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Spectroscopic investigation of fluorinated phenols as pH-sensitive probes in mixed liposomal systems

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Abstract

The pK_a values of three fluorinated phenols, 2,4,6-trifluorophenol (3FP), 2,3,5,6-tetrafluorophenol (4FP) and 2,3,4,5,6-pentafluorophenol (5FP) have been measured by using UV-vis and ^{19}F-NMR spectroscopy at 25°C in water and in the presence of pure POPC, pure DDAB and mixed POPC-DDAB liposomes. The probe-liposomes interaction depends on both the equilibrium between the neutral and ionic form of 3FP, 4FP and 5FP and the charge onto the liposomal surface determined by Zeta Potential measurements in a wide pH range.
The data from the two spectroscopic techniques are in good agreement and show that the incorporation of DDAB into the POPC membrane decreases the pK_a values of the probes second to a non-linear correlation.

Introduction

Interactions between drugs and lipid membranes are directly involved in the cellular uptake [1-3] and mainly depend on hydrophobic and electrostatic forces [4].
Biological binding processes are often pH-dependent [5-7] due to the negative charge onto the membrane surface, hence the sorption and the distribution of a drug are strictly connected to its lipophilic nature and ionization degree under physiological conditions.
Methods to calculate the pH-dependent interactions were widely described and improved [8-12]. Many compounds can be employed as pH indicators by means of their fluorescent or spectroscopic properties [13,14]. Their pK_a values strongly shift in function of pH, producing significant spectra changes that can be used to calculate the pH in the proximity of the bilayer. Fluorinated probes [15,16]

were developed for the determination of pH in biological systems by using ^{19}F-NMR spectroscopy. The introduction of the highly electron-withdrawing atom as fluorine promotes the ionization state of the molecule, decreasing the corresponding pK_a value, as observed for fluorinated alanine derivatives [17].

Liposomes are widely studied both as model membranes [18] and as drug carriers [19]. Moreover, liposomes from phosphatidylcholine were employed as the lipophilic phase [20] to investigate the pH-dependent partition behaviour of (*R*,*S*)-[^{3}H]propanolol, pointing out that the partition coefficient of ionised species is important and can be determined as well as the partition coefficient of neutral molecules [21]. The preparation method [22], temperature [23-25], the bilayer composition [26-28] and the surface charge [29,30] influence the capability of liposomal membrane to interact with an organic compound from the bulk. Recent studies [31,32] have also demonstred that pH plays a fundamental role on the membrane sorption of phenol in cationic vesicular dispersion, besides concentration and vesicle preparation.

The aim of this work has been the determination of the pK_a values of three fluorinated phenols, 2,4,6-trifluorophenol (3FP), 2,3,5,6-tetrafluorophenol (4FP) and 2,3,4,5,6-pentafluorophenol (5FP), employed as pH sensitive probes in aqueous solution in the presence of extruded liposomes formed by the natural zwitterionic phospholipid POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine), the commercial cationic surfactant DDAB (didodecyldimethylammonium bromide) and POPC-DDAB mixed liposomes in 75-25, 50-50 and 25-75 molar ratios.

The presence of fluorine atoms in the structure of phenol increases the lipophilic character and consequently improves its interactions with the liposomal membrane. Moreover, the lower pK_a values of the fluorinated phenols allow to obtain the ionic form under pH conditions easier to reach in comparison to those needed to phenol dissociation.

The pK_a value of the three probes have been measured by using UV-vis and ^{19}F-NMR spectroscopy due to the changes in the spectra at different pH in water and in liposomal solution at 25°C.

The liposomal surface charge was determined by using Zeta Potential measurements.

