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A new ratiometric fluorescence probe as strong sensor of surface charge of lipid vesicles and micelles

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Abstract We report on the ability of a new fluorescent probe, 4-(2-pyren-1-yl-vinyl) pyridine, 1, to respond to micelles and phospholipid vesicles of different surface charge. The probe gets incorporated into micellar and membranous assemblies and shows a large red-shift in the fluorescence emission maxima especially when the surface charge of the organized media is anionic. The effect on the photo-excitation of the probe is very clear and pronounced as it can be easily visualized. The sample color upon photo-excitation changes from blue to orange/red once the probe experiences negatively charged vesicular or micellar surfaces. These results make the probe molecule useful as a reporter for sensing electrostatic environment in biological membranes and related organized assemblies.

Key words: Membrane surface charge; Ratiometric probe; Pyren-1-yl-vinyl pyridine; Anionic surface; Phospholipid vesicle; Micelle

1. Introduction

Membrane surface charge properties regulate several key biological processes, including the rate of membrane fusion, the partitioning of proteins and peptides into biological membranes, the transport of solutes through membrane and the modulation of the activity of certain membrane bound enzymes [1]. The electrostatic character of the individual lipid head groups, lipid intermolecular association and their *trans*-bilayer distribution in membranes also significantly influence the physical properties of membranes [2–4]. Factors such as the lipid hydration and charge repulsion at the lipid head group level are among those that influence the intrinsic membrane curvature and associated strain [5]. This phenomenon is increasingly believed to be responsible for the cellular regulation of a number of biological activities [6,7]. However, determination of the nature of membrane surface is often not easy.

Fluorescence offers a relatively convenient approach to study this phenomenon because of its simplicity, wide scope, and high sensitivity of detection. Several fluorescent probes are known that are being used to probe the structural and dynamic changes associated with membrane surface [8–10]. The use of fluorescent probes in membrane-based studies is however often hampered by certain limitations associated with them. For instance, with the charged probes such as ANS, the association with the membrane bilayers is shallow [11,12], whereas the neutral probes like Laurdan or Prodan are not a sensitive reporter of the ionic environments at the membrane interfaces [13,14]. Similarly, the probes based on excimer and exciplex formation are also not useful as they undergo more complex and less understood structural and conformational changes on excitation [15]. The commonly used polarity-sensitive and electrochromic dyes are also effective only to a limited extent as they provide the response by shifting the broad band already present in the emission spectrum [16,17].

One possibility that has not been fully explored in membranes is the possibility of using the color-changing response against the perturbation in the surface properties of the membranes that can be offered by two-color ratiometric probes. This two band (color) ratiometric response arises on account of the existence of the probe in two different forms as a result of some reaction in the excited state such as proton transfer, electronic charge transfer (CT), isomerization etc. [18–21]. The use of such a probe offers many advantages over other category of probes. Firstly, the probe response is visual due to the change in the color of the emitted light. Secondly, the ratios of the intensities of the two bands become a signal of perturbations in the interfaces. Consequently there is a continuing increase in the demand for two-color ratiometric probes [19,20].

Our laboratory has focussed on diverse membranous assemblies that have very different surface charges and associated properties. Cationic lipid assemblies have been suitably tailored to achieve gene-transfection activity [22–26]. We have also focussed on zwitterionic phosphatidylcholine lipids and the role of fatty acid linked with the glycerol backbone [22]. Clearly the nature of the surface charge of lipid aggregates and their intermolecular association has an important bearing on the ability of the lipid aggregates to mediate various key processes like gene transfection among others [26]. In pursuance of our interest in investigating various lipid aggregates we are now involved in design and development of new probes for membranous assemblies. At present we have targeted new molecular probes that can respond to surface properties of aggregates, especially surface charge. Herein we show a dra-

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Abbreviations: CTAB, cetyl trimethyl ammonium bromide; Tween® 20, polyoxyethylene (20) sorbitan monolaureate; SDS, sodium dodecyl sulfate; DPPC, dipalmitoylphosphatidyl choline; DCP, dicetyl phosphate; DPPA, dipalmitoylphosphatidic acid; CT, charge transfer

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matic variation in fluorescence properties of a newly developed probe, 4-(2-pyren-1-yl-vinyl) pyridine, **1** (Fig. 1), in micelles and vesicles with anionic surfaces. The probe belongs to the general class of donor–acceptor fluorescent molecules [27] that are capable of showing intramolecular CT. The probe is useful since it manifests a differential response at two well-separated wavelengths when incorporated in membranous assemblies rich in negatively charged lipidic species.

