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QUANTITATION OF THE FÖRSTER ENERGY TRANSFER FOR TWO-DIMENSIONAL SYSTEMS. I. LATERAL PHASE SEPARATION IN UNILAMELLAR VESICLES FORMED BY BINARY PHOSPHOLIPID MIXTURES

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An analytical solution is presented for the rate of energy transfer in unilamellar vesicles formed by binary mixtures of phospholipids showing lateral phase separation. The analytical approach developed here is mainly based on geometrical considerations and, therefore, is formally different for lateral phase separation phenomena taking place in the gel and in the liquid crystalline states of the lipid system. The rate of energy transfer among donor and acceptor molecules attached to chemically different phospholipids is mathematically correlated to the average cluster size of the less-rich component of the binary mixture, thus allowing its calculation from experimental measurements. Moreover, the equations derived here permit the calculation of the average cluster size as a function of the concentration of each lipid component within certain ranges, and this can be used to improve our knowledge of the thermodynamics of these processes.

1. Introduction

Förster energy transfer is being increasingly used as a powerful approach to solve biological problems (see Stryer [1] for a recent review). In particular, it may be very useful to solve problems of membrane processes or phenomena altering the average distance among donor and acceptor fluorophores attached to different membrane components. Membrane fusion, lateral phase separation of phospholipids, capping phenomena and changes in the aggregation state of membrane-embedded proteins are a few important examples for which the average distance among different membrane components is being altered.

However, little attention has been directed to this fact, probably because of the evident complications in getting an exact theoretical solution, either of the energy transfer efficiency or of the rate of the energy transfer for the above-mentioned processes. So, although theoretical extensions of the Förster energy transfer have recently been made in an attempt to quantitate both the rate and the efficiency of energy transfer in a two-dimensional space, such as the membrane surface, these theoretical approaches have been derived for the simplest case of a random distribution of membrane-bound donor and acceptor fluorophores [2–4].

Analytical solutions instead of strictly theoretical exact solutions appear to be required to overcome these difficulties as a very recent paper of Wolber and Hudson [5] suggests. In this paper is presented an analytical approach to the quantitation of the rate of energy transfer for binary mixtures of phospholipids undergoing lateral phase-separation. The analytical approach derived here is mainly based on the fact that the rate of energy transfer is mostly determined by the contribution from nearest-neighbor donor-acceptor pairs. It is shown that a quantitative description of the lateral separation phenomena of binary phospholipid mixtures can be achieved by measuring the rate of energy transfer among fluorescent derivatives of their components.

2. Preliminary remarks

The fact that biological phospholipids show a temperature-dependent phase-like transition be-

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tween the so-called gel and liquid crystalline states [6–8] and that lateral phase separation might even be dramatically different depending on the physical state, gel or liquid crystalline, of the lipid mixture [9], and also the different geometrical arrangements of phospholipid molecules depending on their physical state suggest the separate treatment of the quantitation of energy transfer for lateral phase separation phenomena in the gel and in the liquid crystalline states of a lipid mixture.

The gel-to-liquid transition region can, then, be approached as being composed of a mixture of gel and liquid crystalline phases in dynamic equilibrium [10], provided that the proportion of gel to liquid states of this mixture can be estimated as a function of the temperature by scanning calorimetry and/or by measuring the fluorescence polarization of currently used fluorescent probes [8].

3. Quantitation of the energy transfer of binary lipid mixtures undergoing lateral phase separation in the gel state

This case presents the advantage of a known spatial distribution of phospholipids in a triangular lattice [11]. Thus, one can estimate the average energy transfer considering the properties of such a phospholipid distribution without the use of integral continuous methods, but with an analytical discontinuous approach based on the simple geometry of such systems.

Table 1 lists the distribution of lipid molecules around a given lipid molecule in a triangular lattice. Table 1 also gives the value of the relative rate of energy transfer of a phospholipid molecule with each one of the discs of another molecule, $\langle k_r \rangle$, surrounding it. The values of $\langle k_r \rangle$ were calculated using the classical equation of Förster for the rate of energy transfer, $k_T(r)$, between a donor and an acceptor separated by a distance r [12] and the relative number of phospholipid molecules in each disc with respect to the number of phospholipid molecules in the first disc around a given molecule. These equations are:

$$k_T(r) = \tau_0^{-1}(R_0/r)^6 \quad (1)$$

and

$$\langle k_r \rangle = [k_T(r)/k_T(r_1)]n/6, \quad (2)$$

where τ_0 is the excited-state lifetime of the donor in the absence of acceptor; R_0, the distance in Å between the donor and acceptor at which the transfer efficiency is 50%; r_1, the average intermolecular distance of the triangular lattice and n, the number of phospholipid molecules in each disc surrounding a given phospholipid molecule. The assumption implicitly made is that in going from one disc to another, there is no change in the average orientation factor, K^2, contained in R_0 [12].

