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Abstract

The liquid-ordered/disordered-phase domain co-existence in large unilamellar vesicle membranes consisting of phosphatidylcholine:sphingomyelin (2:1) with different amounts of cholesterol has been examined using a concentration dependent self-quenching of a single reporter molecule, C12NBD-PC. A temperature-dependent decrease of fluorescence intensity was associated with the expected formation and increase of l_o-phase membrane fraction in the vesicles. The result is consistent with exclusion of the fluorescent probe from the liquid-ordered phase which partitions preferentially into the liquid-disordered phase membrane domains. This leads to an increase of the local concentration of fluorophore in the liquid-disordered phase and a decrease of the quantum yield. This effect was used to obtain a quantitative estimation of the fraction of the vesicle membrane occupied by the liquid-ordered phase, Φ_o, as a function of temperature and cholesterol content between 0 - 45 mol %. The value of Φ_o was related to the assumed partition coefficient k_p of probe between liquid-ordered/disordered phases. For large unilamellar vesicles containing 20 and 4 mole% cholesterol and probe, respectively, with $k_p = 0$ (probe completely excluded from liquid-ordered phase), $\Phi_o = 0.16$ and with $k_p = 0.2$; $\Phi_o = 0.2$. The results are relevant to the action of detergent in the fractionation of detrergent-resistant membrane from living cells.

Abbreviations used:

LUV, large unilamellar vesicle; GUV, giant unilamellar vesicle; SUV, small unilamellar vesicle; MLV, multilamellar vesicle; PC, egg yolk L-α-phosphatidylcholine; SM, egg yolk sphingomyelin; Chol, cholesterol; PC*, fluorescent lipid analogue C12NBD-PC (1-acyl-2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine); C_M, mean molar concentration of PC*; C_L, local molar concentration of PC*; T_M, main phase transition temperature; l_d and l_o, liquid disordered and liquid ordered (cholesterol containing) L_α phases; Φ_d and Φ_o, membrane fraction occupied by l_d and l_o phases; k_p, l_o/l_d phase partition coefficient; DRMs, detergent resistant membranes.

Footnote 1 (see page 6, second paragraph):

The term "multicomponent membrane" refers to a membrane made of different lipid molecular species; the term "homogeneous lipid membrane" denotes the perfect miscibility of membrane lipids, and "heterogeneous lipid membrane" to indicate that lipid segregation into domains of different molecular composition takes place in the membrane.

1. Introduction

Current interest in membrane microdomains or rafts is due to their putative functional role in processes such as signal transduction [1], protein and lipid sorting [2] cholesterol transport [3] and endocytosis [4]. The biochemical concept of lipid rafts in cell membranes emerged because of isolation of detergent resistant membranes (DRMs) from cell lysates after treatment with detergents at certain temperatures and ratio detergent/cellular mass [5]. The attempt to relate the information acquired from studies of DRMs to the properties of rafts in living cell membranes has led to the investigation of model lipid membranes, exhibiting l_o/l_d phase co-existence under physiological conditions [6]. Phase diagrams of such "pro-raft" lipid mixtures have been constructed to establish the composition and phase boundaries for l_o domains in monolayers as well as a number of vesicular membrane systems including MLV, LUV, GUV [7, 8]. Such systems have been investigated in order to characterize the size of l_o domains and, in the particular case of a ternary lipid mixture consisting of PC/SM/Chol, using time-resolved fluorescence resonance energy transfer methods [9].

In this work we propose a general methodology for quantitative estimation of membrane fraction in l_o phase (Φ_o) in heterogeneous membrane LUVs. The model membrane examined consisted of LUVs of PC/SM/Chol 2:1:X (with cholesterol up to 45 mol %) extruded through 100 nm polycarbonate filters. The concentration self-quenching properties of C12NBD-PC were employed in order to reveal the l_o/l_d phase co-existence and estimate membrane fractions in liquid disordered and liquid ordered phase, Φ_d and Φ_o respectively (under the condition $\Phi_d + \Phi_o = 1$).

The NBD lipid analogues NBD-PE (head labeled) and NBD-PC (labeled at different positions of the chains) have been used for a broad range of studies involving biological and model membranes [10]. It is known that NBD fluorescence intensity in lipid membranes is

modulated by two mechanisms: (i) effect of the environment on the NBD fluorescence, i.e. the membrane composition and structure (in particular, the phase state) [11 - 13] as well as by (ii) concentration-dependent NBD self-quenching, i.e., by the concentration of the NBD fluorophore in the lipid membrane [14]. Mazeres *et al.*, [13] showed in their very comprehensive study that "… the fluorescence response of the NBD group was observed to strongly depend on the chemical structure and physical state of the host phospholipids and on the chemical structure of the lipid probe itself. Among the various fluorescence parameters studied, i.e., Stokes' shifts, lifetimes, and quantum yields, the quantum yields were by far the most affected by these structural and environmental factors, whereas the Stokes' shifts were practically unaffected. Thus, depending on the phospholipid probe and the host phospholipid, the fluorescence emission of the NBD group was found to vary by a factor of up to 5…" In addition, as originally proposed by Hoekstra [15], self-quenching has been recognized as dominant mechanism for change in the quantum yield of NBD in lipid membranes. An important feature of NBD lipid analogues is their unequal partitioning between the different lipid phases coexisting in a lipid membrane. Most studies using NBD-labelled lipid analogues were carried out with the head group labeled NBD-PE or NBD attached to fatty acid chains [16]. On the other hand, the chain labeled NBD-PCs appear to perturb the bilayer structure even more efficiently compared to the head-group labeled NBD-PEs. In the case of chain-linked NBD, the location of the NBD moiety imposes steric and polarity constrains in the hydrophobic region of lipid bilayer depending on the NBD position and the length of PC hydrocarbon chains. It has also been suggested as well that NBD linked to the extremity of an acyl chain tends to approach the lipid water interface inducing chain kink [10,11,17]. The result is the almost perfect exclusion of, e.g. the C12NBD-PCs from ordered lipid phases and preferential accumulation of the labelled lipid into the disordered domains [16]. This was exploited by direct visualization of the segregation of cholesterol-rich domains in monolayers