2. Materials and methods

The phospholipids and the detergents used herein were purchased either from Avanti Polar Lipids, Sigma, or Fluka. The spectra were recorded in buffer prepared in double-distilled water. The absorption and emission spectra were recorded on a Shimadzu UV-Vis 2100 spectrophotometer and a Hitachi F-4500 fluorescence spectrophotometer respectively. The excitation slit width was kept at 10 nm whereas the emission slit width was kept at 5 nm. The quantum yields were estimated by a reported procedure using quinine sulfate in 0.1 N sulfuric acid ($\varphi = 0.55$) as standard [28]. pH was measured on a SCHOTT CG-825 pH meter. The following concentrations were used: probe (5 μM), phospholipid (0.5 mM), and micelles (10 mM).

Small unilamellar vesicles of dipalmitoylphosphatidyl choline (DPPC), dipalmitoylphosphatidic acid (DPPA) and dicetyl phosphate (DCP) were prepared in buffer as previously described [29]. Briefly to a solution of a given lipid in chloroform, a solution of the probe was added in an appropriate amount to maintain the desired concentration. Solvent was evaporated under a gentle stream of N_2 and further under high vacuum (ca. 8 h) to prepare a thin film of the lipid doped with the probe. Buffer solution was added on the film and the suspension was left for hydration (ca. 12 h at 4°C). This was then thawed to 65°C on a water bath for 10 min, vortexed and cooled in water–ice mixture for 15 min. The process was repeated thrice. The resulting suspension was then sonicated for 15 min above the lipid melting phase transition temperature in a bath type sonicator to get the probe-doped vesicles. The vesicles were found to have a unilamellar morphology as revealed by transmission electron microscopy. Micelles were prepared by dissolving each of sodium dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB) and polyoxyethylene (20) sorbitan monolaureate (Tween® 20) in buffer followed by a brief sonication at room temperature.

The probe was synthesized by treating pyren-1-yl-diethyl phosphonate with pyridine-4-carboxaldehyde in the presence of sodium hydride in 1,2-dimethoxyethane. The product was purified by preparative column chromatography over silica gel and characterized by satisfactory FT-IR, ^{1}H-nuclear magnetic resonance, LR and HR mass spectra.

3. Results

The molecular structure of the fluorescent probe, **1**, that has been used in this study is shown in Fig. 1. This consists of pyrene (fluorophore) linked to an appropriately substituted pyridine unit via a *trans*-olefinic bond. The presence of a pyridine moiety renders the probe protonatable and the pyrene unit makes it lipid soluble [30]. The combination of pyrene and pyridine units gives the probe an amphipathic character [31–34]. Because of the direct conjugation of the pyrene aromatic moiety with that of pyridine, any change on the charge characteristics of the pyridine nucleus would be expected to influence the absorption and emission behavior of the probe. Thus the probe offers an opportunity for sensing surface charge of micellar or vesicular aggregates when incorporated therein. The spectroscopic data for the probe in several representative solvents are summarized in Table 1. The relatively high quantum yield of the probe in various solvents of differing polarity indicates that it could be a suitable probe for membrane applications.

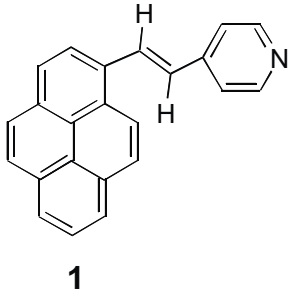

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Fig. 1. Chemical structure of the probe, 4-(2-pyren-1-yl-vinyl) pyridine, **1**, used in the present study.

