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Biochimica et Biophysica Acta 1768 (2007) 2914–2922

Biochimica et Biophysica Acta BBA

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Bilayer polarity and its thermal dependency in the ℓ_o and ℓ_d phases of binary phosphatidylcholine/cholesterol mixtures

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Received 19 June 2007; received in revised form 2 August 2007; accepted 7 August 2007
Available online 24 August 2007

Abstract

Diverse variations in membrane properties are observed in binary phosphatidylcholine/cholesterol mixtures. These mixtures are nonideal, displaying single or phase coexistence, depending on chemical composition and other thermodynamic parameters. When compared with pure phospholipid bilayers, there are changes in water permeability, bilayer thickness and thermomechanical properties, molecular packing and conformational freedom of phospholipid acyl chains, in internal dipolar potential and in lipid lateral diffusion. Based on the phase diagrams for DMPC/cholesterol and DPPC/cholesterol, we compare the equivalent polarity of pure bilayers with specific compositions of these mixtures, by using the Py empirical scale of polarity. Besides the contrast between pure and mixed lipid bilayers, we find that liquid-ordered (ℓ_o) and liquid-disordered (ℓ_d) phases display significantly different polarities. Moreover, in the ℓ_o phase, the polarities of bilayers and their thermal dependences vary with the chemical composition, showing noteworthy differences for cholesterol proportions at 35, 40, and 45 mol%. At 20 °C, for DMPC/cholesterol at 35 and 45 mol%, the equivalent dielectric constants are 21.8 and 23.8, respectively. Additionally, we illustrate potential implications of polarity in various membrane-based processes and reactions, proposing that for cholesterol containing bilayers, it may also go along with the occurrence of lateral heterogeneity in biological membranes.

Keywords: Pyrene; Fluorescence; Ham effect; Py polarity scale; Lipid bilayer; Phospholipid/Cholesterol mixture

1. Introduction

Hydrated pure phospholipid bilayers exist almost in either a highly ordered gel phase (L_β or s_o) or a liquid–crystalline (L_α) depending on whether the temperature is below or above the main phase transition temperature (T_m). An homogeneous fluid phospholipid bilayer in the L_α phase is a complex environment, being a highly organized, cooperative and flexible molecular assembly, largely confined to two dimensions, and exhibiting a considerable degree of anisotropy along the transversal axis to the bilayer surface [1]. This results nearly from the "structure" of a fluid bilayer which is defined operationally as the time-averaged spatial distribution of the component chemical groups of phospholipids, projected along the referred axis [2]. From the wide thermal distribution, different regions can be inferred, with the hydrophobic core consisting of only components from the alkyl chains. Head group components, phosphate and choline, are found within the interface regions, along with waters of hydration. Carbonyls and contiguous methylene groups from the alkyl chains, incorporating some water molecules, are also associated with this region. Accordingly, while the central section of the bilayer is nearly isotropic, the upper portions, only a few angstroms away symmetrically toward both interfaces, are highly ordered [3]. As a result, properties such as the orientational and positional ordering of the alkyl chains [3], polarity [4–6] and extent of water penetration [2,7–9], vary in depth-dependent manner relatively to the interface, e.g., the so-called fluidity and polarity gradients. The polarity profile is essentially similar to the bilayer hydration profile, since it is derived from diverse experimental data (e.g., neutron and X-ray diffraction, EPR, NMR, IR and fluorescence spectroscopies) on water penetration into the hydrocarbon core of the bilayer. Still, the

Abbreviations: DMPC, 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; L_α, liquid–crystalline phase; ℓ_d, liquid-disordered phase; ℓ_o, liquid-ordered phase; MLV, multilamellar vesicles; S.D., standard deviation

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doi:10.1016/j.bbamem.2007.08.012

polarity at a specific depth of the bilayer should depend on the local composition in water, in the presence of polar and apolar groups of constituting lipids, and on their overall relative dynamics [10].

Mixed model membranes containing high- and low-T_m phospholipids can contain coexisting s_o and liquid-disordered (ℓ_d) phases [1], but an intermediate phase, called liquid-ordered (ℓ_o) forms in binary mixtures of phospholipids and cholesterol [11,12]. Cholesterol is the single most abundant lipid species in mammalian cell membranes and roughly 90% of all cellular cholesterol resides in the plasma membrane, where it composes between 25 and 50% of the lipids, depending on the cell type [13]. Cholesterol is probably the most important lipid when it comes to controlling the size and area fraction of ℓ_o phase domains, in model membranes [14]. Binary mixtures of cholesterol and phospholipids in bilayers are nonideal, displaying single or phase coexistence, depending on chemical composition and on other thermodynamic parameters, e.g., temperature or pressure [15]. Additionally, there is a large diversity of fluid lipid bilayer properties that change upon mixing cholesterol into pure phospholipid membranes, such as reduction of water permeability [16], reduction by a factor of about 2–3 in the lipid lateral diffusion [17,18], higher conformational ordering of aliphatic chains of phospholipids [3,19], which influences the modulation of the lateral pressure in depth–manner [20] and thermomechanical and elasticity properties [21,22], as well as increase in bilayer thickness [23], and a general increase in the internal dipole potential [24]. Much of these observations are founded essentially in the condensing effect of cholesterol on phosphatidylcholines, and therefore a phosphatidylcholine/cholesterol mixture occupies a smaller area than that expected from the sum of the molecular areas of both constituents [15]. Besides, the influence of cholesterol content in lipid bilayers polarity profile has also been accessed until recently, by using mostly ESR and EPR techniques [25–27]. In brief, these authors established the steepest sigmoidal-like transmembrane polarity profile upon the addition of cholesterol to phospholipid bilayers, due to augmentation of polarity in the interface and ordered methylene portion, in parallel with a decrease of polarity in the central section.