containing ordered liquid phases that preferentially exclude the fluorescent probe [18]. The l_o/l_d-phase domain co-existence in the bilayers of GUVs and monolayers of different lipid composition has been visualized directly by fluorescence microscopy using a variety of fluorescent probes [6, 7]. The fluorescent molecules usually exhibited a preference for one of the two phases presumably due to the structural perturbation induced by the fluorescent amphiphilic molecule such as steric perturbation resulting from the presence of the bulky fluorophore, hydrophobic mismatch of different hydrocarbon chains, etc. Apparently the l_d-phase more efficiently accommodates the fluorescent lipid analogues. Veatch and Keller [7] observed co-existence of l_o/l_d-phase domains in GUVs comprised of PC/SM/Chol in different proportions using Texas Red di(16:0)PE as a dye which partitions into the cholesterol-poor phase. Our studies of similar mixtures have used C12NBD-PC which by fluorescence microscopy reveals the l_o-phase domains as dark spots within the bright GUV membrane [19, 20].

The results on GUVs suggest that the C12NBD-PC might be a useful tool for investigating membrane heterogeneity of LUVs using only one type reporter molecule. Fluorescence microscopy observations (possibly on GUVs but not on LUVs) revealed the almost complete partitioning of the probe into the l_d-phase domains of the membrane (see footnote 1).

Hitherto fluorescence studies of l_o/l_d-phase membrane micro-heterogeneity of MLVs or LUVs have invariably involved two different reporter molecules. Such methods were based on fluorescent quenching, for example, of DPH by 12SLPC in MLVs [21, 22] or, fluorescence resonance energy transfer (FRET), e.g., between NBD-PE and Rhod-PE in LUVs [9,23]. This approach is complicated by virtue of partition of both reporter molecules between lipid phases.

In this work we investigated the l_o/l_d-phase domain co-existence in the membrane of LUVs using the concentration dependent self-quenching of C12NBD-PC (PC*) alone. We observed a temperature-dependent decrease of fluorescence intensity that we interpret as the formation of and expansion of an l_o-phase domain in LUV membranes. This is rationalized as the effect of the presence of l_o-phase resulting in the effective decrease of membrane space available for PC* species which accumulate preferentially in the l_d-phase. The local concentration of fluorophore in the l_d-phase increases and quantum yield decreases. We used this effect to estimate of the fraction of LUV membrane occupied by l_o- (respectively, l_d-) phase as a function of temperature and PC/SM/Chol 2:1:*X* molar ratio for *X* between 0 – 45 mol %.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (PC), egg yolk sphingomyelin (SM) and cholesterol (Chol) were purchased from Sigma (France). The lipophilic membrane probe C12NBD-PC (1-acyl-2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine) (PC*) was obtained from Avanti Polar Lipids (Alabaster, AL).

2.2. Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared using the extrusion method [24]. Samples were prepared by dissolving and mixing the indicated lipids in chloroform-methanol (9:1) to obtain the desired compositions thereafter the solvent was removed under a stream of oxygen-free dry nitrogen. The residues were subsequently maintained under reduced pressure for one hour and then hydrated in 5 mM HEPES buffer at pH 7.5 to yield a lipid concentration of 1 mM. The samples were then vigorously mixed (vortexed) for 30 seconds at room temperature (23°C) and heated at 65°C for 30 minutes, vortexed again for 30 seconds to ensure more uniform vesicle dispersion and incubated again at 65°C for 15 min. The use of

temperatures above the main phase transition and phase separation of lipids aimed to obtain a homogeneous lipid mixture for the subsequent LUV preparation. The multilamellar vesicles were then extruded with a LiposoFast small-volume extruder equipped with polycarbonate filters (Avestin, Ottawa, Canada) as follows: 12 extrusions through 800 nm, and after that 21 extrusions through 100 nm filters. LUVs samples were kept at 4°C, protected from light, until use. In the binary and ternary systems comprised of PC/SM, or PC/SM/Chol, the ratio PC/SM was always 2:1 mol/mol. C12NBD-PC concentration was 4 mol% unless otherwise stated.

Giant unilamellar vesicles (GUVs) were obtained by the electroformation method [25] in a temperature-controlled chamber following the particular protocol for heterogeneous GUV formation previously described [19]. GUVs were formed from PC/SM/Chol/PC* 51:25:*X*:4 mol/mol, at 34°C (*X* being 0, 10, 20, 35 mol%). The raft-like (l_o) domains were visualized in fluorescence as previously described in [19]. The chain-labeled lipid analogue PC* is excluded from the ordered (l_o) phase and partitions predominantly in the disordered (l_d) phase. That makes the l_o domain appear as a dark round-shaped spot within the bright vesicle membrane.

2.3. Video Microscopy

A Zeiss Axiovert 200M microscope (fluorescent unit fluo arc N HBO 103, Zeiss), equipped with a Lambda 10-2 unit (Sutter Instrument Co.), plus a CCD B/W chilled camera (Cool SNAP HQ), was used for GUV imaging. The set-up was piloted by Methamorph 6.0 software (Ropper Sci.). The morphology transformations and dynamics of the heterogeneous GUV membrane were followed in phase contrast and in fluorescence by Zeiss filter set 16 (Ex/Em=485/>520nm).

2.4. Fluorescence measurements

Steady-state fluorescence measurements were carried out with a Cary Eclipse spectrofluorimeter (Varian Instruments, CA). Quartz cuvettes (1 cm x 1 cm) were used.

Temperature was controlled by a thermostated cuvette holder (Varian Instruments, CA). C12NBD-PC fluorescence was excited at 470 nm, and emitted fluorescence maximum measured at 538 nm. Excitation and emission slits were adjusted to 5 nm. All fluorescence measurements were carried out at a total lipid concentration of 0.5 mM. Temperature scans of heating and cooling were performed with 15 measurements from 4°C to 70°C and back, allowing sample equilibration for 5 min at each temperature.