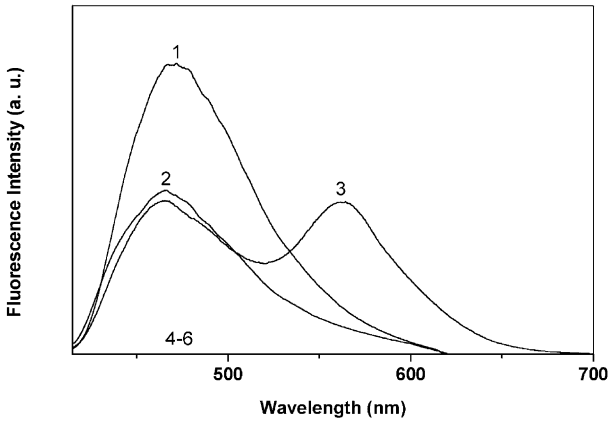

Fig. 2. The emission spectra of **1** ($\lambda_{ex} \sim 380$ nm) in micelles prepared in Tris–HCl buffer (50 mM, pH 7.5). The detergents used (10 mM) are: (1) CTAB; (2) Tween® 20; (3) SDS; (4–6) blanks (traces 1–3 respectively without the probe). The spectrum (trace 3) is presented after three-fold multiplication for clarity.

We first investigated the effect of incorporating the probe in micelles. The micelles incorporating probe were prepared from detergents, Tween® 20, CTAB and SDS in buffer at pH 7.5 that gives neutral, cationic and anionic surfaces respectively. Fig. 2 shows the emission spectra of the probe in various micelles and notably a dramatic change in the fluorescence emission in micelles with an anionic surface (SDS) is clearly evident. At pH 7.5, a new band showing a large red-shift (~95 nm) relative to emission in CTAB can be clearly seen in SDS. The appearance of the new red-shifted emission band is accompanied by a decrease in emission intensity. A similar

Table 1
The absorption (ν_a) and emission (ν_f) maxima, Stokes shift ($\Delta\nu$) and quantum yield (ϕ_f) for **1** (2 μM) in different solvents[a]

Solvents	ν_a (cm^{-1})	ν_f (cm^{-1})	$\Delta\nu$ (cm^{-1})	ϕ_f
Hexane	26 795	22 696	4 098	0.69
Benzene	26 315	21 891	4 424	0.76
Dioxane	26 497	22 123	4 373	0.81
THF	26 638	21 920	4 718	0.72
DCM	26 329	21 626	4 703	0.61
CH_3CN	26 809	21 607	5 202	0.46
MeOH	26 539	21 186	5 352	0.41
EtOH	26 581	21 409	5 177	0.47
Water	25 316	17 985	7 330	0.02

[a]Quantum yields were estimated using quinine sulfate in 0.1 N H_2SO_4 as standard ($\phi = 0.55$). The quantum yields of some related probes are: (1) anthracene, 0.27 (ethanol); 0.30 (cyclohexane); (2) phenanthrene, 0.13 (ethanol); (3) pyrene, 0.65 (ethanol); 0.65 (cyclohexane) [16].

observation was also made for the absorption behavior wherein the spectrum in SDS was broadened and appeared at longer wavelength. It appears that the local acidity near the headgroup of SDS micelles in water helps protonate the pyridine unit of the probe, which is most likely localized at the interfacial region of the micelles. This causes a facile CT in the ground state as seen by the presence of a new absorption band. Absorption into this CT state causes a red-shifted emission.

Further examination of the emission and absorption spectra of the probe in SDS micelles as a function of pH provides additional insights (Fig. 3). The emission spectrum of the probe in SDS at pH ≤ 6 produces only one highly red-shifted emission band at ~ 560 nm. With the increase in pH, dual emission is observed with the appearance of the band at ~ 465 nm. The band at ~ 465 nm that appears at pH > 6 is similar to the emission seen in cationic and neutral micelles. A typical spectrum near physiological pH shows two bands, a CT band for protonated species and another corresponding to non-protonated species of the probe. A plot of the ratios of the intensities of the two bands (I_{560}/I_{465}) against pH shows that the probe undergoes protonation at a pH close to 5.8 in SDS. The absorption spectra of the probe in anionic SDS micelles also show multiple bands at various pHs, with a long wavelength band being prominent at lower pH (Fig. 3A).