We describe herein the equivalent polarity of pure phospholipid bilayers of 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC), as well as of binary mixtures of DMPC/cholesterol and DPPC/cholesterol in the ℓ_o and ℓ_d phases, probed by means of the Ham effect using the pyrene fluorophore [28]. The first approaches to estimate the polarity of lipid bilayers by the pyrene fluorescence were undertaken around the 1980s [29–33] and until now it has been regularly and successfully used in probing microemulsions, ionic liquids, supercritical fluids, surfaces, colloids and polymers, but much more seldom in biological systems such as proteins [34] and lipid bilayers [35]. It is common sense that polarity plays a major role in many physical, chemical, biochemical and biological phenomena. Nevertheless, the term "polarity" (of a solvent or of a microenvironment within an amphiphilic aggregate) appears to be used loosely as one single parameter, to express the complex interplay of all types of solute–solvent interactions. This way, a quantitative evaluation of polarity involves linear solvation energy relationships describing the properties of the solute molecule (dipole moment, polarizability, transition moment, hydrogen bonding capability) and of the solvent molecule (dielectric constant, refractive index, hydrogen bonding capability) [36]. For pyrene, being a nonpolar polyaromatic hydrocarbon, the favorable manner of interaction with its local medium is the dipolarity/polarizability mode. The I_1/I_3 ratio of pyrene (quotient of the first and third vibronic bands intensities, in the fluorescence spectrum) has an extreme sensitivity on the polarity of an environment (I_1 corresponds to a band assigned to a symmetry forbidden transition which depends on the medium polarity, while I_3 corresponds to a band of an allowed transition which is practically insensitive to polarity) [37,38]. Dong and Winnik [39,40] have established that the I_1/I_3 ratio of pyrene correlates exceptionally with the Kamlet-Taft [41] solvatochromic parameter π^* (dipolarity/polarizability) for bulk liquids, introducing the Py empirical scale of polarity. They noticed that the correlation is dependent on the type of liquids, e.g., when dividing solvents by classes (aprotic aliphatics, protic aliphatics, aprotic aromatics), each one gives excellent correlation between the Py scale and the π^* scale. Accordingly, the Py empirical scale appears to be relatively insensitive to the hydrogen bonding ability of protic solvents, being an independent and robust descriptor of polarity of the local media surrounding a pyrene molecule in the excited state, through the static dielectric constant.

Here we give a demonstration on an additional effect of cholesterol in the ℓ_o phase of phosphatidylcholine-based bilayers, revealing that in this phase, the equivalent polarity of the lipid bilayer and its thermal dependencies varies with the cholesterol proportion. We additionally discuss the potential implications of these effects in diverse membrane processes and reactions.

2. Materials and methods

2.1. Chemicals and solvents

The phospholipids DMPC and DPPC (purity higher than 99.9%) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), cholesterol was obtained from Sigma-Aldrich (>99.9%) and were used without further purification. Pyrene (≥99%, for fluorescence grade) was obtained from Fluka (Switzerland). MilliQ water was produced from double-distilled water, by a Millipore Simplicity 185 apparatus (at room temperature, electrical conductivity and pH were systematically 5.4×10^{-6} S m^{-1} and around 6.5–7, respectively). Alcohols (methanol, ethanol, 1-propanol, 2-butanol, 1-hexanol, and 1-octanol) used as standard homogeneous polar liquids and other organic solvents were of the highest purity grade from Merck and/or Riedel-deHaën (Germany).

2.2. Preparation of solutions and samples

Stock solutions of the phospholipids DMPC and DPPC, dissolved in a mixture chloroform:methanol 2:1 (v/v), and of cholesterol in chloroform, were prepared with a concentration of 50 mM and stored at −20 °C. A stock solution of pyrene, with a concentration of around 1 μM ($\varepsilon=5.5\times10^4$ M^{-1} cm^{-1}, at 334 nm [42]), was prepared in ethanol and stored at 6 °C. Small aliquots of this solution were transferred to test tubes, and the solvent was completely evaporated. The pyrene crystalline powder deposited in the bottom of the tubes was then diluted with the standard alcoholic solvents (methanol, ethanol, 1-propanol, 2-butanol, 1-hexanol, and 1-octanol). The absorbance of pyrene at

334 nm ($S_2 \leftarrow S_0$ electronic transition) was always lower than 0.02 for each sample (corresponding to a pyrene concentration below 0.36 μM).

2.3. Preparation of liposomes

Liposomes were prepared according to a modified protocol of the firmly established method revised by Szoka and Papahadjopoulos [43]. For MLV of pure DMPC or DPPC, pyrene was added, at probe to lipid molar ratio of 1:5000, and a final volume of roughly 5 ml was completed with mixture chloroform: methanol 2:1 (v/v). The solvent was evaporated first at a vacuum of 145 mbar during about 15 min, and subsequently at 3 mbar, not less than 1 h, to remove traces of solvent. All the samples were dried using a rotary evaporator Heidolph VV-micro with a vacuum pump Büchi V-500 (coupled to a Büchi V-800 digital control). The resulting lipidic film deposited in the flask walls was hydrated, for about 1 h, using MilliQ water (previously heated at the same temperature of the water bath) well above the main phase transition temperature of DMPC and DPPC, vortexing vigorously and regularly, to produce a suspension of MLV. The final phospholipid concentration in the liposome suspension was 0.5 mM. The total concentration of pyrene in the MLV suspension is determined by the established probe to lipid molar ratio (probe:lipid molar ratio of 1:5000), being therefore 0.1 μM. By using MilliQ water, we also discarded possible spurious and cumulative effects arising from the inorganic and/or organic ionic species ubiquitous to buffer solutions. Additionally, we ensured that there are no noticeable variations in the pH of MilliQ water, along all the duration of the entire spectroscopic measurements. For MLV composed of phospholipid/cholesterol mixtures, DMPC or DPPC and pyrene were dissolved in mixture chloroform:methanol 2:1 (v/v) and the organic solvent was evaporated under a 145-mbar vacuum, during about 15 min. Cholesterol was then added in the settled proportions and all the components were codissolved in chloroform. The samples were vigorously mixed, submitted to water-bath sonication, the solvent was then evaporated and the lipidic film was hydrated, using the same procedure as in the case of pure MLV. The molar proportions of cholesterol used were 5, 35, 40 and 45 mol%, and the final total lipidic concentration was also 0.5 mM.

