Prototropism of 1-hydroxypyrene in liposome suspensions: implications towards fluorescence probing of lipid bilayers in alkaline medium†

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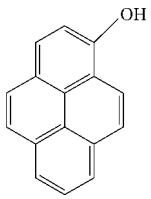
The partitioning efficiency of neutral and anionic prototropic forms of 1-hydroxypyrene in liposome suspensions has been studied. The high partition coefficient value of 1-hydroxypyrene indicates an easy incorporation of the molecule into the lipid bilayer. Detailed pH studies indicate that only the neutral form of 1-hydroxypyrene partitions into the membrane and appreciable spectral changes are observed in the pH range of 9.0–11.5 in Tris–NaOH buffer. However, at pH 11 the spectral changes are maximum. The possibility of using 1-hydroxypyrene as a fluorescent molecular probe for lipid bilayer membranes in alkaline media has been examined, by employing fluorescence intensity and fluorescence anisotropy as probe parameters. The neutral form fluorescence intensity as well as fluorescence anisotropy is sensitive to the changes in the membrane properties and is capable of sensing the phase-transition. This is also capable of monitoring the changes in the membrane due to incorporation of cholesterol and the ethanol-induced interdigitation. The time resolved fluorescence data and the quenching experiments show that 1-hydroxypyrene occupies the water inaccessible interior of the liposome. The high anisotropy value of 1-hydroxypyrene in liposome suggests that it resides in a considerably rigid environment and is very sensitive to the temperature-induced changes in the liposome.

Introduction

Phospholipid molecules form the basis on which cells and cell organelles are constructed. Being amphiphilic in nature they tend to spontaneous aggregation in aqueous solution and form a three-dimensional closed bilayer membrane structure called vesicles, which trap an aqueous volume in their interior. While the majority of applications make use of the biocompatibility of liposomes for use in medically related fields,[1] they also have potential applications in the physical sciences, which may have very little in common with the biological field. The interaction of biological and non-biological systems has opened new horizons in many applications of liposomes in various fields such as food processing, in drug loading, immunology, diagnosis, cosmetics, agro-chemicals and as biosensors.[2,3] A key element in these studies is the detailed understanding of the physical properties of liposomes such as stability, phase-behaviour, fluidity, permeability, order parameters *etc.* even under non-physiological conditions, which has led to a separate area of research.[2,3] The sensitivity of liposomes to various pHs has been studied and a detailed work on pH titration of DPPC and DMPC liposomes has been reported by Cevc and Marsh.[4] In the case of drug delivery systems, preformed liposomes in alkaline pH were used for loading weak acid drugs.[5]

The four important parameters, wavelength, intensity, life-time and polarization/anisotropy of fluorescent probes, provide valuable information about the different aspects of liposome. Conceptually different types of fluorescent probes have been employed to study diverse properties of lipid bilayer membranes.[6,7] Pyrene and pyrene derivatives[8–14] provide powerful tools in terms of their various fluorescence properties such as polarity sensitivity (I_3/I_1 ratio), tendency to excimer formation and reasonable fluorescence anisotropy that have received wide applications in the liposome studies. Substituted pyrenes, in particular have got much attention because of a combination of large solvent polarity induced shift, high fluorescence anisotropy, high quantum yield and emission shift towards the visible region and they offer an excellent probe for liposomes.

The photophysical studies of 1-hydroxypyrene (Scheme 1) show the high pH sensitivity of this molecule and the neutral (PyOH) and anionic (PyO^-) forms have a substantial shift of around 60 nm in their absorption and emission spectra. Its pK_a value being 8.93[15] the prototropic behavior of it can offer a powerful tool for studying the properties of lipid bilayer membranes in the alkaline pHs. The photophysics of 1-hydroxypyrene and the acidity of its singlet-excited state has been a controversial topic of discussion in recent years.[15,16] Although the acidity constant of 1-hydroxypyrene increases substantially upon photoexcitation ($pK_a = 8.93$, $pK_a^* = 4.5$ in unbuffered solution), the acidity of the excited state is still doubtful because of the very short lifetime of the singlet state, during which prototropic equilibrium does not get achieved. The



Scheme 1 1-Hydroxypyrene (PyOH).

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small rate constant for the protolytic photodissociation of 1-hydroxypyrene in bulk water also supports this fact and shows that there is little communication between the two excited forms. Here we have evaluated 1-hydroxypyrene as a fluorescent probe to monitor the membrane (DPPC and DMPC) properties such as thermotropic behavior, lipid–cholesterol interaction and effect of ethanol on lipid bilayers.

Experimental

Materials

1-Hydroxypyrene (Sigma-Aldrich, Bangalore, India) was used after recrystallisation from ethanol. DMPC (dimyristoylphosphatidylcholine), DPPC (dipalmitoylphosphatidylcholine) and cholesterol were purchased from Sigma Chemical Co. (Bangalore, India) and were used as such. All solvents used were of analytical grade and were all distilled before use. Triple-distilled water was always used for the experiments.

