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Does propofol alter membrane fluidity at clinically relevant concentrations? An ESR spin label study

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Abstract

General anesthetics have been shown to perturb the membrane properties of excitable tissues. Due to their lipid solubility, anesthetics dissolve in every membrane, penetrate into organelles and interact with numerous cellular structures in multiple ways. Several studies indicate that anesthetics alter membrane fluidity and decrease the phase-transition temperature. However, the required concentrations to induce such effects on the properties of membrane lipids are by far higher than clinically relevant concentrations. In the present study, the fluidizing effect of the anesthetic agent propofol (2,6-diisopropyl phenol: PPF), a general anesthetic extensively used in clinical practice, has been investigated on liposome dimyristoyl-L-α phosphatidylcholine (DMPC) and cell (erythrocyte, Neuro-2a) membranes using electron spin resonance spectroscopy (ESR) of nitroxide labeled fatty acid probes (5-, 16-doxyl stearic acid). A clear effect of PPF at concentrations higher than the clinically relevant ones was quantified both in liposome and cell membranes, while no evident fluidity effect was measured at the clinical PPF doses. However, absorption spectroscopy of merocyanine 540 (MC540) clearly indicates a PPF fluidizing capacity in liposome membrane even at these clinical concentrations. PPF may locally influence the structure and dynamics of membrane domains, through the formation of small-scale lipid domains, which would explain the lack of ESR information at low PPF concentrations.

Keywords: ESR; Microviscosity; Membrane; Propofol; Neuro-2a

1. Introduction

Several drugs can be solubilized inside the membrane bilayer and some of them induce lipid perturbations such as a decrease in membrane fluidity, order and permeability. Propofol (PPF) is a nonbarbiturate intravenous anesthetic agent currently used for the induction and maintenance of general anesthesia in clinical practice [1]. It is characterized by a unique phenolic structure not present in any other conventional anesthetic. Due to its lipophilic property, PPF is presumed to penetrate into membranes and to interact with lipids inducing changes in membrane fluidity [2]. Electron spin resonance (ESR), fluorescence spectroscopy or differential scanning calorimetry techniques have allowed to qualitatively demonstrate that PPF modifies membrane fluidity and reduces the phase-transition temperature [3,4]. Although, the $GABA_A$ receptor is now identified as a key target for propofol [5], lipid theory of anesthesia is not totally discarded and a correlation between its action and lipid perturbations is possible. Indeed, Cantor in his lateral pressure profile model

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); 5-DSA, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy; 16-DSA, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy; τ_c, correlation time parameter; *S*, order parameter.

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connects general anesthesia with anesthetic anisotropic effects on the lateral pressure profile in membranes which are very large even at low anesthetic concentrations [6–8]. The membrane fluidizing effect of PPF has been seen as a concept enclosing a major quantitative problem [5]. Indeed, anesthetic concentration necessary to cause changes in the properties of membrane lipids has been reported to be of several orders of magnitude higher than the clinically relevant concentrations [9,10]. Blood PPF concentrations in the range of 10^{-6} M are commonly used in clinical practice and assumed as effective concentrations for providing anesthesia [4]. Modern techniques for delivering intravenous anesthesia use perfusion systems that include pharmacokinetic models and allow the practitioner to target effect-site concentrations of a given drug, defined as the concentration supposed to be measured at the site of action in the central nervous system. It has been shown that, PPF free aqueous concentrations about 0.4 μM for the effective concentration 50% (EC_{50}) are appropriate benchmarks for quasi steady state concentration required for anesthesia [5,11]. However, given the nature of the different media in the central nervous system which do not share the same biophysical properties, one can reasonably assume that the concentrations of PPF are not necessary similar inside and outside the lipid membranes. Unfortunately, there is no technique available to measure such effective concentrations at the cellular level. Therefore, quantifying the change in membrane fluidity induced by PPF especially at clinically relevant concentrations could be important for a better understanding of the molecular mechanism of its anesthetic action. Hence, this study covers a wide range of PPF concentrations which should include real concentrations at the cellular membrane scale corresponding to effective blood concentration ranges.

Based on one previous work [12], ESR with nitroxide spin probes was used as a method to probe the membrane microviscosity. Indeed, the relative anisotropy observed in an ESR spectrum is directly related to the rotational mobility of the probe, and can be correlated with the probe microenvironment. The change in probe mobility allows to study the membrane fluidity and often yields useful information on the dynamic state of membrane phospholipids [13]. In particular, nitroxide labeled fatty acid probes (*n*-doxyl stearic acid) have been shown to be useful to determine these parameters within lipids and cell membranes [14]. Absolute values of microviscosity can be obtained after prior calibration of the ESR spectra of nitroxide probes in mixtures of known viscosities [12]. In these experiments, the microviscosity is defined as the homogenous solution viscosity, which results in a spectrum identical to the one recorded in the explored microenvironment [15]. Moreover, absorption spectroscopy of merocyanine 540 (MC540) was also used as a second method to probe the fluidity change on membranes. Indeed, the lipophilic dye MC540 has been used as a sensor of molecular events either in model membrane systems or in biological membrane [16] because its absorption spectrum is very sensitive to the solubilizing surrounding properties. MC540 is shown to be located at the membrane interfacial region under dimeric and monomeric forms [17,18]. It preferentially binds to membranes with loosely packed lipids and binds to fluid-phase vesicles rather than to gel-state vesicles. Indeed, in the fluid-state vesicles, the dye molecules have an absorption spectrum characteristic of molecules in a hydrophobic environment. In contrast, at a similar concentration in the gel-state vesicles, the absorption spectrum changes and reflects molecules in aqueous solvent [19]. Thus, any change, even small, on membrane fluidity state could be seen as a spectrum evolution towards one of the above-sited configurations. Moreover, neither the PPF nor the DMPC liposome absorbs in the MC540 absorption wavelength range (300–600 nm).

In the present study, the effects of PPF (diluted in ethanol) and its commercial form Diprivan® have been investigated on liposome and cell membrane fluidity, using the ESR technique with two *n*-doxyl stearic acid spin probes. Microviscosity values at two different depths inside membranes were monitored as a function of PPF concentration in the range 0–10^{-2} M. PPF fluidizing potency was also demonstrated qualitatively by the use of the MC540 as a probe of the DMPC lipid bilayer microviscosity.

