

Quantification of lipid bilayer effective microviscosity and fluidity effect induced by propofol

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Received 8 June 2004; received in revised form 3 November 2004; accepted 3 November 2004

Available online 21 November 2004

Abstract

Electron spin resonance (ESR) spectroscopy with nitroxide spin probes was used as a method to probe the liposome microenvironments. The effective microviscosities have been determined from the calibration of the ESR spectra of the probes in solvent mixtures of known viscosities. In the first time, by measuring ESR order parameter (S) and correlation time (τ_c) of stearic spin probes, we have been able to quantify the value of effective microviscosity at different depths inside the liposome membrane. At room temperature, local microviscosities measured in dimyristoyl-L- α phosphatidylcholine (DMPC) liposome membrane at the different depths of 7.8, 16.95, and 27.7 Å were 222.53, 64.09, and 62.56 cP, respectively. In the gel state (10 °C), those microviscosity values increased to 472.56, 370.61, and 243.37 cP. In a second time, we have applied this technique to determine the modifications in membrane microviscosity induced by 2,6-diisopropyl phenol (propofol; PPF), an anaesthetic agent extensively used in clinical practice. Propofol is characterized by a unique phenolic structure, absent in the other conventional anaesthetics. Indeed, given its lipophilic property, propofol is presumed to penetrate into and interact with membrane lipids and hence to induce changes in membrane fluidity. Incorporation of propofol into dimyristoyl-L- α phosphatidylcholine liposomes above the phase-transition temperature (23.9 °C) did not change microviscosity. At 10 °C, an increase of propofol concentration from 0 to 1.0×10^{-2} M for a constant lipid concentration mainly induced a decrease in microviscosity. This fluidity effect of propofol has been qualitatively confirmed using merocyanine 540 (MC540) as lipid packing probe. Above 10^{-2} M propofol, no further decrease in microviscosity was observed, and the microviscosity at the studied depths (7.8, 16.95, and 27.7 Å) amounted 260.21, 123.87, and 102.27 cP, respectively. The concentration 10^{-2} M was identified as the saturation limit of propofol in dimyristoyl-L- α phosphatidylcholine liposomes.

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Keywords: ESR; Liposome; Viscosity; Propofol

Abbreviations: DMPC, dimyristoyl-L- α phosphatidylcholine; n-DSA, n-doxyl stearic acids spin probe; ESR, electron spin resonance; MC540, merocyanine 540; PPF, 2,6-diisopropylphenol (Propofol); DPPC, dipalmitoyl phosphatidyl choline; 5-DSA, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy; 12-DSA, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy; 16-DSA, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy; MLV, multilamellar large vesicle; τ_c , correlation time parameter; W_0 , central line width at half-height; h_0 , amplitude of the center line; h_{-1} , amplitude of the high-field line; S , S , order parameter; $T_{||}$ and T_{\perp} , anisotropic hyperfine splitting; η , viscosity; ρ , density of liquid; P_{gly} , percent of glycerol; SDS, sodium dodecyl sulphate; CTAB, cetyltrimethyl-ammonium bromide.

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1. Introduction

Many physiological functions associated with biological membranes are known to be profoundly affected by the dynamic properties of the phospholipid matrix. It is believed that the changes in such physical properties of bilayers as phase transitions temperature, lateral phase separations, local dielectric properties, and rotational diffusion modulate the biological activity of many membrane-associated receptors, transport proteins, and enzymes. One of the most widely techniques available to study these properties is fluorescence spectroscopy. It has been shown that environmental fluorescent probes of varying structural complexity report on most of these dynamic phenomena, both in natural as well as in model membrane and micellar systems [1]. One important goal of the work has consisted in determining the apparent or effective membrane microviscosity [2]. However, many membrane microviscosity values measured with different fluorophores depend often on the molecular properties of the probe [3]. Generally, when the fluorophore requires a large volume for motion, whether it be for excited-state formation or nonradiative deexcitation, a wide range of microviscosity values is obtained. It has been suggested that the large probe may cause membrane bilayer distortion [2].

Fluorescent probes report, not only on bilayer microviscosities and phase behaviour, but they also provide information on the local dielectric properties of their environment [4,5]. Usually, the fluorescence emission characteristics can be related to the dielectric constant of a solvent in a standard solvent scale.