Liposome preparation

Both unilamellar as well as multilamellar vesicles (MLVs) were used in our studies. For the absorption studies unilamellar vesicles are preferred for minimum scattering interference. However, for cholesterol and ethanol-induced interdigitation experiments only the MLVs were used. Unilamellar vesicles were prepared by ethanol injection method.[1] The ethanolic solution of the lipid was injected rapidly with the help of a fine needle to the buffer, maintained at 50 °C (optimised condition). The volume of ethanol injected is always less than 1% v/v in order to avoid any damage to the liposome by ethanol. This method gives rise to small unilamellar vesicles (SUVs) in the size range ~15–25 nm. For the MLVs (~100–1000 nm), the lipids (DMPC and DPPC) were dissolved in chloroform at a molar concentration of 1×10^{-3} M. The solution was evaporated with the help of a rotary evaporator and the residual solvent was removed by applying vacuum. The lipid film was left under vacuum to ensure complete dryness. Liposome vesicles were prepared by adding appropriate volumes of Tris–buffer [2-amino-2-(hydroxymethyl) propane-1-3 diol] at desired pH, to the dry lipid film with vigorous vortexing and then warming at 10 degree above phase transition temperature for complete hydration. The concentration of the liposome was varied by subsequent dilutions. DMPC-cholesterol liposomes were prepared by adding same volume of lipid to different volumes of cholesterol stock in chloroform; such that the molar ratio of cholesterol is varied from 0 to 50 mole% of the lipid.

Labeling

A stock solution of 1-hydroxypyrene was prepared in 1×10^{-4} M of Tris–buffer at desired pH. Except for the partition coefficient experiments, the probe was always added to the lipid during the hydration step while vortexing the solution for all experiments. For the partition coefficient experiments, however a fixed volume of the probe was added to the liposome of varying concentration (0 to 4.5×10^{-4} M) so that the final concentration of the probe was 1×10^{-6} M and the solution was equilibrated for an hour above the phase transition temperature. For all the experiments, a control solution containing the same concentration of lipid in absence of the probe was used as a blank. For the ethanol-induced interdigitation studies in DPPC, the labelled lipid was kept in a refrigerator for one day. Then desired amount of ethanol was added to it in cold and was incubated at 50–55 °C before taking fluorescence measurements.

Fluorescence measurements

Absorption spectra were collected with a Perkin Elmer lambda 25 spectrophotometer. Fluorescence measurements were carried out with a Hitachi F-4500 spectrofluorimeter, with a 150 W xenon lamp as the light source. The excitation and emission spectra were recorded with 5/5 nm slit widths. The temperature dependence and fluorescence anisotropy experiments were carried out with a Jobin-Yvon-Spex Fluorolog II spectrofluorimeter, with a 450 W xenon lamp as light source. The temperature was controlled by circulating water through a jacketed cuvette holder from a refrigerated bath (INSREF Ultra Cryostat, India). The temperature was also checked inside the cuvette before and after the experiments and the variation was negligible. The steady-state fluorescence anisotropy (r_{ss}) values were obtained using the expression $r_{ss} = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + 2GI_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities when the emission polarizer is parallel and perpendicular, respectively, to the direction of polarization of the excitation beam, and G is the factor that corrects for unequal transmission by the diffraction gratings of the instrument for vertically and horizontally polarized light.

Time resolved fluorescence measurements

Lifetime measurements were carried out using the IBH single-photon counting fluorimeter in a time-correlated single-photon counting arrangement consisting of ps/fs Ti-Sapphire Laser system (Tsunami Spectra Physics, Bangalore, India). The pulse repetition rate was 82 MHz and the full width half maximum is less than 2 ps. The emission was collected at magic angle polarization (54.7) to avoid any polarization in the emission decay. The instrument response time is approximately 50 ps. The decay data were further analysed using IBH software. A value of χ^2, $0.99 \leq \chi^2 \leq 1.4$ was considered as a good fit.

Results and discussion

Determination of the partition efficiency of 1-hydroxypyrene into the membrane

In order to find out the efficiency of partitioning of 1-hydroxypyrene in its neutral (PyOH) and anionic (PyO^-) form, we conducted a detailed pH study of the molecule in Tris buffer in the absence and presence of liposome. But before proceeding into the liposome work we need to have an idea of the lipid to probe ratio that should be maintained during the experiments in order to have minimum interference from the probe remaining in the bulk water, which is very crucial in such studies. The pK_a value of 1-hydroxypyrene is reported to be 8.93 in unbuffered NaOH solution.[15] Thus at pH 7, 1-hydroxypyrene is expected to be in the undissociated neutral form where as around pH 12 it will be predominantly in the anionic form. In order to check which form of 1-hydroxypyrene partitions into the membrane we carried out experiments at pH 7 and pH 12.

Fig. 1 shows the UV-Visible absorption and emission spectra of 1-hydroxypyrene at (A) pH 7 (a, a′) and (B) pH 12 (b, b′), respectively. It is clear that in the presence of liposome there is a red shift of around 5 nm in the absorption maxima at pH 7, whereas the maxima remain the same at pH 12. But the molar absorptivity (ε) value remains almost the same in the absence and presence of liposome at both pHs. Thus absorption spectra alone are not much informative in this case. However, it is clear from the emission spectra at pH 7 that although the peak shapes remain the same, there is a continuous increase in the fluorescence intensity with a little red shift as the liposome concentration is increased and finally it gets saturated indicating the complete partitioning of the probe to the membrane. But at pH 12 it is found that the fluorescence spectra is predominantly from the anionic form and there is a very small increase in the fluorescence intensity of the neutral form with increasing liposome concentration. This indicates negligible partitioning of 1-hydroxypyrene to the membrane at pH 12.