2. Materials and methods

2.1. Liposome preparation

Stearic acid derivatives (Aldrich, USA) labeled by stable doxyl radical ring at the C-5 (5-DSA) or at the C-16 (16-DSA) position (counted from the carboxylic group of the stearic acid derivative) were added to a chloroform solution (Merck, Germany) of 5 mg/mL DMPC (Sigma, USA). 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 (1/15 M; Na_2HPO_4–KH_2PO_4, from Riedel-de Haën, Belgium) at a temperature above the DMPC phase-transition temperature (23.9 °C), and stirred by a vortex mixer in order to obtain large multilamellar vesicles (MLV) [20]. After this hydratation, 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, CA), under a pressure up to 6895 Pa of nitrogen. The procedure was repeated ten times at a temperature above the phase-transition temperature of the phospholipids and resulted in unilamellar liposomes with a mean size of about 90 nm and a very low polydispersity [21,22]. Propofol (PPF) (Acros, Belgium) was first dissolved in ethanol (Merck, Germany) in order to obtain stock solutions. Two μL of the stock solutions was added to 250 μL of liposome solution in order to achieve the desired concentration of PPF. The final percentage of ethanol was less than 1% in all experiments. The commercial form of PPF (Diprivan®: PPF 10 mg/mL) is formulated in intralipid, a lipid vehicle emulsion (10% soya bean emulsion, egg phosphatides and glycerol). Diprivan® (Zeneca, Belgium) or equivalent volumes of intralipid (IL) (Kabi Pharmacia, Belgium) were added to DMPC liposomes

solution and compared to the results obtained in the absence of Diprivan® and IL as well as to the results obtained with pure PPF in DMPC liposomes.

2.2. Erythrocyte

Stearic spin labels (5- and 16-DSA) were used as a probe to quantify erythrocyte membrane fluidity. An ethanol solution of *n*-DSA (2.0×10^{-4} M) was dried in a test tube (0.1 mL/tube) under nitrogen gas to form a thin film. The erythrocyte suspension in phosphate buffer saline solution (PBS) (hematocrit 50%) was then added to test tubes and incubated at 37 °C for 20 min with gentle shaking to incorporate the spin label into the membranes. The labeled erythrocytes were washed twice with PBS to remove unbounded spin label and resuspended in PBS (hematocrit 50%). The spin-labeled erythrocytes were then incubated with various concentrations of propofol at 37 °C for 10 min. Finally, 50 μL of suspension was transferred to a glass capillary for ESR experiments.

2.3. Cell culture

Neuro-2a cells, a subclone of the C1300 murine neuroblastoma derived spontaneously in the A/J mouse (American Type Culture Collection, Rockville, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Belgium) supplemented with 5% fetal calf serum (Invitrogen, Belgium), 0.01% penicillin–streptomycin (Invitrogen, Belgium), and 0.002% gentamicin (Invitrogen, Belgium) and were maintained in a humidified 37 °C, 5% CO_2 incubator.

An amount (60 μL) of spin label stock solution (10^{-2} M) prepared in ethanol was added to the culture medium (10 mL) containing cells and then incubated at 37 °C for 3 h in humidified atmosphere with 5% of CO_2. Incubation was ended by washing cells with PBS in order to remove unbounded spin label. After dilution of the pellets in PBS (final hematocrit 50%), an amount of PPF solution was added to achieve the desired concentration. Added PPF solution was less than 1% of total volume and never exceeded 10^{-4} M as PPF concentration. The cell viability was determined by trypan blue dye exclusion essay [23]. Final solutions were incubated at 37 °C for 10 min and then transferred into a microcapillary tube (50 μL) for ESR measurements.

2.4. Absorption measurements

Absorption spectra of merocyanine 540 (Sigma, Belgium) in lipid suspensions were carried out on a Uvikon 941 spectrophotometer (Kontron instruments, Italy). These measurements were performed in a quartz cuvette of 1 cm optical path length thermostatized by a circulating bath connected to the cuvette holder.

2.5. ESR experimental conditions

All ESR experiments were performed at 9.5 GHz using a Bruker ESR 300E spectrometer (Bruker, Germany) equipped with a variable temperature controller accessory and operating at a center field strength of 3360 G with 120 G as scan range, a modulation amplitude of 2.0 G and 20 mW microwave power. Order parameter (S) and correlation time (τ_c) were calculated according to McConnell [24].

2.6. Microviscosity determination

The mobility of *n*-DSA in the explored medium (liposomes, erythrocyte and Neuro-2a) was quantified by correlation time (τ_c) or order parameter (S) as described elsewhere [25,26]. In the case of weakly to moderately immobilized probes ($\tau_c<3\times10^{-9}$ s) [27], τ_c is used, whereas, for strongly immobilized labels ($\tau_c>3\times10^{-9}$ s), S is better suited [13]. Previously established standard curves of microviscosity vs. correlation time or order parameter [12] were used to convert measured parameters into membrane microviscosity. Each measure was repeated at least four times and microviscosity standard deviation was calculated to be 4%.

3. Results

Stearic acid spin labels have been used as probe to analyze the membrane fluidity of liposomes as well as of cell membranes [14,28,29]. Fig. 1 shows the ESR spectra of 5-DSA embedded: in DMPC lipid bilayer at 33 °C (Fig. 1a), in erythrocyte membrane at 25 °C (Fig. 1b), and in Neuro-2a membrane at 25 °C (Fig. 1c). The absence of any free peak on these spectra indicates the total incorporation of the spin label in the membranes. In liposomes, the probe-to-phospholipid molar concentration ratio is of 1/100 ensuring a complete incorporation of probes. Moreover, any unbounded spin label was removed from cell suspension by washing twice before measurement as described in Material and methods.