The bilayer microviscosity can be studied by use of luminescence techniques such as steady-state or dynamic depolarization and diffusion-dependent inter- and intramolecular excimer formation [1,5]. Unfortunately, these techniques have limitations. For example, the concentrations required for effective intermolecular pyrene excimer formation in lipid bilayer often lead to probe aggregation because of nonuniform probe distribution [5]. Microviscosities obtained from intramolecular excimer formation are more dependent on the structural characteristics of the probe rather than on the environment itself [3]. To calculate the microviscosities from steady-state depolarization, we must assume that probe motion in the bilayer is isotropic. This assumption may not be valid for most nonpolar fluorophores [6]. Electron spin resonance (ESR) technique is also currently used to investigate the microenvironment of nitroxide spin probes in membrane liposome by measuring the nitrogen-coupling constant and ESR spectra line width [7]. The line widths are governed by rotational and lateral diffusion of the spin probes, which in turn is affected by viscosity (η) and temperature of the local environment [7]. Thus, the relative anisotropy observed in an ESR spectrum is directly related to the rotational mobility of the probe, a term that can be correlated with the probe's microviscosity [8]. This correlation allows

measuring the local microviscosity at different depths inside a liposome membrane. Chandar et al. have determined the microviscosity within sodium dodecyl sulphate (SDS) micelles by comparing the ESR nitroxide spectra in SDS micelles to the spectra of the probe in ethanol–glycerol mixture at known viscosity. They suggest that the microviscosity can effectively be defined as the homogenous solution viscosity, which results in the same spectrum as that in the microenvironment [8].

In this work, we have established standard curves of effective microviscosities by calibration of the ESR spectra of three *n*-doxyl stearic acids (*n*-DSA; $n=5, 12, 16$) probes in glycerol–ethanol mixtures of known viscosities. These curves allow us to quantify the effective microviscosity at different depths inside liposomes by measuring the order parameter (S) and the correlation time (τ_c) on *n*-DSA ESR spectra. Local microviscosity of the probe environment in the SDS and 2-propanol were calculated using our method. Our results proved to be consistent with values obtained by other techniques and reported in previous studies. Hence, we have applied our method to samples of biological interest such as 2,6-diisopropylphenol (propofol; PPF) solubilized in liposome solutions.

Several drugs can be solubilized inside the membrane bilayer. Some of them induce a decrease in membrane microviscosity and order. Propofol (PPF) (Fig. 1) is a nonbarbiturate anaesthetic agent commonly used for induction and maintenance of general anaesthesia in clinical practice [9]. It is characterized by a unique phenolic structure not present in any other conventional anaesthetic. Due to its lipophilic property, PPF is presumed to penetrate into and interact with membrane lipids, inducing changes in membrane fluidity [10]. Therefore, quantifying the change in viscosity induced by PPF could be important to better understand the molecular mechanism of its anaesthetic action. It has been previously shown by fluorescence and ESR that PPF promotes the formation of fluid phase domains in membranes [11] and decreases the gel-to-fluid state transition temperature [12]. In this study, we have quantified the membrane microviscosity change induced by PPF incorporation into dimyristoyl-L- α phosphatidylcholine (DMPC) by measuring ESR S parameter and correlation time of *n*-DSA. A fluidity effect of PPF in DMPC liposomes below the phase transition temperature has been evidenced. The limit of PPF incorporation in DMPC has also been determined. The PPF fluidity effect has been qualitatively

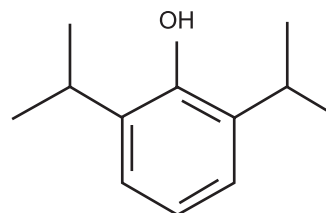


Fig. 1. Chemical structure of 2,6-diisopropylphenol (propofol).

confirmed by using Merocyanine 540 (MC540) as another lipid packing probe.

2. Materials and methods

2.1. Chemicals

Absolute ethanol and chloroform from Merck (Germany), glycerol, dipalmitoyl phosphatidyl choline (DPPC) and dimyristoyl phosphatidyl choline (DMPC) from Sigma (USA), stearic spin probes; 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy (5-DSA), 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy (12-DSA) and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy (16-DSA) from Aldrich (USA) were with analytical grade and used without further purification.