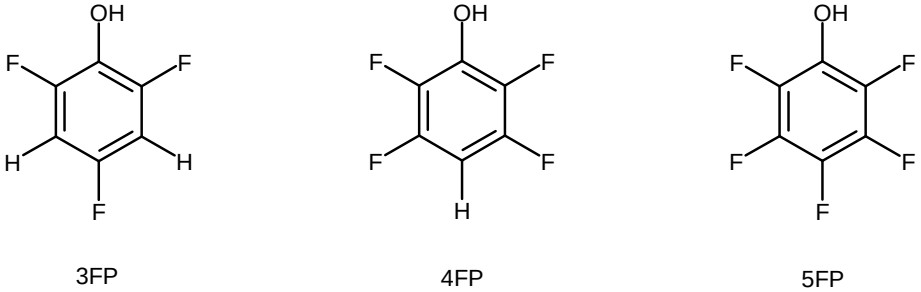

Figure. 1 Structures of the investigated probes.

Experimental Section

Materials

POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine) was purchased from Avanti Polar Lipids (Alabaster, AL). DDAB (didodecyldimethylammonium bromide), 3FP (2,4,6-trifluorophenol) 4FP (2,3,5,6-tetrafluorophenol) and 5FP (2,3,4,5,6-pentafluorophenol), deuterated water (D_2O 99.5%), NaOH and HCl 1 M were obtained from Aldrich. Milli-Q water was employed and all purchased materials were used without further purification.

Instruments

The ^{19}F-NMR spectra were acquired at 25.0 ± 0,1°C on a Varian Mercury spectrometer at 282.32 MHz in 5 mm tubes, and were referenced against internal standard: 2,2,2-trifluoroethanol (3 mM) at -77.26 ppm. Typical experimental parameters were flip angle 26°, interpulse delay 1 s, collecting 128 transients, and spectral width 31 kHz using 15K data points. Each FID was zero filled to 128K and multiplied with an exponential function (line broadening 1 Hz) prior to Fourier transformation.

The UV-vis absorption spectra were recorded on a Varian Cary 1E spectrophotometer at 25.0 ± 0.1°C, in standard quartz cells (10 mm), in the range 200-400 nm.

For the pH measurements was used a Radiometer pH meter with a Hanna combination electrode InLab 423 calibrated at two pH values by using the following buffers: 1.68, 4.00, 7.00 and 10.01.

The Dynamic Laser Light Scattering data were extrapolated by using the Stokes-Einstein relationship for the calculation of the hydrodynamic radius with a Brookhaven (90PLUS BI-MAS) digital correlator at a scattering angle of 90°, equipped with a 35 mW He-Ne laser at the wavelength of 660 nm.

The surface charge of each probe-liposome systems was measured by using a Brookhaven Zeta Plus Potential Analyzer at an angle of 15°, the mobility of liposomes, μ, was converted into the zeta potential value, ζ, by means of the relation $\mu = \varepsilon\zeta/4\pi\eta$, where ε is the permittivity and η is the viscosity of the medium.

Extrusion was performed by using an extruder from Lipex Extruder (Lipex Biomembranes Inc., Vancouver, B.C., Canada) through polycarbonate filters (Whatmann) with pore sizes of 200 nm.

Preparation of liposome and probe solutions

A $CHCl_3$ stock solution (50 mg/ml) of POPC was employed for the liposome preparation by the thin film hydration method. One ml solution of the lipids was rotary evaporated to dryness and the resulting film was further dried under vacuum. The lipid films were hydrated with an aqueous

solution containing NaOH 3.30 x 10^{-3} M to a total lipid concentration of 5 mg/ml. The lipid dispersion was stirred for 30 min at room temperature. The liposomes were sized down by sequential extrusion at 25°C, well above the gel-to-liquid crystalline phase transition temperature (T_m) of the lipid POPC and DDAB membrane. The obtained large unilamellar vesicles were then diluted with heavy water to the concentration of 6 x 10^{-3} M in the case of ^{19}F-NMR titrations and to 0.25-0.5 x 10^{-3} M in the case of UV-vis titrations.

The 1 M probe solutions were prepared by dissolving 3FP, 4FP and 5FP in dioxane. The dissociation of phenol into phenate were reached by adding 50 x 10^{-3} mL of the organic solution in NaOH 3.3 x 10^{-3} M to achieve the final probe concentration of 0.05 M.