2.4. Spectroscopic measurements

Absorption spectra were recorded at room temperature using a Shimadzu UV-2401 PC spectrophotometer (Shimadzu, Japan), and fluorescence emission spectra were performed on a Spex Fluoromax-3 spectrofluorimeter (Jobin Yvon – Horiba, France) equipped with a thermostated cell holder with magnetic stirring accessory (coupled to a refrigerated/heated circulator Julabo F12-ED — precision of 0.1 °C). All emission spectra were carried out in 1-cm quartz cuvettes for fluorescence, using λ_{exc}=334 nm, collecting the emission from 360 to 510 nm (increment of 1 nm), using 1-nm slit widths in excitation and emission (wavelength resolution of 1 nm), and corrected for nonlinear instrument response. The MLV suspensions were stirred continuously during the measurements. For all pyrene alcoholic solutions, the results are averaged over 5 independent scans, corrected for the Raman band in the emission spectra, and the respective standard deviation (±S.D.) are calculated accordingly. The spectra were collected incrementing the temperature in steps of 10 °C, and respecting a thermal stabilization time of 15 min between each measurement, in each series of heating scans. For pure phospholipid bilayers, data were collected incrementing the temperature in 2 °C, respecting a thermal stabilization period of 10 min between every measurement, in each series of heating scans. The same procedure was followed for the phosphatidylcholine/cholesterol mixtures, and all the results presented for MLV aqueous suspensions are the average±S.D., for at least 4 independent emission scans.

The use of the Py empirical polarity scale requires fluorescence spectra free of physical, chemical and instrumental artifacts that may lead to imprecise values of dielectric constant. As follows, the pyrene concentration in solutions and suspensions must be kept lower than 0.36 μM (Abs<0.02), to avoid for primary and secondary inner filter effects, as well as for pyrene excimer formation [42]. Additionally, the slit widths should be at most 1 nm and all scans of alcoholic solutions have to be corrected for blank emission, because the Raman band from the alcohols overlaps the first vibronic band of pyrene emission spectra at about 373 nm, leading to significantly higher polarity values [44]. Besides, it is also known that in alcoholic and aqueous solutions, pyrene emission spectra display subtle changes as function of the time and/or light intensity to which the sample is submitted [45], because pyrene is photochemically unstable and the appearance of photoproducts (mainly 1-hydroxypyrene) may influence the results. We precluded this possible nuisance by using a spectrofluorimeter equipped with a 150-W xenon lamp, a very narrow slit width (1 nm) in excitation, and limiting the irradiation time to less than 3 min, for each fluorescence scan. We also controlled rigorously the temperature of the measurements, in order to present the linear relationship in Fig. 1.

3. Results

3.1. Py polarity scale in alcoholic solvents

We measured the I_1/I_3 values for pyrene dissolved in six alcoholic solvents: methanol, ethanol, 1-propanol, 2-butanol, 1-hexanol, and 1-octanol. For these alcohols, a very good linear dependence (R=0.9985) of the I_1/I_3 ratio as a function of the static dielectric constants at 20 °C of each solvent [46] is seen in Fig. 1. The standard deviations (S.D.) associated to each experimental point are not shown in Fig. 1, because they are very low: S.D. is ±0.02 for methanol and ethanol, being ±0.01 for the remaining alcohols. In addition, the I_1/I_3 values are in perfect accordance with the fundamental studies on the polarity of homogeneous solvents quantified by means of the pyrene Ham effect [37,39,40]. Given that pyrene is essentially insoluble in water (solubility of about 6.5×10^{-7} M, at 25 °C [47]), but fairly soluble in aliphatic hydrocarbons (e.g., solubility in dodecane 7.4×10^{-2} M, at room temperature [48]), it is readily incorporated into the hydrophobic acyl chain region close to the glycerol moiety and first methylenic groups in phospholipid bilayers [49–52], probing this way the average polarity corresponding to its location inside the bilayer. Even if the incorporation of molecular pyrene is putatively not complete (from the experimental solubility values, one can estimate a very high value for the partition constant into the hydrophobic interior of bilayers, of about 10^5), the remaining free molecules in aqueous medium, besides being at very low concentration, have also a low down quantum yield in water [42], interfering this way