2.2. Liposome preparation

Spin-labeled stearic acids were added to a chloroform solution of DMPC (or DPPC; 5 mg/ml). The mixture was stirred for 5 min, and the solvent was evaporated under vacuum. The formed lipid film was suspended in phosphate buffer solution (pH 7.0) at a temperature above the DMPC or DPPC phase transition temperature 23.9 and 41.4 °C, respectively, and stirred by vortex mixer to obtain multilamellar large vesicles (MLV) [13]. After this hydration, five freeze–thaw cycles using liquid nitrogen were carried out to allow a better incorporation of the labeled stearic acid into the liposome phospholipid bilayer. The MLV suspensions were transferred into an extruder (Lipex Biomembrane, Canada) in which the unilamellar liposomes were formed by passing the suspensions through polycarbonate filters (0.1 µm pore size, Nucleopore, Pleasanton, CA) under a pressure of up to 6895 Pa of nitrogen. The procedure was repeated 10 times at a temperature above the phase transition temperature of the phospholipids and induced unilamellar liposomes which had a mean size of about 90 nm with very low polydispersity [14,15].

2.3. Absorption spectra

Absorption spectra of MC540 in lipid suspensions were carried out on a Uvikon 941 spectrophotometer (Kontron instruments, Italy). These measurements were performed in quartz cuvette (1 cm length×0.4 cm width) thermostated by a circulating bath connected to the cuvette holder.

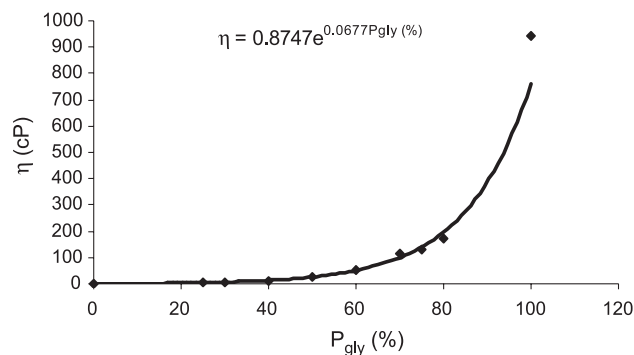


Fig. 2. Viscosity vs. percent of glycerol (P_{gly}) in glycerol–ethanol mixture.

2.4. Glycerol–ethanol mixtures and viscosity measurements

Glycerol–ethanol mixtures were obtained by adding ethanol to preheated glycerol (35 °C) and by stirring the solution for several minutes at room temperature.

Viscosity (η) of the glycerol–ethanol mixtures was measured at room temperature using a standard Ostwald viscosimeter [16] and was calculated by Eq. (1)

$$\eta_2 = \eta_1 \frac{\rho_2 * t_2}{\rho_1 * t_1} \quad (1)$$

where ρ_1 , η_1 , t_1 are the known density, known viscosity, and the measured liquid flow time of ethanol, respectively; ρ_2 and t_2 are the measured liquid flow time and density of the glycerol–ethanol mixtures, and η_2 is its unknown viscosity.

The measured values are listed in Table 1.

The viscosity against the percent of glycerol (P_{gly}) in glycerol–ethanol mixtures is plotted in Fig. 2. The experimental data were fitted to an exponential law. The isolated point in Fig. 2 represents the value of viscosity in a solution of 100% glycerol reported from literature [17]. This value cannot be measured using Ostwald viscosimeter with a sufficient accuracy.

2.5. Electron spin resonance spectroscopy measurements

The ESR measurements were performed at 9.56 GHz using a Bruker ESR 300E spectrometer (Bruker, Germany) equipped with a variable temperature controller accessory. The labeled liposome suspension was introduced into a 50-µl capillary tube. All measurements without liposome were made at room temperature and using an aqueous flat cell. Spectra were recorded by using an 80-G scan range at 20-mW microwave power and 2-G modulation amplitude.

Table 1
Viscosity and density of glycerol–ethanol mixtures at room temperature

Percent of glycerol in ethanol	0	25	30	40	50	60	70	75	80	100
Density	0.785	0.926	0.944	0.993	1.041	1.090	1.130	1.153	1.173	1.256
Viscosity (cP)	1.083	4.689	5.987	11.523	23.775	51.482	112.133	129.105	171.133	945.00

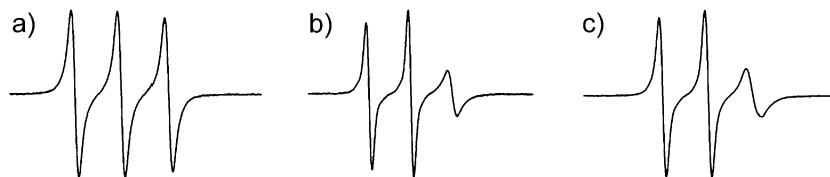


Fig. 3. ESR spectra of 16-DSA in (a) ethanol, (b) in DMPC liposome at 25 °C, and (c) in a mixture of 35% ethanol and 65% glycerol.

