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# Interaction of fluoxetine with phosphatidylcholine liposomes

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**Abstract**

Fluoxetine (Prozac) is one of the latest of a new generation of antidepressants, approved by FDA in 2002.

The interactions of fluoxetine with multilamellar liposomes of pure phosphatidylcholine (PC) or containing cholesterol 10% molar were studied as a function of the lipid chain lengths, using differential scanning calorimetry and spin labelling EPR techniques.

The DSC profiles of the gel-to-fluid state transition of liposomes of DMPC (C14:0) are broadened and shifted towards lower temperatures at increasing dopant concentrations and, with less than 10% fluoxetine, any detectable transition is destroyed. The broadened profiles and the lowered transition temperatures demonstrate that both the size and the packing of the cooperative units undergoing the transition are modified by fluoxetine, leading to a looser and more flexible bilayer. No phase separation was observed.

The effects of fluoxetine on the thermotropic phase behaviour of DPPC (C16:0) and, even more, of DSPC (C18:0) are different from that of DMPC. In fact, in the former cases, two peaks appeared at increasing dopant concentrations, suggesting the occurrence of a phase separation phenomenon, which is a sign of a binding of fluoxetine in the phosphate region.

In cholesterol containing membranes, fluoxetine, even at low concentrations, leads to a general corruption of the membrane, both in terms of packing and cooperativity, and the formation of any new phase is no longer observable.

EPR spectra reflect the disordered motion of acyl chains in the bilayer. It was found that fluoxetine lowers the order of the lipid chains mainly in correspondence of the fifth carbon position of SASL, indicating a possible accumulation near the interfacial region.


## 1. Introduction

Prozac (fluoxetine, FLX) is one of the latest of a new generation of antidepressants, known as the selective serotonin uptake inhibitors (SSRI) [1]. It is used medically in the treatment of depression, obsessive–convulsive disorder, bulimia nervosa, and many other disorders. Chemically, fluoxetine is unrelated to tricyclic and tetracyclic antidepressant agents such as imipramine and its analogs, being a secondary amine with one phenyl and one tolyl group in its structure (Fig. 1) [2]. It can be considered an amphipathic molecule having a pKa = 10.7 [3] and therefore, at physiological pH, a polar head and a hydrophobic body.

A high membrane permeability is usually associated with amphipathic drugs, antidepressant included, which makes the interactions of these drugs with biomembranes of wide interest [4–7] because their effects may influence the cellular processes; in fact a demonstrated large cooperativity between proteins and lipid moiety exists, so that the protein-specific membrane functions can be affected by agents able to alter the bilayer structure. In the light of these remarks, the investigation of the drug–phospholipid bilayer system is important because, if from one side the aspecific interaction with the lipid moiety may be a determining factor of the pharmaceutical effects of the drugs, from the other it may be responsible of the drugs toxicity as well. In the case of fluoxetine, for example, it was found that the drug alters the functions of the liver mitochondria [8]. We investigated the interactions of fluoxetine with multilamellar liposomes of phosphatidylcholine (PC) of various chain lengths, determining the partition coefficients of the drug, its preferred

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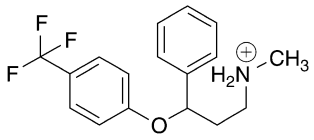


Fig. 1. Molecular structure of fluoxetine.

distribution inside the bilayer, and the decay of the ordered lipid organisation that it produces, using differential scanning calorimetry (DSC) and spin labelling EPR techniques. The results confirmed the strong and peculiar destabilising effect on the lipid organisation which could be expected on the basis of general considerations [9] concerning the relatively large dimensions of the molecule and the presence of the polar group.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals, of the highest available quality, were obtained from Sigma Chemical Co. (St. Louis, USA), while the solutions were prepared with quality milliQ water.