The measurements were performed at 25°C by mixing 20 µL of the 0.05 M aqueous probe solution and 2 mL of 1 x 10^{-3} M liposomal solution directly in a quartz cell with a path length of 1 cm. The lipid/probe ratio was kept constant to 2/1.

The internal reference CF_3CH_2OH from a stock D_2O solution was added for the ^{19}F-NMR titrations.

^{19}F-NMR titrations

The ^{19}F-NMR spectra of 3FP, 4FP and 5FP in the neutral and ionic state were carried out in a wide range of pH by adding HCl 1 M. The pK_a values of the probes in H_2O/D_2O solutions (90/10 v/v) and in the presence of liposomes were measured as previously described [33] by using Eq.1

Eq. 1
$$\delta = \frac{\delta_1 10^{-pH} + \delta_2 10^{-pK_a}}{10^{-pH} + 10^{-pK_a}}$$

where δ_1 and δ_2 are the chemical shift of the probe in the neutral and ionic state, respectively.

UV-vis titrations

The UV-vis spectra of the probes in the neutral and ionic state were carried out in a wide range of pH by adding HCl 1 M and the absorption at 282, 266 and 290 nm have been used for the pK_a calculations [34] of 3FP, 4FP and 5FP, respectively, in aqueous solution and in the presence of liposomes by using Eq.2

Eq. 2
$$ABS = \frac{A_1 10^{-pH} + A_2 10^{-pK_a}}{10^{-pH} + 10^{-pK_a}}$$

where A_1 is the absorbance of the probe in the neutral and A_2 is the absorbance of the probe in the ionic state.

Results and Discussion

The probes 3FP, 4FP and 5FP present an higher lipophilic degree and a lower pK_a in comparison to phenol (Table 1).

	Log *P*	**pK_a**
Phenol	1.48 ± 0,30[a]	10.0 ± 0.10
3FP	2.36 ± 0,30[a]	7.17 ± 0.05 [35]
4FP	2.86 ± 0,30[a]	5.53 ± 0.05 [36]
5FP	3.06 ± 0,30[a]	5.41 ± 0.05 [36]

[a] from ACDlabs 5.0 software calculations.

Table 1. Log *P* and pK_a of 3FP, 4FP and 5FP

The presence of fluorine atoms, with a nuclear spin $I = 1/2$, the natural isotopic abundance of 100% and high receptivity (a measure of the ease of detecting a nucleus; ^{19}F is 0.83 of that of protons) offer an opportunity to use ^{19}F-NMR spectroscopy for the examination of ionization equilibria.

The chemical shift range of a ^{19}F-NMR signal is intrinsically very wide and therefore the fluorine nucleus is an excellent, highly sensitive probe of its environment. From the titration curve is possible to determine the pK_a of 3FP, 4FP and 5FP.

The ^{19}F-NMR spectra at pH 2.6 and 11.0 of 3FP in D_2O are reported in Figure 2. as an example.