The spectra of 5-, 12-, and 16-DSA spin labels in glycerol–ethanol were taken at different percentages of glycerol. All these spectra were interpreted in terms of the correlation time parameter τ_c and the order parameter S which were defined successively as

$$\tau_c = (6.5 \times 10^{-10}) \times W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{\frac{1}{2}} - 1 \right], \text{ expressed in second} \quad (2)$$

where W_0 is the width of the central line at half-height, and h_0 and h_{-1} are the amplitudes of the center- and high-field lines [18], and

$$S = \frac{(2T'_{\parallel} - 2T'_{\perp})}{(2T_{\parallel} - 2T_{\perp})}, \quad (3)$$

where T'_{\parallel} and T'_{\perp} are the anisotropic hyperfine splitting measured from the ESR spectra, and $2T'_{\parallel} - 2T'_{\perp} = 52G$ [19].

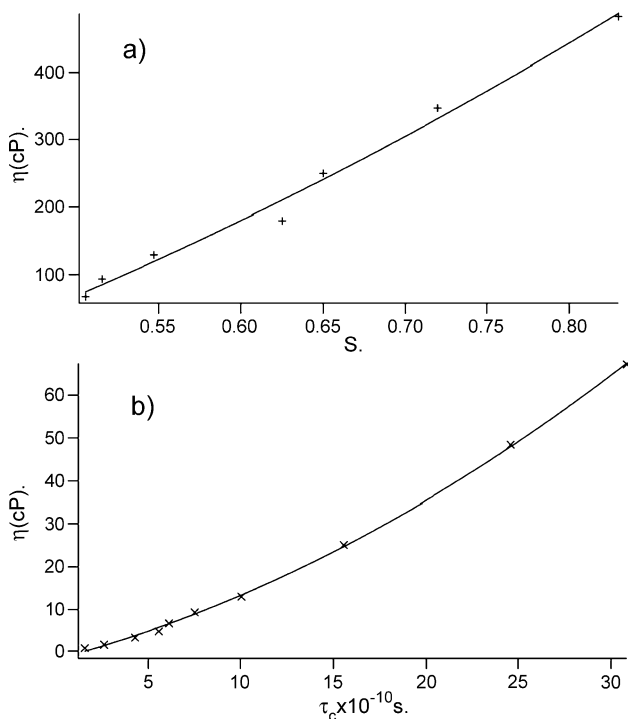


Fig. 4. (a) Viscosity vs. S parameter in glycerol–ethanol mixture with 5-DSA at increasing glycerol percentage content ($P_{\text{gly}} \geq 65\%$). (b) Viscosity vs. correlation time (τ_c) in glycerol–ethanol mixture with 5-DSA at increasing glycerol percentage content ($P_{\text{gly}} \leq 60\%$).

This value corresponds to the anisotropic hyperfine splitting measured from the ESR spectra of radical species completely rigid oriented in a parallel or perpendicular way, respective to the applied magnetic field [19]. The mobility of n-DSA can be quantified as τ_c in the case of weakly to moderately immobilized labels ($\tau_c < 3 \times 10^{-9}$ s), whereas, for strongly immobilized probe ($\tau_c > 3 \times 10^{-9}$ s), S is better-suited [7,20].

3. Results

3.1. Standard viscosity plots

Spin labels were used as viscosity probes because their ESR spectra are very sensitive to polarity and mobility [21]. Fig. 3 shows the spectrum of 16-DSA in ethanol (Fig. 3a), in DMPC liposomes (Fig. 3b), and in glycerol–ethanol mixture (Fig. 3c) at 25 °C. Spin-labeled stearic acids n-DSA were solubilized in glycerol–ethanol mixture of increasing viscosity. In each case, S and τ_c were calculated from the measured ESR spectra. For strongly immobilized probes corresponding to η above 70, 80, and 180 cP, respectively, for 5, 12, and 16 DSA (Fig. 4a), S was used to determine or calculate the microviscosity. For weakly immobilized probes corresponding to η below these values (Fig. 4b), microviscosity was determined using τ_c .

For the sake of clarity, 5-DSA curves only are presented in Fig. 4 because 12- and 16-DSA curves are very similar to the 5-DSA curve. However, the corresponding fitted formulas are gathered in Table 2.

Using these standard curves, the local microviscosities within sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB), and 2-propanol at 25 °C were calculated, and the values are 14, 23.59, and 1.37 cP, respectively.