3.1. Fluidity of DMPC unilamellar liposomes

3.1.1. Propofol effects probed by ESR

In DMPC unilamellar liposomes, ESR spectra were collected for 5- and 16-DSA probes. The S and τ_c parameters were measured at a temperature above (33 °C) and below (10 °C) the gel-to-fluid transition temperature (23.9 °C) of DMPC [30–32]. In the fluid state (33 °C), local microviscosities of DMPC membrane measured at depths of 7.8 (5-DSA) and 27.7 Å (16-DSA) were 177.92 and 42.29 cP, respectively. At 10 °C, these values increased to 472.56 and 243.37 cP. The microviscosity in the gel state was calculated for both probes using the S parameter only, whereas in the fluid state, S parameter and correlation time were used for 5-DSA and for 16-DSA, respectively.

The addition of PPF up to 10^{-2} M into DMPC liposomes at 33 °C did not modify the microviscosity (Fig. 2a). However, in the gel state (10 °C), two distinct regions were revealed (Fig. 2b). The first one (I) was characterized by a constant value of the microviscosity obtained for a concentration of PPF up to 10^{-4} M (Fig. 2b). The second one (II) corresponded to PPF concentrations ranging from 10^{-4} to 10^{-2} M (Fig. 2b), and was characterized by a decrease of the microviscosity values.

The variation of the DMPC bilayer microviscosity with and without PPF was also investigated as a function of temperature (Fig. 2c). In the gel state ($10 \leq T < 20$ °C), the microviscosity decreased slightly with the temperature. However, near the gel-to-fluid transition temperature (23.9 °C), an abrupt decrease of microviscosity was noticed. When the temperature ($T > 25$ °C) became higher than the transition temperature, the lipid bilayer reached a constant microviscosity value of about 200 cP. Furthermore, the addition of 5.0×10^{-7} or 10^{-5} M PPF did not seem to change the lipid bilayer microviscosity (Fig. 2c).

3.1.2. PPF effects probed by MC540

The lipophilic dye MC540 was used to monitor the effects of PPF in the DMPC lipid bilayer. The spectrum of MC540 in DMPC aqueous solution at 10 °C (gel state) showed a bulge at 501 nm and two peaks at 532 and 568 nm (Fig. 3a, b and c; solid line). The increase in temperature from 10 °C to 20 °C of the DMPC–MC540 solution showed a progressive disappearance of the bulge and a decrease of the peak at 532 nm with a concomitant increase of the peak at 586 nm (Fig. 3a; dashed lines). The addition of high PPF concentrations (5×10^{-4} and 10^{-3} M) to DMPC–MC540 solution led to a modification of MC540 absorption spectrum (Fig. 3b) similar to that induced by an increase in temperature only. At low PPF concentrations (10^{-7}–10^{-6} M), a tiny but similar spectral modification was noted (Fig. 3c). This spectral change was more visible in the difference plots (Fig. 3d), which represent the difference between the spectra of DMPC/MC540 solutions with PPF and spectrum of DMPC/MC540 solutions without PPF.

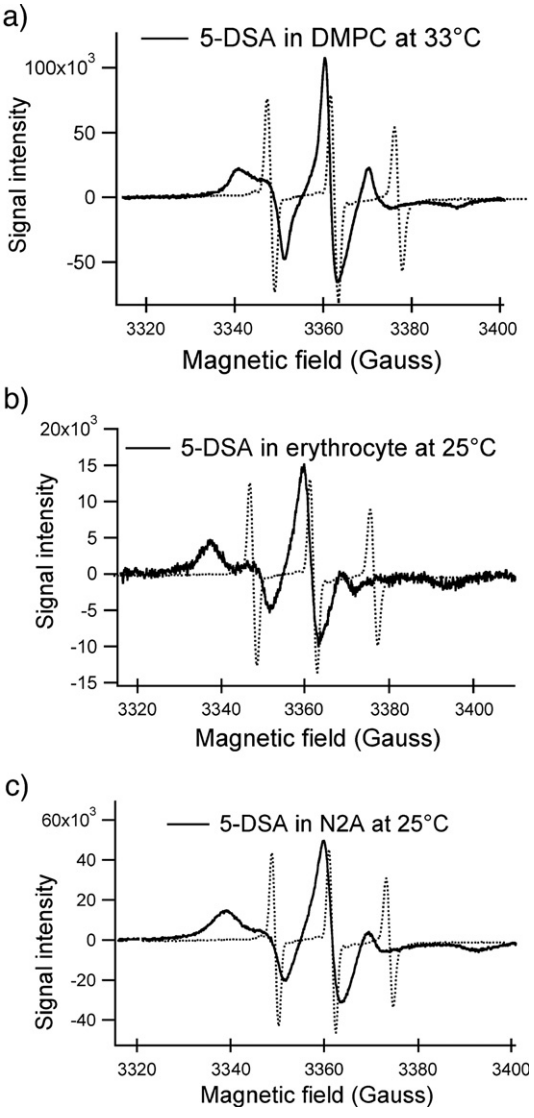

Fig. 1. ESR spectra of 5-DSA in PBS (dotted lines) and (solid lines) (a) in DMPC liposomes at 33 °C, (b) in erythrocytes at 25 °C, and (c) in Neuro-2a cells at 25 °C.