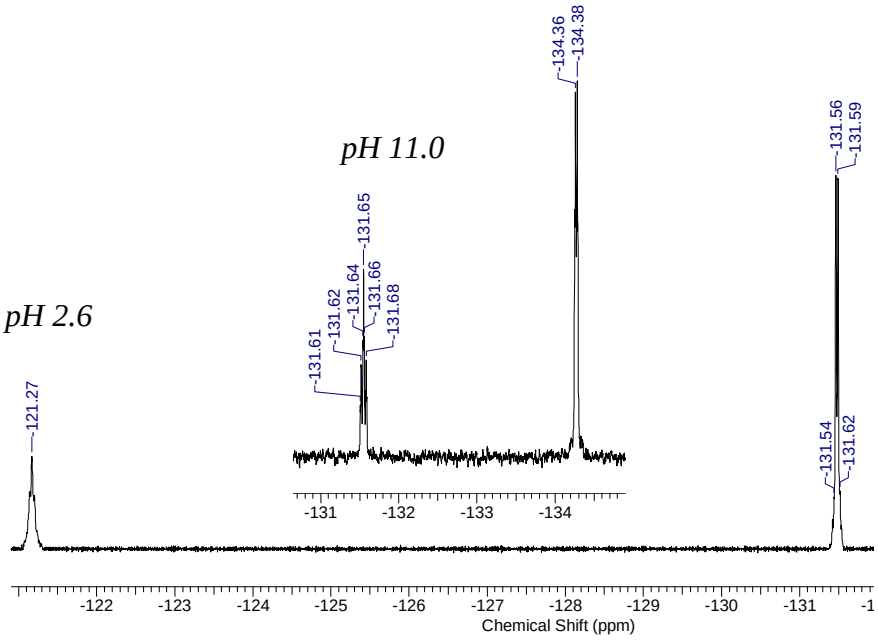

Figure 2. ^{19}F-NMR spectra of 3FP in D_2O at pH = 2.6 and 11.0.

The corresponding titration curve of 3FP in D_2O is shown in Figure 3.

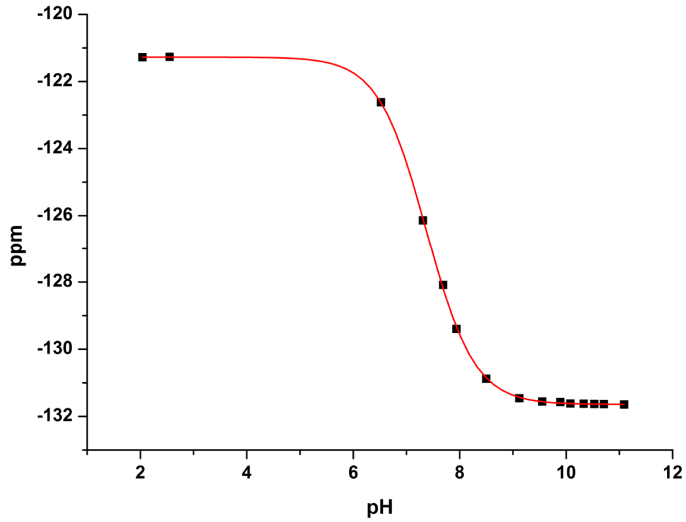

Figure 3. ^{19}F-NMR Titration of 3FP in D_2O.

The probe-liposome interaction is pH-dependent because is related to the affinity of neutral form of 3FP, 4FP and 5FP for the inner region of the bilayers.

^{19}F-NMR titrations in the presence of liposomes have shown that the probes are associated to the bilayer at pH values closer to the corresponding pK_a. The ^{19}F-NMR spectra of 3FP in the presence of POPC liposomes at pH = 11.0 and pH = 7.2 are reported in Figure 4.