Table 1

Distribution of molecules around a given phospholipid molecule in a triangular lattice and their relative rate of energy transfer $\langle k_r \rangle$ with the central molecule

Disc number	Distance (r_1)	Number of molecules (n)	$\langle k_r \rangle$
1	1	6	1.000
2	$\sqrt{3}$	6	0.037
3	2	6	0.0156
4	$(5+2\sqrt{3})^{1/2}$	12	0.0033
5	3	6	0.00137

These data show that to a good first approximation, one can consider only the contributions to the observed rate of energy transfer arising from the nearest-neighbor molecules. In fact, the error is less than 6% in doing so. However, the problem is greatly simplified. Also, it can be seen that the error in the average rate of energy transfer, neglecting the contributions of the discs higher than the third, is less than 1%. However, simplification of the problem and consideration of the operational errors suggest the analysis of energy transfer as if it arises only from the nearest-neighbor molecules. These assumptions still hold for binary lipid mixtures in unilamellar vesicles, since the effect of the bilayer curvature on the energy transfer efficiency is negligible [3] and the minimum distance among polar head groups of the phospholipid of the inner and outer leaflets of the bilayer is clearly larger than $3r_1$.

3.1. Analysis of ideal cases

3.1.1. Ideal mixture of phospholipids

This case implies a random distribution of both lipids, i.e., that no preferential self-association of phospholipid molecules of each class occurs. If N_A and N_B are the numbers of molecules of phospholipids A and B, respectively, the number of A-B contacts, C_{AB}, is given by

$$C_{AB} = 6\left[1 - N_A/(N_A + N_B)\right]N_A. \quad (3)$$

Hence, the average rate of energy transfer for this case can be written as

$$\langle k_T \rangle = \theta_{AB} f_a k_T(r_1),$$

with $\theta_{AB} = C_{AB}/6N_A$, i.e., the fraction of the total A contacts involved in interactions with B molecules.

$$\langle k_T \rangle = \left[1 - N_A/(N_A + N_B)\right] f_a k_T(r_1), \quad (4)$$

where f_a is the fraction of B molecules labelled with the acceptor fluorophore.

3.1.2. Completely immiscible phospholipids

This case can be mathematically expressed as

$$\Delta G_e = \tfrac{1}{2}(\Delta G_{AA} + \Delta G_{BB}) - \Delta G_{AB} \gg RT, \quad (5)$$

where ΔG_e is the excess Gibbs free energy associated with the formation of one A-B contact from A-A and B-B contacts. As a general rule, it follows that in these cases the more compact (closest) structures must be formed, since for this kind of structure the number of contacts, C_{AB}, is the lowest possible. Hence, the expression for the average rate of energy transfer has to take this into account. Considering what has been established in the preceding sections of this paper, for the case in which donor and acceptor groups are attached to completely immiscible A and B molecules, respectively, the average rate of energy transfer is given by

$$\langle k_T \rangle = \theta_{AB} f_a k_T(r_1). \quad (6)$$

where θ_{AB} is now $C_{AB}/6n$, in which n is the number of lipid molecules in the cluster. Therefore, we need to know the dependence of C_{AB} upon the total number of molecules of the cluster to correlate $\langle k_T \rangle$ with the actual average cluster size of the less-rich component of the mixture. This is derived in appendix A. Substituting the value of $C_{AB}(s, n_{exc})$ into eq. (6), we obtain, for clusters up to six molecules:

$$\langle k_T \rangle = \left[(2n+6)/6n\right] f_a k_T(r_1), \quad 2 \leq n \leq 6 \quad (7)$$

and for larger cluster sizes:

$$\langle k_T \rangle = (6n)^{-1}\left\{6 + 12\left[s + n_{exc}/6(s+1)\right]\right\} \times f_a k_T(r_1), \quad (8)$$

where s is the number of complete lipid shells of the cluster which is an integer ≥ 1 and n_{exc} the number of lipid molecules in the incomplete outer shell of the cluster (see appendix A for more details).

Eqs. (7) and (8) give the dependence of the rate of energy transfer for the case of complete lateral phase separation as a function of the cluster size of the less-rich component of the lipid mixture, the cluster size being expressed in terms of its average number of shells in eq. (8) and determined in this situation just by the molecular ratio of both lipid components and the average size of the vesicle.

3.2. The real case: partial lateral lipid phase separation

For this case, the fraction of the less-rich phospholipid component of the mixture, C_A, is related to the concentration of each cluster by

$$C_A = \sum_{i=1}^{m} iC_i, \quad (9)$$

where C_i is the concentration of the cluster containing i phospholipid molecules of class A and C_A is the total fraction of the A molecules.

Thus, according to eq. (6) we can write for the average rate of energy transfer:

$$\langle k_T \rangle = C_A^{-1} f_a k_T(r_1) \times \sum_{i=1}^{m} C_i \theta_{AB}(s_i, n_{exc,i}) \quad (10)$$

Substituting for $C_{AB}(s_i, n_{exc,i})$ the value derived in appendix A, the summation of eq. (10) can be

written as follows:

$$\sum_{i=1}^{m} C_i\theta_{AB}(s_i, n_{exc,i}) = C_1 + \sum_{j=2}^{6}[(2j+6)/6j]C_j + \sum_{k=7}^{m}(C_k/k)\{1+2[s_k + n_{exc,k}/6(s_k+1)]\} = \sum_{i=1}^{m} C_i/i + \frac{1}{3}\sum_{j=2}^{6} C_j + 2\sum_{k=7}^{m}(s_{k,app}/k)C_k. \quad (11)$$