The derivation of quantitative fluorescence parameters was found to be dependent on small variations in the experimental procedure. The factors that give rise to variations include the method of preparation of LUVs, lipid concentration and bleaching and solvent evaporation during introduction of the fluorescent probe. Storage of LUVs for several days at 4^oC also resulted in vesicle aggregation. Reproducibility of the specific fluorescence intensity measurements between carefully prepared samples was ± 8%. This error increased to ± 15% in LUV containing 25 mole% cholesterol possibly because the lo-phase dominates the bilayer of the LUV and small variations in cholesterol content of different vesicles may produce significant differences in total fluorescence intensity.

3. Results

3.1. C12NBD-PC fluorescence within homogeneous lipid membranes

It may be expected that for a homogeneous lipid bilayer LUV the mean membrane molar concentration of PC*, C_M, would be equal to the local membrane molar concentration, C_L, of the probe at any location in the membrane. The same assumption should hold for the fluorescence intensity.

3.1.1. Effect of the environment within homogeneous membranes

The effect of the environment on the fluorescence intensity of C12NBD-PC embedded in homogeneous lipid bilayer membranes was examined first. Fig.1 shows temperature dependence over the range 4^o to 70^oC of specific fluorescence (recorded as intensity per μM

of PC*) for 4 mol % PC* in LUVs of different lipid composition and phase state: PC, and PC/SM 2:1 mol/mol – in liquid disordered (l_d) phase; SM, which undergoes a transition between gel and liquid disordered (l_d) phase in the temperature range 35^o to 40^{o}C; SM/Chol 1:1 mol/mol presenting only liquid ordered (l_o) phase over the entire temperature range [8]. Only heating curves are presented as the cooling curves can be superimposed on the heating curves. It can be seen that the NBD intensity in LUV of PC is similar to that of LUV consisting of PC/SM 2:1. Thus, the difference in lipid composition of the LUV does not have a significant effect on the fluorescence intensity for these two l_d-phase membranes. Furthermore, the main l_β to l_d phase transition of egg SM is clearly detected at about 38^{o}C [26]. Fluorescence is very low for SM/Chol 1:1 LUV (only l_o phase membranes). The NBD specific fluorescence intensity is higher in liquid disordered (l_d) phases than in gel or in liquid ordered (l_o) phases. A possible explanation might be that the NBD moiety at the extremity of the kinked PC hydrocarbon chain is expelled more efficiently from the hydrophobic region of bilayer when the latter is highly structured (as it is the case in gel or l_o phase) thereby exposing NBD closer to the water interface. It should be noted that the quantum yield of NBD fluorophore is close to zero in water and increases with decreasing polarity of the environment [27]. With increasing temperature the bilayer becomes more disordered and is reflected as a monotonous decrease of fluorescence intensity (see [16]). This disorder presumably results in greater penetration of water molecules into disordered lipid bilayer, thereby increasing the polarity in the NBD environment with a consequent decrease in NBD fluorescence intensity. It is noteworthy that increasing the temperature of bilayers in an ordered physical state (gel for SM below T_M, or liquid ordered l_o phase for SM/Chol 1:1 for temperatures between 4°C and 70°C) does not change fluorescence intensity suggesting that ordered phases are relatively stable structures.

3.1.2. Concentration dependent quenchingof C12NBD-PC in homogeneous membranes

The specific fluorescence of C12NBD-PC in PC/SM 2:1 mol/mol LUVs plotted as a function of the mean concentration (C_M) of fluorophore at different temperatures between 4°C and 70°C is shown in Fig. 2. The concentration dependent self-quenching of C12NBD-PC is clearly seen, for example, at 20°C where the specific fluorescence intensity for 3 mol% probe is 3-fold greater than for a probe concentration of 9 mol%. The effect is less pronounced at higher temperatures. The experimental points are well fitted with exponential curves [14]:

$I = I_0.e^{-\alpha C}$, with $C = C_M = C_L$ for homogeneous lipid membranes (1)

In Fig. 2, I and I_0 are in a. u. per μM of PC*, and C is in mol %. The corresponding values for I_0 and α are given in Table 1. Thus, the emitted specific fluorescence intensity, I, can serve as a "reporter" of C:

$C(I) = 1/\alpha \ln (I_0/I)$, (2)

3.2. C12NBD-PC fluorescence in heterogeneous lipid membranes

3.2.1. Experimental

If we consider a PC* containing vesicle of lipid composition in which there is (within a designated range of temperature) formation of l_o-phase domains then provided the temperature is above the miscibility transition temperature, the membrane is homogeneous and the mean molar membrane concentration of PC* in the membrane, C_M, is equal to the local membrane concentration, C_L, of the probe at any particular location in the membrane. The same assumption should hold for the specific fluorescence intensity. As temperature decreases below the miscibility temperature l_o-phase domains form spontaneously as illustrated in GUVs in Fig. 3A (A1 →A2). Lowering the temperature leads to a further increase of the domain size, Fig. 3A (A2 →A3 →A4). This means that the membrane fraction in l_o-phase (Φ_o), increases and the membrane fraction in l_d-phase (Φ_d)

correspondinglydecreases, $\Phi_o + \Phi_d = 1$. On the other hand, at a given temperature, higher proportions of cholesterol induce larger Φ_o, see Fig. 3B for a qualitative illustration (temperature 20°C and cholesterol content 0, 10, 20, and 35 mol%). Indeed it can be seen that Φ_o is greater than Φ_d in GUV comprised of PC/SM/Chol 41:20:35, (Fig. 3B4). The coexistence of the two liquid phases is observed as bright domains of l_d-phase within the dark l_o-phase membrane rather than dark lo-phase domains within the bright l_d-phase membrane, ie the indispersion l_d/l_o phases occurs in the GUV PC/SM/Chol 2:1:*X* membranes for cholesterol content *X* between 20 and 35 mol%.

Phase separation of lo-phase in LUV preparations has been examined by the fluorescent method during temperature scans and a summary of the results is presented in Fig. 4. It can be seen that increasing the proportion of l_o-phase in the cholesterol containing LUVs results in a corresponding decrease of membrane space into which PC* may partition. An inflection is observed at about 39°C which may correspond to the miscibility transition and formation of l_o-phase domains upon cooling the sample. Below the miscibility transition the local concentration, C_L, of fluorophore in the l_d-phase will be higher than the mean concentration, C_M. In this interpretation the specific fluorescence from heterogeneous LUV (PC/SM/Chol 2:1:*X*) will be lower compared with that of homogeneous LUV (PC/SM 2:1) measured at the same temperature. Moreover, the higher the molar fraction of cholesterol in the bilayer (*X* between 10 - 45 mol %) the lower is the specific fluorescence intensity, I, bought about by the concentration dependent self-quenching of the probe.