### 2.2. Liposome preparation

Multilamellar vesicles were prepared following the method of Kusumi et al. [10]. Phospholipids were dissolved in a 2:1 chloroform methanol mixture, then dried with a stream of nitrogen gas and kept under vacuum for at least 14 h. The dried lipids were suspended, when not otherwise specified, in a HEPES 0.1M buffer, pH 7.2, solution containing fluoxetine. The lipid dispersion, with a 101 mM final lipid concentration, was warmed over the transition temperature, mixed repeatedly with a vortex for 30 s and used just as obtained for DSC measurements. A solution of tempo, 1 mM final concentration, was added to the phospholipid dispersion for the determination of the partition to tempo.

When required, spin labels (SASL) were added to the chloroform methanol mixture (final concentration of SASL = 1.8 mM).

### 2.3. Determination of the association constant

For the determination of the phospholipid–fluoxetine association constant, the lipid dispersion, maintained for an hour at 6 °C, was then centrifuged at 12 000 $\times g$ for 15 min at the same temperature; the supernatant was carefully drained from the pellets, and the amount of the FLX in the solution was calculated from its absorbance value at $\lambda = 232$ nm, $\varepsilon = 9900$. The low temperature was mandatory for a good separation of the supernatant, so the association constant is relative to the gel phase for all the three phospholipids.

### 2.4. UV-VIS spectroscopy

Spectrophotometric measurements were recorded on a UV-VIS Beckman DU 640 instrument equipped with a thermostated quartz cell. All measurements were taken in triplicate.

### 2.5. DSC measurements

Calorimetric measurements were performed on a Setaram DSC 92.

About 50 mg of phospholipid dispersion was placed in an aluminium crucible. An identical crucible was filled with an equivalent weight of HEPES solution and placed in the reference cell.

The temperature scanning rate was 0.5 °C $min^{-1}$. The transition temperature $T_c$ from the gel ($L_\beta$) -to- fluid ($L_\alpha$) phase of liposomes was taken at the peak of the DSC profiles.

### 2.6. EPR measurements

EPR measurements were performed on a Bruker ER 200 D, 9 GHz spectrometer at microwave power range from 0.1 to 220 mW. Samples were placed in a gas permeable TPX tube 1 mm i.d. (Wilmad, N.J. USA) and centered in the resonant cavity, then deoxygenated under nitrogen flow above $T_c$ for 10 min.

## 3. Results and discussion

### 3.1. Partition coefficients

The logarithm of octanol–water partition coefficient (log $P$) is used as a measure of the hydrophobicity of compounds and it is assumed to represent the general tendency of a chemical to partition between an aqueous and an organic phase. $P$ is defined as $P = (n_{oct}/V_{oct})/(n_w/V_w)$, where $n$ denotes the number of moles of fluoxetine, $V$ the volume, and $_{oct}$ and $_w$ refer to octanol and water phase respectively. The tabulated log $P$ of fluoxetine at 20 °C is 3.75 [11].

It must be said that the notion of partition coefficient, when extended to liposomes/water dispersions, can be vague for a number of reasons, not least the difficulty of defining the volume of liposomes and considering it like a homogeneous phase; anyway it is commonly accepted and log $P$ is used to quote the fraction of dopant adsorbed into the membrane. Instead, the concept may be misleading when, contrary to molecules which more or less uniformly diffuse in the lipid region of the bilayer, the drug establishes stechiometric bonds most likely with the polar groups of the phospholipid heads; in this case an association constant $K$ may be more appropriate to describe the situation.

There are evidences, as discussed in the following, that fluoxetine binds to the negatively charged phosphate group,

Table 1
Association constant $K$ for the binding of fluoxetine with phospholipids

| | DMPC | DPPC | DSPC |
|---|---|---|---|
| $K$ ($M^{-1}$) | 61 | 55 | 55 |
| % | 74 | 70 | 70 |

%: percent fraction of fluoxetine (5 mM) bound to liposomes (100 mM) at 6 °C (gel state).
$\Delta K = \pm 5$, $\Delta\% = \pm 2$.

so we quote in Table 1 the values of a hypothetical association constant $K$; anyway, $K$ and log $P$ are nothing but different parameters expressing the molar drug fraction bound to or adsorbed into the bilayer. The percent fraction of fluoxetine, at the adopted experimental conditions (6 °C, gel phase), is about 80% and is given in Table 1 as well. The experimental conditions represent in some sense a worst case, so the data demonstrate that fluoxetine is a rather hydrophobic molecule which, in a water dispersion of liposomes, is almost exclusively present in the lipid phase.