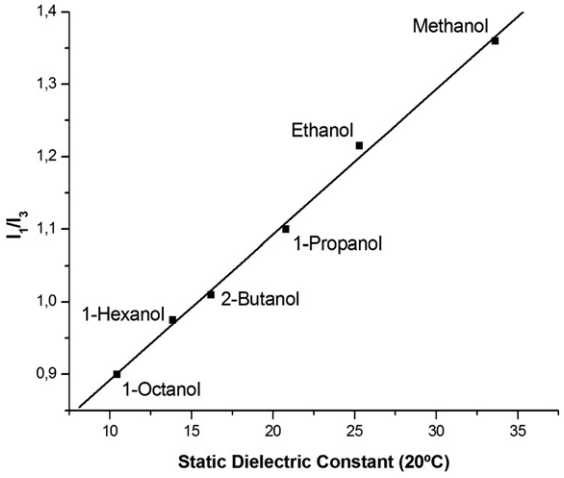

Fig. 1. Calibration plot indicating a very good linear dependence (R=0.9985; y=0.0201x+0.6916) for the ratio I_1/I_3, calculated from the respective pyrene emission intensities, as a function of the static dielectric constant of various alcohols at 20 °C, ranging between 0.9 for 1-octanol, until 33.6 for methanol.

insignificantly in the measurements of fluorescence intensity. Therefore, calibration plots such as that depicted in Fig. 1 can be used as a polarity index to estimate equivalent dielectric constants with pyrene incorporated within model membranes [31].

Also, the study of polarity of lipid bilayers composed by lipidic mixtures, at defined points of their respective phase diagrams, involves a wide range of temperatures, which confers a great importance to the determination of the correspondent variation of I_1/I_3 in homogeneous solvents. It is important, therefore, to determine how the values of I_1/I_3 in homogeneous alcoholic solutions vary with temperature. This way, since the dependence of the static dielectric constant of homogeneous solvents on temperature is known in detail [62], we evaluated the variation of I_1/I_3 as a function of temperature for each alcoholic solvent, within a defined temperature range, e.g., temperature varying from 11 °C until 51 °C. These results are depicted in Fig. 2, and they are in accordance with previous assessments [62,53,54], showing a decrease of I_1/I_3 values with the increase of temperature for more polar solvents (methanol and ethanol), and a relative insensibility to temperature for less polar alcohols (2-butanol and 1-hexanol). The variation of slopes varies directly with the polarity of the solvent, e.g., higher slope for methanol, and nearly absence of slope for 2-butanol and 1-hexanol. The highest values for S.D. were obtained for ethanol (at most ±0.03), and the lowest for 1-hexanol (±0.01, for all the measurements). The thermal variations observed for 2-butanol and 1-hexanol are equal, within the experimental error.

3.2. Polarity and its thermal variation in pure phospholipid bilayers

Based on the solvents and temperature dependences of the Py polarity scale in alcoholic solvents, we performed analogous studies in the model membrane systems, represented in Fig. 3A and B, for DMPC-based and DPPC-based bilayers matrixes,

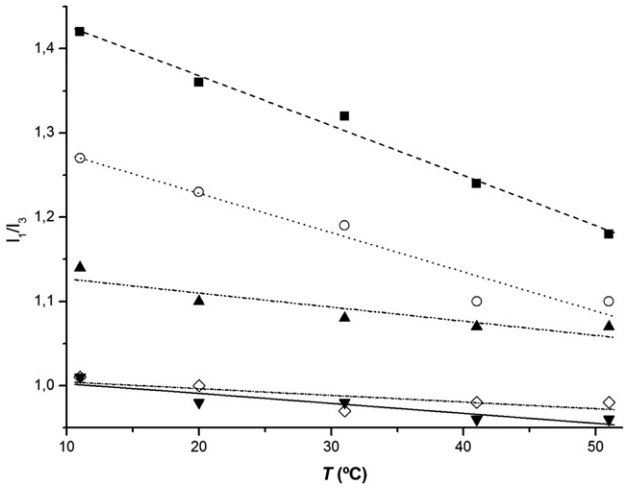

Fig. 2. Thermal dependence of the ratio I_1/I_3 of pyrene diluted in each alcoholic solvent: methanol (■), ethanol (○), 1-propanol (▲), 2-butanol (◇), and 1-hexanol (▼), as a function of temperature, from 11 °C until 51 °C. Within the temperature range analyzed, all the linear fittings are good. The thermal variations observed for 2-butanol and 1-hexanol are equal, within the experimental error.