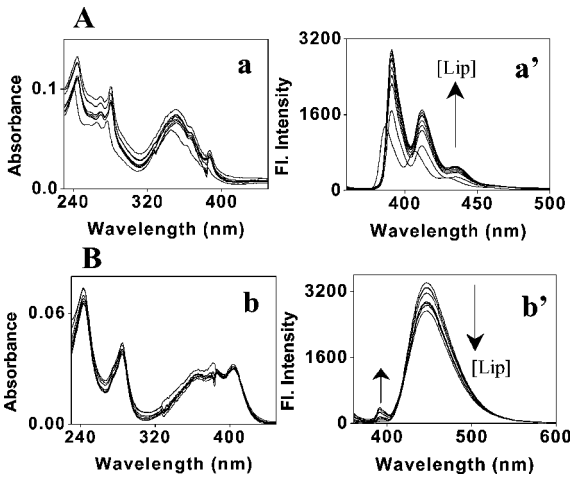

Fig. 1 UV-Visible absorption spectra (a, b) and emission spectra (a′, b′) of 1-hydroxypyrene (1.5×10^{-6} M) in DMPC liposome ($0–5 \times 10^{-4}$ M) at (A) pH 7 and (B) pH 12, $\lambda_{ex} = 350$ nm.

The change in the steady state fluorescence anisotropy with increasing liposome concentration is rather significant and clearly shows the 1-hydroxypyrene partitioning. Fig. 2A shows the change in anisotropy of 1-hydroxypyrene *versus* the liposome concentration at (a) pH 7 and (b) pH 12. The anisotropy values were measured at $\lambda_{em} = 392$ nm at pH 7 and at $\lambda_{em} = 447$ nm at pH 12. As is seen in the figure the anisotropy at pH 7 keeps increasing with the liposome concentration and finally around 0.3 mM of liposome it levels up, whereas there is a negligible change in the anisotropy value at pH 12 in the absence or presence of liposome. This is more clear from the emission anisotropy spectrum of 1-hydroxypyrene in a 0.2 mM concentration of liposome at pH 11 as seen in Fig. 2B. Under this condition the emission spectrum shows contributions from both PyOH and PyO^- forms. The emission anisotropy spectrum shows a high value of 0.14 in the PyOH emission range and the value decreases to almost zero in the PyO^- emission range. Hence, it is clear that only the neutral form of 1-hydroxypyrene partitions into the membrane and not the anionic form.

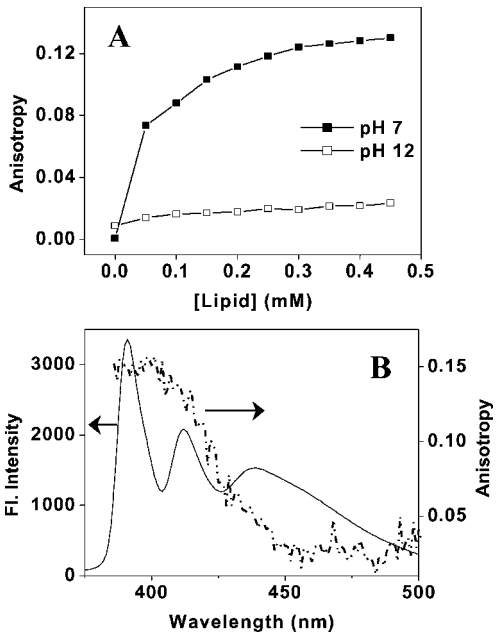

Fig. 2 (A) Plot of fluorescence anisotropy of 1-hydroxypyrene *versus* DMPC concentration at pH 7 ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 392$ nm) and pH 12 ($\lambda_{ex} = 403$ nm, $\lambda_{em} = 447$ nm), (B) emission spectrum (solid line) and emission anisotropy spectrum (dotted line) of 1-hydroxypyrene in 2×10^{-4} M DMPC liposome at pH 11 ($\lambda_{ex} = 360$ nm, isobestic point).

Determination of the partition coefficient of 1-hydroxypyrene at pH 7

An essential physico-chemical parameter to understand the behaviour of a compound towards membranes is its partition coefficient value, which is a measure of its lipophilicity and gives information about the fraction of it associated with the lipid. The partition coefficient (K_p) value of any fluorescent probe between the aqueous and liposome media can be evaluated by fluorescence spectroscopy as long as there is a difference in a fluorescence parameter of the partitioning molecule (*e.g.*, quantum yield, fluorescence anisotropy or fluorescence lifetime) when in the aqueous solution and after incorporation in the membrane.[17] As it is clear from Fig. 1 and 2, at pH 7 although there is an increase in the fluorescence intensity upon incorporation into the liposome, the change in anisotropy is more remarkable in the absence or presence of liposome. Hence, we have taken the fluorescence anisotropy as the parameter to calculate the K_p value at pH 7 by using the equation given below, similar to that used for fluorescence intensity[18]

$$r = r_0 \, L/(55.6/K_p + L)$$

where r_0 represents the maximum anisotropy resulting from total probe incorporation into the membrane and L is the lipid molar concentration. The above equation gives rise to a saturation curve, which on non-linear curve fitting gives the value of K_p to be 8×10^5 at pH 7 at 15 °C.