### 3.2. DSC studies

The DSC profiles of the gel-to-fluid state transition ($T_c$) of DMPC (C14:0), DPPC (C16:0), DSPC (C18:0) of

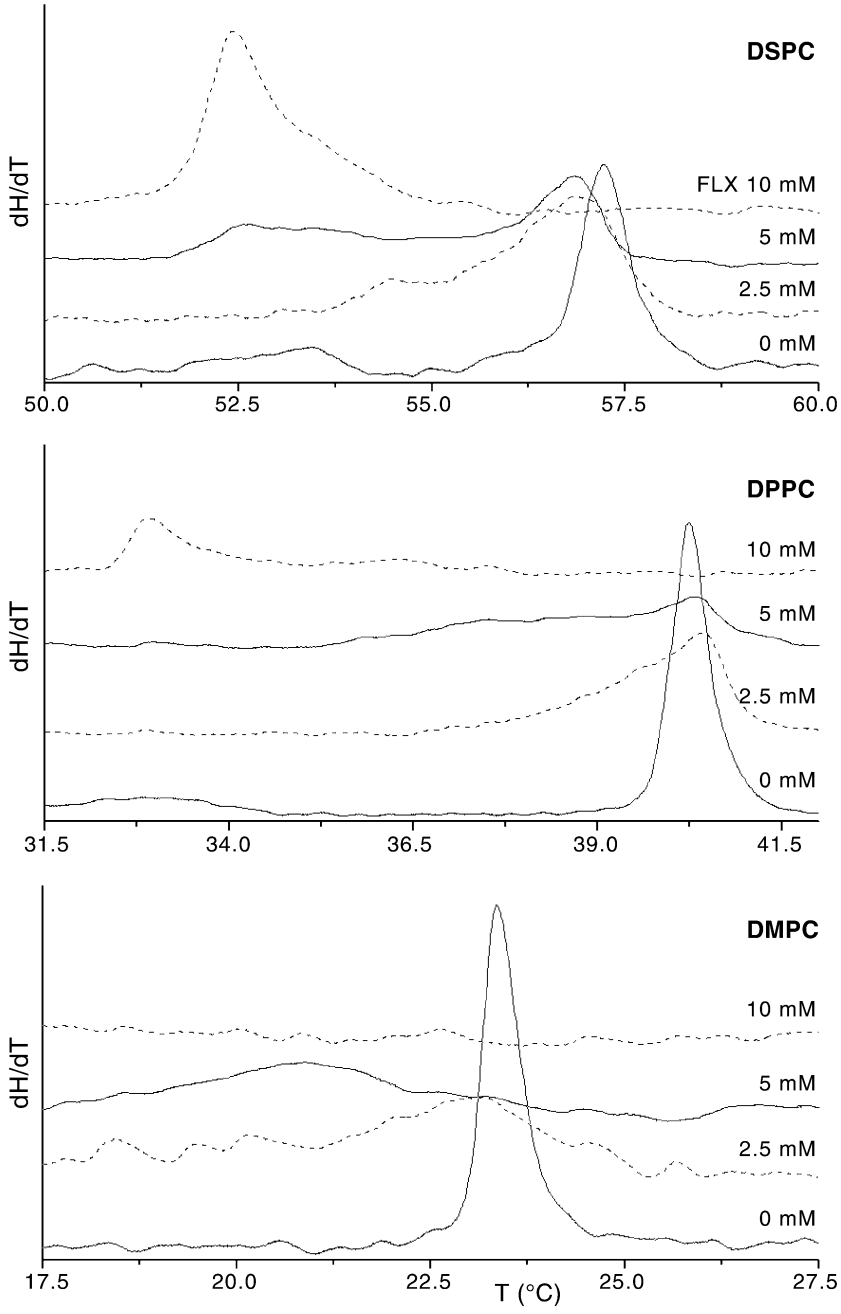


Fig. 2. DSC profiles of the gel-to-fluid state transition of multilamellar liposomes 100 mM in PC at increasing fluoxetine (FLX) concentrations.

multilamellar liposomes at increasing concentrations of fluoxetine are plotted in Fig. 2.