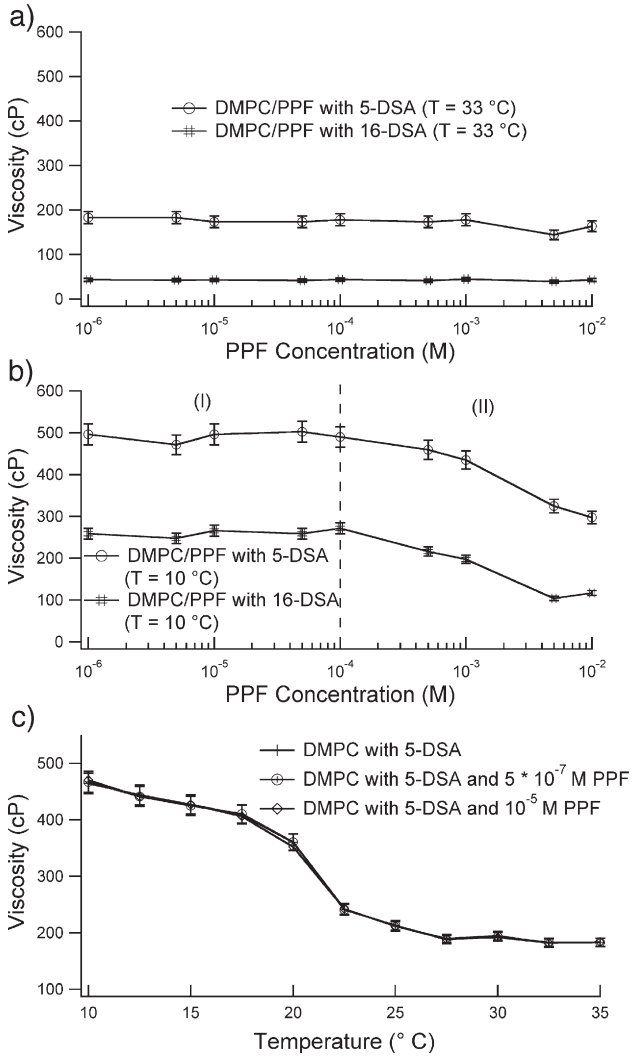

Fig. 2. (a) and (b) DMPC bilayer microviscosity vs. PPF concentration as determined by 5- and 16-DSA, at 33 and 10 °C, respectively, and (c) DMPC bilayer microviscosity vs. temperature as determined by 5-DSA, with and without the addition of PPF.

3.1.3. Effects of Diprivan

Fig. 4 shows the variation of the lipid bilayer microviscosity as a function of PPF concentration formulated as Diprivan® (PPF (Diprivan®)). To reach high PPF concentrations, the added volume of Diprivan® may reach 20% of the total volume of the tested sample. Therefore, the microviscosity of DMPC lipid bilayer as a function of Diprivan® equivalent intralipid (IL) volume at 33 °C and 10 °C was also investigated (Fig. 4a

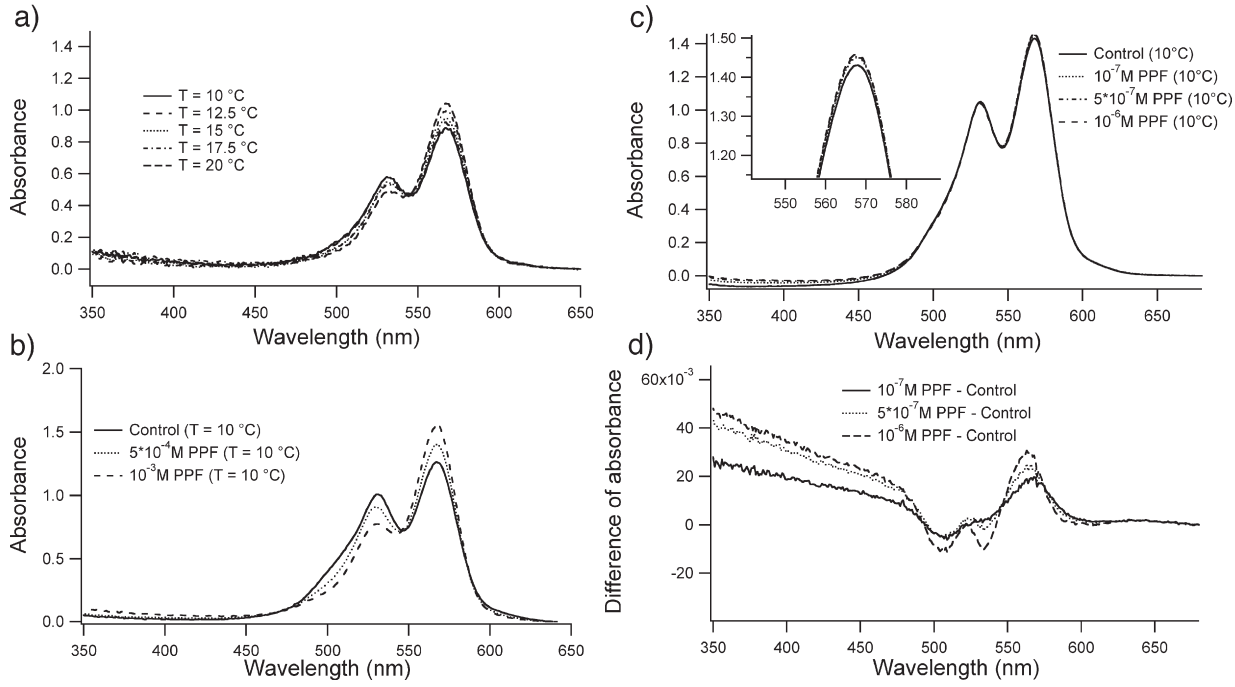

Fig. 3. Absorption spectra of MC540 (10^{-5} M) in DMPC liposomes (1.843×10^{-3} M). (a) Absorbance vs. temperature, (b) absorbance vs. high PPF concentrations (10^{-4}–10^{-3} M) at 10 °C, (c) absorbance vs. low PPF concentrations (10^{-7}–10^{-6} M) at 10 °C, and (d) difference between DMPC/MC540/low PPF concentration (10^{-7}–10^{-6} M) spectra and DMPC/MC540 spectrum without PPF.

and b). In the fluid state (33 °C), neither the PPF (Diprivan®) nor the IL had any effect on the local microviscosity of DMPC liposomes (Fig. 4a). However, in the gel state (10 °C), plots of microviscosity showed a tiny decrease starting from 10^{-4} M and extending to 10^{-2} M PPF (Diprivan®) concentration (Fig. 4b). Fig. 4c shows the plots of the microviscosity of DMPC