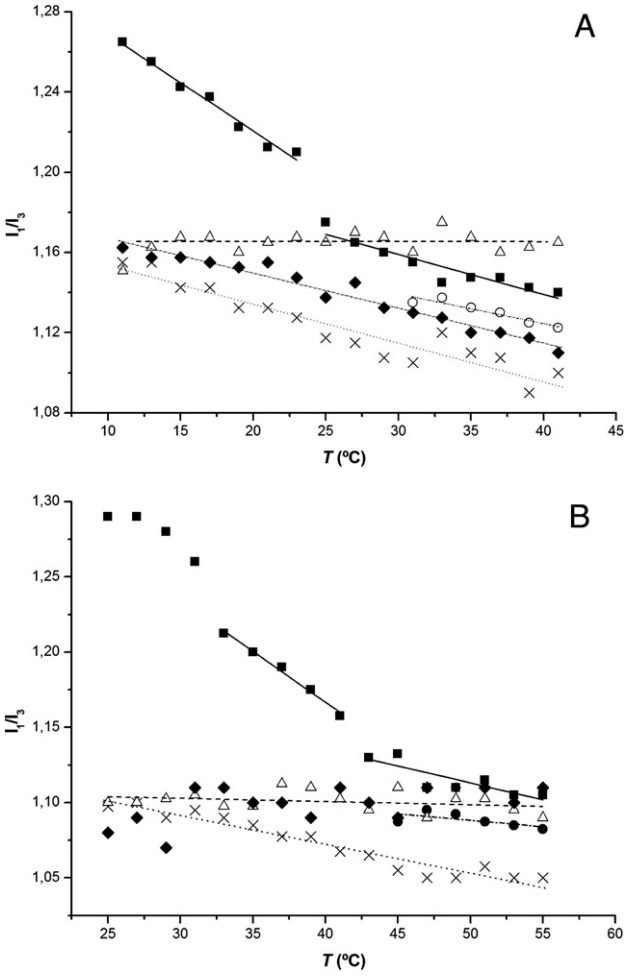

Fig. 3. Thermal dependence of the ratio I_1/I_3 as a function of temperature for: bilayers of pure DMPC and of DMPC/cholesterol mixtures (in panel A), ranging the temperature from 11 °C until 41 °C; (panel B) bilayers of pure DPPC and of DPPC/cholesterol mixtures, varying the temperature from 25 °C to 55 °C. In both panels, (■) states for the pure DMPC and DPPC bilayers, respectively; (○) for DMPC with 5 mol% of cholesterol; (●) for DPPC with 5 mol% of cholesterol; (×) for DMPC and DPPC with 35 mol% of cholesterol; (♦) for DMPC and DPPC with 40 mol% of cholesterol; (△) for DMPC and DPPC with 45 mol% of cholesterol. The various trend lines, each having a distinctive trace corresponding to every data set, are included with the purpose of eye guiding. In panel B, for the data points of DPPC with 40 and 45 mol% of cholesterol, only one trend line is presented, since the thermal dependence is equivalent in both cases, within the experimental error.

respectively. For DMPC containing lipid bilayers the temperature varied from 11 °C to 41 °C, and for the DPPC bilayers, temperature varied between 25 °C and 55 °C. In panels A and B, the points referring to pure DMPC and DPPC are both labeled by black squares (■). The S.D. associated to the experimental points are not shown in both panels, since they are very low: S.D. being typically ±0.02 and ±0.01, observing the value of ±0.03 in very few cases.

For pure phospholipid bilayers, the results are in accordance with previous studies using the pyrene Ham effect and with EPR methodologies which had put forward that the polarity in near-surface region in fluid bilayers (i.e., above the respective T_m) is comparable to that of ethanol at room temperature [25,31,33]. It is also shown that the I_1/I_3 values decreased with

the increase of temperature, and the pyrene Ham effect is in fact sensitive to the phase transition temperature of the bilayer from a gel phase ($P_{\beta'}$, in the case of phosphatidylcholines there is a rippled gel phase) to the liquid crystalline L_α phase for both DMPC and DPPC. These bilayers display significantly different polarities above and below their respective T_m (41.5 °C for DPPC [12], and 24.1 °C for DMPC [17]). Equivalent polarity is higher in the $P_{\beta'}$ phase, since above T_m there is an expansion in the phospholipid molecular area and lower hydrophobic thickness, due mainly to the increased conformational freedom of methylenic chains, which results in a dynamic squeezing out of water molecules from the bilayer. When comparing DMPC with DPPC bilayers above their T_m, it could seem that DMPC displays relatively higher polarity values. But, paying attention to the thermal behavior of homogeneous solvents one should justly conclude that they are nearly equivalent. This is because above their T_m, both DMPC and DPPC bilayers display equivalent polarities analogous to ethanol, and in this case, we can estimate from the thermal dependence in ethanol in Fig. 2, a differential of roughly 0.09 in I_1/I_3 values, when varying the temperature from 31 °C (≈7 °C above the T_m of DMPC) to 48 °C (≈7 °C above the T_m of DPPC). This is a larger difference, than the variation observed when comparing DMPC bilayers at 31 °C ($I_1/I_3 = 1.15 \pm 0.02$) and DPPC at 48 °C ($I_1/I_3 = 1.11 \pm 0.02$), which may afford a practical equivalence, at about the same temperature above their respective T_m. Lastly, in Fig. 3B it is also displayed that the pyrene Ham effect is as well sensitive to the pre-transition (from the lamellar phase L_β, to the lamellar rippled phase $P_{\beta'}$) of DPPC, at T around 33 °C.

3.3. Polarity and its thermal dependency in bilayers containing cholesterol

We studied the polarity properties of pure DMPC and DPPC bilayers and their respective mixtures with cholesterol, in regions of their phase diagrams corresponding exclusively to liquid-disordered (ℓ_d) and liquid-ordered (ℓ_o) phases. We must highlight, that in this work we just aim to analyze the equivalent polarity properties of lipid bilayers in defined and homogeneous areas of the established phase diagrams, that is to say in the absence of phase separation, although the derived main conclusions may also be impedingly extended to lipid bilayers displaying coexistence of phases.

When adding 5 mol% of cholesterol to DMPC (experimental points represented by hollow circles (○) in panel A) and DPPC (represented by black circles (●) in panel B), obtaining the ℓ_d phase above their respective T_m, we observed I_1/I_3 values systematically slightly lower than those obtained in pure phosphatidylcholine bilayers, as seen in Fig. 3A and B. However, these results and the respective thermal variations in both cases are, within the experimental precision of the Py empirical polarity scale, undoubtedly homologous to their respective thermal profiles obtained for the pure phospholipid membranes.