Here $s_{k,app} = s_k + n_{exc,k}/6(s_k+1)$ and gives the value of the total closed shells plus the fraction of the one being formed. Let us now neglect the population of clusters containing less than six lipid molecules because this has to be very low for the systems under consideration. Using eq. (11) and bearing in mind the above-mentioned simplifications, we finally obtain:

$$\sum_{i=1}^{m} C_i\theta_{AB}(s_k, n_{exc}) = (C_A/\langle i\rangle)(\langle i\rangle^{-1} + 2\langle s_i/i\rangle), \quad (12)$$

since

$$C_A = \langle i\rangle \sum_{i=1}^{m} C_i, \quad (13)$$

$$\sum_{i=1}^{m} C_i/i = \langle 1/i\rangle \sum_{i=1}^{m} C_i \quad (14)$$

and

$$\sum_{i=1}^{m}(s_{i,app}/i)C_i = \langle s_i/i\rangle \sum_{i=1}^{m} C_i. \quad (15)$$

Eq. (13) introduces the average cluster size, $\langle i\rangle$, in terms of its number of phospholipid molecules, and eq. (15) introduces the average cluster size, $\langle s_i/i\rangle$, in terms of the ratio of cluster shells to lipid molecules in the cluster.

Combining eqs. (10), (12)–(15) we finally obtain for the average rate of energy transfer:

$$\langle k_T\rangle = f_a k_T(r_1)\langle i\rangle^{-1}(\langle 1/i\rangle + 2\langle s_i/i\rangle). \quad (16a)$$

The last term in eq. (16a) can be written as $\langle(1+2s_i)/i\rangle = \frac{1}{2}\langle D_i/i\rangle$, where D_i is the diameter of cluster i, which is assumed to be circular, in r_c units, r_c being the functional radius of the ideal circular surface element occupied by one phospholipid molecule (note that this value should be close to $\frac{1}{2}r_1$, but not necessarily equal to it). Since the total surface area, S, of a circular cluster of i phospholipid molecules can be written as $S = \pi i r_c^2 \approx (1+2s_i)^2\pi r_c^2$, it turns out that $\langle D_i/i\rangle \approx \langle D_i^{-1}\rangle$.

On the other hand, we can visualize the cluster distribution at equilibrium, on the basis of its dynamic nature [10], as a group of clusters of A molecules of a size practically constant in terms of D_i and isolated A molecules which are being interexchanged among clusters. Then, we can write $\langle D_i^{-1}\rangle \approx 1/D_{\langle i\rangle}$ and, therefore:

$$\langle k_T\rangle = \tfrac{1}{2} f_a k_T(r_1)\langle i\rangle^{-1} D_{\langle i\rangle}^{-1}. \quad (16b)$$

Due to the monotonic character of the function $\langle i\rangle^{-1} D_{\langle i\rangle}^{-1}$, the value of $\langle i\rangle$ is determined once the value of this function is known. Thus eq. (16b) allows us to calculate the average cluster size of the less-rich component of the phospholipid mixture, termed phospholipid A, in the gel state by measuring the average rate of energy transfer among donor and acceptor molecules attached to phospholipids A and B, respectively. In addition, as shown in appendix B, the calculation of the average cluster size of phospholipid A in the gel state as a function of its concentration contains in itself the necessary information to evaluate the excess free energy $\Delta G_{AB} - \frac{1}{2}(\Delta G_{AA} + \Delta G_{BB})$. Therefore, the thermodynamic description of the phase separation of a phospholipid mixture in the gel state can be greatly improved from energy transfer measurements.

4. Quantitation of the rate of the energy transfer of binary lipid mixtures undergoing lateral phase separation in the liquid crystalline state

Above T_m an important feature is the loss of the triangular arrangement of phospholipid molecules [11]. The distribution of phospholipid molecules around a given phospholipid molecule can then be estimated using continuous integral methods instead of the discontinous one used above. For these cases the average rate constant of energy

transfer between an excited donor molecule and its surrounding acceptor molecules can be derived (see appendix C) and is given by

$$\langle k(r)\rangle \approx -\frac{1}{16}\epsilon(r^{-4}-\sigma^{-4}), \tag{17}$$

where $\epsilon = \alpha B\pi/n_p$, α being a normalization factor such that $\alpha\int_{\sigma}^{R_g}P(r)dr = N_B/N_A$, and B is the constant of the Förster equation $k_T(r) = Br^{-6}$, giving the rate of energy transfer between a donor-acceptor pair at a distance r [12].

This equation holds only if no preferential self-association of each class of phospholipids, i.e., phase separation, occurs. However, when phase separation takes place the average distance between two chemically different phospholipid molecules is dependent upon the cluster size of each kind of phospholipid. Nevertheless, at low concentrations of one phospholipid, this can be seen as if one phospholipid is dissolved in the other and consideration of clusters of only one of the phospholipids becomes necessary. For simplicity, as will be seen below, it is assumed here that this 'dissolved' phospholipid is the one carrying the donor fluorescent group. Again, the A molecules are the ones being dissolved or undergoing phase separation from the solvent phospholipid B. If this is the case, the average rate of energy transfer, $\langle k_T\rangle$, can be written

$$\langle k_T\rangle = f_a\sum_{i=1}^{m} iC_i\bar{k}_i, \tag{18}$$

where $\bar{k}_i$ is the average rate of energy transfer per phospholipid molecule in a cluster of i molecules.

Evidently, $\bar{k}_i$ must be a function of the cluster size, i, and we will derive its expression next.