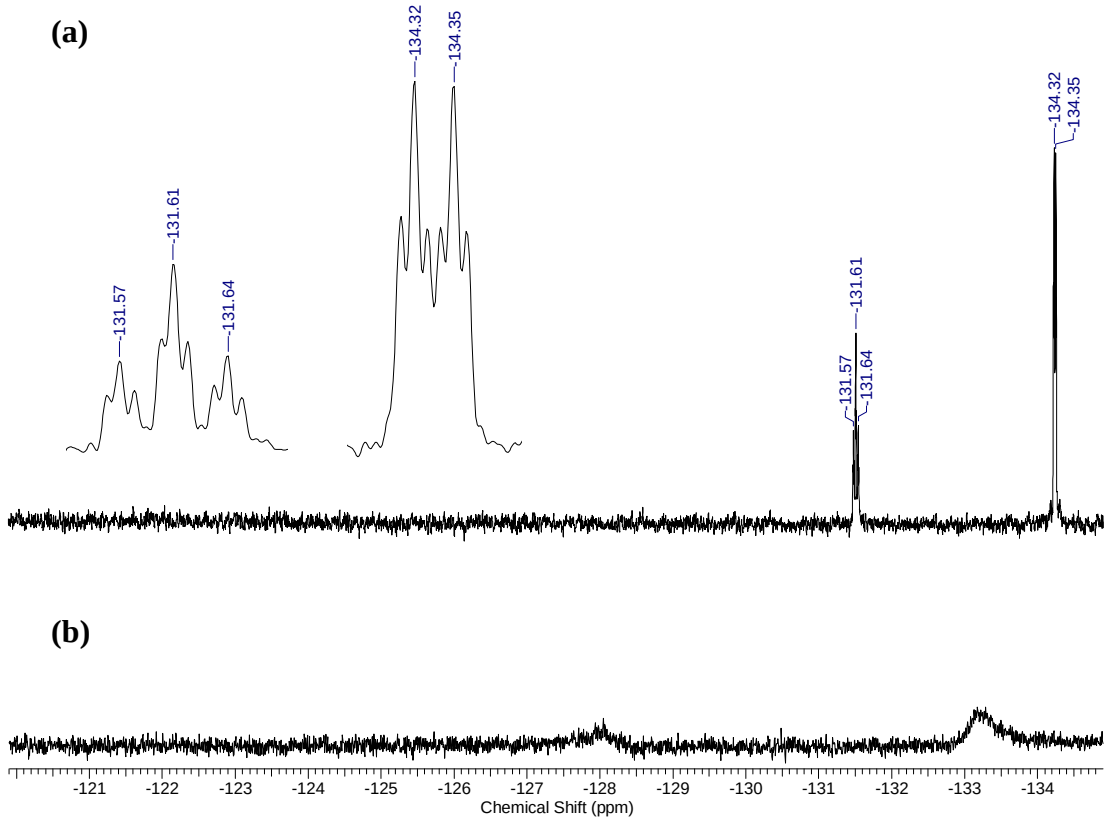

Figure 4. ^{19}F-NMR spectra of 3FP in the presence of POPC liposomes at pH = 11.0 (**a**) and pH = 7.2 (**b**).

The increasing broadening of the peaks and a totally lack of resolution at pH = pK_a hamper the calculation of the pK_a values so ^{19}F-NMR have been replaced by UV-vis spectroscopy.
In the presence of liposomes the UV-vis spectra change second to the charge of the probe at different pH as can be observed in Figure 5.

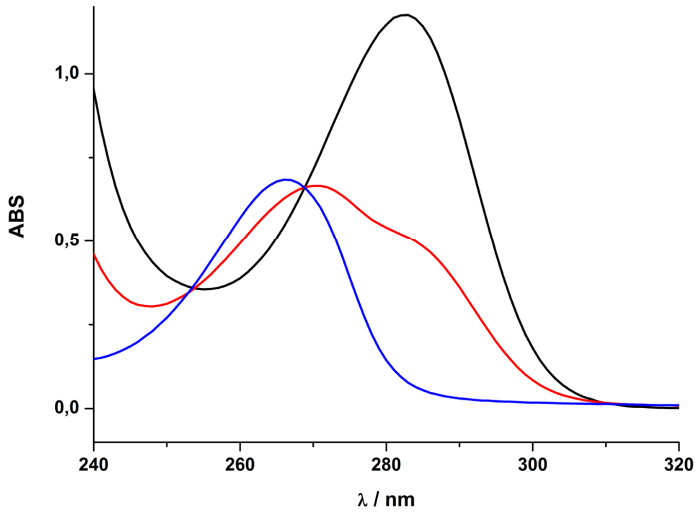

Figure 5. UV-vis spectra of 3FP in the presence of POPC liposomes at pH = 11.4 (dark) 7.3 (red) and 3.1 (blue).

The pK_a values of the investigated probes have been obtained by the corresponding titration curve. The UV-vis titration curve of 3FP in the presence of POPC liposomes is reported in Figure 6.