For a ℓ_o phase with 35 mol% of cholesterol, the results for DMPC and DPPC (both represented by cruces (×) in Fig. 3A and B) are significantly different from those obtained for L_α and ℓ_d phases, for the same ranges of temperature. As expected, the sensitivity to the phase transition temperatures is completely eliminated. Additionally, when increasing the cholesterol proportion to 45 mol% (both represented by hollow triangles (△) in Fig. 3A and B), the equivalent polarity values augmented and the thermal variation amplitudes disappear, when compared to the 35 mol% of cholesterol mixtures. Taking the proportion of 45 mol% as a reference, it is concluded that this mixture in the DPPC matrix is characterized by an equivalent polarity of roughly 20.8 along the thermal range analyzed, being the DMPC mixture more polar with an equivalent dielectric constant of 23.3, in all the extent of temperatures analyzed. Hence, as depicted in Fig. 3A and B, in general the equivalent polarity values not only decrease when the temperature increases, as this dependence varies at higher cholesterol proportions in the lipidic mixtures, for the ℓ_o phase regions of the phase diagrams analyzed. When comparing these results, with the variation of the relative polarity with temperature for homogeneous solvents (see Fig. 2), we observe that for the pure L_α phases, for the ℓ_d phases, for the ℓ_o phases with 35 mol% of cholesterol and for the ℓ_o phase of DMPC with 40 mol% (represented by black rhombus (◆) in panel A), all these systems display thermal behavior analogous to homogeneous polar solvents, i.e., the polarity decreases with temperature. Contrastingly, the results for the ℓ_o phases of DMPC and DPPC with 45 mol% and the ℓ_o phase of DPPC with 40 mol% of cholesterol (represented by black rhombus (◆) in panel B) display thermal variations that are more alike to the expected dependence for less polar alcohols. In the case of 40 mol% of cholesterol, both in mixtures with DMPC and DPPC, the thermal dependence is different for each the phospholipid matrix. In the case of DMPC, the thermal dependence is intermediate between 35 and 45 mol%, but much more alike to the case of 35 mol%, contrasting with DPPC which is nearly identical to the case of 45 mol%. In the absence of secure experimental and theoretical grounds, one can ascribe this observation to the differences in the structural properties of DPPC- and DMPC-based bilayers. The DPPC lipid bilayers have a slightly longer hydrophobic thickness, allowing the pyrene to be located on average in regions with less sensitivity to variations of polarity due to possible small vertical displacements. Consequently, the thicker DMPC is more influenced by this potential occurrence than the wider DPPC counterpart. Furthermore, using the equivalent polarity values for these microenvironments and the calibration plots for the polarity of alcoholic solvents at 20 °C (Fig. 2), one can estimate equivalent dielectric constant values as for example for DMPC mixed with 35 mol% and 45 mol% of cholesterol, as being 21.8 and 23.8, respectively. In the case of 35 mol%, the polarity is slightly lower to 1-propanol, but at 45 mol%, the lipid bilayer is displaying an equivalent polarity much closer to ethanol. That is to say, varying the chemical composition in cholesterol in the ℓ_o phases, there is a correspondent variation in the equivalent polarity of lipid bilayers and of its thermal dependence.

4. Discussion

The most important functions of a biological membrane are twofold: defining boundaries and serving as a permeability

barrier. This characteristic is fundamental for the cells and subcellular organelles to form and maintain their internal environment that may be significantly different from the exterior. The permeability of lipid bilayers is strongly determined by the polarity and hydration/hydrophobicity gradients, as they are responsible for the formation of the surface and dipole potentials, which control processes like transport across membrane, ion conductance and insertion of proteins and other molecules in the bilayer. These barrier properties, as well as other membrane physical and chemical properties, such as membrane fusion and the kinetics of enzymatic and redox reactions at the membrane surface and within lipid bilayers, are affected by the level of sterols in the bilayer [25,26,55–57]. These studies suggest that the presence of cholesterol induces an increase of the polarity and hydrophobicity, in the headgroup and hydrophobic core regions, respectively. Our studies demonstrate variations in the equivalent polarity as a function of cholesterol proportion in the lipidic mixture, in the more ordered region of the methylenic palisade, since this is the observable location of pyrene inserted parallel to the ordered portion of acyl chains, from direct NMR and molecular dynamics findings [49,51,52] and by means of indirect arguments from two-dimensional kinetics analysis [50]. It should be pointed out that due to the relative dimensions of pyrene itself (longest axis of 9.2 Å [42]) and being this half of the typical hydrophobic thickness of fluid DMPC and DPPC bilayers (ca. 13–15 Å), this probe is actually reporting average values of the established polarity gradient along the hydrophobic thickness, in the ordered section of acyl chains, that is to say, in the more polar region. We used a very low probe to lipid ratio (e.g., 1:5000), so we can fairly neglect the alleged effects of pyrene in the dynamic and structural properties of lipid bilayers. From a recent molecular dynamics study [52], it was suggested that the effects in ordering the nearby acyl chains are of local nature and do not significantly affect those lipid properties that are computed over the whole system, such as density profiles. Interestingly, this molecular dynamics study also proposes a similarity between the influences of pyrene in lipid bilayers, and the effects induced by cholesterol on structural membrane properties, in both cases above and below the T_m of phospholipid bilayers. This way, the quantification of equivalent polarity in lipid bilayers through the Py polarity scale, certainly has its disadvantages, such as the limited spatial resolution in measuring the gradient of polarity, but on the other hand, it is superior in what concerns the chemical and photophysical properties of pyrene. Besides the referred advantage as an independent and robust descriptor of polarity through the dipolarity/polarizability mode, in the fundamental state pyrene is apolar, and should actually display a relatively minimal dipole moment in the excited state. Since the ground state of highly symmetric aromatic hydrocarbons is definitely non-polar, there should be a characteristic shift in the emission spectra in solvents with high dielectric constant. This occurrence is not observed. There are minor variations in pyrene structure in the excited state, from the nuclear motions associated with vibrational modes [38] and electron density differences [58]. Additionally there is a minimal change in the quadrupole moment on the promotion to the excited state [59], it can be estimated that the excited state dipole is just slightly higher than the ground state counterpart. This is also highlighted from the analysis of solvation in supercritical CO_2, which reveals that the magnitude of local density enhancement surrounding an excited state pyrene molecule is only 1.5 times that of the ground state [60]. As well, unexcited pyrene induces effects in phospholipid bilayers analogous to those exerted by cholesterol [52], contrarily to the observed effects in the structure and dynamics of lipid bilayers associated with the widespread use of some lipidic fluorescent (e.g., NBD- and anthroyloxy-labeled methylenic chain lipids) and EPR probes (e.g., DOXYL-labeled methylenic chain lipids) that are more polar even in the ground state. Therefore, pyrene probes are expected to mimic more closely the natural sterol components of membranes, as well as introducing negligible impacts in the phospholipid bilayer structure and dynamics at low concentration in bilayers. The present arguments reinforce the positive expectations about these probes, provided that they are used adequately. When in the excited state, the possible consequences of pyrene in lipid bilayers are very limited, as depending on the slightly more polar characteristics in the first electronic excited level. Nevertheless, since we cannot exclude the possible occurrence of transversal displacements of pyrene along the hydrophobic length during its excited state lifetime, resulting from small differences in location imposed by the small excited state dipole, as well as a function of the bilayer chemical composition, we cannot exclude the possible occurrence of subtle variations in the comparison of the averaged equivalent polarities in lipid bilayers.

