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Substituted phenols as pollutants that affect membrane fluidity

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Abstract: *In a toxicological context, the cellular effects of a variety of molecular compounds interacting with membranes may be understood in terms of their ability to affect and modulate lipid-membrane physical properties and even slight changes in membrane fluidity may cause aberrant function and pathological processes. Different model systems (mice splenocytes and liposomes) have been used in modelling studies of the physical effects on lipid bilayers underlying the action of membrane active phenolic compounds, considered by EPA (Environmental Protection Agency) as priority pollutants (phenol; 2-chlorophenol; 2,4-dichlorophenol; 2,4,6-trichlorophenol; pentachlorophenol; 2-nitrophenol; 2,4-dinitrophenol; 2-methyl-4,6-dinitrophenol). Membrane fluidity was assessed by fluorescence steady-state anisotropy of a fluorescent probe 1,6-diphenil-1,3,5-hexatriene (DPH). The substituted phenols increased the fluidity of cells and liposome membranes in a concentration dependent manner and the nitro substituted phenols were the most efficient perturbing the biophysical properties of the membrane. A good parallelism has been established between the results obtained with cell models and artificial liposome model systems, implying that liposomes are useful alternative systems in membrane modification studies and can be conveniently used in order to evaluate the potential toxic effect of phenol derivatives that are common environmental pollutants.*

Key words: *Phenols, Membrane fluidity, Fluorescence anisotropy, Liposomes, Splenocytes*
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Introduction

Substituted phenols are widely used, with a great variety of functions, in the production of many and different products (Martinez *et al.*, 1996, Spain, 1995; Tilak *et al.,* 2007), plastics, explosives, medicines, paints, detergents, pesticides, anti-oxidants, among others. Despite their general use, substituted phenols are contaminants that are of considerable environmental concern. In fact, they present severe environmental contamination of soil, ground water and air and thus, US Environmental Protection Agency (EPA) rated some substituted phenols as priority pollutants and recommended restricting its concentration in the natural water bodies (EPA-US, 1976). Their toxicity also affects many organisms by interfering with basic cell functions . By simple accumulating in the membrane, some phenols can non-specifically perturb the membrane functions, causing a so-called narcotic effect (Brecken-Folse *et al.*, 1994; Bueno de Mesquita *et al.*, 1993; Escher *et al.*, 1996; Hardell, 1993; Rana and Verma, 2005; Tilak *et al.*, 2007). Additionally, substituted phenols act as uncouplers that destroy the electrochemical proton gradient by transporting protons across the membrane and/or by inhibiting the electron flow (Escher *et al.*, 1996).

The inevitable contact of these compounds with life beings and their toxicity by bioaccumulation implies the need of a better knowledge of their interactions, particularly with the biological membranes. These interactions depend on the properties of the membranes, as well as the physicochemical properties of the compounds. Interactions between compounds and biological membranes can have numerous results, including an induced perturbation of physicochemical state of the membrane with possible consequences in cell functionality (Knazek *et al.*, 1981). Membrane fluidity has been reported as an important physicochemical membrane requirement (Knazek *et al.*, 1981) and can be estimated by various methods (Lacowicz, 1999; New, 1990). The method most widely used is the measurement of fluorescence steady-state anisotropy, because of its great sensitivity (Lucio *et al.*, 2004). In this study, the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used for monitoring structural changes and proximity relationships in biomembranes. When compounds are incorporated into lipid bilayers, values of fluorescence anisotropy reveal the extent of restriction of probe motion and thus reflect the nature of its immediate microenvironment (Vincent *et al.*, 1982) making possible to monitor the effect of phenols studied in membrane fluidity.

Several times, the complexity of natural membranes has conditioned research studies. As a result, liposomes have been progressively used to mimic the structure and anisotropy of biological membranes, for which they are considered to be valuable cell models (Lacowicz, 1999; New, 1990; Cevc, 1990; Fendler, 1980; Fendler, 1983; Lasic, 1993; Lasic, 1996). Moreover, liposomes where already used to study many parameters of substituted phenols (Escher *et al.*, 1996). For all the pointed reasons in the current study both cellular and liposome systems were used aiming to highlight the similarity of these models and to present liposomes as advantageous alternative systems in membrane studies.