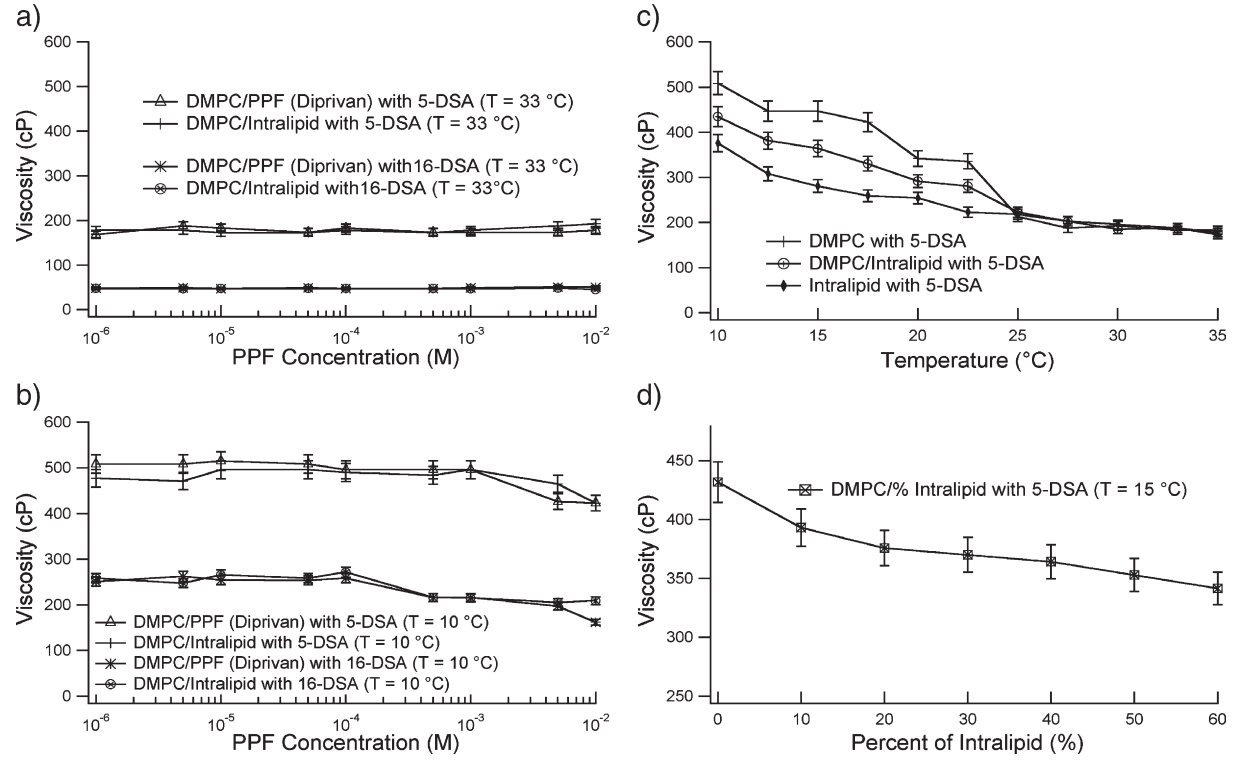

Fig. 4. (a) and (b) DMPC bilayer microviscosity vs. PPF (Diprivan®) concentration and equivalent IL quantity as determined by 5- and 16-DSA, at 33 and 10 °C, respectively, (c) DMPC, DMPC–IL mixture and IL microviscosities vs. temperature (20% of IL is equivalent to the percentage of IL in solution with 10^{-2} M PPF (Diprivan®)), and (d) DMPC bilayer microviscosity vs. IL percentage, at 15 °C.

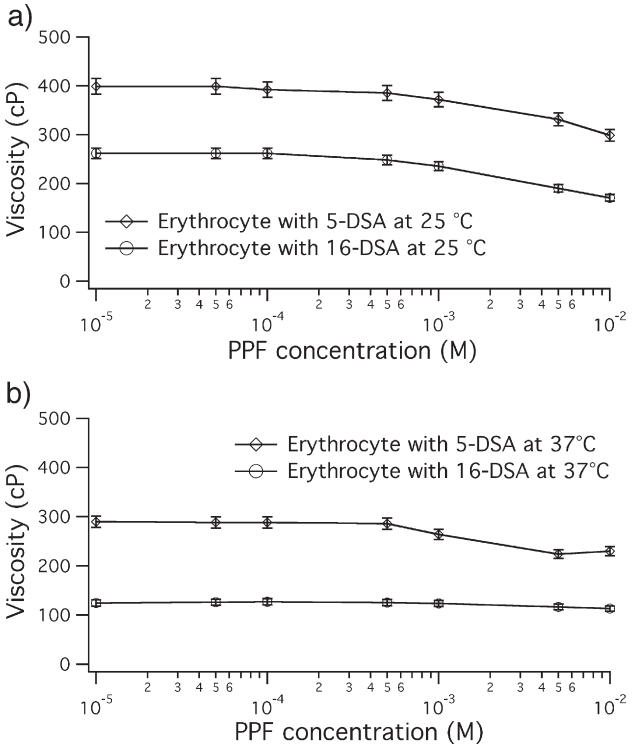

Fig. 5. Erythrocyte membrane microviscosity vs. PPF concentration as probed by 5- and 16-DSA. (a) At 25 °C, and (b) at 37 °C.

lipid bilayer, IL and the mixture of the two components as a function of the temperature. It is important to note that 35.7 μL of IL and 164.3 μL of DMPC were used for the mixture, which corresponds to the quantities used to obtain a PPF (Diprivan®) concentration of 10^{-2} M in DMPC. The addition of an increasing percentage of IL in DMPC lipid solution showed a decrease of the measured microviscosity (Fig. 4d).

3.2. Effects of propofol on the erythrocytes membrane fluidity

As PPF is an intravenous anesthetic agent, it seems of primary interest to investigate its effect on the erythrocyte membrane. In these experiments we used equine erythrocytes due to their availability in our laboratory. With PPF concentration ranging from 10^{-7} to 10^{-5} M (data not shown), no change in local membrane microviscosity was observed, neither at 37 °C nor at 25 °C. A tiny decrease of microviscosity was noted at PPF concentrations between 5×10^{-5} and 10^{-3} M (Fig. 5a and b). Above this concentration range, a clear decrease of microviscosity was observed at the two different membrane depths (5- and 16-DSA) and at 25 °C temperature. However, only 5-DSA showed a clear decrease of microviscosity at 37 °C (Fig. 5a and b). It is important to note that above 10^{-3} M PPF concentration, microscopic examination of tested samples revealed a partial hemolysis of erythrocytes. The PPF-to-erythrocyte solution volume ratio did not exceed 1/100 and we did not note any equivalent ethanol volume effect on erythrocytes. This hemolysis was clearly produced by PPF, averaging 50% at 5×10^{-3} M PPF and 100% at 10^{-2} M PPF.