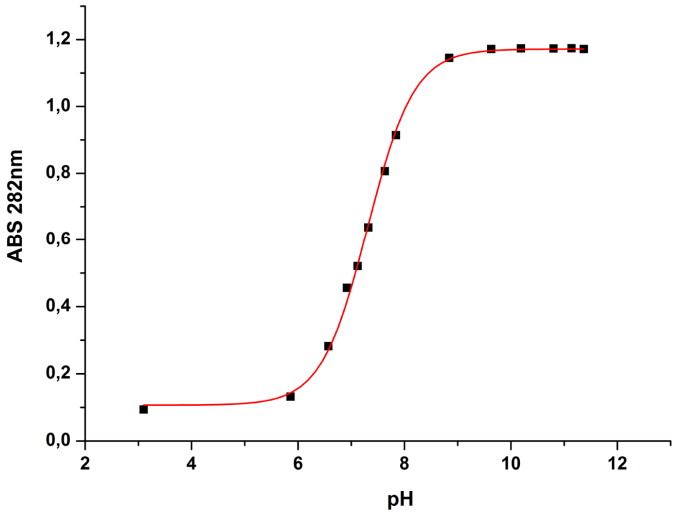

Figure 6. UV-vis titration of 3FP in the presence of POPC liposomes.

In this case the measurement of pH of the liposomal surface becomes independent on the probe concentration by plotting the ratio between the absorbances at two wavelengths in function of the

pH values. The UV-vis titrations of 0.5 x 10^{-3} and 1.0 x 10^{-3} M 3FP in aqueous solution are shown in Figure 7.

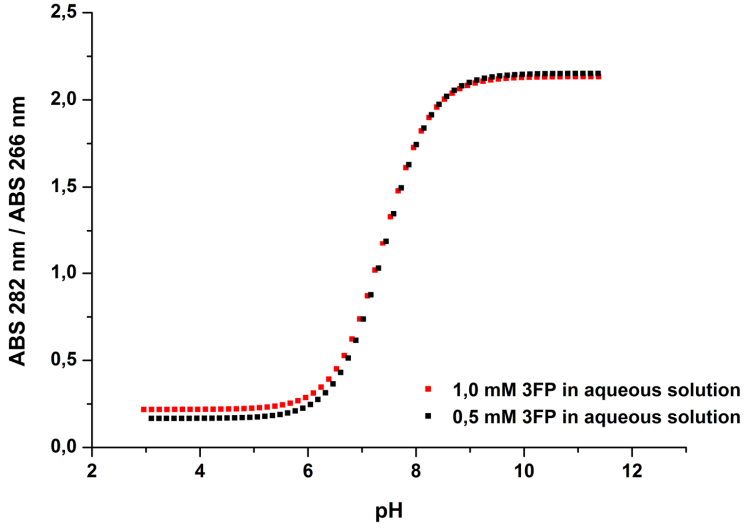

Figure 7. UV-vis titrations of two different concentrations of 3FP in aqueous solution

UV-vis analysis allows to obtain different pK_a of 3FP, 4FP and 5F second to the liposomal surface charge, by adding an appropriate amount of H^+ to reach the same concentration of neutral and ionic form in solution for each probe.

The pK_a and the Zeta Potential values obtained for the investigated systems are reported in Table 2.

	pK_a from UV-vis titrations			Zeta Potenzial at pH 11/ mV		
System	**3FF**	**4FF**	**5FF**	**3FF**	**4FF**	**5FF**
Aqueous solution	7.38 ± 0.01 (7.36 ± 0.03)[a]	5.60 ± 0.05 (5.59 ± 0.01)[a]	5.41 ± 0.06 (5.47 ± 0.01)[a]	-	-	-
POPC liposomes	7.31 ± 0.02	5.60 ± 0.03	5.49 ± 0.05	-31.3 ± 1.4	-32.3 ± 0.8	-38.6 ± 1.8
POPC/DDAB 75/25 liposomes	7.09 ± 0.03	5.18 ± 0.03	5.08 ± 0.04	1.2 ± 1.9	-3.5 ± 2.7	-5.9 ± 2.5
POPC/DDAB 50/50 liposomes	7.10 ± 0.01	5.19 ± 0.05	5.13 ± 0.07	4.8 ± 1.7	-10.1±1.0	-7.4 ± 0.4
POPC/DDAB 25/75 liposomes	6.92 ± 0.04	4.58 ± 0.06	4.22 ± 0.08	20.6 ± 1.8	16.0 ± 1.0	15.5 ± 1.4
DDAB liposomes	6.32 ± 0.03	4.15 ± 0.06	3.78 ± 0.03	53.4 ± 2.0	52.5 ± 2.5	52.7 ± 0.5

[a] Data from ^{19}F-NMR titrations.