Fluoxetine modifies the thermotropic phase behaviours of DPPC and DSPC in a rather heavy and similar way: in fact, in both cases, two peaks appear at increasing dopant concentrations, demonstrating the occurrence of a new different phase. This fluoxetine-rich phase is not immediately generated from the pure phospholipid phase and its formation can be followed through the DSC profiles. We observe firstly the appearance of a large shoulder at the low temperature side of the main peak, and a plausible hypothesis for explaining it is that, at low concentrations (2.5 mM), fluoxetine provokes the separation from the pure phospholipid phase of a range of domains with slightly different packing characteristics and transition temperatures, always lesser than those of the parent peak; the lack of cooperativity suggests that the domains should not be contiguous but dispersed throughout the whole lipid matrix. These domains grow at the expense of the main peak at intermediate dopant concentrations (5 mM) and, finally, a new cooperative phase is restored when the fluoxetine/phospholipid ratio is sufficiently high (10%). The process is not complete, and the new phase coexists with the low cooperative domains which formed first; the formation of the new, fluoxetine-rich, phase is favoured in DSPC liposomes, with respect to DPPC.

Fluoxetine proved to be a strong perturbing agent which, even at low concentrations, determines the partial disaggregation of the lipid matrix in domains of lower cooperativity and packing.

Such effects can be expected when a relatively large and polar molecule is located in the polar region and alters the overall polar head organisation up to the glycerol backbone region; in our case, these effects can be reasonably ascribed to a stechiometric like binding of the positively charged fluoxetine to the phosphate group. The hypothesis that the perturbation may be generated in the polar region is also supported by the total annihilation of the pretransition, because the ripple gel phase is very sensitive to the presence of dopants in this side of the membrane.

The DSC profiles of DMPC are broadened and shifted towards lower temperatures at increasing dopant concentrations and at 10% fluoxetine level an extremely broad transition centered at 15 °C is hardly detectable; the broadened profiles and the lowered transition temperatures demonstrate that both the size and the packing of the cooperative units undergoing the transition are modified by fluoxetine, leading to a much looser bilayer, while any clear phase separation phenomenon is not observed.

There are no grounds to think that the interaction of fluoxetine with DMPC is not the same of DPPC and DSPC, and, in particular, that the drug does not bind to the phosphate group, so the different behaviour of DMPC liposomes must be related to the length of acyl chains.

The destabilising effect of fluoxetine is most likely due to its binding to the phosphate group which determines a strong weakening of the polar heads organisation; when not sufficiently counterbalanced by van der Waals interactions between alkyl chains, which assure the packing of the lipid matrix, the fluidising effect is such as to virtually destroy the main transition at drug concentrations above 5 mM. The formation of an ordered and cooperative gel phase, implied by the observation of a DSC peak, is made possible only by chains longer than 16 carbon atoms, and, in this perspective, the formation mechanism are expected, as they are, to be more efficient for DSPC than DPPC.

As cholesterol is a natural component of biomembranes, we tested the bilayers modifications in liposomes containing cholesterol 10% molar: the results, plotted in Fig. 3, show that the destabilising effects of fluoxetine are even stronger.