We have chosen alcohols (protic aliphatic solvents) to establish a linear relationship between I_1/I_3 and the static dielectric constant, because this class of solvents is expected to mimic more closely the local polarity resulting from the aqueous penetration into the methylenic chain palisade of lipid bilayers. We have found that the equivalent dielectric constant is higher in the L_β, phase than in the L_α (and ℓ_d) phase, of both pure DMPC and DPPC bilayers. This has also been observed, previously, by other authors, together with the thermal behavior of the pyrene Ham effect, namely the sensitivity to the bilayer phase transition temperature [61], showing a decrease along a broad temperature range prior to T_m, becoming even abruptly lower above T_m, but still displaying a linear thermal dependence, relatively similar to what was observed in homogeneous polar solvents [62]. Since we used the conventional film deposition method, in order to avoid for artifactual demixing of cholesterol in lipid bilayers [63], the maximum proportion of cholesterol used in this work was 45 mol%, which is within the maximum solubility limit of cholesterol in phosphatidylcholine bilayers. In addition, we have chosen the proportion of 5 mol% of cholesterol mixed with DMPC and DPPC to obtain the respective ℓ_d phases, and 35, 40 and 45 mol% of cholesterol to get hold of the ℓ_o phases. We chose these temperature ranges in order to scan a thermal extent that included the values of room and main physiological temperatures. Although the low- and high-temperature limits are also of importance for diverse living organisms, the characteristic temperatures for the vast majority

of the life environments are included in the temperature range under study in this work.

For 5 mol% of cholesterol, in the ℓ_d phase, the lateral ordering of the acyl chains of the phospholipids is not very pronounced. The cholesterol molecules are arranged perpendicular to the bilayer surface, with the hydroxyl group positioned near the middle of the headgroup region of the PC molecule, but at this proportion their rigid ring system does not seem capable of inducing a higher degree of lateral ordering, as it happens when they are present in larger amounts. Consequently, the quantity of water in the bilayer is not substantially modified and the polarity results are similar to those obtained for L_α phases in pure phospholipid bilayers [25,27].

For cholesterol mixtures with DMPC and DPPC, both in the ℓ_o phase, the polarity clearly decreases for a 35 mol% of cholesterol, when compared with corresponding bilayers in L_α and ℓ_d phases. Since the polarity of lipid bilayers is largely determined by the water penetration into the bilayer, as the chain ordering and close packing increase when raising the cholesterol concentration, there is a consequent decrease on the water molecules present at the level of the headgroup region [27]. At 35 mol% proportion of cholesterol, the bilayer is near the situation of twice the quantity of phospholipid component and somehow the cohesive properties of the lipidic bilayer should be enhanced, consequently limiting the penetration of water. These observations are due to the condensing effect of cholesterol on phosphatidylcholines, by reducing average orientational and positional ordering of the alkyl chains and consequently reducing the free volume. One explanation is that cholesterol forms reversible, condensed complexes of defined stoichiometry with phosphatidylcholines [15,64]. Another possibility is that the phospholipid headgroups shield hydrophobic cholesterol from contact with the membrane–water interface [65]. In performing this umbrella function, the acyl chains become more ordered to allow a dual closer packing of the lipid headgroups. Additionally, we found that with 40 and 45 mol% of cholesterol, the polarity tends to increase, when compared with the situation at 35 mol%. This should result from a more accentuated water penetration in the bilayer, as the presence of a higher proportion of cholesterol may induce a wider separation of the phospholipid headgroups and/or less efficient acyl chains packing. This can be understood either as resulting from different and/or loosely condensed complexes, as ensuing from the phospholipid headgroups being stretched to their limit and thus lowering the efficiency in shielding for all cholesterol molecules. In both cases our interpretation is based in the increasing of the proportion of cholesterol in the ℓ_o phase from 35 to 45 mol%, resulting in extended water penetration into the bilayer due to a diminished efficiency in the overall cohesive properties in these mixed bilayers. This lowering results from the variation in chemical composition, from a situation of almost twice the phospholipid in relation to cholesterol (35 mol% of cholesterol), to the situation of nearly equimolar proportions (45 mol% of cholesterol). In addition, for 40 and 45 mol% of cholesterol, although the bilayer displays an elevated equivalent polarity, the thermal dependence is more alike to an apolar homogeneous solvent in the case of DPPC at 40 and 45 mol%, and DMPC at 45 mol%, being the DMPC bilayers at 40 mol% in an intermediate situation (Fig. 3A and B). On the other hand, for 35 mol% of cholesterol in both phospholipid matrixes, the lipid bilayers still demonstrate a thermal dependence typical of more polar homogeneous solvents. We may interpret these observations as resulting from the highly anisotropic nature of lipid bilayers, in which small changes in hydrophobic thickness may imply large variations in the transversal variation of polarity, conversely to homogeneous alcoholic solvents. Consequently, in DMPC/cholesterol and DPPC/cholesterol phase diagrams, although the ℓ_o phase is usually considered uniform from the point of view of molecular dynamics [12,14,17], the polarity properties and their thermal variations depend on the cholesterol proportions in the total lipid composition of the bilayer.