Table 2. The pK_a and the zeta potential values obtained for the investigated probes in aqueous solution and in the presence of pure and mixed POPC liposomes.

The pK_a values for 3FP, 4FP and 5FP in aqueous solution from UV-vis analysis are close to the corresponding values from NMR and are in agreement with previously reported data. (Table 1).

The pK_a values of the probes tend to remain constant in the presence of POPC liposomes (Table 2).

Zeta Potential measurements have shown that POPC liposomes are negatively charged and unable to influence the pH of the solution, as demonstred by the linear correlation obtained by increasing pH from 3.0 to 11.6 (Figure 8).

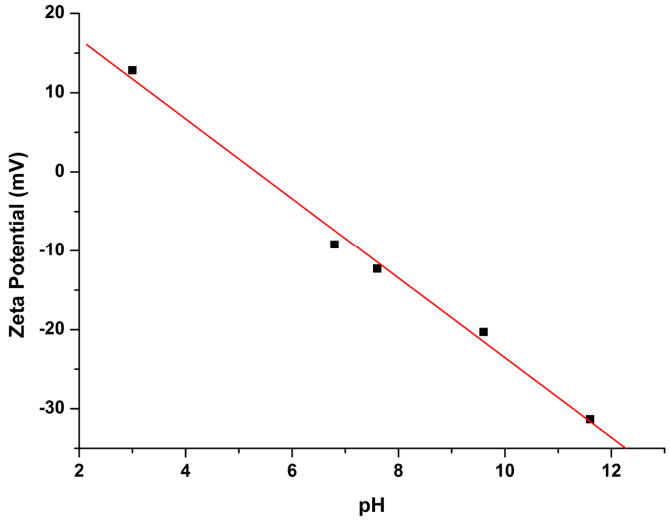

Figure 8. Zeta potential measurements of 3FP in the presence of POPC liposomes (the line is drawn as a visual guide).

The incorporation of DDAB turned into cationic the surface of POPC liposomes. The surfactant is located both in the inner and in the external layer of the membrane following an asymmetrical distribution. At high pH DDAB promotes an anion exchange [37] by which a sufficient amount of OH^- replaces Br^- up increasing the pH in the liposomal surface: an higher concentration of HCl is needed to reach the equilibrium between the neutral and ionic form of the investigated probes [38] and consequently lower pK_a can be observed.

Moreover, a significative effect can be observed in the presence of pure DDAB liposomes: the surface charge is approximately the same (Table 2) so the decreasing of the pK_a up to 10.9, 19.9 and 25.6 % for 3FP, 4FP and 5FP respectively, is correlated to the lipophilic degree of the probe.

Conclusions

The pK_a values of 3FP, 4FP and 5FP depend on the lipophilic degree of the molecules as shown by ^{19}F-NMR and UV-vis spectroscopy, but are not affected by the presence of POPC liposomes, as pointed out by Zeta Potential measurements.

The decreasing of the pK_a values by increasing the DDAB concentration follows a non-linear correlation as highlighted by UV-vis analysis. This effect is probably due to the asymmetrical

distribution of the cationic surfactant between the inner and the external layers of the liposomal membrane.

The comparison of the UV-vis spectra of the three investigated probes can be a useful method to determine the pH of the liposomal surface. In this case reliable values can be obtained in the range pK_a ±1, by monitoring the UV-vis spectra changes.

The data present herein could contribute to the understanding of the properties of liposomes as reaction media in the acid-base catalysis and the interactions that occur in the presence of molecules in neutral and charged form.

Acknowledgments

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