Table 2
Fitted formula of viscosity curves of glycerol–ethanol mixture for the three spin labels

Spin label position	Correlation time (τ_c) vs. viscosity (η)	Order parameter (S) vs. viscosity (η)
5-DSA	$\eta = 0.0357 \tau_c^2 + 1.1411 \tau_c - 1.6844$	$\eta = 672.16 S^2 + 371.47 S - 284.38$
12-DSA	$\eta = 0.0381 \tau_c^2 + 2.251 \tau_c - 4.1345$	$\eta = 1562.4 S^2 + 1251.40 S + 301.49$
16-DSA	$\eta = 0.0838 \tau_c^2 + 6.5755 \tau_c - 4.5234$	$\eta = 11861 S^2 - 2607.30 S + 242.91$

Table 3

Viscosity of DMPC liposomes at three different bilayer depths and at two different temperatures

n-DSA in liposome	Viscosity (cP) at 25 °C	Viscosity (cP) at 10 °C
5-DSA	222.53	472.56
12-DSA	64.09	370.61
16-DSA	62.56	243.37

3.2. Effects of propofol on DMPC unilamellar liposomes

In DMPC unilamellar liposomes, ESR spectra were collected for incorporated stearic acids spin labeled at the fifth, twelfth, and sixteenth carbon positions. The S and τ_c parameters were measured at temperature above (25 °C) and below (10 °C) the gel-to-fluid transition temperature (23.9 °C) of DMPC.

Microviscosity values at room temperature are displayed in Table 3. At this temperature, the DMPC liposomes are in the fluid state. In this condition, the microviscosity at the level of 5-DSA nitroxyl radical was calculated using S . For 12- and 16-DSA situated in the fluid region of the lipid bilayer, the microviscosity was calculated using τ_c .

The microviscosity of the three spin labels incorporated in DMPC liposomes at the gel state (10 °C) are presented in Table 3. These microviscosity values were calculated using the S parameter.

The addition of PPF in DMPC at room temperature did not change S and τ_c parameters (data not shown).

We further investigated the variation of the microviscosity for different PPF concentrations (ranging from 0 to 10 mM) and at a temperature (10 °C) below the gel-to-fluid state transition temperature of DMPC.

Microviscosity values presented in Fig. 5 were obtained by using the viscosity standard plots (Fig. 4), which enabled us to transform the ESR spectrum parameters into

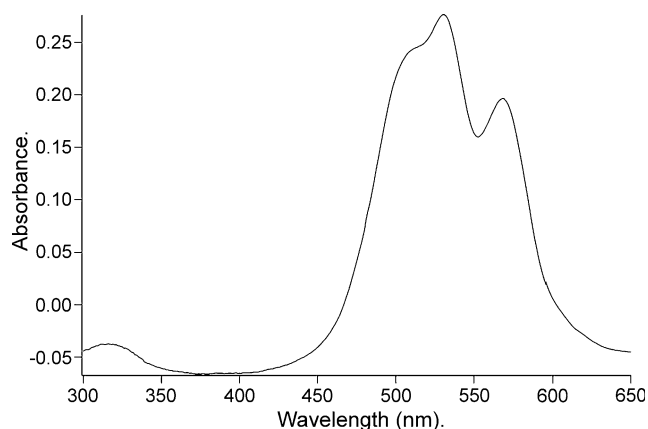


Fig. 6. Absorption spectra of MC540 (10^{-5} M) in DPPC ($1.7 \cdot 10^{-3}$ M) liposome at 16 °C.

corresponding microviscosity value. Fig. 5 shows the influence of increasing PPF concentrations on the membrane microviscosity at 10 °C in the presence of the three different spin labels 5-, 12- and 16-DSA. Addition of PPF at concentrations ranging from 10^{-3} to $5 \cdot 10^{-2}$ M into DMPC liposomes containing 5-DSA provoked a rapid decrease of the microviscosity in a concentration-dependent manner. At PPF concentration higher than 10^{-2} M, no further microviscosity decrease was observed (Fig. 5). When the experiment was performed in the presence of 16-DSA, PPF addition provoked a lower decrease in microviscosity compared to that observed with 5- and 12-DSA. In both cases, addition of high PPF concentrations (from 10^{-2} to $5 \cdot 10^{-2}$ M) did not affect the decrease in microviscosity, as described above. The mean microviscosity values calculated at the different depths (C5, C12, and C16) were 260.21, 123.87, and 102.27 cP, respectively, for the PPF concentration range from 10^{-2} to $5 \cdot 10^{-2}$ M.