Cholesterol by itself modifies the DSC profiles of liposomes in different ways and degrees, according to their lipid composition; however, at the experimental concentration, the main phase transitions, at least of DPPC and DSPC, are still clearly observable. The contemporary presence of fluoxetine and cholesterol leads to a general corruption of the membrane, both in terms of packing and cooperativity, and prevents, at higher fluoxetine contents, the formation of any observable new phase. Most likely cholesterol acts as an obstacle to the hypothesised process that the DSC peak at low temperature is generated by the rearrangement as a whole, in a new gel phase, of the many domains which, at lower fluoxetine concentrations, are sparse.

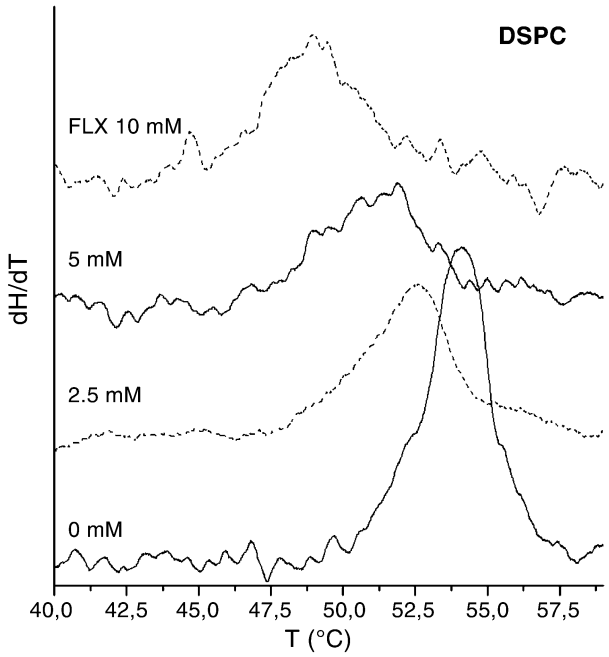


Fig. 3. DSC profiles of the gel-to-fluid state transition of multilamellar liposomes 100 mM in DSPC, containing cholesterol 10% molar, at increasing fluoxetine (FLX) concentrations.

## 4. EPR measurements

### 4.1. Hyperfine splitting

EPR spectra have been collected for stearic acids, spin labeled at the 5th, 7th, 10th carbon positions (n-SASL) and incorporated in liposomes, with and without dopant.The order parameter was measured over and below the gel-to-fluid transition temperature of liposomes. It appeared that above the transition temperature the lipid matrices can accommodate rather high concentrations of dopant without aggravating the disordered motions of the lipid chains (data not shown) [12,13]. In the conditions of very slow motion, characteristic of the gel state, $A_{max}$ (the separation between the outer hyperfine lines) is the most appropriate parameter for monitoring the effects of dopants on the ordered organisation and motion of the alkyl chains [14]. The differences $\Delta A_{max}$ between the values of $A_{max}$ in pure liposomes and in the presence of fluoxetine 10 mM are reported in Fig. 4.

The largest values of $\Delta A_{max}$ occur for all phospholipids in correspondence of the fifth carbon position of SASL, and, from this point of view, spin labeling confirms the results of DSC and their interpretation, indicating a possible accumulation of fluoxetine near the interfacial region. At the same time, the intrinsically low values of $\Delta A_{max}$ at the deepest positions demonstrate that the corruption of the phase transition profiles is effectively due to modifications of the phospholipids aggregation state at the glycerol level, more than to a weakening of the acyl chains order.

The values of 5-SASL's $A_{max}$ and order parameter $S$ in lipid matrices containing 10 mol% of cholesterol are quoted in Table 2. Given that the fluidising effects of fluoxetine are more marked below the transition temperature, while the opposite effects of cholesterol are more evident over it, the data, for all phospholipids, both in the gel and fluid state, show that fluoxetine is able to counter the rigidifying action of cholesterol.