It is noteworthy that the self quenching effect is not linear with respect to increasing cholesterol content. For example, I is similar for 40 and 45 mol % of cholesterol, suggesting that the amount of SM available to complex with cholesterol is limiting in LUVs containing such high proportions of cholesterol.

An analysis of the fluorescence data has been performed to provide an estimate of the fraction of the membrane in l_o-phase, Φ_o. The difference between the specific fluorescence intensities, I, measured for l_o/l_d-phases LUVs has been used and, for LUVs only in l_d-phase, measurements were recorded at the same temperature. It is assumed that, as long as the probe remains imbedded in l_d-phase lipid bilayer, the changes induced in NBD fluorescence due to its environment are negligible. Indeed, the l_d-phase observed in bilayers where there is coexistence of l_d/l_o-phases does not have a defined phospholipid composition as SM will preferentially complex with cholesterol leaving a PC-rich l_d-phase. Nevertheless, as seen in Fig. 1 the specific fluorescence intensity changes in LUV bilayers are small where the SM/PC molar ratio is between 0 and 1:2. The specific fluorescence intensity of C12NBD-PC can therefore be used to provide a quantitative estimate of the membrane fraction of LUVs occupied by l_o phase as a function of temperature and PC/SM/Chol 2:1:*X* molar ratio. A theoretical analysis is described below.

3.2.2. Theoretical

If we consider a homogeneous lipid vesicle of surface S, with a mean area per molecule A, containing a given number, N, of PC* molecules. The mean molar concentration of PC* (the number of PC* probe molecules per molecule) is $C_M = N / N_T$. (The total number of molecules in the vesicle, N_T, being $N_T = S / A$). The presence of l_o phase domains in the vesicle bilayer restricts the membrane space available for the PC* molecules which partition preferentially into the l_d phase, as explained in the previous section for a heterogeneous vesicle. This corresponds to a higher local PC* concentration, C_L, in the l_d phase. The local molar concentration of PC* will be $C_L = N_d / N_{dT}$, where N_d is the number of PC* molecules in the l_d phase and N_{dT} the total number of molecules in the l_d phase domain. $N_{dT} = S_d / A_d$

where S_d / A_d are respectively the membrane surface and the mean area par molecule in l_d phase. In practice, N_d is lower than N, due to non-ideal exclusion of PC* molecules from the l_o phase. Indeed, certain number of PC* molecules would be expected to be present in the l_o phase.

For a vesicle containing N molecules of PC* it follows that:

$N = C_M . (S / A)$, when $C_L = C_M$ for the case of a homogeneous membrane, (1)

$N_d = C_L . (S_d / A_d)$, when $C_L \neq C_M$ for the case of l_o / l_d co-existence in the membrane, (2)

Dividing (2) by (1) one obtains:

$S_d = S . (C_M / C_L) . (N_d / N) . (A_d / A)$, (3)

Here (N_d / N) is ≤ 1, and $(A_d / A) \cong 1$, therefore, $S_d \leq S . (C_M / C_L)$.

$S_d = S . (C_M / C_L)$ represents an upper limit for the membrane surface occupied by l_d phase. Accordingly, an upper limit for the membrane fraction, $\Phi_d = S_d / S$, occupied by l_d phase is defined and a lower limit obtainedfor the membrane fraction, $\Phi_o = S_o / S$, occupied by l_o phase domains respectively:

$\Phi_d = (C_M / C_L) . (N_d / N) . (A_d / A) \leq (C_M / C_L)$, (4)

$\Phi_o = 1 - (C_M / C_L) . (N_d / N) . (A_d / A) \geq 1 - (C_M / C_L)$, (5)

$(\Phi_o + \Phi_d = 1)$

When there is ideal partitioning of PC* in the l_d-phase domains (all PC* molecules are restricted in the l_d-phase domains, i.e., $N_d / N = 1$), and assuming $A_d = A$, one gets:

$\Phi_d = (C_M / C_L)$, (6)

$\Phi_o = 1 - (C_M / C_L)$, (7)

It can be seen that the experimental measurement of the local concentration of PC*, C_L, in the l_d phase permits the estimation of the membrane fractions, Φ_o and Φ_d, occupied by l_o or l_d phase, respectively.

3.2.3. Quantitative estimation of membrane fractions Φ_o and Φ_d, occupied by l_o and l_d phase using calibration curves

The experimental curves presented in Fig. 2 are calibration curves for the specific fluorescence of C12NBD-PC in LUV membrane of PC/SM 2:1 mol/mol, for probe concentrations between 3 and 9 mol% over the temperature range 4° to 70°C.

Let us now consider the curves in Fig. 4 in this context. All LUV samples contain 4 mol % PC* mean concentration, C_M. Above the miscibility temperature, all the samples have $C_L = C_M = C$. Below the miscibility temperature, cholesterol containing LUVs show evidence of coexisting l_o/l_d membrane domains. In the latter case, the global specific intensity of a sample, I, will be the sum of the intensities originating from the different types of membrane domains bearing different PC* local concentrations and specific fluorescence intensities. If it is assumed there is ideal partitioning of PC* in the l_d phase all the fluorescence will originate from l_d-phase domains of the membrane, $S_d = \Phi_d$. S, with PC* concentration C_L higher than 4 mol %.

According to the theoretical model developed the same specific fluorescence intensity I would be emitted by homogeneous l_d-phase LUV sample, see eqs. (1, 2), with PC* mean concentration $C_M = C_L$:

$$I = I_0.e^{-\alpha C_L}, \quad \text{or,} \quad C_L(I) = 1/\alpha \ln(I_0/I), \qquad \text{where } C_L > 4 \text{ mol \%} \tag{8}$$

where α and I_0 are given in Table 1.