4.1. Calculation of $\bar{k}_i$

For a cluster of only one molecule, $i = 1$, it immediately follows that $k_1(\sigma) = \langle k(R_g)\rangle$, where $\langle k(R_g)\rangle$ is given by eq. (17).

The value of $\bar{k}_i$ for clusters of i molecules, $2 \leq i \leq 7$, is given by the expression:

$$\bar{k}_i = \tfrac{1}{4}\gamma_i\epsilon\int_{\sigma}^{R_g}\exp(-A/r^6kT)r^{-5}dr + \tfrac{1}{4}(1-\gamma_i)\epsilon\int_{2\sigma}^{R_g}\exp(-A/r^6kT)r^{-5}dr, \tag{19}$$

where γ_i is the average fraction of the disc surrounding a lipid molecule of cluster i which is directly accessible to this lipid molecule. This value can be calculated approximately, assuming that each polar head group of a lipid molecule occupies a circular area of the vesicle surface and that the most compact structures are always formed. The formation of the most compact structures is based on purely thermodynamic grounds, since these structures have the minimum number of contacts between chemically distinct phospholipids, thus minimizing the ΔG value of the whole system. Values of γ_i equal to 7/9, 2/3, 7/12, 24/45 and 1/2 are obtained for cluster sizes from 2 to 6 with these assumptions. The remaining symbols in eq. (19) are defined in appendix C.

It should be noted that the second term of eq. (19) represents less than 8% of the value of $\bar{k}_i$ in all these cases and that, since for lipid systems undergoing phase separations the total population of these clusters must be very low, the error introduced by neglecting this term in the average $\langle k_T\rangle$ of the system can be considered negligible.

For a cluster of i molecules in which $i > 7$, this term can be written as

$$\bar{k}_i = 1/i\sum_{j=1}^{s_i} n_j\bar{k}_j, \tag{20}$$

where the summation is extended over all the shells of the cluster, s_i; n_j is the number of molecules in each shell and $\bar{k}_j$ the value of the average rate of energy transfer for each molecule of shell j.

Assuming a circular shape of the cluster, since this is the most compact structure, the following formula for $\bar{k}_j$ can be derived

$$\bar{k}_j \approx \tfrac{1}{2}k_1(\sigma) + \rho^4_{s_i-j} - \rho^4_0, \tag{21}$$

where

$$\rho^4_{s_i-j} = \tfrac{1}{32}\epsilon\left[\sigma_{AB} + (s_i - j)\sigma_{AA}\right]^{-1}, \tag{22}$$

$$\rho^4_0 = \tfrac{1}{32}\epsilon\,\sigma_{AB}^{-4}. \tag{23}$$

The factor $\frac{1}{2}$ has been introduced in eqs. (21)–(23) to account only for the surface fraction of each A molecule closer to the outer surface of the cluster.

Eq. (21) gives a highly complex dependence of $\bar{k}_j$ upon the shell number, but it can be greatly simplified by physical considerations. For a donor phospholipid molecule in the third shell from the outer side of the cluster, the closest distance to an acceptor phospholipid molecule is approximately three times that to an acceptor molecule for a donor molecule of the outer shell (assuming that $\sigma_{AB}/\sigma_{AA} \approx 1$). On the other hand, the number of A molecules in the outer shell of a cluster is larger than that in the third shell from the outside of the cluster. Hence, the probability of energy transfer between an A donor molecule of the outer shell and a B acceptor molecule is clearly higher than the probability of this process taking place with one A donor molecule of the referred third shell. Thus, the average rate of energy transfer, $\langle k_T \rangle$, for a molecule of the outer shell is greater than 81 times that for a molecule of the third shell from the outside. So, the contributions to the average rate of energy transfer from the molecules of the inner shells up to and including the third one from the outside can be neglected. Then, eq. (21) need only be applied to the two external shells without incurring an appreciable error (< 1%).

Bearing in mind these considerations, we can write

$$\bar{k}_i \approx i^{-1}\left(n_{s_i}\bar{k}_{s_i} + n_{s_i-1}\bar{k}_{s_i-1}\right). \tag{24}$$

On the other hand, the number of lipid molecules in shell j, n_j, can be calculated using the equation

$$n_j = \frac{\Delta S_j}{S_{PL}} = \frac{S_j - S_{j-1}}{S_{PL}} = 8(j-1), \quad j \geq 2, \tag{25}$$

where S_j and S_{j-1} are the areas of the clusters having j and $j-1$ shells, respectively, and S_{PL} is the 'effective' area occupied by the phospholipid molecule in the cluster ($S_{PL} = \pi R_A^2$). It can be easily shown that $S_j = \pi r_j^2$, with $r_j = (2j-1)R_A$.

Substituting the values of n_{s_i} and n_{s_i-1} given by eq. (25) and the values of k_{s_i} and k_{s_i-1} given by eq. (21) into eq. (24) we obtain:

$$\bar{k}_i \approx (8/i)\left\{(s_i - 1)\left[k_1(\sigma) + \rho_1^4 - \rho_0^4\right] - \tfrac{1}{2}k_1(\sigma) - \rho_1^4 + \rho_0^4\right\}. \tag{26}$$

4.2. Average rate of energy transfer

Substituting the values of $\bar{k}_i$ derived in section 4.1 into eq. (18):

$$\langle k_T \rangle = f_a C_A \left\{ k_1(\sigma) \sum_{j=1}^{7} (\gamma_j j) C_j / C_A + 8 \sum_{i=8}^{m} (C_i/C_A)\left[(s_i - 1)\left(k_1(\sigma) + \rho_1^4 - \rho_0^4\right) - \tfrac{1}{2}k_1(\sigma) - \rho_1^4 + \rho_0^4\right]\right\}, \tag{27}$$

where f_a, C_A, $k_1(r)$, ρ_1, ρ_0 and γ_j are known.