From the K_p value the membrane-bound mole fraction (x_L) of 1-hydroxypyrene was calculated using the equation[17]

$$x_L = K_p \, L/55.6 + K_p \, L$$

and the lipid to probe ratio was calculated to be ≈250. This ratio was maintained for further work, where there will be maximum partitioning of 1-hydroxypyrene into the membrane.

Determination of the pK_a value of 1-hydroxypyrene in aqueous and liposome media

The pK_a value of 1-hydroxypyrene has been reported to be 8.93 in an unbuffered solution and it is known to be sensitive to the presence of a buffer.[15] Hence, it was desirable to carry out absorption as well as fluorescence studies to find out the pK_a value of 1-hydroxypyrene in Tris buffer in the absence and presence of liposome before proceeding further. The pH was varied from 7 to 13 by addition of either HCl or NaOH to 1×10^{-4} M Tris solution in water. Fig. 3 represents the UV-Visible absorption and fluorescence emission spectra of 1-hydroxypyrene in (A) homogeneous solution (a, a′) and (B) in the presence of DMPC liposome (b, b′) at various pHs. For the emission spectra the excitation wavelength was fixed at the isobestic point so as to have equal contribution from the neutral as well as the anionic species.

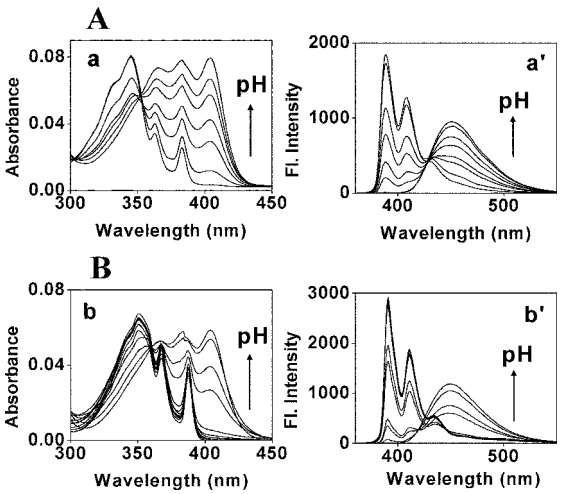

Fig. 3 UV-Visible absorption spectra (a, b) and emission spectra (a′, b′) of 1-hydroxypyrene in (A) homogeneous (Tris buffer) and (B) liposome media at varying pHs. The pH was varied from 7 to 13 at a step of 0.5; a few spectra have been removed for clarity. $\lambda_{ex} = 352$ nm for homogeneous studies and $\lambda_{ex} = 360$ nm for the liposome studies (isobestic point).

As it is clear from the absorption spectra, the neutral and the anionic form absorption spectra are not widely apart and there always remains a significant contribution of ε from both the species at any wavelength. The absorbance corrected normalized optical density values of the two species at corresponding wavelengths are plotted as a function of pH both in absence and presence of liposome and is seen in Fig. 4A. Fig. 4B represents similar plot constructed from the fluorescence intensity data after all the corrections were made.

According to Henderson's equation at any pH, pK_a = pH − log[salt]/[acid], here [salt] = [PyO^-] and [acid] = [PyOH]. Thus the pK_a is equal to the pH where [PyO^-] = [PyOH], and from the figure it is clear that for the homogeneous study the pK_a is 9.3 and for the liposome medium it is shifted to 11.3. This shift of 2 pH units as we go from homogeneous to liposome

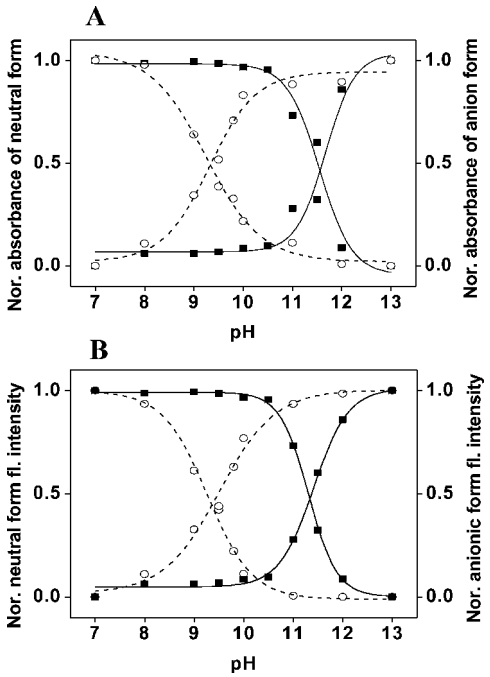

Fig. 4 Plot of normalized (A) absorbance and (B) emission intensity of the neutral and anionic species of 1-hydroxypyrene as a function of pH in homogeneous (dotted line) and liposome (solid line) media.

medium is a consequence of the incorporation of the neutral form of 1-hydroxypyrene into the liposome membrane. In the homogeneous medium there exists an equilibrium between the undissociated 1-hydroxypyrene and the dissociated form depicted as [PyOH] ⇌ [PyO^-] + [H^+] in the pH range of our study, in presence of liposome a fraction of the neutral form gets partitioned into the liposome hence shifting the equilibrium towards the left which is reflected in the apparent pK_a value of 11.3. It is note worthy that the pK_a values obtained are the same irrespective of whether it is calculated from the absorption study or fluorescence study. As has been demonstrated earlier in the literature[15] this is due to the absence of prototropic equilibrium in the excited state. From the Fig. 4A and 4B it is expected that significant spectral changes between the homogeneous and liposome media will be observed in the pH window of 9 to 12.