A graphic method to obtain $C_L = C_L(I)$ is exemplified in Fig. 2 (see dashed lines) for LUVs consisting of PC/SM, 2:1 and 20 mol% cholesterol at 4 °C. For the measured I = 37.16

a.u./μM, and, from the calibration curve (i.e., from eq. (8) at 4 °C), the corresponding C_L (I) = 4.577 mol % is obtained**.** That gives, using eqs. (6, 7): $\Phi_d = 0.874$, and $\Phi_o = 0.126$. The experimental error for the estimate of membrane fractions was about ± 0.035.

The local PC* concentration in l_d phase resulting from the creation of lo-phase domains can also be evaluated in another way. Since the experimentally measured specific fluorescence intensity of homogeneous LUVs depends on PC* concentration, C_M, then for a measured specific fluorescence, I, of a LUV of defined composition the corresponding local concentration $C_L = C_L$ (I) can be derived. In the example where $C_M = 4$ mole%:

$$C_L (I) / C_{4\%} = \ln (I_0/I) / \ln (I_0/ I_{4\%}), \qquad (9)$$

In fact both eq. (9) and eq. (8) give $C_L = C_L$ (I), and can be used to obtain Φ_d and Φ_o from (6) and (7).

Using (9) permits the expression of the phase membrane fraction Φ_o as a function of cholesterol content assuming $\Phi_o = 0$ for the sample with 0% cholesterol. For example, applying (9) for the graphic case in Fig. 2 one obtains:

I = 37.17 a.u./μM; $I_{4\%}$ = 42.6 a.u./μM; $C_L / C_{4\%} = 1.19$; C_L = 4.75 mol %; $\Phi_d = 0.842$; $\Phi_o = 0.158$.

By way of illustration, and assuming ideal partitioning of PC* in the l_d-phase domains, Fig. 5A shows the fraction of l_o-phase membrane, Φ_o (as Φ_o x 100 %), for PC/SM/Chol 2:1:*X* LUVs containing between 0 and 45 mol% cholesterol, at 4, 20 and 37 °C. Φ_o calculated using (7) with C_L(I) from (9).

4. Discussion

This study demonstrates in the case of LUV that fluorescence emission from a single fluorescent probe partitioning between liquid domains in the membrane can be fit to a simple

model. The model can be applied to obtain a quantitative estimation of membrane fractions characterizing the co-existence of the two liquid phases, the liquid disordered (l_d) phase, and the liquid ordered (l_o). The approach is similar to that reported by Brown and coworkers [14] who explained, in quantitative, terms the observed changes in relative intensity and life-time properties of the NBD probe in lipid membranes by the self-quenching of this probe at high local concentrations due to energy migration and trap-site formation processes. The model membranes chosen for their study were small unilamellar vesicles (SUV) and monolayers of egg PC containing different concentrations of NBD-PE and were intended to provide a homogeneous distribution of the fluorophore. They noted that effective or "local" concentrations of fluorophore in heterogeneous membrane structures could be derived from the fluorescence intensity measurements.

In the present work a heterogeneous membrane structure, LUV model membrane consisting of a ternary lipid mixture of PC, SM and cholesterol was used for the investigation. In the quantitative estimation of the fraction of the membrane in l_o phase, Φ_o, presented in Fig. 5A the probe is assumed to partition ideally into the l_d-phase domains and be excluded completely from the l_o-phase domains. Accordingly, a lower limit for the membrane fraction, $\Phi_o = S_o / S$, occupied by l_o phase domains is obtained. In practice, the PC* molecules partition in different molar concentrations between the two phases but with a preference for the l_d-phase membrane. This is seen by direct observation of fluorescence from GUV (Fig. 3). Therefore, the actual membrane l_o fractions, Φ_o are somewhat greater than those shown in Fig. 5A. How much greater depends on the ratio between the molar concentrations of PC* in the co-existing l_o and l_d phases, i.e., on the partition coefficient k_p. A systematic study of partitioning of amphiphiles between coexisting ordered and disordered phases in two-phase

lipid bilayer membranes has been reported [16] in which a partition coefficient $k_p \cong 0.2$ was obtained for different NBD-labeled lipids in a similar membrane system.

Taking into account $k_p \neq 0$, eqs. (6) and (7) are replaced respectively by:

$$\Phi_d = [(C_M / C_L) - k_p] / (1 - k_p), \quad (10)$$

$$\Phi_o = [1 - (C_M / C_L)] / (1 - k_p), \quad (11)$$

Assuming $k_p \cong 0.2$ for C12NBD-PC, and, e.g., for LUVs (PC/SM) / Chol (2:1) : 20 mol%, at 4 °C, one obtains $\Phi_d = 0.842$ and $\Phi_o = 0.158$, instead of $\Phi_d = 0.874$ and $\Phi_o = 0.126$, obtained above in the case of ideal partitioning (when $k_p = 0$), and experimental error about ± 0.035.

One of the possible applications of the present work is in the characterization of detergent-resistant membrane preparations isolated from membranes that contain putative domains of l_o-phase. Furthermore, the quantitative approach to estimation of domain fractions may be useful to establish the relationship between formation, stability and coexistence of liquid lamellar phases, l_o and l_d in model membranes and, in turn, on the dynamics of lipid rafts in biological membranes.

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Figure Legends:

Figure 1. Temperature dependence of C12NBD-PC (PC*) specific fluorescence intensity maximum I (in a.u. per μM of PC*) for 4 mol % PC* containing LUVs of different lipid compositions and phase states: PC, and PC/SM 2:1 mol/mol in liquid disordered (l_d) phase; SM in which there is the main phase transition between gel and liquid disordered (l_d) phase at about 38^{o}C [26]; SM/Chol 1:1 mol/mol forming a liquid ordered (l_o) phase over the range of temperature examined. The data was recorded from heating curves and no significant hysteresis was observed in the corresponding cooling curves.

Figure 2. Specific fluorescence of C12NBD-PC in homogeneous membrane LUVs of PC/SM 2:1 mol/mol as a function of mean concentration (C_M) of fluorophore at different temperatures. A close fit of the data to exponential functions is observed [14]. The intersecting dashed lines represent the value of an unknown local concentration C_L corresponding to an experimentally obtained specific fluorescence I. The example shown is for LUVs comprised of PC/SM, 2:1 containing 20 mol% cholesterol at 4 °C: with a value I = 37.16 a.u./μM, then $C_L = C_M$ (I) = 4.577 mol %, from eq. (8).