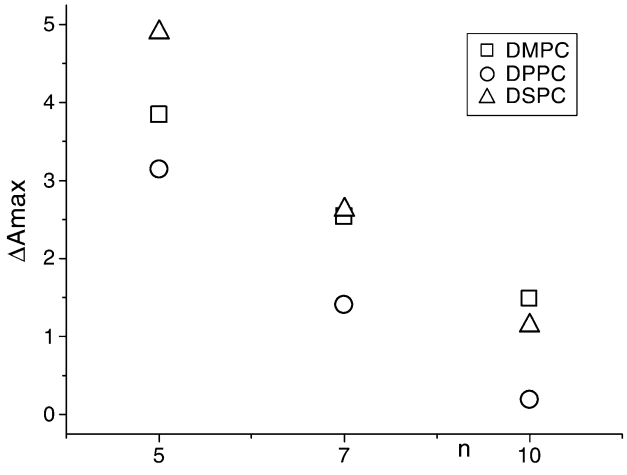


Fig. 4. $\Delta A_{max}$ values (in $10^{-4}$ Tesla) vs. spin label position $n$ in multilamellar liposomes 100 mM of DMPC, DPPC and DSPC doped with FLX 10 mM.

Table 2
Effects of fluoxetine on the values of $A_{max}$ (in $10^{-4}$ Tesla) and $S$

| | Pure lipid | | +Fluoxetine | | +Cholesterol | | +Fluox. and chol. | |
|---|---|---|---|---|---|---|---|---|
| | $A_{max}$ | $S$ | $A_{max}$ | $S$ | $A_{max}$ | $S$ | $A_{max}$ | $S$ |
| DMPC | 59 | 0.63 | 56 | 0.62 | 60 | 0.67 | 55 | 0.65 |
| DPPC | 62 | 0.57 | 58 | 0.56 | 62 | 0.61 | 60 | 0.59 |
| DSPC | 62 | 0.54 | 59 | 0.53 | 62 | 0.57 | 60 | 0.55 |

$A_{max}$ was measured at 16 °C, below the transition temperature of all the phospholipids.
$S$ at 26 °C (DMPC), 44 °C (DPPC), and 58 °C (DSPC), over the respective transition temperatures.
Values are quoted with significant figures; $\Delta A_{max}=\pm 0.5$, $\Delta S=\pm 0.005$.

In principle the above observation, that a bulky group (fluoxetine) in the interfacial region may affect the mobility of cholesterol, could be of some interest, but, as we will hint in the following, it is also possible that, preferentially, the spin labels simply relocate themselves near fluoxetine.

A second point, underlined by EPR experiments, concerns the interaction of fluoxetine with SASL's, and will be discussed because may have a general validity, demonstrating that fluoxetine is able to modify the binding and the distribution of other molecules in the polar region.

A general interfacial property of liposomes is the reduced polarity at the surface with respect to the bulk of the solution, which implies a shift $\pm\Delta pK_a$ of titrable compound, where the sign is positive for the dissociation of an acid, and negative for the dissociation of a cationic base. The shift, at the headgroup level, is something over unit [15,16], and is irrelevant in the case of fluoxetine whose $pK_a$ in solution is 10.7 and higher than 9.0 in liposomes. Cholesterol, when present, increases the hydration of the liposomes surface [17] and reduces the shift of $pK_a$ relative to the bulk.

Instead, the $pK_a$ of the carboxyl group of SASL's in PC membranes is about 6.5 [18], and pH 7.2 both the protonated $-COOH$ and unprotonated $-COO^-$ carboxyl forms are present and produce a clear inhomogeneous broadening of the spectrum. The result is interpreted in terms of two anchoring sites in the polar region of PC bilayers, which are related to the possible forms of the carboxyl group: the unprotonated $-COO^-$ group is anchored in the positively charged choline region while the protonated $-COOH$ is anchored in the phosphate region by hydrogen bonds [19]. As a consequence, the nitroxide label is located at different depths inside the bilayer: with respect to the unprotonated form, the position of the protonated forms is about two carbon atoms deeper, and can be identified as the more fluid component of the EPR spectrum. The composite structure of the spectrum is removed by fluoxetine which causes the disappearance of the component relative to $-COOH$. Most likely, the hydrogen bonds formed by the $-COOH$ groups are broken by the stronger ion–ion interaction of the negatively charged phosphate group with the positively charged amino group of fluoxetine, and SASL's are forced to leave their anchoring point, relocating themselves in the choline region.