Further simplification of eq. (27) can be done, bearing in mind that we are concerned with systems undergoing phase separation, i.e., with a strong tendency to self-association. So, the total population of very small clusters containing up to seven phospholipid molecules must be almost negligible. Therefore, we can assume that the contribution to $\langle k_T \rangle$ from these terms can be described approximately by the higher cluster contribution term without an appreciable error. In addition, $R_g \gg \sigma$ and then $k_1(\sigma) \approx 2\rho_0^4$. With these simplifications and combining eqs. (13), (15) and (27) we obtain:

$$\langle k_T \rangle = \left\{8\left[\tfrac{1}{2}k_1(\sigma) + \rho_1^4\right]\langle s \rangle / \langle i \rangle - 8\left[\tfrac{1}{2}k_1(\sigma) + 2\rho_1^4\right]\langle i \rangle^{-1}\right\} f_a C_A. \tag{28}$$

Eq. (28) allows the calculation of the average cluster size in terms of the ratio of its average number of shells, $\langle s \rangle$, to its average number of lipid molecules, $\langle i \rangle$, for binary mixtures of lipids undergoing phase separation in the lipid crystalline state directly from the measured rate of energy transfer among donors and acceptors attached to chemically different phospholipid molecules as a function of the concentration of the less-rich lipid component of the mixture. It is also clear that for these systems $\langle s \rangle / \langle i \rangle \gg \langle i \rangle^{-1}$ and, hence, that the first term of eq. (28) will provide the most important contribution to the average rate of energy transfer in these situations.

Finally, it seems worthwhile to remark here that the calculated average cluster size of the less-rich

lipid component of the binary mixture as a function of its concentration can be used to improve our understanding of the thermodynamics of the lateral phase separation process (see appendix B and also section 3.2).

5. Concluding remarks

An approach to quantitate the average rate of energy transfer for binary lipid mixtures undergoing phase separation has been developed in the preceding sections. The generality of this approach is restricted by the following assumptions:

(i) A triangular lattice for lipids in the gel state.

(ii) A negligible relative population of small clusters.

(iii) A random orientation among donor and acceptor molecules, i.e., an orientation factor of $K^2 = 2/3$.

(iv) The existence of a large number of acceptor molecules per donor molecule.

Assumption (i) is justified by the very well known phospholipid arrangements in the gel state [11], while assumption (ii) is a direct consequence of the very nature of the phase separation phenomena. Assumption (iii) suggests the use of donor and acceptor molecules attached to the polar head group of the phospholipids, since for these cases $K^2 \approx 2/3$, as a result of the simultaneous averaging over space and time, and of the very low concentrations of fluorescently labelled phospholipids to be used in these studies ($< 5\%$) [2,3]. Assumption (iv) merely establishes a condition for the experimental design of the experiments.

Perhaps, it should be mentioned here that assumption (i) has to be changed for problems other than those analyzed in this paper, as has been done in the following paper for problems dealing with the distribution and average aggregation state of intrinsic membrane proteins, and eventually it can be used to gain insight into unknown geometric arrangements of membrane components by comparison of the experimental results with those predicted theoretically according to different geometric models.

It seems worthwhile to analyze here in some detail the major criticism in the use of energy transfer for obtaining information about lateral phase separation phenomena, namely, the perturbation introduced in the lipid system by the fluorescently labelled phospholipids. Firstly, the small amount of fluorescently labelled fractions of each lipid of the mixture needed for doing these kind of studies [2,3]: Fung and Stryer [3] have demonstrated the absence of morphological alterations of lipid vesicles containing up to 2% phospholipids fluorescently labelled in the polar head group with respect to control ones formed under the same conditions without labelled phospholipids. Two extreme kinds of perturbations can then be attributed to the labelled phospholipids; these are: (a) the fluorescent derivatives might undergo phase separation by themselves even from the same unlabelled phospholipid and (b) steric factors owing to the large size of donor and acceptor molecules attached to the phospholipid polar head group can promote cluster disruption. The first possibility can, at least, be ruled out for various donor-acceptor pairs because in a recent study Fung and Stryer [3] have demonstrated that these labelled phospholipids are randomly distributed when incorporated into dipalmitoylphosphatidylcholine vesicles and thus this can always be tested as these investigators have shown in the above-cited study. Furthermore, scanning calorimetry of mixtures of normal and fluorescent phospholipid derivatives can be used as an additional control test to choose fluorescent derivatives promoting a negligible alteration in the melting profile of the phospholipid [6–8]. The second possibility can be disregarded because of the very low concentration of labelled phospholipids used in these experiments.