3.3. Effects of propofol on the Neuro-2a cell membrane fluidity

The investigation of membrane microviscosity and the effect of PPF was finally conducted on neuronal cells (Neuro-2a), which are the main target of PPF. Preliminary experiments were

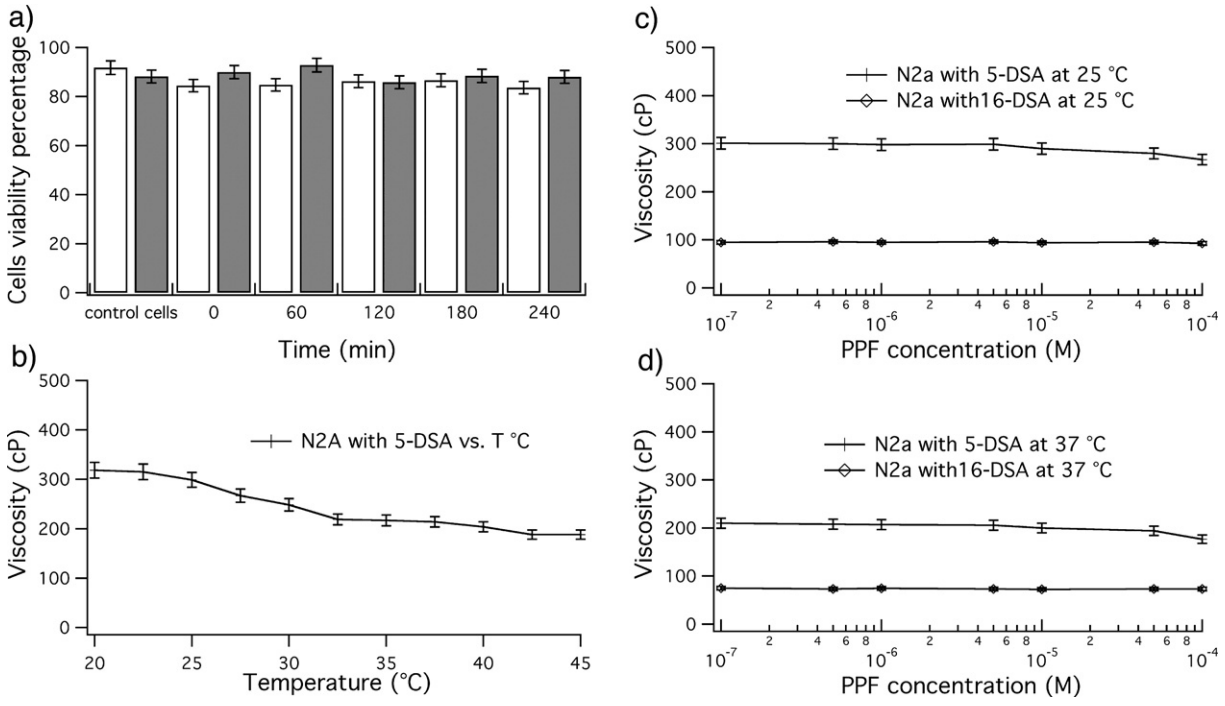

Fig. 6. (a) Mean (±S.D.) of living cells percentage in PBS (control), in PBS with DSA and 100 μM of PPF (filled box), or in PBS, DSA and ethanol (equivalent PPF quantity) (empty box), at increasing incubation time, (b) Neuro-2a membrane microviscosity vs. temperature as probed by 5-DSA, (c) and (d) Neuro-2a membrane microviscosity vs. PPF concentration as probed by 5- and 16-DSA, at 25 and 37 °C, respectively.

set up to evaluate the toxicity of PPF and of stearic spin probes on the cells. Fig. 6a shows that preincubation with various concentrations of PPF ranging from 1 to 100 μM, for several hours, did not induce any cytotoxic effect as measured by cell viability test based on trypan blue. Likewise, at the concentration used to obtain a good ESR signal (10^{-5} M DSA), no cytotoxic effect of the spin probe was observed (data not shown).

Fig. 6b shows the microviscosity sensed by 5-DSA probes incorporated in Neuro-2a cell membrane as a function of temperature. At 20 °C, the microviscosity measured at the depth of 7.8 Å, was 318.27 cP. This value decreased with temperature until reaching 229.94 cP at 33 °C. Between 33 °C and 39 °C, i.e. the range of physiological temperatures, the microviscosity remained nearly constant around a mean value of 207.86 cP. Above 40 °C, the microviscosity showed a decrease accompanied by the appearance of peaks of free 5-DSA in water on the ESR spectra, indicating a likely membrane destruction, that could be expected as cells were dying at this high temperature (Fig. 6b).

Fig. 6c and d shows the variation of microviscosity sensed by 5- and 16-DSA spin probes incorporated in Neuro-2a cells at 25 °C and 37 °C and as function of the PPF concentration. The microviscosity sensed by 5-DSA was lowered by 30% when temperature was increased from 25 °C to 37 °C whereas the microviscosity sensed by 16-DSA was lowered by only 20%. Therefore, it can be suspected that the effect of temperature was more pronounced on the outer membrane leaflet than on the inner one. At PPF concentrations ranging between 10^{-7} and 5×10^{-6} M, microviscosity showed no significant variation, neither in the inner membrane layer nor in the outer one. From 10^{-5} M up to 10^{-4} M, a tiny fluidizing effect of PPF was observed in the outer layer of neuronal cell membranes, both at 25 and 37 °C.