The above hypothesis from one side finds a confirmation in the fact that the EPR spectrum of the near fully unprotonated 5-SASL's at pH 8.5 is superimposable to the spectrum of the same spin labels at pH 7.2 in the presence of fluoxetine (Fig. 5), from the other side implies two more reflections.

In fact the simple relocation of protonated SASL's in the outward region of the membrane should produce a broader lineshape of EPR spectra than that observed, which is more consistent with the hypothesis of a deprotonation of SASL's after their removal from the phosphate region. We can only suggest that the apparent $pK_a$ near 7, assigned to SASL's in the polar head region, is a mean value representative of a distribution which, in the vicinity of choline, should have lower values, closer to that of SASL's in solution. With this assumption, SASL's, relocated by fluoxetine in the choline region, should also deprotonate.

The second point is the very unpolite behaviour of fluoxetine, which competes with SASL's for parking: there must be a tendency of the two molecules to join, and a possible reason is that, in the acyl chains region, the space perturbed by the presence of fluoxetine may be more favourably occupied by the nitroxide ring of SASL's.

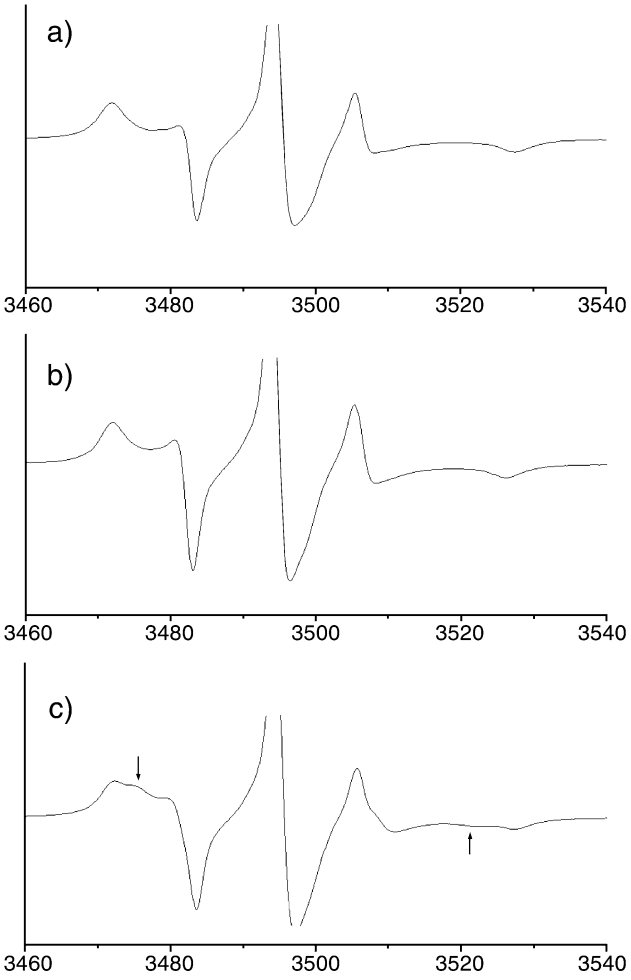


Fig. 5. EPR spectra of 5-SASL in an aqueous dispersion of DSPC liposomes at 57 °C: a) pH=8.5; b) pH=7.2 in the presence of FLX 10 mM; c) pH 7.2; *x*-axis: magnetic field in $10^{-4}$ Tesla. The arrows indicate the position of the outer hyperfine lines of the protonated fraction of 5-SASL.