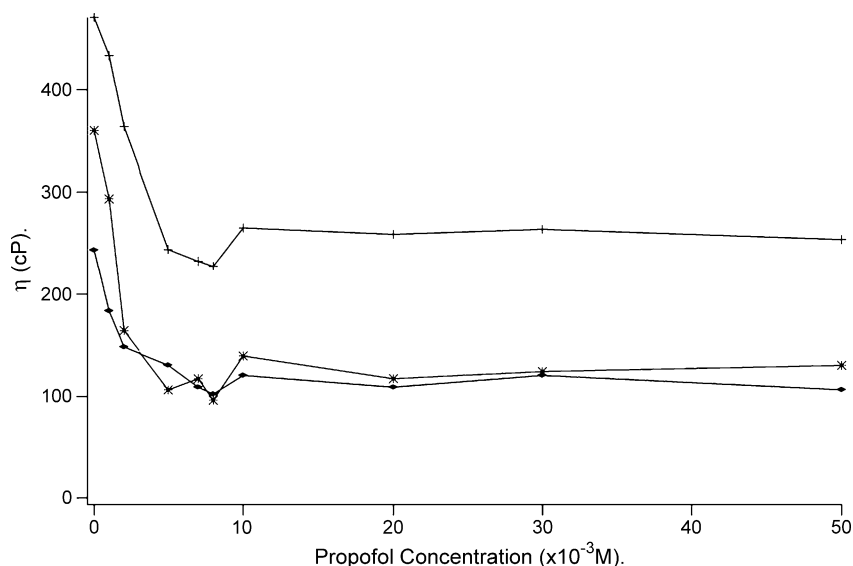


Fig. 5. Viscosity values vs. PPF concentration for different spin label (+: 5-DSA, *: 12-DSA, ♦: 16-DSA) in DMPC ($1.7 \cdot 10^{-3}$ M) at 10 °C.

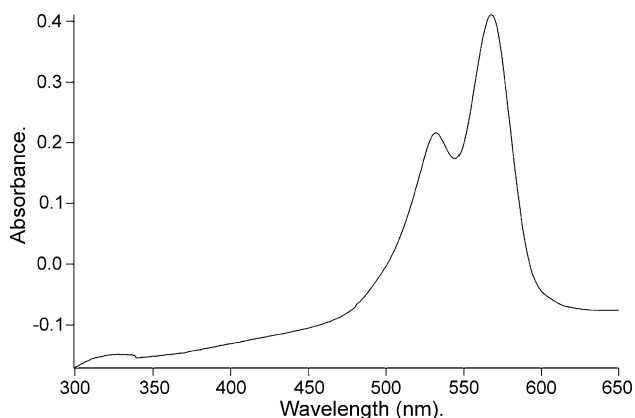


Fig. 7. Absorption spectra of MC540 (10^{-5} M) in DPPC ($1.7 \cdot 10^{-3}$ M) liposome at 55 °C.

3.3. Effect of PPF on DPPC unilamellar liposomes probed by MC540

The lipophilic dye MC540 was used as a sensor of molecular events either in model membrane systems or in biological membranes [22] because its absorption spectrum is very sensitive to the solubilizing surrounding properties. We used MC540 to monitor the effects of PPF in the lipid DPPC bilayer. The spectra of MC540 in the presence of DPPC liposomes at 16 °C (gel state) showed a bulge at 501 nm and two peaks at 532 and 568 nm (Fig. 6). The phase transition temperature of DPPC is 43.1 °C [23]. At 55 °C, DPPC liposomes were in the fluid state. At this temperature, the spectrum obtained when adding MC540 to these liposomes showed one peak at 568 nm with a shoulder at 532 nm (Fig. 7). The spectrum of MC540 obtained after addition of PPF to DPPC liposomal dispersions at 16 °C is shown in Fig. 8. Addition of PPF to DPPC–MC540 solution yielded a transformation of MC540 absorption spectrum (Figs. 6 and 8) similar to that induced by the temperature increase alone (Figs. 6 and 7).