The average efficiency of energy transfer, $\langle E_T \rangle$, should be mentioned. This is given by the equation

$$\langle E_T \rangle = \langle k/(k_0 + k) \rangle, \tag{29}$$

where k_0 is the rate constant of the overall deexcitation process of the donor fluorophore in the absence of acceptor molecules. It turns out that the evaluation of $\langle E_T \rangle$ using this equation in terms of those derived previously presents serious mathematical problems. An approximation to overcome these difficulties is to work with low

levels of energy transfer, i.e., using conditions under which the extent of energy transfer is lower than or about 10%, because under these experimental conditions $k \ll k_0$ and we can write:

$$\langle E_T \rangle \approx \langle k/k_0 \rangle = k_0^{-1} \langle k \rangle. \quad (30)$$

Evidently, this assumption will tend to give a slight overestimation of $\langle k \rangle$. Using the equations derived previously, iterative computer methods can be devised in order to obtain a more reliable value for $\langle k \rangle$. Briefly, with the first estimation of $\langle k \rangle$, obtained using eq. (30), a first approximate value for the average cluster size, $\langle i \rangle$, of the less-rich component of the lipid mixture can be calculated using eqs. (16a), (16b) and (28); the value of $\langle i \rangle$ so derived can be used to generate the distribution of lipid cluster by means of eqs. (B.12) and (B.5) (see appendix B) and the theoretically expected value of $\langle E_T \rangle$ for such a cluster distribution can be obtained with the equations derived previously and with eq. (29). An iterative computer method can then be applied to match the experimentally measured $\langle E_T \rangle$ and that calculated, using $\langle k \rangle$ as the fitting parameter.

In addition, for the cases being analyzed here, statistical thermodynamic theory provides a rather nice alternative approach. For a very large number of systems under observation in a canonical ensemble, we can substitute the average value of a property of the ensemble for its most probable value [13], i.e., for the property being analyzed here: $\langle E_T \rangle \approx E^*$, with E^* being the extent of energy transfer in the most probable state of the system. Therefore, eq. (29) can be written as

$$\langle E_T \rangle \approx E^* = k^*/(k_0 + k^*), \quad (31)$$

where k^* represents the rate of energy transfer among donor fluorescent molecules within the most probable cluster size of the less-rich component of the phospholipid mixture and acceptor fluorescent molecules of the other lipid component of the mixture. Using eq. (31), k^* can be estimated from the experimental value of $\langle E_T \rangle$. With known k^*, the most probable cluster size can be obtained using the equations derived in this paper.

Simultaneously, the evaluation of the average cluster size and cluster distributions using both approaches, whenever possible, may be used to check the validity of the hypotheses and approximations developed here to describe the physical phenomena under consideration.

In conclusion, the average efficiency of energy transfer may allow us to determine the average lipid cluster size as a function of the concentrations of both lipids in the mixture, within limited molar fraction ranges, directly from experiment. These data can be used to improve our knowledge of the thermodynamics of lateral phase separation (see appendix B) without using any specific kinetic model.

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Appendix A. Evaluation of C_{AB}

Let n be the total number of molecules of the generic cluster. We assume in the following that the most compact cluster possible is always formed. Then, for $2 \leq n \leq 6$, C_{AB} can be empirically determined:

$$C_{AB} = 2n + 6. \quad (A.1)$$

For larger values of n, C_{AB} can be more easily related with the number of closed shells.

Note that the number of molecules in a closed shell of triangular lattice is equal to $6s$ where s refers to the order number of the shell starting from the inside of the cluster ($s = 1$ corresponds to the smallest hexagon). Also, it can be noted that the total number of contacts A-B of a closed shell

is

$$C_{AB}(s) = 6(2s+1). \quad (A.2)$$

However, a cluster size of n molecules will have some excess of molecules, n_{exc}, over those forming closed shells. Then,

$$C_{AB}(s, n_{exc}) = n_{exc}(6-2r) + C_{AB}(s). \quad (A.3)$$

where r represents the 'average' number of contacts of a molecule of an incomplete shell with the same class of molecules (phospholipids). The properties of $C_{AB}(s, n_{exc})$ allow us to calculate r: $C_{AB}(s, n_{exc}) = C_{AB}(s+1)$, when $n_{exc} = 6(s+1)$. Hence:

$$r = 3 - 1/(s+1). \quad (A.4)$$

Thus, substituting the value of r given by eq. (A.4) into eq. (A.3), we obtain:

$$C_{AB}(s, n_{exc}) = 6 + 12[s + n_{exc}/6(s+1)], \quad (A.5)$$

where

$0 < n_{exc}/6(s+1) < 1$, by definition of n_{exc}

On the other hand, the relation between the total number of molecules, n, in the cluster and the values of s and n_{exc} can easily be derived:

$$n = 1 + 6(1 + 2 + \ldots + s) + n_{exc} = 3s(s+1) n_{exc} + 1, \quad (A.6)$$

so, with known s and n_{exc}, the value of n is also determined.

Appendix B. Evaluation of the excess free energy of A-B contacts and cluster size distribution

$$C_A = \sum_{i=1}^{m} i c_i, \quad (B.1)$$

where c_i is the concentration of the cluster containing i phospholipid molecules of class A and C_A is the total phospholipid A concentration.