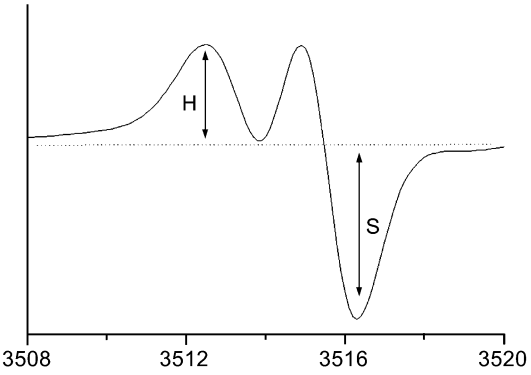


Fig. 6. EPR spectrum of TEMPO in an aqueous dispersion of DSPC liposomes above the gel-to-fluid state transition; *x*-axis: magnetic field in $10^{-4}$ Tesla. *H*, *S*: 1/2 height of the high field hyperfine lines of the spin label in the lipid and aqueous phase.

### 4.2. Partition to tempo

The EPR spectra of the spin label TEMPO in an aqueous dispersion of phosphatidylcholine liposomes is made up by the superposition of two distinct components that are partially resolved only in correspondence of the high field hyperfine lines (Fig. 6). Of the two, the outer is assigned to the spin label in the aqueous phase (*S*), the other to the spin label in the hydrophobic region of the lipid (*H*); then the parameter $f=H/(H+S)$ represents approximately the fraction of TEMPO dissolved in the membrane.

Data show that the dopant increases the permeability of liposomes to Tempo especially below the transition temperature and for the longest phospholipids (Fig. 7).

The effect is a consequence of the already demonstrated destabilization of the lipid structure: fluoxetine reduces the van der Waals' interactions between acyl chains and the energy required to Tempo for diffusing in the bilayer.

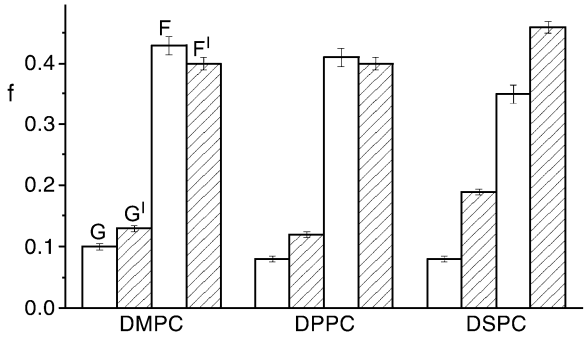


Fig. 7. Fraction *f* of TEMPO dissolved in pure multilamellar liposomes 100 mM in PC, in the gel ( *G*) and fluid ( *F*) phase, and in the presence of FLX (8 mM) ( $G^l$ and $F^l$). The $T_G$ and $T_F$ for each phospholipids were: DMPC: 16 and 26 °C; DPPC: 30 and 49 °C; DSPC: 40 and 58 °C. Standard deviations were calculated by repeated measurements (minimum 4) on DPPC liposomes.

## 5. Conclusions

The paper presents a first study of the chemical physical interactions of fluoxetine with phospholipid membranes. Fluoxetine proved to be a powerful perturbing agent, able to destabilise the membrane at molar ratio of about 1% and a mechanism of interaction was hypothesised which is the result of the balance between the disorder introduced by the drug bound to the polar region, and the packaging forces of van der Waals type which originate in the lipid moiety and depend on the acyl chain lengths. Moreover, it was shown that the drug increases the membrane permeability to small organic solutes like Tempo and can alter the permeability and distribution inside the membrane of charged and polar compounds.

In fact, the structure and the presence of a polar group make a good candidate of fluoxetine to be a member of that large class of molecules whose amphiphilic character allows let them locate at the polar–lipid interface of the bilayer, position which seems the first responsible of the capability to alter the membrane associated functions of cells.

The interference with mitochondrial energy metabolism, with loss of respiratory control and uncouple oxidative phosphorylation, or the induction of haemolysis are widely reported exempla of alterations which are common to fluoxetine as well as tricyclic antidepressants like imipramine or known uncoupler like phloretin [7–9,20]. In all these cases, the interaction with the membrane is related to the amphiphilic character more than to the specific chemical properties of the molecules, making important the investigation of the effects on the phospholipids organisation.

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