We were particularly interested in the behavior of the probe

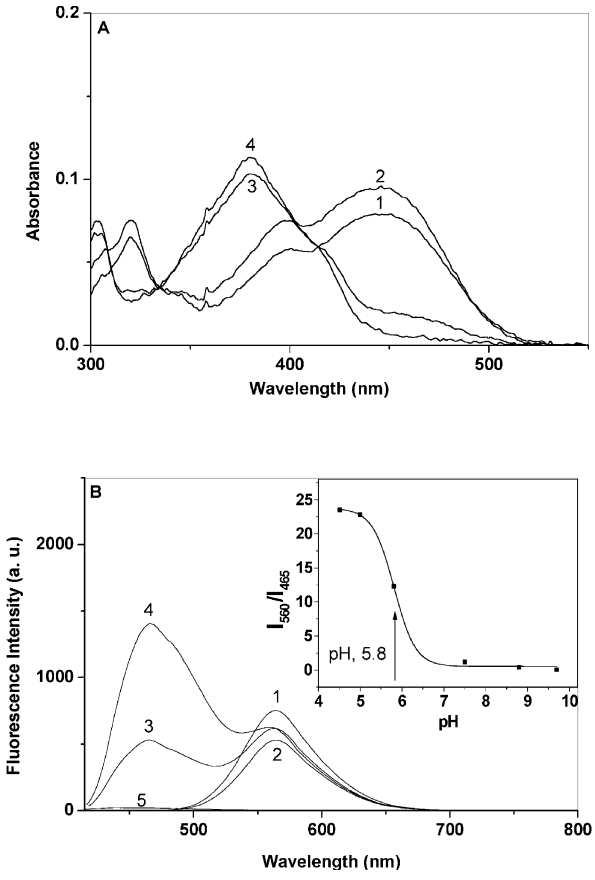

Fig. 3. A: The absorption spectra of **1** in SDS micelles (10 mM) prepared in different buffers. B: The corresponding emission spectra, the inset shows the plot of I_{560}/I_{465} against pH. The buffers (50 mM) used are: (1) acetate, pH 5.0; (2) citrate-phosphate, pH 5.8; (3) Tris–HCl, pH 7.5; (4) glycine-NaOH, pH 8.8; (5) blank.

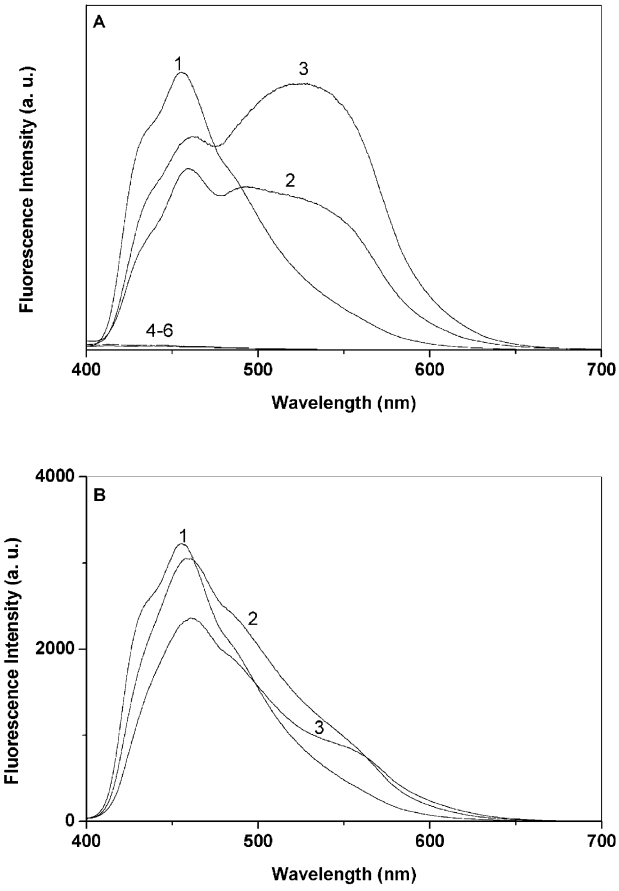

Fig. 4. A: The emission spectra of **1** ($\lambda_{ex} \sim 380$ nm) in different phospholipid vesicles (0.5 mM) in Tris–HCl (50 mM, pH 7.5) buffer. (1) DPPC; (2) DCP; (3) DPPA; (4–6) blank (traces 1–3 respectively without the probe). The emission spectra (traces 2 and 3) are presented after 2.5-fold multiplication for clarity. B: The effect on the fluorescence spectra of the probe in DPPC on gradual addition of SDS; (1) DPPC; (2) SDS (1 mM); (3) SDS, (2 mM).