Let us define the following parameters:

$$A + A \overset{K_{AA}}{=} A_2, \quad \Delta G_{AA} = -RT \ln K_{AA},$$

$$A + B \overset{K_{AB}}{=} AB, \quad \Delta G_{AB} = -RT \ln K_{AB},$$

$$B + B \overset{K_{BB}}{=} BB, \quad \Delta G_{BB} = -RT \ln K_{BB}$$

and the excess free energy of an A-B contact

$$\Delta G_c = \tfrac{1}{2}(\Delta G_{AA} + \Delta G_{BB}) - \Delta G_{AB} = -RT \ln K_0,$$

$$K_0 = K_{AA}^{1/2} K_{BB}^{1/2} / K_{AB}. \quad (B.2)$$

Now, let us consider the case of a cluster size of n molecules of the A phospholipid, $2 \leq n \leq 6$. In this case, during the process

$$A + A_n \overset{K}{=} A_{n+1}, \quad \Delta G(n \rightarrow n+1),$$

the net loss of two A-B contacts occurs. Thus, we can write

$$\Delta G(n \rightarrow n+1) = -RT \ln K = 2\Delta G_c, \quad K = K_0^2. \quad (B.3)$$

On the other hand, for clusters of more than six phospholipid molecules, during the process

$$A + A_p \overset{K'_p}{=} A_{p-1} \quad (p > 6), \quad \Delta G(p \rightarrow p+1),$$

an average of r contacts between the newly incorporated A molecule and the A_p cluster are established (see appendix A). Since it can be assumed that these contacts come from earlier A-B contacts, it can be written:

$$\Delta G(p \rightarrow p+1) = -RT \ln K'_p = r\Delta G_c, \quad K'_p = K_0^r. \quad (B.4)$$

From eqs. (B.2)–(B.4) it can easily be shown that

$$c_2 = p_2 K_0^2 c_1^2, \ldots, \qquad c_6 = p_6 K_0^{10} c_1^6,$$
$$c_7 = p_7 K_0^{10+r} c_1^7, \ldots, \qquad c_i = p_i K_0^{10+r(i-6)} c_1^i,$$

where p_i takes into account the overall ways to form c_i from c_{i-1} and vice versa, i.e., essentially it is an entropic factor. It can be seen that $p_i = C_{AB}(i-1)/C_{AB}(i)$, where $C_{AB}(i-1)$ and $C_{AB}(i)$ are the total number of A-B contacts of clusters of $i-1$ and i A molecules, respectively, given by eq. (A.5). Therefore, $p_i \approx 1$ for clusters containing two or

more complete shells of phospholipid molecules.

Then, we can write

$$C_A = \sum_{j=1}^{6} p_j j K_0^{2(j-1)} c_1^j + \sum_{i=7}^{m} i p_i K_0^{10+r(i-6)} c_1^i \quad (p_1 = 1). \tag{B.5}$$

On the other hand, for a system undergoing significant phase separation for which populations of clusters containing less than 7 phospholipid molecules can be neglected without an a priori appreciable error, and using eqs. (13) and (B.5).

$$\frac{d(C_A/\langle i\rangle)}{dC_A} = \frac{d}{dC_A}\sum_i p_i K_0^{10+r(6-i)} C_1^i = \frac{dC_1}{dC_A}\left(\sum_i (p_{i-1}/p_i) K_0^{6r} C_i i + \sum_i (p_{i+1}/p_i) K_0^{6r} C_i\right). \tag{B.6}$$

Since for these systems we can assume $p_{i+1}/p_i \approx 1$ (see above) and $r =$ constant, this equation can be transformed into

$$\frac{d(C_A/\langle i\rangle)}{dC_A} = K_0^{6r}(\langle i\rangle + \langle i\rangle^{-1}) dC_1/d\ln C_A. \tag{B.7}$$

Moreover, the free energy change accompanying the growth of a large cluster can be considered independent of its size (see eq. (B.5) and the discussion above on p_i and r). Hence, we can approach the system as follows:

$$pC_1 \overset{K_p}{\rightleftharpoons} C_p, \quad K_p = K_0^{10+r(p-6)} = [C_p]/[C_1]^p,$$

with $C_1 + pC_p = C_A$, and if $K_p \gg 1$, as has to be expected for the systems under consideration, then $p \approx \langle i\rangle$ and also

$$C_1 \approx (C_A/\langle i\rangle K_{\langle i\rangle})^{1/\langle i\rangle}. \tag{B.8}$$

Taking the derivative dC_1/dC_A and bearing in mind that $\langle i\rangle \gg 1$, we find:

$$\frac{dC_1}{dC_A} = \frac{1}{C_A\langle i\rangle} - \frac{d\langle i\rangle}{dC_A}\left(\frac{1 + \ln K_0 r\langle i\rangle}{\langle i\rangle^2}\right). \tag{B.9}$$

Combining eqs. (B.7) and (B.9) and taking the derivative of the first term of eq. (B.7) we obtain:

$$\langle i\rangle K_0^{6r}\left(1 - \frac{1}{\langle i\rangle}\frac{d\langle i\rangle}{d\ln C_A} - r\ln K_0 \frac{d\langle i\rangle}{d\ln C_A}\right) = 1 - \frac{1}{\langle i\rangle}\cdot\frac{d\langle i\rangle}{d\ln C_A}. \tag{B.10}$$

Considering that the assumption $K_p \gg 1$ implies $K_0 > 1$, then, for this case: $\langle i\rangle K_0^{6r} \gg 1$, eq. (B.10) becomes

$$d\ln C_A = (r\langle i\rangle \ln K_0 + 1) d\ln\langle i\rangle. \tag{B.11}$$

Hence

$$\ln(C_A/\langle i\rangle) = r\langle i\rangle \ln K_0 + M, \tag{B.12}$$

where M is the integration constant. Thus, a plot of $\ln(C_A/\langle i\rangle)$ versus $\langle i\rangle$ will yield a slope equal to $r\ln K_0$, from which K_0 can be evaluated. With known K_0, ΔG_c is immediately determined by eq. (B.2). Furthermore, the size distribution of clusters of A molecules can then be calculated (see (B.5)).