4. Discussion

The ESR spectra of 16-DSA in ethanol, with and without glycerol, and in DMPC liposome are shown in Fig. 3. The spectrum obtained in the presence of DMPC liposome is quite different from that of ethanol alone. When the nitroxide molecule can rotate rapidly and randomly, then all the anisotropy averages out and a sharp three-line spectrum is obtained, as in the case of ethanol (Fig. 3a). In the presence of the DMPC liposomes, the nitroxide apparently cannot rotate freely, suggesting its immobilization inside the liposome bilayer. The ESR spectrum of 16-DSA in DMPC liposome (Fig. 3b) resembles the spectrum of the same probe in a mixture of 35% ethanol and 65% glycerol (Fig. 3c). The spectra resemblance inferred that the mobility of nitroxide is restricted on the same way in glycerol–ethanol mixture and DMPC liposomes. The degree

of mobility as monitored by the spectral anisotropy, which is calculated in terms of rotational correlation time and order parameter, can be correlated with the probe's microviscosity [8]. Using this correlation, we established standard curves and obtained for the first time a quantitative evaluation of local microviscosity. ESR S and τ_c parameters of fatty acid spin labels 5-, 12-, and 16-DSA were expressed as a function of a glycerol–ethanol mixture known viscosity. Then, these plots or their corresponding fitting formula enabled us to easily calculate the local microviscosity of any given solution from measured S and/or τ_c parameters. The measured microviscosity within SDS, CTAB, and 2-propanol cited proved to be consistent with values obtained by other techniques and reported in previous studies [24].

Spin-labeled stearic acids 5-, 12-, and 16-DSA have their nitroxyl group NO^\bullet attached at various positions along the alkyl chain. In the DMPC unilamellar liposome bilayers, the three spin labels 5-, 12-, and 16-DSA are oriented with their long axes parallel to the phospholipid chains and NO^\bullet located at different positions on the carbon chain, yielding information on the environment at different depths in the bilayer (7.8, 16.95, and 27.7 Å) [25]. Using measured S or τ_c (according to their degree of immobilization) of these spin labels, we were able to determine the microviscosity value at selected probe positions in liposome bilayer using our standard viscosity curves or their corresponding fitted formula.

At 25 °C, DMPC was in the fluid state, and the measured microviscosity values at C5, C12, and C16 carbon position were 222.53, 64.09, and 62.56 cP, respectively. However, at a temperature (10 °C) below the phase transition DMPC temperature (23.9 °C), microviscosity values at the same positions were 472.56, 370.61, and 243.37 cP, respectively. These results clearly indicated that the central region of the lipid bilayer explored by 12- and 16-DSA was more “fluid” than the hydrophobic region near the polar head groups explored by 5-DSA, which is in agreement with reported results [19]. We also showed that decreasing the temperature

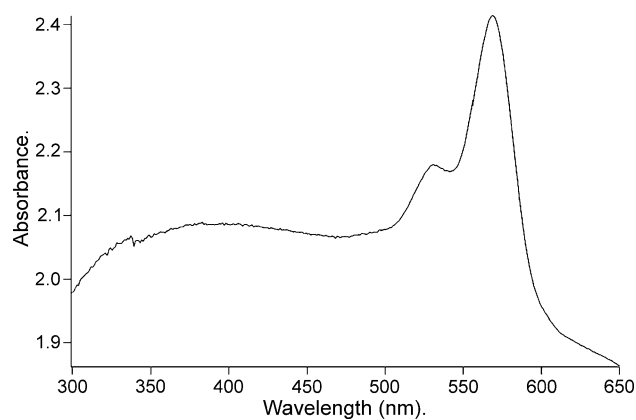


Fig. 8. Absorption spectra of MC540 (10^{-5} M) in DPPC ($1.7 \cdot 10^{-3}$ M) liposome after addition of PPF (10^{-2} M) at 16 °C.

by 13 °C below the DMPC phase transition temperature (23.9 °C) induced a large increase in the microviscosity: about 200 cP near polar head group (5-DSA) and about 150 cP at central region of the lipid bilayer (12- and 16-DSA). It is important to note that 16-DSA may undergo vertical fluctuations in the liposomal membrane, reflecting flexibility of the fatty acid alkyl chain [21,26,27].

Using this method that makes possible to quantify the variations of local microviscosity, we have investigated the effect of incorporation of the anaesthetic agent PPF in DMPC liposomes on membrane microviscosity.