Determination of working pH

In order to get the best working pH, where there will be maximum spectral changes, we carried out the fluorescence studies of 1-hydroxypyrene in DMPC liposome at pH 9–12 and the spectra are shown in Fig. 5 (a–c) for pH 9, 10 and 11, and in Fig. 1b′ for pH 12. The lipid concentration is varied from 0 to 5×10^{-4} M keeping the 1-hydroxypyrene concentration fixed at 1 μM. As it is seen from the figure at pH 9, there are not many spectral changes upon addition of liposome except for an increase in intensity with a slight red shift. At pH 10 both PyOH (392, 411 nm) as well as PyO^- (447 nm) emissions are observed from the aqueous solution whereas at pH 11 the PyO^- form fluorescence is predominant. But as the lipid concentration increases there is a progressive increase in the intensities of the neutral peaks with a simultaneous decrease in the anionic peak intensity observed at all the above three pHs, whereas there is very little change in the spectra at pH 12. As

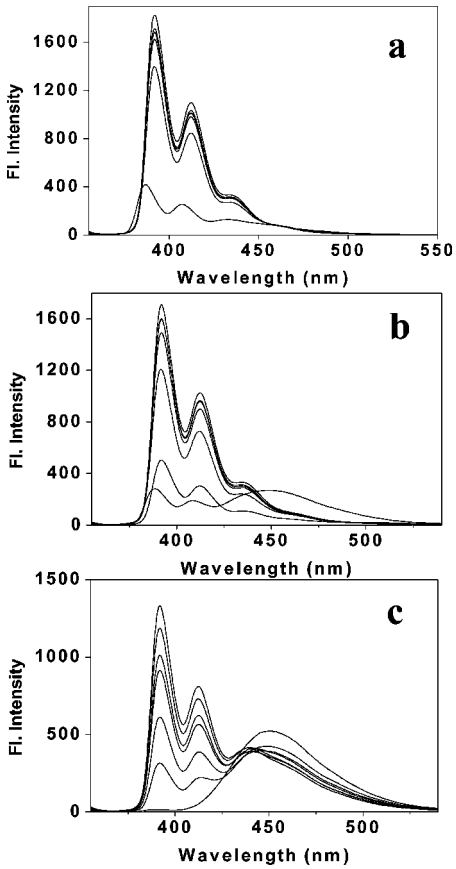

Fig. 5 Emission spectra of 1-hydroxypyrene (1×10^{-6} M) in DMPC vesicles (0–4.5×10^{-6}M) at (a) pH 9, (b) pH 10 and (c) pH 11 (λ_{ex} = 350 nm).

observed here at pH 9 and pH 10 the total intensity at the neutral peaks is always a combination of the fraction in liposome and that in the aqueous medium, whereas at pH 11 there are altogether two different species being responsible for the fluorescence in the absence and presence of liposome. Thus, we can always rule out any spectral impurity due to the neutral form of 1-hydroxypyrene from the aqueous medium at pH 11 and it becomes convenient to monitor the change in intensity at the neutral peak to get complete information about the probe that is in the liposome. Hence for our further work we have chosen pH 11 as the appropriate pH.

Determination of the partition coefficient at pH 11

The K_p values for 1-hydroxypyrene in DPPC liposome were calculated using a steady-state fluorescence method[17,18] by using the equation (details are given in electronic supplementary information, ESI†)

$$F = F_0 \, L/(55.6/K_p + L)$$

F_0 represents the maximum fluorescence intensities of neutral form of 1-hydroxypyrene. The K_p values were determined at three temperatures, at 30 °C and 50 °C, where DPPC is known to exist in the solid gel and liquid crystalline phases, respectively and at 42 °C *i.e.* the phase transition temperature. The values are found to be 1.98×10^5, 3.9×10^5 and 3.1×10^5 at 30 °C, 42 °C and 50 °C, respectively. This high value of K_p indicates the easy incorporation of 1-hydroxypyrene into the lipid bilayer, hence qualifying it as a suitable probe for liposomal studies. A large K_p ensures that most of the probe has partitioned into the membrane and the observed fluorescence parameters reflect clearly the membrane property. However, it is observed that at pH 11 the K_p values are smaller as compare to that at pH 7, which can be due to the relatively more availability of the neutral form of 1-hydroxypyrene to partition at pH 7. K_p is low at solid gel phase and increases with increase in temperature till the phase transition temperature and drops slightly in the liquid crystalline phase. These changes are attributed to the permeability changes in the lipid bilayer with the temperature changes.[19,20] At solid gel phase the membrane is compact with low permeability hence K_p is low, whereas at liquid crystalline phase the K_p value increases due to the increase in the permeability as a result of the disordered orientation of the acyl chains. The large increase at the phase transition temperature can be attributed to the large increase in the membrane disorder and permeability because of the coexistence of the solid and liquid domains.