Appendix C

In the following discussion, it is assumed that the system is in thermodynamic equilibrium and that the number of B molecules, N_B, some of which are labelled with acceptor molecules, is greater than the number of A molecules, N_A, some of which are labelled with donor molecules, i.e., $N_B \gg N_A$.

On the other hand, according to Förster, energy transfer is promoted by interactions of a dipole-dipole nature; thus, $\Psi(r) = A/r^6$, where A is a constant previously evaluated by Förster [12]. Because of the short lifetime of the excited donors, of the order of 10 ns, when compared to the two-dimensional diffusion coefficient of phospholipid molecules [3], diffusion effects on energy transfer among phospholipid pairs can be neglected. Hence, the distribution of B molecules, $\rho(r)$, around each of the randomly distributed A molecules can be written as

$$p(r) = \exp[-\Psi(r)/kT] = \exp(-A/r^6 kT). \tag{C.1}$$

The probability, $P(r)\mathrm{d}r$, of finding an acceptor molecule at a distance between r and $r+\mathrm{d}r$ from the donor can then easily be evaluated for a two-dimensional system,

$$P(r)\,\mathrm{d}r = 2\pi r \exp(-A/r^6kT)\,\mathrm{d}r. \qquad \text{(C.2)}$$

This function must be normalized to give

$$\alpha\int_{\sigma}^{R_g} P(r)\,\mathrm{d}r = n_B, \qquad \text{(C.3)}$$

where $n_B = N_B/N_A$, α is the normalization factor, r the sum of A and B radii and R_g the average radius of the two-dimensional vessels, the vesicles, assuming these are spherical. Eq. (C.3) implies that we are averaging over half the surface of the sphere.

The average rate constant for energy transfer of the system composed of the donor A molecule and n_B acceptor molecules is given by

$$\langle k_T\rangle = \alpha\int_{\sigma}^{R_g} k_T(r)P(r)\,\mathrm{d}r/\alpha\int_{\sigma}^{R_g} P(r)\,\mathrm{d}r$$
$$= \frac{1}{n_B}\int_{\sigma}^{R_g} k_T(r)P(r)\,\mathrm{d}r, \qquad \text{(C.4)}$$

where $k_T(r)$, according to Förster [12], can be written as

$$k_T(r) = Br^{-6}; \qquad \text{(C.5)}$$

where B is a constant for each donor-acceptor pair and is independent of the distance.

Combining eqs. (C.2), (C.4) and (C.5), one obtains

$$\langle k_T\rangle = \frac{B\pi}{n_B}\int_{\sigma}^{R_g}\frac{\exp(-A/r^6kT)}{r^5}\,\mathrm{d}r. \qquad \text{(C.6)}$$

The integral can be solved by a Taylor expansion of the exponential, considering that r will never be lower than σ, the sum of the A and B radii, and that A/r^6kT is always small. Taking only the first two terms of the Taylor expansion

$$\langle k_T\rangle \approx \epsilon\int_{\sigma}^{R_g} r^{-5}(1-\delta r^{-6})\,\mathrm{d}r$$
$$= \epsilon\left(-\tfrac{1}{4}r^{-4} + \tfrac{1}{10}\delta r^{-10}\right)_{\sigma}^{R_g} \approx -\tfrac{1}{16}\epsilon(r^{-4})_{\sigma}^{R_g}, \qquad \text{(C.7)}$$

where $\epsilon = B\pi/n_B$ and $\delta = A/kT$.

References

[1] L. Stryer, Ann. Rev. Biochem. 47 (1978) 819.
[2] N. Shacklai, J. Yguerabide and H.M. Ranney, Biochemistry 16 (1977) 5585.
[3] B.K.K. Fung and L. Stryer, Biochemistry 17 (1978) 5241.
[4] D.E. Koppel, P.J. Fleming and P. Strittmatter, Biochemistry 18 (1979) 5450.
[5] P.K. Wolber and B.S. Hudson, Biophys. J. 28 (1979) 197.
[6] H. Hinz and J. Sturtevant, J. Biol. Chem. 247 (1972) 6071.
[7] S. Mabrey and J. Sturtevant, Proc. Natl. Acad. Sci. USA 73 (1976) 3862.
[8] Y. Barenholz, J. Suurkuusk, D. Mountcastle, T. Thompson and R. Biltonen, Biochemistry 15 (1976) 2441.
[9] W. Kleeman and H.M. McConnell, Biochim. Biophys. Acta 419 (1976) 206.
[10] T.Y. Tsong and M.I. Kanehisa, Biochemistry 16 (1977) 2674.
[11] H. Trauble and E. Sackmann, J. Am. Chem. Soc. 94 (1972) 4499.
[12] T. Forster, Ann. Physik 2 (1949) 55.
[13] T.L. Hill, Introduction to statistical thermodynamics (Addison-Wesley, Reading, 1962).