Materials and Methods

Chemicals: Phenols were provided from the following companies: Aldrich – 2-chlorophenol (2CP), 2,4-dichlorophenol (24DCP), 2,4,6-trichlorophenol (246TCP), pentachlorophenol (PCF), 2,4-dinitrophenol (24DNP); Fluka – 2-methyl-4,6-dinitrophenol (2M46DNP); Riedel-de-Haen – 2-nitrophenol (2NP); Sigma Aldrich – phenol (P). All chemicals were of highest purity available (≥97%) and used without additional purification. The fluorescent probe DPH was from Molecular Probes. All other chemicals were supplied by Merck. Solutions were prepared with double-deionised water (conductivity less than 0.1 $\mu S\ cm^{-1}$), and for all solutions studied, the ionic strength was adjusted to 0.1 M with NaCl.

Mouse splenocyte isolation and fluorescence labelling: Balb/c mice were obtained from Harlen Iberica (Spain). Mice were sacrificed by cervical dislocation; spleens were removed and homogenized in RPMI 1640 culture medium. Splenocytes were washed with RPMI and adjusted to $1x10^7$ cells ml^{-1} in culture medium supplemented with 2 mM glutamine, 100 U ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 2 mM Hepes, and 10% of heat inactivated FCS.

This suspension was then submitted to a centrifugation (260 g for 10 min at 4°C) and the supernatant was rejected. The cells pellet was resuspended in 0.8% ammonium chloride solution (4-5 min) to lyse the red blood cells.

The cells were centrifuged (260 g for 10 min at 4°C) and resuspended in Hepes buffer before the incorporation oh the fluorescent probe, DPH.

For cell labelling, DPH was dissolved in DMSO and diluted 100 fold in Hepes buffer to obtain a solution with a concentration of $1x10^{-5}$ M. An aliquot of the fluorescent probe solution was added to the same volume of the cellular suspension ($1x10^7$ cells ml^{-1}). To ensure complete incorporation of the probe, cellular suspensions were left in the dark for 1 hr at room temperature (Ferreira *et al.*, 2005).

Phenols in Hepes buffered solutions (Hepes 10 mM, *I* = 0.1, pH =7.4) were added to the labelled cells. The final phenol concentrations were always in a range that did not compromise the cellular viability: 0 – 3.5 mM for P, 0 – 3 mM for 24DCP, 0 – 2.5 mM for 246TCP, 0 – 1.0 mM for 2CF and 24DCF, 0 – 0.5 mM for 2NP, 0 – 0.15 mM for 24DNP and 0 – 0.1 mM for 2M46DNP. The resultant suspensions were left for one hour, in the dark at room temperature to achieve a complete distribution of phenol derivatives in the membrane.

Trypan blue viability assay: Trypan blue 0.4% (1:1) assay was performed to determine splenocyte citoxicity. The viable cells were counted with a hemacytometer, and the results were given as a percentage of viable cells obtained for each drug concentration used. This assay was performed before the samples preparation and at the end of each measurement to assure cellular viability (>70%) (Cordeiro-da-Silva *et al.*, 2004) and monitor the toxicity of the compounds studied.

Labelled liposome preparation: Liposomes were prepared by the classical method of the lipid film hydration (Lasic, 1993; Hope *et al.*, 1985). An EPC solution, prepared with chloroform-methanol (9:1), was evaporated using a dry nitrogen stream in a fume hood yielding a thin lipid film on the sides of a round bottom flask. Then, the lipid film was thoroughly dried to remove residual organic solvent by placing the flask on a vacuum pump overnight The resultant dried lipid film was dispersed with a buffer (Hepes 10 mM, I = 0.1, pH =7.4) and the mixture was vortexed to yield multilamellar liposomes (MLV). The mixture was then extruded 10 times through polycarbonate filters with a pore diameter of 100 nm to obtain unilamellar liposomes (LUV) (Lasic, 1993).

The fluorescence probe dissolved in methanol was added to the LUV suspension, in a ratio of 100:1, to prevent changes in the structure of liposome membranes (New, 1990). To ensure complete incorporation of the probe in lipid bilayers, the suspensions were left in the dark for 30 min (New, 1990).