Figure 3. Images of GUVs illustrating the effect of the temperature (A1-A4) or cholesterol content (B1-B4) on the membrane fraction, Φ_o, of l_o phase present in the vesicle membranes. A1-A4, GUV formed from PC/SM/Chol/PC* 51:25:20:4 show formation of lo-phase domains and as the temperature is lowered through the miscibility temperature. A1, 47 °C, homogeneous membrane; A2, 29 °C, the l_o-phase domains have formed in the membrane, and are seen as dark spots; A3, 26 °C, and A4, 23 °C, the fraction of l_o-phase membrane (Φ_o) increases, and the membrane fraction in l_d-phase (Φ_d) decreases upon cooling. B1-B4, GUV formed from PC/SM, 2:1, containing 4 mole% PC* and, B1, 0 mol%; B2,10 mol%; B3, 20

mol%; B4, 35 mol% cholesterol recorded at 20°C. Note that Φ_o is greater than Φ_d in GUVs containing the highest proportion of cholesterol (B4).

Figure 4. Temperature dependence of C12NBD-PC (PC*) specific fluorescence intensity maximum I (in a.u. per μM of PC*) for 4 mol % PC* in LUVs containing different proportions of cholesterol, as indicated in the figure.

Figure 5. The l_o-phase membrane fraction, Φ_o (as Φ_o x 100 %), for PC/SM/Chol 2:1:*X* LUVs containing between 0 and 45 mol% cholesterol, at 4, 20 and 37 °C. Φ_o was calculated using (7) with $C_L(I)$ from (9). (A) The case of ideal partitioning of PC* in the l_d-phase domains ($k_p = 0$); (B) A real partitioning case: PC* molecules partition at different molar concentrations between l_o and l_d phases, favoring the l_d phase membrane with $k_p = 0.2$, eq. (11). The absolute experimental error for the membrane fractions estimation (in %) was about ± 3.5 %.

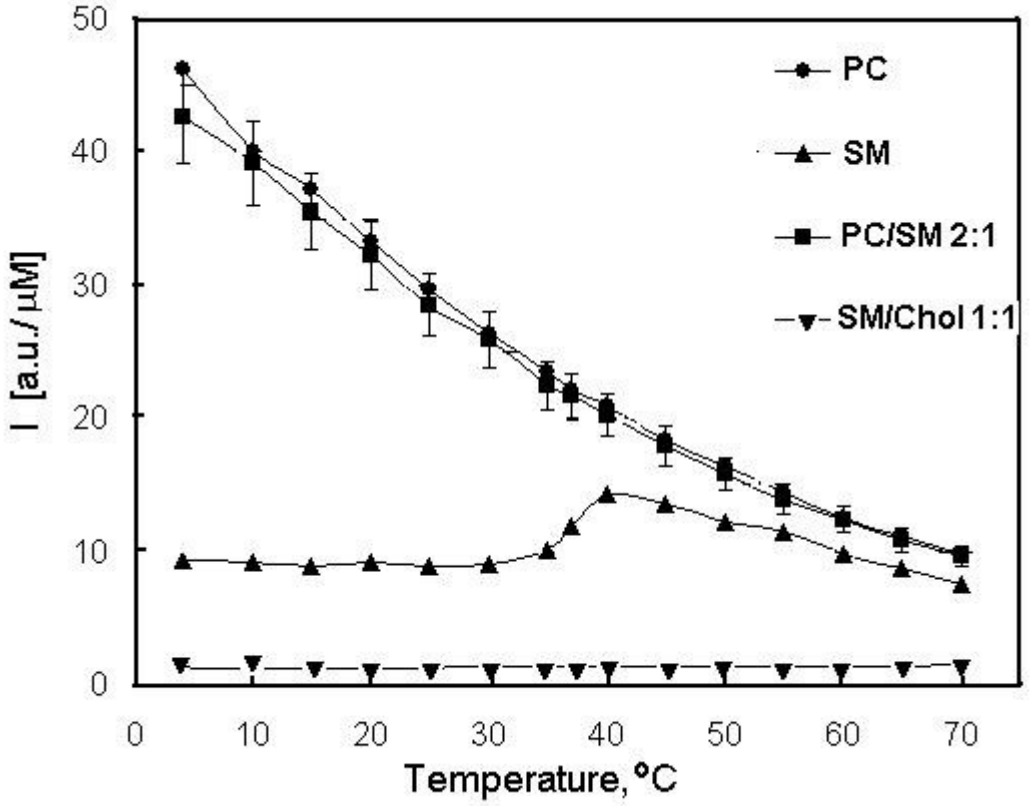

50
40
30
20
10
0
I [a.u./μM]
0
10
20
30
40
50
60
70
Temperature, °C
PC
SM
PC/SM 2:1
SM/Chol 1:1