Effect of temperature

Lipid membranes are known to exist in different phases depending on the temperature. The vesicles of DMPC and DPPC are known to undergo a pretransition from the lamellar gel phase to the rippled gel phase, and successively the main transition from the rippled phase to the liquid crystalline phase. The temperatures of main transition and pretransition for DMPC and DPPC are 23 °C, 14 °C and 42 °C, 35 °C,[21,22] respectively. Thermotropic phase transition involves significant changes in the physical properties of the lipid bilayer. The phase behaviour of a lipid membrane determines its properties such as permeability, fluidity, fusion, aggregation and protein binding; all of which affect the stability of liposome. The response of a potential molecular probe to the phase transition is often taken as a convenient test for their applicability for the liposome studies.

The temperature profile for the change in the intensity of the neutral form (at 392 nm) of 1-hydroxypyrene is shown in Fig. 6A. For DMPC the intensity of neutral form is low below 15 °C and increases steeply in the temperature range between 15 °C to 23 °C, beyond which it decreases. For DPPC the intensity increases between 25 °C to 42 °C and then decreases. Therefore a local maximum in the intensity is observed at 23 °C for DMPC and at 42 °C for DPPC. In the case of DPPC the pretransition at around 35 °C is also seen. In the solid gel phase, the membrane is tightly packed and the capacity of the hydrocarbon interior to accommodate 1-hydroxypyrene is less; this results in a decrease in the fluorescence intensity of the neutral form. Near the phase transition temperature lower resistance pathways are produced in the membrane because of the microscopic regions of disorder.[22] This leads to an increased permeability of membrane resulting in greater population of 1-hydroxypyrene inside the membrane, which explains the local maximum in the intensity of the neutral form near the phase transition temperature. Above this temperature, in the pure liquid crystalline phase, the regions of local disorder vanish and the permeability decreases hence decreasing the neutral form intensity. Thus it shows that the spectral behaviour of 1-hydroxypyrene is very sensitive to the membrane property and is capable of sensing the thermotropic changes in the membrane.

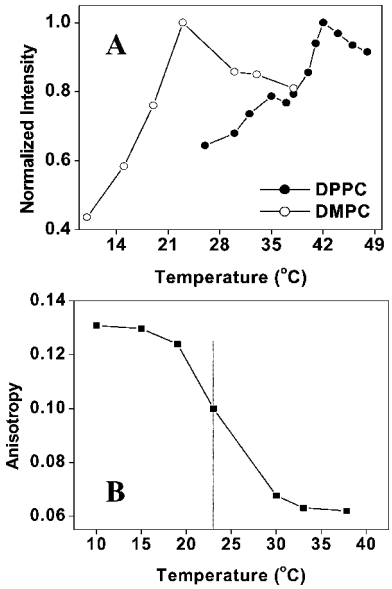

Fig. 6 (A) Temperature profiles for the variation of the neutral form fluorescence intensity of 1-hydroxypyrene (1×10^{-6} M) in DMPC and DPPC liposomes (3×10^{-4} M) at pH 11, (B) plot of fluorescence anisotropy variation of 1-hydroxypyrene in DMPC liposome (3×10^{-4} M) as a function of temperature, ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 392$ nm).

Anisotropy studies

The anisotropy value of 1-hydroxypyrene in liposome was measured as a function of temperature. Fig. 6B shows the variation of anisotropy of 1-hydroxypyrene at $\lambda_{em} = 392$ nm, in DMPC liposome. The anisotropy value at 10 °C is found to be 0.14 that decreases with increase in temperature; at phase transition temperature (23 °C) it shows a sudden drop and becomes 0.07 at 30 °C where it almost remains constant. However, the anisotropy observed at $\lambda_{em} = 447$ nm does not vary with the temperature. The observed nature of variation in anisotropy is similar to that of DPH, which is a well-studied anisotropy probe in liposomes.[23] Thus it can be assumed that like DPH, 1-hydroxypyrene also orients rigidly in the liposome interior and since with increase in temperature the membrane fluidity increases and the molecule gets more rotational freedom, the anisotropy value decreases, hence sensing the phase transition in the bilayer.

Thus both the fluorescence intensity as well as anisotropy properties of 1-hydroxypyrene successfully monitor the temperature-induced changes in the lipid bilayer.

Time resolved fluorescence and quenching studies

The fluorescence decay of 1-hydroxypyrene in DMPC liposome at pH 11 was monitored both at the neutral as well as anionic form emission wavelengths and the data at the two temperatures are tabulated in Table 1. The monoexponential decay of both forms shows the uniqueness in the distribution of 1-hydroxypyrene *i.e.* the neutral form fluorescence arises from the probe in liposome where as the anionic form is from the fraction remaining in the aqueous medium. The decrease in lifetime of both forms with increase in temperature is due to the increase in the fluidity of the membrane in the liquid crystalline phase and the increase in k_{nr} in the aqueous medium, respectively.

Table 1 Fluorescence lifetime data of 1-hydroxypyrene in DMPC liposome at pH 11

	Neutral form		Anionic form	
Temperature/°C	τ/ns	χ^2	τ/ns	χ^2
15	11.40	1.24	3.45	1.14
35	10.07	1.30	3.20	1.19

The quenching experiments by using I^- as the quencher further supports the lifetime data. I^- is an hydrophilic quencher and quenches that part of neutral probe, which is either in aqueous medium or in the water accessible part of the liposome. In our experiment it was found that there was no appreciable change in the neutral form fluorescence intensity of 1-hydroxypyrene even on addition of 0.4 M of KI, which indicates that 1-hydroxypyrene occupies the water inaccessible liposome interior.