4. Discussion

The *n*-doxyl stearic acid (*n*-DSA) chains have been shown to orient themselves approximately in the direction of the liposome surface normal and parallel to the phospholipid molecules [33]. Thanks to the nitroxide radical brought on by its chain at the fifth and sixteenth carbon position, *n*-DSA ($n=5$, 16) probes explore the bilayer lipid microenvironment at two different depths of 7.8 (5-DSA) and 27.7 Å (16-DSA). Samples of ESR spectra of 5-DSA probes embedded in DMPC liposomes, in erythrocytes and in Neuro-2a cells, are shown in Fig. 1. These spectra represent the spin probes in rapid anisotropic motion [29]. ESR spectra are known to be very sensitive to the probe anisotropic motion degree [15] and any motional restriction caused by local viscosity is directly seen as a spectrum modification. These spectral modifications are related to the probe microviscosity [12,15]. Based on a previous work [12], the correlation between *n*-DSA probes rotational mobility and the microenvironment viscosity was used to monitor quantitatively the effect of the PPF on the membrane microviscosity of liposomes and cells.

4.1. DMPC unilamellar bilayer

Propofol effects on membrane fluidity have been the focus of many studies [3,10,34,35]. A clear membrane fluidity effect at PPF concentrations higher than clinically relevant concentrations has been evidenced [34–36], but no evident fluidity effect has been observed at the clinical PPF doses [10,35]. In a previous work, we investigated the effect of incorporation of PPF in DMPC liposomes on the lipid bilayer fluidity by quantifying the microviscosity as a function of PPF concentration [12]. Throughout the tested PPF concentration range (10^{-3}–5×10^{-2} M), which is much higher than clinically relevant concentrations, we evidenced the fluidizing effect of PPF and identified 10^{-2} M as the saturation limit of PPF in DMPC liposomes [12]. In the present work, we extended the study to lower PPF concentrations ($\leq 10^{-3}$ M) in order to know if PPF has also a membrane fluidizing effect at clinical doses.

The addition of PPF ranging from 0 to 10^{-2} M into DMPC liposomes at a temperature (33 °C) above the gel-to-fluid state changed neither S (5-DSA) nor τ_c (16-DSA) parameters and consequently did not modify microviscosity values (Fig. 2a). This absence of PPF effect on lipid bilayer fluidity even at high concentrations is explained by the fact that 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 [37]. In the gel state (10 °C), the incorporation of PPF concentration up to 10^{-4} M into these liposomes did not provoke any measurable change in the bilayer fluidity (Fig. 2b). However, PPF concentrations ranging from 10^{-4} to 10^{-2} M clearly increased membrane fluidity, as already reported [3,36,38]. At the explored depths of 7.8 Å (5-DSA) and 27.7 Å (16-DSA), PPF exhibited a somewhat similar effect on membrane microviscosity. This could suggest that PPF is situated both in the outer and in the inner layers of the bilayer membrane. However, this idea has been ruled out [39,40]. Indeed, it has been reported that PPF almost uniformly diffuses in the region near the polar head groups [3]. PPF can also establish hydrogen bonds based on the interactions between its phenolic hydroxyl group and carbonyl oxygen or the hydroxyl group coming from aqueous solvent and other molecules within liposome bilayers [41]. PPF could be seen as bulky groups, when intercalated in the lipid moiety, through its steric hindrance and collisions, and is able to reduce the van der Waals forces between the ordered chains [3]. Consequently, the microviscosity decrease could be explained by the fact that the intercalated PPF molecules increase the distance between phospholipid molecules and therefore the freedom degree of hydrocarbon chain, giving consequently more fluidity at 5- and 16-DSA nitroxide radicals located at the inner emplacement than PPF which probably remains near the polar heads region thanks to the hydrogen that binds to the lipid bilayer.

The variation of the DMPC bilayer microviscosity in the absence and in the presence of PPF (5.0×10^{-7} and 10^{-5} M) as a function of temperature (Fig. 2c) indicates again the absence of any detectable PPF fluidizing effect at low concentration in both states of DMPC lipid bilayer. This absence of PPF fluidizing effect could be related to the sensitivity limit of our ESR method. Thus, in an attempt to confirm or discard this result, we used merocyanine 540 (MC540) as a membrane probe. This molecule is very sensitive to the membrane microviscosity and was already used to probe the effect of PPF on DPPC bilayer fluidity [12].

The increase of DMPC solution temperature leads to a progressive change in the lipid membrane vesicle from gel state to fluid state [42]. The modification of the bilayer fluid state can be effectively monitored using MC540 absorption spectrum change (Fig. 3). Indeed, the progressive decrease of the 501 nm bulge and the 533 nm peak along with the increase of the 568 nm peak of MC540 are indicative of the penetration of the dye into the DMPC lipid bilayer when this later becomes more fluid [43]. This behavior was clearly observed when DMPC/MC540 solution temperature was increased (Fig. 3a) as well as when PPF at high concentrations (10^{-4}–10^{-3} M) was added to DMPC/MC540 solution in the gel state (T=10 °C) (Fig. 3b). At low PPF concentrations (10^{-7}–10^{-6} M), we noted a similar tiny spectral modification (Fig. 3c). These spectral modifications were more visible in the difference plots (Fig. 3d), which represent the difference between the spectra of DMPC/MC540 with PPF low concentrations (10^{-7}–10^{-6} M) and the spectrum of DMPC/MC540 without PPF. Moreover, the presence of the isobestic points in all absorption plots is a clear indication of the exchange of the two forms of the dye (dimer and monomer) between the bulk solution and bilayer membrane. These results qualitatively confirm the ESR results observed at high PPF concentrations but also indicate a fluidizing effect of PPF even at clinically useful concentrations, which was not detectable using the ESR technique. Absorption spectroscopy seems to be more appropriate to detect a small fluidizing effect of PPF than the ESR technique. Indeed any fluidizing effect, even locally, modifies the dimer–monomer repartition in the two explored media (bulk solution, bilayer membrane), which in turn changes the dye absorption spectrum. In contrast, a small fluidizing effect can be averaged out in ESR signal especially if the repartition of these effects is not uniform on the bilayer membrane.