Fig. 1

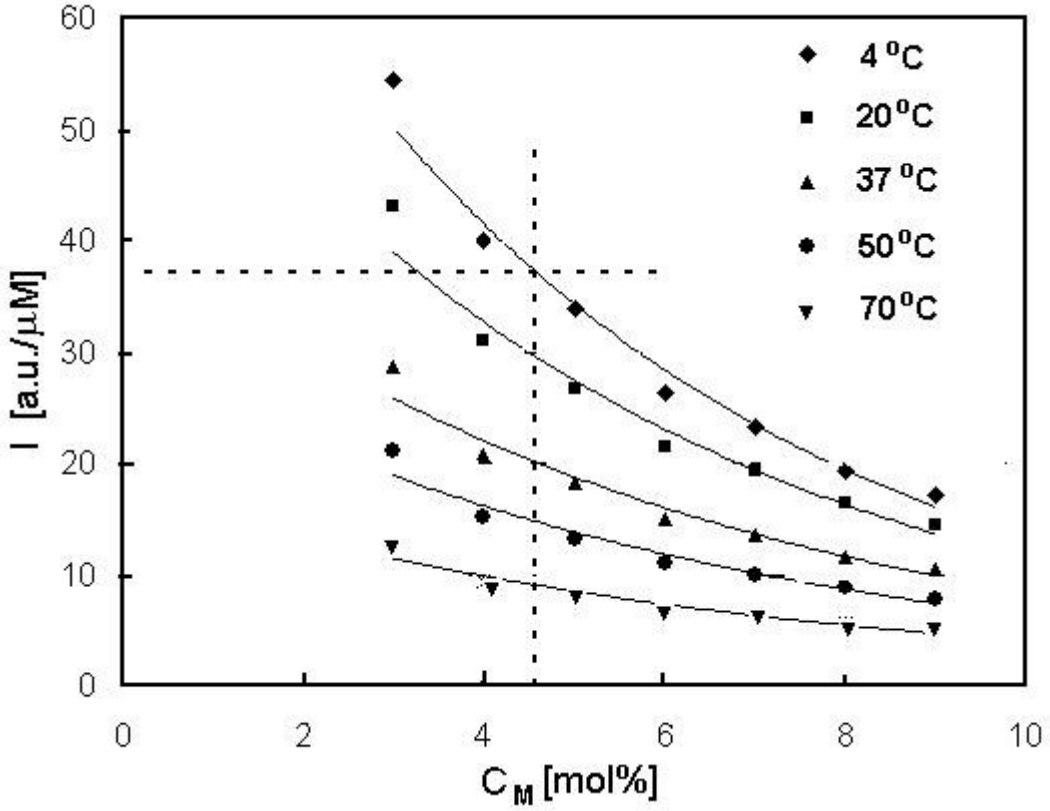
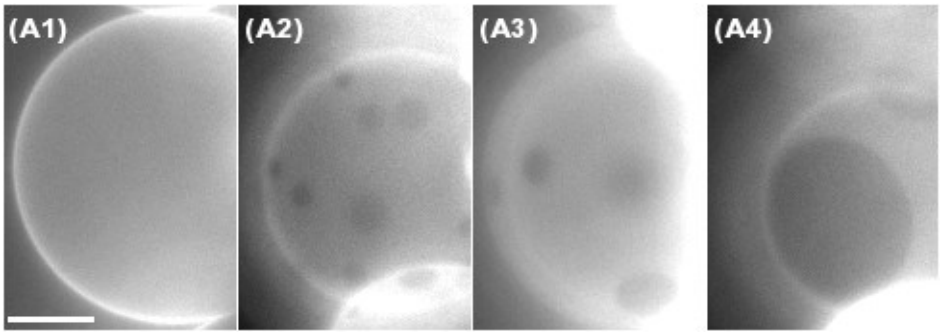


Fig. 2

A



B

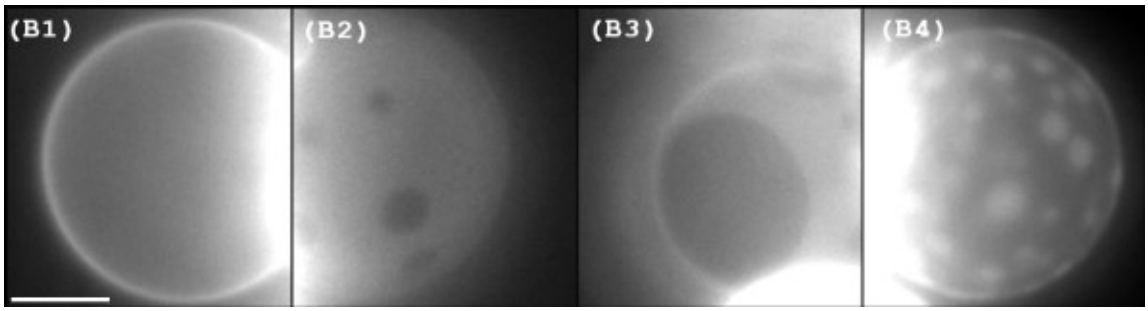


Fig. 3

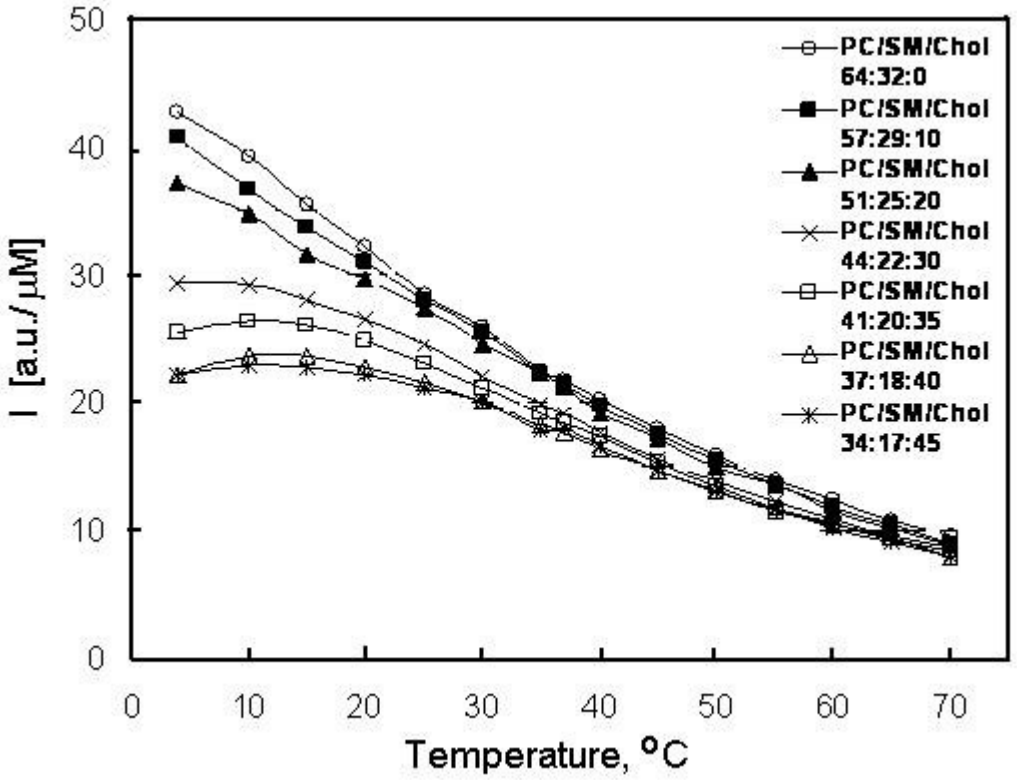

I [a.u./μM]
Temperature, °C
PC/SM/Chol 64:32:0
PC/SM/Chol 57:29:10
PC/SM/Chol 51:25:20
PC/SM/Chol 44:22:30
PC/SM/Chol 41:20:35
PC/SM/Chol 37:18:40
PC/SM/Chol 34:17:45