Effect of cholesterol

Incorporation of cholesterol into liposomes brings about major changes in the properties of these vesicles. The effect of cholesterol on lipid membrane has been widely studied by various techniques.[24–28] Cholesterol has a very high affinity towards phospholipids and it can be incorporated in concentrations as high as 1 : 1 or even 1 : 2 molar ratios of cholesterol to lipid. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxy group oriented towards the aqueous surface, and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer, thus inducing more efficient packing of the lipid bilayer, and alters the fluidity of the membrane. Cholesterol reduces the enthalpy of phase change and with increasing concentration it becomes zero, thus with increasing addition of cholesterol the phase transition gradually gets blurred and is completely eliminated by 50 mole%.[1]

The variation of neutral form intensity of 1-hydroxypyrene with temperature in presence of various mole% of cholesterol in DMPC is given in Fig. 7. The sharp increase in the intensity of the neutral form observed at the phase transition temperature is very much suppressed on addition of higher mole percent cholesterol and by 40 mole% the variations are minimum. Thus the effect of cholesterol on the properties of DMPC membrane is also reflected in the spectral behavior of 1-hydroxypyrene. The increased compactness of the membrane upon addition of cholesterol prevents the redistribution of 1-hydroxypyrene

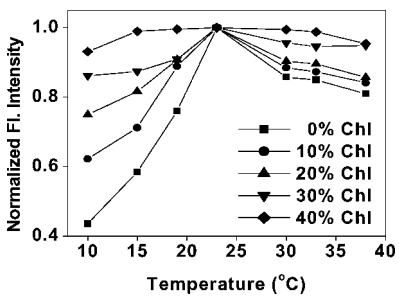

Fig. 7 Effect of addition of various mole percent of cholesterol on the variation of neutral form fluorescence intensity of 1-hydroxypyrene (1×10^{-6} M) with temperature in DMPC liposome (3×10^{-4} M).

between the membrane and the aqueous medium thus sensing the cholesterol-induced effect in the membrane.

Effect of EtOH

It has long been recognised that a wide variety of structurally unrelated small amphipathic organic molecules induce interdigitation in lipid bilayers.[29] Short chain alcohols are one such category of molecules. Alcohols ranging from methanol to 1-heptanol have been reported to induce interdigitation in phosphatidylcholine bilayers; ethanol being the most widely studied molecule.[30]

In the interdigitated state, lipid molecules from opposite monolayers interpenetrate with the terminal segments of their alkyl chains facing the aqueous phase and contacting water thus altering the membrane properties. Compared to non-interdigitated lipid bilayers fully interdigitated structures have shorter membrane thickness, a reduced surface charge density and a high molecular order in the acyl chains, an increased mobility in the head group and a decrease in the membrane solubility for water. Several physical techniques[31] such as X-ray diffraction, neutron diffraction, high-resolution electron cryomicroscopy, scanning density meter, Raman, infrared, NMR, ESR and luminescence spectroscopy have been used extensively. Recently it has been shown that ESPT property of 1-naphthol can be used as a very convenient technique for monitoring the ethanol-induced changes in lipid layers.[32] Since 1-hydroxypyrene is found to be very promising in sensing the solid gel to liquid crystalline phase change we extended our studies to investigate its sensitivity towards the interdigitation phase change.

In pure ethanol only the neutral form emission of 1-hydroxypyrene is seen, whereas in pH 11 buffer only the anionic form emission is observed. With increasing addition of ethanol to the buffer there is a gradual increase in the neutral form intensity with corresponding decrease in the anionic emission. But when ethanol is added to 1-hydroxypyrene labelled liposome, a gradual decrease in the neutral form intensity is observed which is in contrast to that observed in the homogeneous solution of buffer and ethanol (Fig. 2 in ESI†).

Fig. 8 shows the plot of neutral form intensity of 1-hydroxypyrene in DPPC liposome as a function of ethanol concentration. This shows a gradual decrease in the intensity till 0.8 M ethanol, with a sudden drop near 0.9 M and beyond which it remains almost constant. It is reported that the ethanol-induced interdigitation in DPPC liposome membrane occurs in the range of 0.8 to 1.2 M ethanol concentrations.[33] Thus the sudden drop observed in the neutral form intensity at 0.9 M can be attributed to the onset of interdigitation.

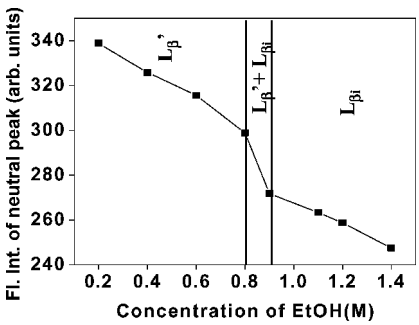

Fig. 8 Plot of variation of neutral form fluorescence intensity of 1-hydroxypyrene (1×10^{-6} M) in DPPC liposome (3×10^{-4} M) *versus* ethanol concentration.