The effect of the commercial form of propofol (Diprivan®) on the fluidity of the same membrane model was also investigated. Compared to the results obtained with PPF (Fig. 2b), Diprivan® exerted a very small effect on DMPC membrane fluidity (Fig. 4b). Moreover, experiments of microviscosity of DMPC as a function of Diprivan® equivalent intralipid (IL) volumes showed results similar to those obtained with Diprivan®. This would indicate that ESR signal modification is caused by the presence of IL in Diprivan® solution. Therefore it might be that the fluidizing effect of PPF observed in the gel state of DMPC when using pure PPF is averaged out when using Diprivan® by a possible partitioning of DSA probes between lipid bilayer and Diprivan® lipid vesicles. The plots of the microviscosity of DMPC lipid bilayer, IL and DMPC/IL against temperature show the same value in fluid state (Fig. 4c). However, in the gel state, the DMPC/IL microviscosity seems to be the average of DMPC lipid microviscosity value and IL one. Furthermore, the addition of an increasing percentage of IL in DMPC lipid solution at 15 °C decreases the measured microviscosity (Fig. 4d). These findings further support the idea of probes partitioning between the two media where the probes took a different freedom degree. Diprivan® lipid vesicles cross probably into DMPC hydrophobic region, enclose a part of probe molecules and consequently, the ESR spectrum is the average of both DMPC probes signal and IL probes signal. This effect is observed only in the gel state where the DMPC lipid bilayer and lipid emulsion have a different microviscosity. In conclusion, the presence of IL in Diprivan® composition seems to disturb ESR measurements. For this reason, the effect of Diprivan® was not further investigated in cell membrane.

4.2. Erythrocyte membrane

At temperatures of 25 °C and 37 °C, PPF concentrations below 10^{-5} M did not induce any erythrocyte membrane fluidity change (data not shown). However, a small fluidity effect was noticed at PPF concentrations higher than 5×10^{-4} M especially near the membrane surface explored by 5-DSA (Fig. 5). Above 10^{-3} M, PPF had a clear hemolysis effect.

At 25 °C, local microviscosities measured with 5- and 16-DSA probes were 397.09 and 261.75 cP, respectively. Those microviscosity values decreased to 292.38 and 126.36 cP, when erythrocytes are maintained at the physiological temperature (37 °C). Comparing these values to those measured in DMPC membrane below and above its gel-to-fluid phase transition (5-DSA: 472.56 cP at 10 °C and 222.53 cP at 33 °C) (16-DSA: 243.37 at 10 °C and 42.29 cP at 33 °C), we clearly point out the importance of membrane molecular composition in its stability [25,44]. In contrast to DMPC membrane which contains only saturated phospholipids, one half of the erythrocyte membrane is represented by proteins and the other half mainly by unsaturated phospholipids and cholesterol. The molar ratio between cholesterol and phospholipids is about 0.9 [25]. The presence of polyunsaturated phospholipids leads to an accumulation of trans-gauche isomerizations, causing a decrease of order towards the end of the acyl chains [45,46]. This can play a role in the fluidity state of erythrocyte membrane, especially in the inner layer of the membrane explored by 16-DSA, which explains the difference in microviscosity noted between the inner and the outer membrane layers. This accumulation of trans-gauche isomerizations can also play a great role in erythrocytes hemolysis process by PPF. As PPF seems to be kept near the polar heads region as described above, its action on reducing van der Waals forces between phospholipids could have a drastic effect on the membranes formed by unsaturated phospholipids due to their conformational disorder, and could explain the hemolysis effect induced by high PPF concentrations.

4.3. Neuro-2a membrane

A PPF effect on neuronal cell membranes was only observed at PPF concentrations ranging from 10^{-5} to 10^{-4} M (Fig. 6c and d). The effect was tiny and was especially noted in the outer layer of neuronal cell membranes, both at 25 and at 37 °C. This finding reinforces the hypothesis that due to its phenolic hydroxyl group, PPF is kept near the outside membrane. The absence of the fluidizing effect on the inner membrane layer even at high PPF concentrations could also be explained by the fact that neuronal cell membranes contain more cholesterol than the other membranes, giving them greater depth stability [47].

As far as our ESR results did not reveal any ESR measurable membrane fluidizing effect of PPF at clinical doses, one may question if PPF promotes small-scale fluid domains as described by Balasubramanian et al. [48], and how can the small-scale domains microviscosity effects be observed with our paramagnetic probes? Balasubramanian et al. observed that propofol promotes the formation of small-scale domains in large-scale fluid phases and they estimated their size to be 20–50 Å in a DPPC–DMPC mixture model membrane. Hence, they speculate that anesthetic activity may involve an effect on the protein–lipid coupled system through formation of small-scale lipid domain structure. This idea is shared by several authors attributing the activity of certain proteins both to their conformational aspect in membrane and their sensitivity towards the surrounding lipids [49,50]. The liposoluble *n*-doxyl stearic acid spin labels consist in carboxyl group bearing a skirt stearic acid chain to which a nitroxide moiety is attached [25]. In the membrane, the probe's carboxyl group positions itself near the phospholipid's polar head, whereas its stearic acid chain plunges in the membrane parallel to phospholipid chains [33]. Nitroxide moiety is located at the position $n=5$ or $n=16$ of the stearic acid chain [25]. ESR spectrum is the average of all absorption signals representing all probes, in other words representing all probed microenvironment microviscosities. Therefore, in an attempt to see a significant ESR signal modification, two criteria should be fulfilled. The first one is the number of probes situated in small-scale domains that should be enough to modify the ESR averaged signal. The second one is the magnitude of the perturbation, which should be sufficient to reach the nitroxide moiety microenvironment. Hence, the addition of small concentrations of anesthetic may locally influence the structure and dynamics of membrane domains, through the formation of small-scale lipid domains, but not in a sufficient way to be detected by the ESR technique.

5. Conclusion

In the present work, the effect of propofol on the microviscosity of DMPC bilayer, erythrocyte membrane and neuronal cell (N2a) membrane was investigated by using the ESR spin probing technique. Propofol membrane fluidizing effect was monitored throughout a wide PPF concentration range. Our ESR results revealed a clear effect at concentrations higher than the clinically relevant concentrations. However, absorption spectroscopy of MC540 clearly indicated a propofol fluidizing potency even at clinical concentrations. PPF may locally influence the structure and dynamics of membrane domains, through the formation of small-scale lipid domains, which would explain the lack of ESR information at low PPF concentrations. The effect of Diprivan® on membrane fluidity could not be studied because of the ESR measurement disturbance caused by the intralipids contained in its composition.

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