Hepes buffered solutions of the substituted phenols were added to the liposomes and left for two hr, in the dark at room temperature for incorporation. The final phenolic concentrations were in the range of 0 – 3.0 mM for P, 0 – 2.24 mM for 24DCP, 0 – 2.0 mM for 2CF and PCF, 0 – 1.4 mM for 246TCP, 0 – 0.6 mM for 2NP, 0 – 0.15 mM for 24DNP and 0 – 0.1 mM for 2M46DNP.

Fluorescence quenching studies: Fluorescence quenching studies were carried out in a Perkin-Elmer LS 50B steady-state fluorescence spectrometer equipped with a constant temperature cell holder with 1-cm path length cuvettes. Excitation wavelength was set to 361 nm and emission wavelength to 432 nm (Ferreira *et al.*, 2005).

Fluorescence intensity values were corrected for absorbance of the quencher at the excitation wavelength (Coutinho *et al.*, 1993).

Fluorescence anisotropy measurements: Steady-state anisotropy measurements (r_{ss}) were performed in the same Perkin-Elmer LS 50B steady-state fluorescence spectrometer after polarizers have been inserted. The sample was automatically excited with vertically polarized light and fluorescence intensities were recorded with the analysing polarizer oriented parallel (I_{VV}) and perpendicular (I_{VH}) to the excitation polarizer. These values were used to calculate steady-state anisotropy (r_{ss}) -

$$r_{SS} = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where *G* is an instrumental correction factor (Lacowicz, 1999).

Experimental steady-state anisotropy measurements were corrected using the following equation (Lucio *et al.*, 2004):

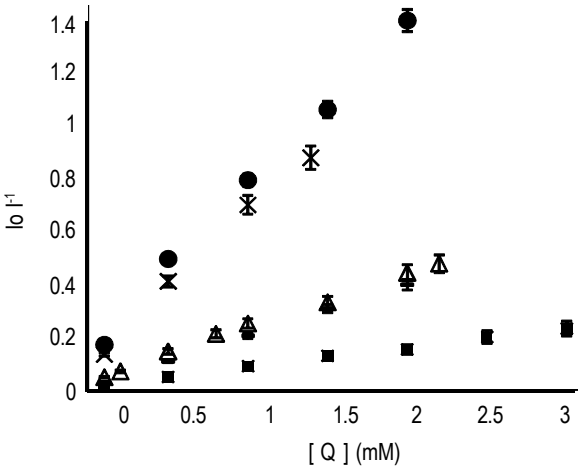

Fig. 1: Stern-Volmer linear plots for increasing concentrations of P (■), 2CP (▬), 24DCP (△), 246TCP (X) and PCP (●) (Hepes 10mM, pH=7.4 e *I*=0.1M) obtained for liposomes of EPC (500 mM) labelled with DPH probe.

