{\theta_\infty}{1 - \theta_\infty} \tag{16}$$

This equation resembles the Langmuir adsorption isotherm for lipids [25,27], with $K \equiv k_f / k_b$ as the adsorption coefficient. This Langmuir-type behaviour arises from the assumption of maximum coverage in the kinetic mechanism (eq. 2) of the model.

c_L^* can be related to the volume of liposomes injected in every experiment and the initial concentration of lipids present in the subphase according to the existence of an initial surface pressure π_0. Thus, c_L^* can be written as the contribution of two terms:

$$c_L^* = c_{L,inj} + c_{L,0} \tag{17}$$

where $c_{L,inj}$ denotes the concentration of lipids injected at the initial time point (proportional to V_{inj}) and $c_{L,0}$ the initial concentration of lipids in equilibrium with the monolayer (proportional to π_0). Therefore:

$$k_f c_L^* = k_f c_{lip} \frac{V_{inj}}{V_s} + \beta \pi_0 \tag{18}$$

where V_s represents the volume of the solution in the experimental vessel and c_{lip} the concentration of liposomes injected. Thus, eq. 15 becomes:

$$\delta + \pi_0 = \Gamma_m RT \left\{ \frac{\alpha V_{inj} + \beta \pi_0}{k_b + \alpha V_{inj} + \beta \pi_0} \right\} \tag{19}$$

where $\alpha \equiv k_f c_{lip} / V_s$ and β are the parameters needing to be determined from the experimental values. A nonlinear regression test [31] between the variables δ, π_0 and V_{inj} (summarized in table 1), employing the values of Γ_m and k_b obtained above (eq. 13), yields the following values for α and β:

$$\left.\begin{aligned} &\alpha = (7.4 \pm 1.0) \times 10^{-2} \text{ min}^{-1} \text{ ml}^{-1} \\ &\beta = (8.6 \pm 0.7) \times 10^{-3} \text{ min}^{-1} \text{ mN}^{-1} \text{ m} \\ &r^2 = 0.9452 \end{aligned}\right\} \tag{20}$$

This nonlinear regression test results in a good agreement and permits the evaluation of k_f. From the definition of parameter $\alpha \equiv k_f c_{lip} / V_s$, with $c_{lip} = 20$ mM and $V_s = 180$ ml, we obtain:

$$k_f = (6.3 \pm 0.9) \times 10^{-2} \text{ M}^{-1} \text{ min}^{-1} \tag{21}$$

In summary, the proposed model yields theoretical values that are in close agreement with the experimental data, leading, as $t \to \infty$, to behaviour similar to that of Langmuir equilibrium adsorption for lipids at the water-air interphase, with the following adsorption coefficient at $T = 25\,^\circ$C:

$$K \equiv \frac{k_f}{k_b} = (1.1 \pm 0.3) \times 10^4 \text{ M}^{-1} \tag{22}$$

The above value is characteristic for cases where extensive adsorption occurs, in agreement with expectation, i.e., the strong tendency of lipids to pass from the aqueous solution to monolayer.

6.2. *Presence of Ca^{2+} in solution*

On addition of Ca^{2+} to the bulk phase (fig. 4), an increase in surface pressure is observed. Fig. 4 also depicts in graphical form the general equation, eq. 8, with the fitted values listed in table 2. The values of the minimized sum of squares in table 2 are indicative of poor quantitative agree-

Table 2

Fitted parameters of eq. 18 (in the presence of Ca^{2+}) [a]

π_0 (mN m^{-1})	$[Ca^{2+}]$ (mM)	α (min^{-1})	δ (mN m^{-1})	Sum of squares
5	5	0.2301	16.76	10.76
	10	0.2671	19.51	13.36
	20	0.2371	22.72	16.76
	40	0.2756	27.03	22.42
	60	0.2463	28.24	36.02
10	5	0.1555	11.69	8.459
	10	0.1225	14.42	9.715
	20	0.2601	16.42	6.398
	40	0.1620	19.90	27.63
	60	0.1578	21.02	25.96
20	5	0.4224	7.583	7.982
	10	0.4237	7.787	7.416
	20	0.2894	8.683	6.483
	40	0.2580	9.801	5.765
	60	0.2249	10.62	8.974
30	5	0.04138	3.155	0.6243
	10	0.07821	3.476	0.5612
	20	0.07331	7.226	1.635
	40	0.07139	7.722	2.367
	60	0.1002	7.243	2.159

[a] 0.5 ml liposome solution (20 mM) injected into subphase at the initial time point.

ment between the experimental values and fitted curves. Furthermore, the shape of the experimental curves demonstrates poor qualitative agreement.