A further insight on the interpretation of the varying equivalent polarity in the ℓ_o phase, as a function of the cholesterol content, may be ascribed to an approach based on the orientational polarizability, that appeared very recently [66]. Accordingly, it is known that the static dielectric constant is composed by two components: the distortional polarizability (electronic redistribution), which is determined by the various chemical groups forming the molecular systems under analysis (solutes and solvents), and the orientational polarizability (molecular reorientation), which is determined by the diverse molecular movements, e.g., rotations, vibrations and translational mobility. Since orientational polarizability is known to be temperature-dependent whereas the distortional component has a negligible dependence, it is likely that the increased equivalent polarity at high cholesterol content in lipid bilayers might be due to an increase in the distortional polarizability component, e.g., from the more polarizable π orbitals within the cholesterol structure.

Henceforth, in the ℓ_o phase, lipid bilayers can display different polarity values as being more or less polar, as well as dissimilar thermal variations, simply by varying the proportion of cholesterol. It is interesting to note that cholesterol can constitute up to 50% of lipid in membrane microdomains [14,15], such as those values that occur in the area of coexisting ℓ_d and ℓ_o phases in the phase diagrams for DPPC/cholesterol [12] and DMPC/cholesterol [17]. This way, microdomains in biological membranes can provide different solvation polarities for membrane components, only by slight differences in the cholesterol content in the ℓ_o phase. It is likely that the microdomains having higher cholesterol contents, e.g., above roughly 40%, could be more enriched in membrane components whose activity or reactivity should be maintained by a state of membrane solvation with a polarity dependence on temperature which is nearly nil, although at apparently higher polarity values than at little lower cholesterol contents (35 mol%). Also, the immiscibility of ℓ_d and ℓ_o phases can be related to the occurrence of non-mixing among conventional liquid solvents characterized by dissimilar polarities, provided that one must restrict the comparison, due to the obvious differences in a wide diversity of structural and dynamic properties between two-dimensional fluid bilayers and three-dimensional liquids.

The present study shows bilayer polarity as an additional factor that should be taken into account when examining diverse phenomena occurring in lipid bilayers, that may or are found to

be dependent on properties modulated by cholesterol content, such as the redox and radical chemistry in bilayers [67], function and distribution of membrane proteins [68], which in turn may influence reactions and processes involving membrane components [69], as well as the activity of membrane enzymes, such the Na^+/K^+-ATPase [70,71]. With respect to the last issue, it was found that at low concentrations, cholesterol stimulates the activity of Na^+/K^+-ATPase, whereas at higher proportions it is inhibitory, correlating with a change in hydration at the protein–lipid interface [70]. Furthermore, the activity in a mixture of DOPC (di(C18:1)PC) and cholesterol was found very considerably greater than in DEPC (di(C22:1)PC) in the absence of cholesterol, showing that cholesterol must exert its effects in the ionic pump through additional variations in other physical–chemical parameters, besides the simple change in bilayer thickness, which influences the hydrophobic matching between the length of the transmembrane segment of the enzyme and the hydrophobic thickness of the bilayer [71]. As discussed by Starke-Peterkovic et al. [24], founded on the changes in water penetration into the mixed lipid bilayer, it would be expected to induce significant variations in the internal dipolar potential, but given that these effects are interwoven, it is very difficult to state definitely which effect is dominating, as well as their relative contributions. On the other hand, there is a common link between both effects, which is the polarity of the lipid bilayer, since it is mainly determined by the extent of water penetration into the bilayer interior [26] and it is determining if one wants to estimate the intrinsic dipole moments within bilayers through the Helmholtz equation for parallel capacitors [24]. Consequently, it is liable that the activity of Na^+/K^+-ATPase can actually be influenced by the polarity of the bilayer, through the changes in the interface hydration or because of the modification in the dipole potential, or even by both. For instance, this work also aims to emphasize that one must acknowledge as commonsensical that there are a number of ways to describe the physical features on the effects of cholesterol in simple lipid bilayers and none of these descriptions is absolute, nor are they independent of each other. And so, the polarity of mixed phospholipid/cholesterol bilayers, namely in the ℓ_o phases of phosphatidylcholine matrixes, should be taken as a further quantitative parameter in the analysis and comprehension of various biological membrane processes dependent on the solvation properties of lipid bilayers, along with the occurrence of lateral heterogeneity phenomena.

Acknowledgments

We thank Professor Eurico Melo for the worthwhile discussions and suggestions.

This work was supported through project POCTI/QUI/45090/2002 from the POCTI program of the Fundação para a Ciência e a Tecnologia (FCT), Portugal.

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