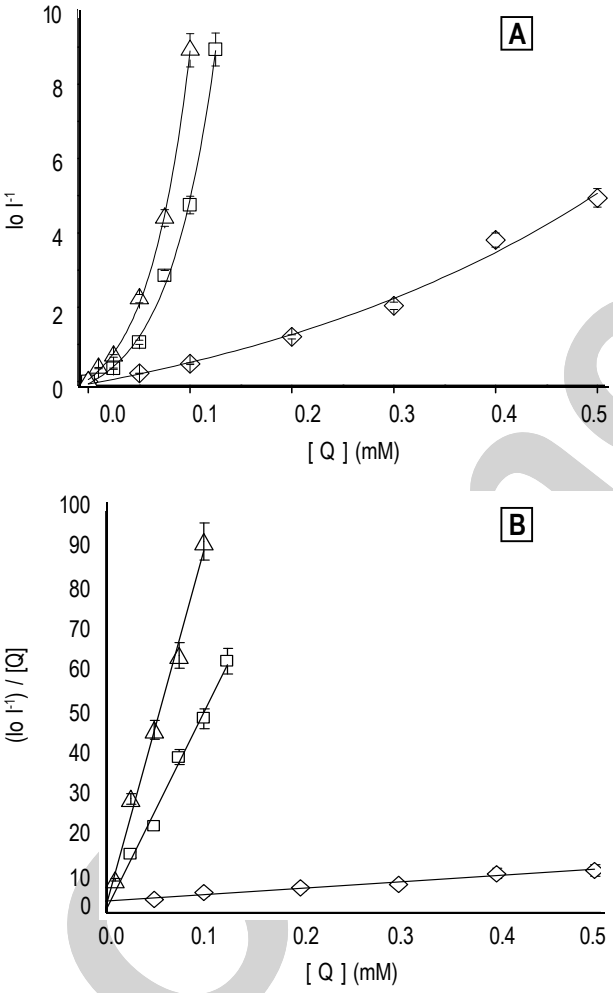

Fig. 2: (A) Graphic representation of (I_0 I^{-1}) in function of increasing concentrations of 2NP(◇),24DNP (□) and 2M46DNP (△) (Hepes 10 mM, pH = 7.4 e *I*=0.1M) and its resultant linearization by the sphere of action model (B)

Table - 1: Values of Stern-Volmer constants (K_{SV}), obtained for the studied substituted phenols in cells and in liposomes

Compounds	Splenocytes	Liposomes
	K_{SV} (mM^{-1})	K_{SV} (mM^{-1})
P	0.16 ± 0.02	0.09 ± 0.04
2CP	0.21 ± 0.01	0.177 ± 0.009
24DCP	0.72 ± 0.02	0.19 ± 0.02
246TCP	0.26 ± 0.01	0.591 ± 0.008
PCP	0.53 ± 0.02	0.75 ± 0.03

[a]The reported values are the mean of at least three independent measurements; the error that affects each value is the standard deviation

Table - 2: IC_{25} values obtained from the concentration (in mM) of phenols (Hepes 10mM, pH=7.4 e *I*=0.1M) required to increase membrane fluidity ratio by 25% in lipossomes (500 μM) and cells (1 X 10^6 cells ml^{-1}) labelled with DPH probe

Compounds	IC_{25} cells (mM)	IC_{25} liposomes (mM)
P	2.48 ± 0.06	3.68 ± 0.02
2CP	n.r.	2.76 ± 0.01
24DCP	0.604 ± 0.009	2.45 ± 0.01
246TCP	1.32 ± 0.04	1.74 ± 0.03
PCP	0.618 ± 0.006	1.04 ± 0.02
2NP	0.0592 ± 0.0003	0.0443 ± 0.0002
24DNP	0.0204 ± 0.0002	0.0117 ± 0.0001
2M46DNP	0.00906 ± 0.0009	0.00977 ± 0.00007

[a]The reported values are the mean of at least three independent measurements; the error that affects each value is the standard deviation, n.r. = not reached

$$r' = \frac{\theta + \tau_0}{\theta + \tau'} \times r_{ss} \quad (2)$$

Where r' is the corrected steady-state anisotropy; t_0 and t' are the fluorescence lifetime of the fluorophore in the absence and presence of the compound, respectively, and q is the rotational correlation time (Lúcio *et al.*, 2004). The values of t' where calculated from the fluorescence intensity of the fluorophore in the absence (I_0) and in the presence (I) of the phenolic compound (Lucio *et al.*, 2004):

$$\frac{I_0}{I} = \frac{\tau_0}{\tau'} \quad (3)$$

Results and Discussion

Quenching studies: All studied compounds were able to quench the fluorescence of the DPH probe incorporated in cells and liposomes. For phenol and chlorophenol derivatives the fluorescence quenching profiles were described by the Stern-Volmer equation (Lucio *et al.*, 2004) as can be observed in Fig. 1.

The slope of the linear plots obtained corresponds to the Stern-Volmer constant (K_{SV}) which reflects the efficiency of quenching in presence of the phenolic compounds and provides a useful tool to predict the location of the phenolic compounds in the membrane (Table 1).