when incorporated into membranes. It was of interest to us to see whether the ability of the probe towards anionic micellar surface is applicable to membranes. Fig. 4 shows the fluorescence spectra of the probe in vesicles prepared from different phospholipids. The emission profile in DPPC membrane at 25°C shows a slightly structured band peaking at ~ 455 nm. The emission spectrum of the probe shows a dramatic change when incorporated into negatively charged vesicles. The change is well evident in vesicles prepared from anionic lipids DCP and DPPA with one emission band at ~ 460 nm and a new broad and highly red-shifted band at ~ 530 nm. This behavior is very similar to that observed in micelles showing the general ability of the probe to sense negative surface charge. Also, subtle differences in emission spectra obtained for the probe in DPPA and DCP vesicles were observed. The new red-shifted band was more pronounced in vesicles of DPPA as compared to DCP. To further investigate the response of the probe towards anionic amphiphiles, fluorescence spectra were studied in DPPC membrane upon gradual addition of SDS. The typical changes are shown in Fig. 4. The probe senses the presence of anionic amphiphiles getting incorporated in DPPC vesicles, which is clearly evident from the red-shift in the emission spectrum on SDS addition. Similar

but less pronounced changes were seen with increase in fatty acid content of the phospholipid vesicles (not shown).

In order to find rational explanation for our observations, we carried out emission studies at different pH values (1.2–7.1) and in dioxane–water binary mixtures (Fig. 5). The pH studies can provide us with the pK_a of the probe and also elucidate the changes that take place in emission behavior on protonation (Fig. 5A). In the entire pH range above pH 5, the probe shows a very week emission with its peak around ~555–560 nm. As the pH is decreased below 4.6 there is a sharp increase in emission with peaks around ~570 nm. The absorption spectra also showed similar changes (not shown). The plot of the emission λ_{max} versus pH (inset Fig. 5A) provides the pK_a for the probe to be 4.4. The sudden increase in emission intensity also corresponds to this pH.

Dioxane–water mixture helps us to understand the behavior of probe in relatively apolar environments that may resemble the microenvironment in lipidic aggregates [35,36]. Fig. 5B shows that in pure dioxane, the probe gives a 'structured' emission band peaking at ~431 and 454 nm, which gradually broadens, becomes featureless and gets red-shifted on water addition. This is accompanied by a decrease in the emission intensity of the probe and finally in neat water, the probe shows almost negligible fluorescence. However, dramatic changes are seen in the emission spectra in the presence of a small amount of acid. Trace 6 in Fig. 5B shows the emission spectrum in dioxane–water mixture (80:20) along with a trace amount of HCl. In this condition, it is possible to see a new pronounced emission band at around 560 nm. Thus the presence of acid in the solvent–water mixture or acidic pH causes protonation of the pyridine unit leading to the development of the red-shifted band. The absorption spectra were found to be consistent with our results obtained with fluorescence data.