Interdigitation increases the compactness of the membrane and the loss of bilayer midplane environment with increased membrane packing in the presence of ethanol forces the 1-hydroxypyrene occupying the lipid interior to move towards the interfacial water accessible site, hence reducing the neutral form intensity. From Fig. 8 it is clear that the maximum change in the neutral form intensity happens at 0.9 M ethanol. Thus it is inferred that the bilayer is present only as the gel phase till 0.8 M, both gel and interdigitated phases occur between 0.8 and 0.9 M and above 0.9 M only the interdigitated phase exists. 0.9 M is the critical ethanol concentration, where interdigitation occurs in DPPC liposomes, which agrees very well with the literature value.

Determination of K_p in the presence of ethanol

Since interdigitation brings about tighter acyl chain intermolecular packing and increase in membrane surface density, it is expected that with interdigitation the solute partitioning should decrease. And it is indeed observed in the partition coefficient values of 1-hydroxypyrene. The K_p value is found to decrease from 3.9×10^5 to 2.2×10^5 at 0.5 M and to 8.0×10^4 at 1.0 M ethanol concentration at 50 °C. The drastic decrease of K_p at 1.0 M of ethanol is due to the decrease in permeability of the bilayer as a result of interdigitation. This decrease of K_p with ethanol concentration also explains the observed reduction in the neutral form intensity of 1-hydroxypyrene in DPPC with increasing ethanol.

Effect of temperature in the presence of ethanol

It is well documented that ethanol interacts with lipid bilayers by two mechanisms showing a biphasic effect.[34] At low concentration ethanol interacts preferentially with the fluid hydrophobic region of the bilayer, hence shifting the phase transition to a lower temperature. But at higher ethanol concentration the secondary interaction dominates that favours the gel phase thus shifting the phase equilibrium towards higher temperature and reverting back the phase transition temperature.

Temperature dependent studies were carried out for 1-hydroxypyrene in DPPC liposomes by adding different concentrations of ethanol. The plot of neutral form intensity of 1-hydroxypyrene with temperature is shown in Fig. 9A. The main transition temperature that is observed at 42 °C in the absence of ethanol, decreases with addition of ethanol till 1.0 M of ethanol concentration and on further increasing the ethanol concentration the phase transition temperature starts increasing till 2.0 M ethanol, where it reverts back to 42 °C. This is clearly seen in Fig. 9B.

This shows that 1-hydroxypyrene can be successfully employed for monitoring ethanol-induced changes in DPPC bilayers.

Conclusion

The present work shows that the prototropic behavior of 1-hydroxypyrene can be used as a very efficient tool for the liposomal studies in alkaline pH. This is mainly due to the large shift in the absorption and emission spectra of the two forms PyOH and

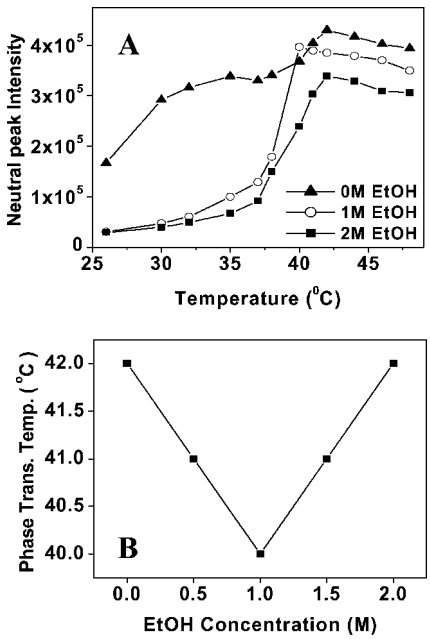

Fig. 9 (A) Temperature profile for the variation of neutral form fluorescence intensity of 1-hydroxypyrene (1×10^{-6} M) in DPPC liposome (3×10^{-4} M) in the absence and presence of ethanol (1.0 M and 2.0 M). (B) Plot of variation of phase transition temperature of DPPC liposome as sensed by 1-hydroxypyrene *versus* ethanol concentration.

PyO^-. Since there is only ground state prototropism the absorption spectral changes can be used to study the 1-hydroxypyrene and liposome interaction. But fluorescence technique has got the advantages of double selectivity, availability of fluorescence anisotropy as an independent parameter and high sensitivity so that a very low concentration of 1-hydroxypyrene can be used; which is an important criterion for an extrinsic probe for such studies. The detailed pH study shows that only the neutral form of 1-hydroxypyrene partitions into the membrane and not the anion. Spectral changes due to prototropism are significant in the pH range of 9–11.5; hence fluorescence intensity as a probe parameter can be used in this pH window. The highest sensitivity is seen at pH 11. The neutral form fluorescence intensity of 1-hydroxypyrene is found to be very sensitive to the changes in the membrane such as solid gel to liquid crystalline phase transition and the effect of cholesterol and ethanol on it. The lifetime data as well as the quenching data suggest that 1-hydroxypyrene occupies the interior water inaccessible site of the liposome. The appreciable steady-state anisotropy value of neutral form suggests that the orientation of 1-hydroxypyrene is considerably rigid inside the lipid bilayer and it is sensitive to the thermotropic changes. Hence, 1-hydroxypyrene can also be used as an anisotropy probe similar to DPH at any pH.

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