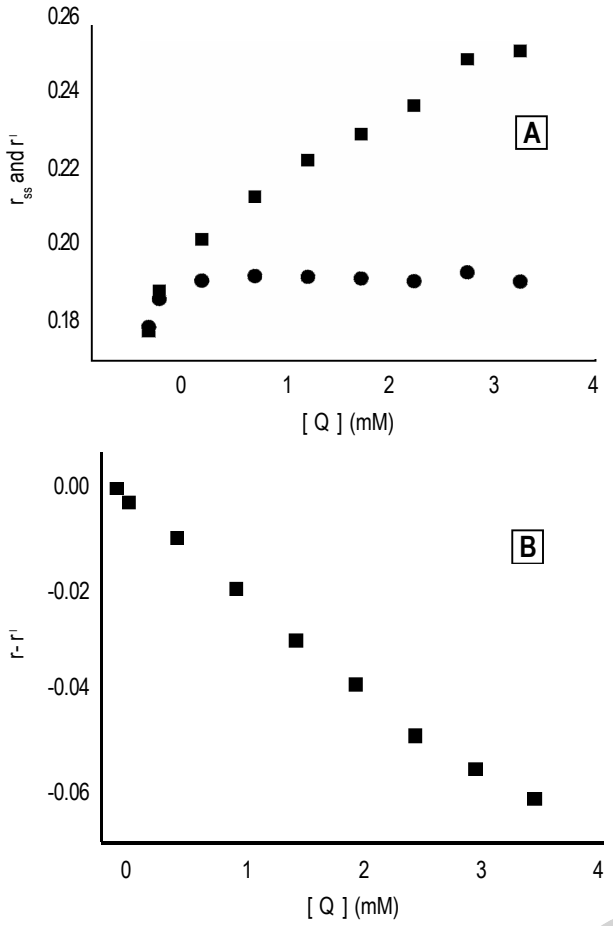

Fig. 3: Effect of phenol (P) concentration in corrected anisotropy, r' (■); experimental steady-state anisotropy, r_{ss} (●) (A) and corresponding r_{ss}-r' (B) membrane of splenocytes labelled with DPH probe

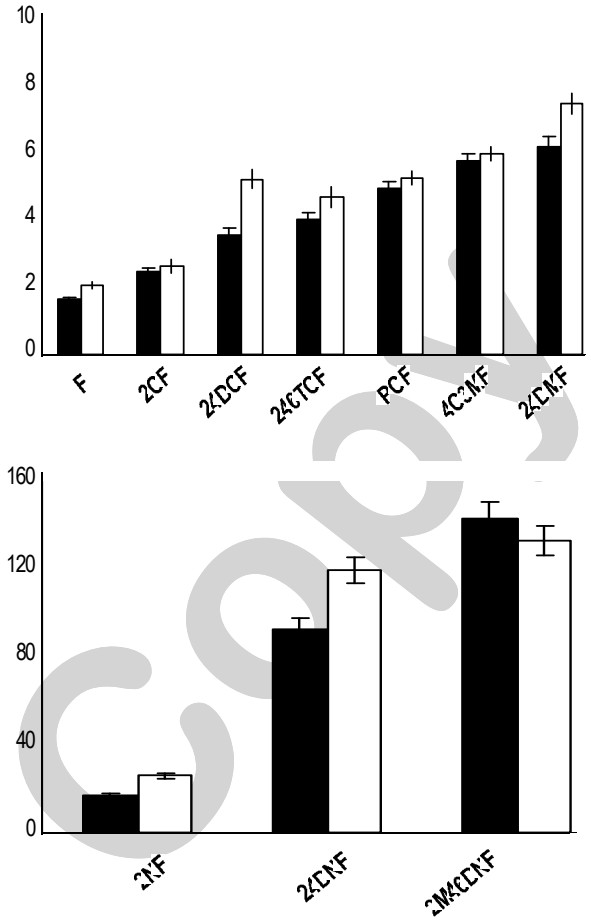

Fig. 4: Fluidizing effect provoked by a concentration of 0.1 mM for P, 2CP, 24DCP, 246TCP and PCP and 0.05 mM for 2NP, 24DNP and 2M26DNP, in splenocytes (□) ($1x10^6$ cells ml^{-1}) or in liposomes of EPC (■) (500 μM) labelled with DPH probe

By observation of the K_{SV} values, it is possible to conclude that all the compounds studied are able to penetrate into the membrane and affect the microenvironment surrounding the probe.