Some anaesthetics have been shown to alter the membrane fluidity and depress the phase transition temperature [12]. The fluidity alterations have been previously described qualitatively by measuring a decrease in the order parameter [28]. Adding PPF (≤ 20 mM) in DMPC at 25 °C, i.e., above the DMPC phase transition temperature, did not change the local microviscosity. Obviously, the lipid matrices in the fluid state can accommodate high (up to 20 mM) concentrations of the dopants without aggravating the disordered motions of the lipid chains, as previously reported [29]. In the gel state (10 °C), the local microviscosity for n-DSA in DMPC liposomes was increased (Table. 3). However, the incorporation of PPF into these liposomes caused a decrease in the microviscosity. This decrease was studied as a function of PPF concentration at constant DMPC concentration (7.4×10^{-3} M). An increase in PPF concentration ranging from 0 to 1.0×10^{-2} M induced a rapid decrease in microviscosity value (Fig. 5). Above 10^{-2} M PPF, no further decrease was observed, and the local microviscosity at the depth C5, C12, and C16 averaged 260.88, 123.87, and 102.27 cP, respectively. Above 10^{-2} M PPF concentration, any additional amount of PPF seems to remain outside the unilamellar vesicles. Therefore, 10^{-2} M PPF concentration appears as the saturation limit of PPF in DMPC liposomes. PPF clearly increases the membrane fluidity (Fig. 5). We also found that, at the depth of 5- and 12-DSA, PPF exhibited a higher effect on the membrane microviscosity compared to that observed at other depths. This discrepancy can be explained by the fact that PPF can act either with fatty acids within liposomes or aqueous molecules. Indeed, PPF can easily cross membranes given its high lipophilicity [30]. However, it can also establish hydrogen bonds based on the interactions between its phenolic hydroxyl group and carbonyl oxygen or hydroxyl group coming from aqueous solvent and other molecules within liposome bilayers. Consequently, the strong microviscosity decrease observed in the presence of C5 label is due to the fact that this label is located at the inner membrane, and hydrogen bonds keep PPF near the outside membrane. Therefore, we showed that PPF can also act at other sites even if the most likely targets for general anaesthetics are thought to be neuronal ion channels, especially integral membrane proteins (GABA A receptors) [31].

Merocyanine 540 (MC540) is a photosensitizer used in the field of photodynamic therapy [32,33], which has also

been extensively employed as sensor of transmembrane potential [34,35] and lipid packing [36]. It is a solvatochromic dye whose absorption spectrum shifts with polarity [34,35,37]. As many amphipathic molecules, it has a strong tendency to dimerize in aqueous solutions [26]. The absorption spectrum of the dye in water has a double-banded structure (501 and 533 nm), and the extinction coefficient is concentration-dependent, indicating dimerization of the dye [38]. In lipid bilayers, MC540 is located at the membrane interfacial region under dimeric and monomeric forms [36,39]. It has been shown that MC540 binding is influenced by the degree of lipid packing in the bilayer and that the dimerization equilibrium is sensitive to liposome curvature, composition, phase state, and packing [39,40]. MC540 binds preferentially to membranes with loosely packed lipids and binds to fluid-phase vesicles rather than to gel-phase vesicles. This MC540 property can be used to monitor the viscosity modifications induced by PPF inside liposome bilayers. Indeed, in fluid-phase vesicles, the dye molecules have an absorption spectrum characteristic of molecules in a hydrophobic environment. In contrast, at a similar concentration in gel-phase vesicles, the absorption spectrum changes and reflects molecules in aqueous solvent [22,41].

Increasing temperature above the phase transition temperature of DPPC leads to a change in vesicle from gel state to fluid state [42]. The modification of the bilayer fluid state can be monitored using MC540 absorption spectrum change (Figs. 6 and 7). Indeed, when DPPC bilayer is heated to 55 °C, the 501-nm bulge and the peak at 533 nm disappear. This behaviour is indicative of the penetration of dye into the unilamellar liposomes when the bilayer goes through the phase transition [43]. These results are in good agreement with the literature [36].

The disappearance of the bulge at 501 nm and the peak at 533 nm was also seen when PPF was added to MC540–DPPC solution in the gel state. The comparison of the spectra of Figs. 7 and 8 allows us to infer that MC540 spectra obtained when PPF is added to dye/liposome solution maintained below the phase transition are similar to the spectrum observed when the temperature of DPPC liposomes is increased above the transition temperature without adding PPF. This result confirms qualitatively our ESR results showing that PPF induces a modification in the membrane fluidity.

In conclusion, the methodology developed in this work seems to be adequate for studying microviscosity at different depths inside liposome membrane or micellar solution. Microviscosity change induced by drug addition or by temperature can be monitored.

Acknowledgment

We would like to thank Doctor Michel Tricot for his kind help in operating viscosimeter.

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