Fig. 4

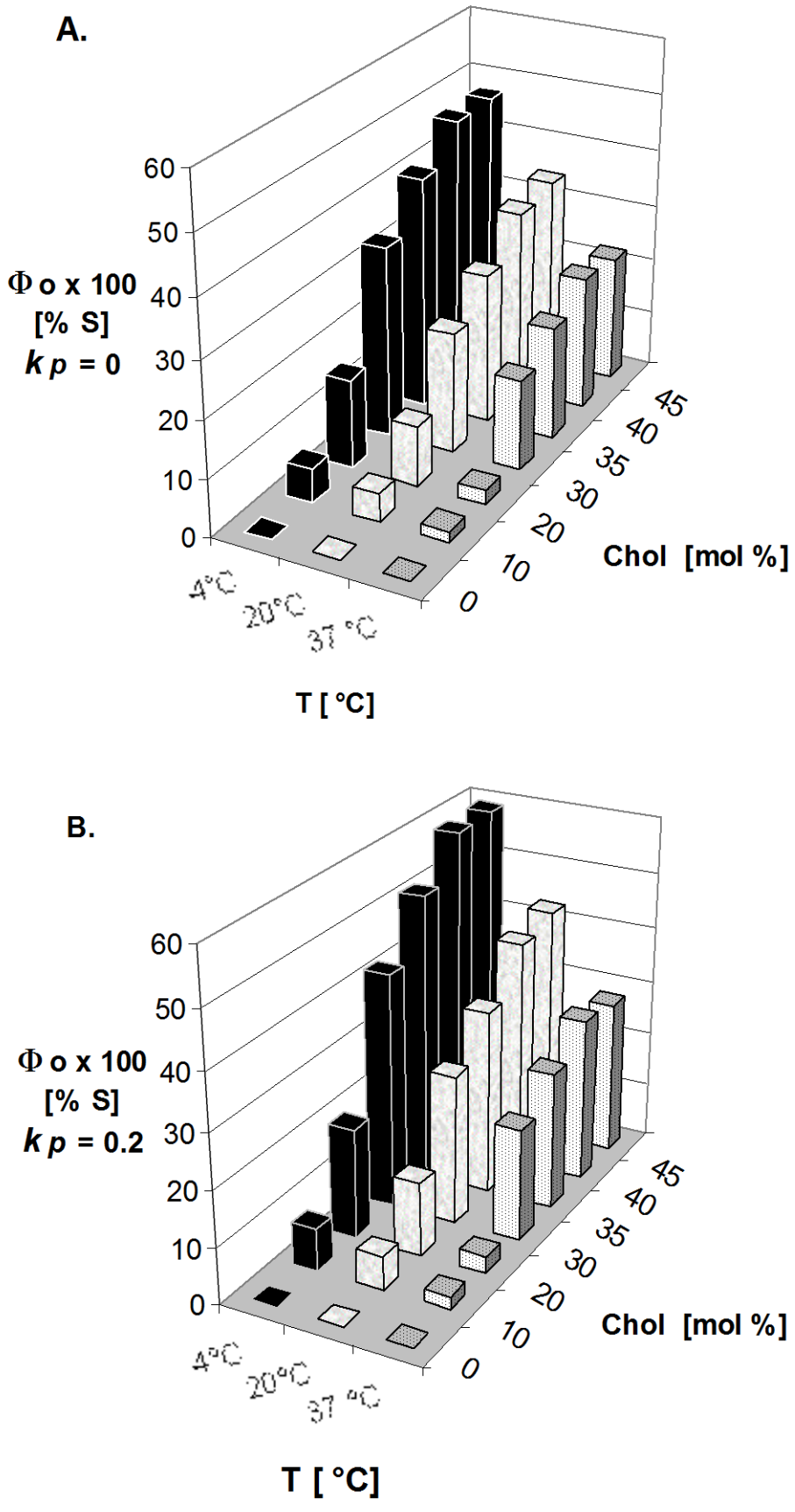

A.
Φ o x 100
[% S]
k p = 0
60
50
40
30
20
10
0
4°C
20°C
37 °C
T [°C]
0
10
20
30
35
40
45
Chol [mol %]
B.
Φ o x 100
[% S]
k p = 0.2
60
50
40
30
20
10
0
4°C
20°C
37 °C
T [°C]
0
10
20
30
35
40
45
Chol [mol %]

Fig. 5

Table 1

C12NBD-PC fluorescence in homogeneous lipid membranes consisting of PC/SM 2:1 mol/mol.

T [^{o}C]	I_0 [a.u. / μM of PC*]	α [1 / mol%]	R^2
4	**88.705**	**0.1901**	**0.9824**
20	**65.672**	**0.1746**	**0.9743**
37	**41.695**	**0.16**	**0.9697**
50	**30.058**	**0.1558**	**0.9612**
70	**17.769**	**0.1481**	**0.9497**

Parameters of the calibration curves (exponential fits), $\mathbf{I\,(C) = I_0.e^{-\alpha C}}$ of experimental data shown in Fig. 2 for the specific fluorescence of C12NBD-PC in homogeneous membrane LUVs of PC/SM, 2:1 mol/mol as a function of fluorophore mean concentration, $C_M = C_L = C$ [mol %], at different temperatures. R^2 is the fit correlation coefficient. Thus, the emitted fluorescence intensity, I, is the designator for C in the case of $C = C\,(I) = 1/\alpha \ln (I_0/I)$.