Nitro phenol derivatives were also able to quench the fluorescence of DPH probe incorporated in cells and liposomes, but present a positive deviation to the Stern-Volmer relation, which is a consequence of a larger extent quenching (Lacowicz, 1999). The upward Stern-Volmer plots were analyzed according to the sphere of action model as described elsewhere (Lacowicz, 1999). Fig. 2 is a graphic representation of $(I_0/I-1)$ as a function of total quencher (phenol) concentration [Q] and its resultant linearization by the sphere of action model. Similar profiles were obtained in liposome systems.

The values of the Stern-Volmer constants obtained were 15 ± 1 for 2NP, 483 ± 20 for 24DNP and 870 ± 30 for the 2M46DNP meaning that the quenching efficiency increases with the number of the substituent groups.

Anisotropy measurements: Steady-state fluorescence anisotropy measurements have been widely used to study the effect of several compounds in cellular membrane fluidity (Lacowicz, 1999).

All studied substituted phenols were able to increase the fluidity of the cells and liposomes membranes in a concentration dependent manner.

The experimental anisotropy data, r_{ss} were used to calculate the corrected anisotropy, r' (Lucio *et al.*, 2004). The difference between r_{ss} and r' is a measure of the fluidizing effect caused by the substituted phenols, without the effect of the intrinsic decrease of the fluorescent lifetime of the probe. In Fig. 3 is depicted a comparison between r_{ss} and r' for increasing concentrations of phenol in splenocytes membranes.

Similar profiles were obtained for all the other compounds studied both in cellular and liposome systems.

Since r_{ss}-r' decreases with increasing concentrations of all substituted phenols, it can be concluded that a membrane fluidization occurred, by the presence of the studied compounds.

The difference in potency of the compounds studied to increase membrane fluidity is reflected in their IC_{25} values, which are defined as the concentration of each compound required to increase the fluidizing effect ratio $[100(r'-r_{ss})/r'_0)]$ by 25% (Table 2). The determination of IC_{25} values was obtained from the plot of the % of increase of membrane fluidity as a function of compound concentration [(Q) in mM]. For all the compounds tested in cellular membrane and liposome systems a good linear fit was obtained ($R^2 \geq 0.999$).

From IC_{25} values, it is possible to conclude that the effect of phenol compounds in membrane fluidity is dependent at a large extent by the number and type of substituents. The IC_{25} values obtained for nitro phenols are significantly smaller than the obtained for chlorophenols. And for both types of phenol derivatives the fluidizing effect ratio increases with the number of substituent groups.

In Fig. 4 are displayed the fluidity effect of phenolic compounds in cells and liposomes, obtained for the same concentration of compound.

Fluidity effect observed was approximately the same regardless the system used. Therefore, for these types of compounds results obtained in both systems tested (cells and liposomes) prove that liposomes can be used as biomembrane models aiming to have a simpler reference system which obviates the complexity of the cells. The inherent advantages of these biomembrane models, namely their easy preparation and manipulation, as well as low cost associated to their overall utilization, makes them a possible alternative tool to study pollutants environmental effects, not only in the research of new compounds, but also in current control analysis that are performed in a regular basis.

Additionally, the results described in this work prove that the fluidity effect can be measured even before reaching the concentration that compromises cellular viability.

This is a valuable observation considering the fact that the toxicity of the lipophylic compounds is frequently related to their bioaccumulation, and their fluidity effect in cellular membranes can be a supplementary parameter to evaluate their pollutant properties. Indeed, lipid bilayers are structured fluids with dynamic heterogeneity, both in space and time. A growing body of evidence has shown that lipid-bilayer structure, composition and dynamics play a key role in membrane functionality and pollutant compounds which change any of these membrane characteristics either by modifying membrane lipid content (Singh and Singh, 2006), or membrane fluidity (Martins *et al*., 2003) or even by causing lipid peroxidation (Rana and Verma, 2005) may have important consequences in passive and active membrane functions. Hence, the molecular mechanisms involved in the pharmacological/toxicological effects of pollutants upon membrane interaction are a fundamental question in environmental toxicology studies.

Acknowledgments

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