The above-described observations show that the general equation, eq. 8, is inapplicable for describing experiments in the presence of Ca^{2+}; nevertheless, it is possible that the model provides a qualitative test of the role played by Ca^{2+} in solution during the process of exchange of lipids between liposomes and the monolayer.

As can be seen in table 2 for each value of the initial pressure π_0, the value of the corresponding fitted parameter δ increases as $[Ca^{2+}]$ increases, indicating that the lipid content in the monolayer, on thermodynamic equilibrium being attained, is directly proportional to parameter δ (eqs. 6 and 10), increasing with rise in $[Ca^{2+}]$. Therefore, it appears that Ca^{2+} exerts its effect by perturbing the equilibrium of lipid exchange between liposomes and the monolayer, thereby facilitating the passage of lipids from liposomes in the subphase to the monolayer.

In experimental studies of the interaction between Ca^{2+} and DPPC membranes [19,20], lateral compression of the lipid bilayer by bound Ca^{2+} was suggested to occur and the proposal was put forward of long-range attraction between bound Ca^{2+} and the head groups of the surrounding lipid molecules. This could explain the increase in surface pressure with $[Ca^{2+}]$. Hence, we consider the present model to be suitable for extension to the case where Ca^{2+} is present in solution, provided an appropriate, new state equation is derived to replace the expression based on conditions of ideality (eq. 5), where the interaction between lipid molecules in the monolayer plays an important role. Work aimed in this direction is underway in our laboratory, and it is hoped that a simple non-ideal state equation for the monolayer can explain this effect.

7. Concluding remarks

A simple physico-chemical model to describe the lipid exchange between liposomes and monolayer has been proposed. This model allows us to study the congruency of the hypothesis used, especially the kinetic mechanism of exchange of lipids and the surface state equation of the monolayer. Thus, the kinetic mechanism (eq. 2) with the maximum surface concentration of lipids in the monolayer and the ideal state equation (eq. 5), derived in order to build the mathematical model are in close agreement with the experimental results, for the situation where no Ca^{2+} is present in solution. Moreover, the physico-chemical parameters of the model can be obtained from the experimental values.

When Ca^{2+} is added to the bulk phase, an increase in surface pressure is observed. If the model that is valid for the case where $CaCl_2$ is absent in the subphase is applied in this situation, poor qualitative and quantitative agreement is obtained, possibly due to the inappropriate use of the ideal state equation for the monolayer.

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Appendix

For an isothermal process, if the variation with time of the superficial pressure is taken into account solely, the system of differential equations (eqs. 3 and 6) becomes:

$$\frac{d\pi(t)}{dt} + \{k_b + k_f c_L^*\}\pi(t) = k_f c_L^* \Gamma_m RT \quad \text{(A1)}$$

with the following initial condition:

$$\pi(t=0) = \pi_0 \quad \text{(A2)}$$

The most general solution of the inhomogeneous differential equation, eq. A1, is:

$$\pi(t) = A \exp\{-(k_b + k_f c_L^*)\} + \frac{k_f c_L^*}{k_b + k_f c_L^*}\Gamma_m RT \quad \text{(A3)}$$

where A is obtained from the initial condition (eq. A2):

$$A = \pi_0 - \frac{k_f c_L^*}{k_b + k_f c_L^*}\Gamma_m RT \quad \text{(A4)}$$

The general solution, eq. A3, can then be expressed as:

$$\Delta\pi(t) \equiv \pi(t) - \pi_0 = \delta(1 - e^{-\alpha t}) \quad \text{(A5)}$$

with the following definitions of the parameters:

$$\left.\begin{aligned} \alpha &= k_b + k_f c_L^* \\ \delta &= \frac{k_f c_L^*}{k_b + k_f c_L^*}\Gamma_m RT \end{aligned}\right\} \quad \text{(A6)}$$

where one can observe that a dimensional analysis yields the correct dimensions for the parameters:

$$[\alpha] = [k_b] = [k_f c_L^*] = T^{-1}\ (\text{min}^{-1})$$

$$[\delta] = [\pi_0] = MLT^{-2}\ (\text{N m}^{-1})$$

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