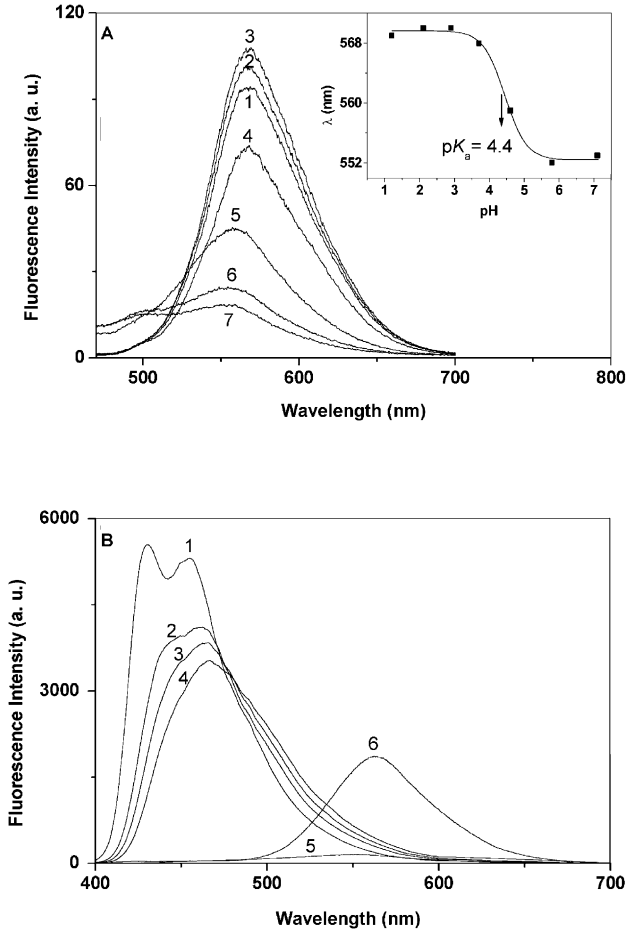

Fig. 5. A: The emission spectra of **1** in buffers of different pH. The buffers (50 mM) used are KCl (1–2) and citrate-phosphate (3–7). The pH values are (1) 1.2; (2) 2.1; (3) 2.9; (4) 3.7; (5) 4.6; (6) 5.8; (7) 7.1. The inset shows the plot of emission maxima against pH. B: The emission spectra (λ_{ex} ~380 nm) of the probe in various dioxane–water mixtures. The compositions are: (1) 100:0; (2) 95:5; (3) 90:10; (4) 80:20; (5) 0:100; (6) 4 containing a trace amount of HCl. The emission spectra (traces 5 and 6) are presented after five-fold multiplication for clarity.

4. Discussion

Design of membrane interactable fluorescence probes for various applications is a topic of current interest because of the need to improve on the probes available in this category and to offer complementary strategies toward involved fluorescence probing of a complex, biologically relevant environment [18–20]. For a probe to be effective in membrane-related studies, it should produce large changes in the wavelength of emission. This is advantageous, as it can then be possible to carry out ratiometric fluorescence sensing [37]. Such strategies do not depend on measuring merely the fluorescence intensity at one particular wavelength, but involve measuring the ratio of fluorescence intensities at more than one wavelength. This is important, as measurement of intensity change is susceptible to interference by quenching, scattering, intrinsic auto-fluorescence of samples etc. Also, it is important to develop probes that show emission changes in the visible/near infrared range as they avoid the problem of auto-fluorescence of biological samples and are ideally suited for microscopic, multi-color applications in cell biology.

The probe reported in the present work satisfies most of the above criteria for the new probe development. The probes are suited for applications in micelles and membranes because of their amphipathic nature. The pyrene–pyridine conjugate aligns itself in a way that the pyrene unit remains embedded in the hydrophobic core whereas the pyridine moiety gets localized near the lipid–water interface [12,31–34]. It may be mentioned here that for this type of donor–acceptor conjugates there is a possibility of CT from pyrene (donor) to pyridine (acceptor). This modular design along with its preferred alignment makes the molecule suitable for probing the interfacial environment. The CT process is in general expected to make the probe extremely sensitive to the nature of the interface.

The study in dioxane–water was carried out to investigate the protonation of the probe in a largely non-polar environment similar to heterogeneous systems used in the present study. The results show two principal effects to be operational. One, the emission is sensitive to the polarity of the medium, especially the presence of water as evident from the emission spectra of the probes in dioxane–water mixtures. But more pertinently, the probe spectral behavior is strongly modulated by acid–base equilibria. In the presence of acid, the pyridine unit gets protonated and a fairly strong red-shifted emission is seen. There is a concomitant change in its absorption behavior. These observations may be explained by con-

sidering the CT character of the probe. As the pyridine unit gets protonated, it becomes a strong acceptor and facile CT takes place between pyrene and the pyridinium moiety [38,39]. The results with pH titration concur with the above suggestion. As the probe gets protonated, even in water, it begins to show enhanced and highly red-shifted emission.

In keeping with its acid–base behavior, the fluorescence property of the probe is expected to be strongly responsive to the surface charge of the micelles and the vesicles. The large red-shift observed in the emission spectra of the probe in anionic micelles and vesicles conclusively suggests that the pyridine probes undergo protonation at the interface of these aggregates. This is consistent with the knowledge that the local pH at anionic surfaces is acidic. The protonation leads to a facile CT in anionic detergents as observed with solvent–water mixtures. The pK_a of the probe as determined from the plot of emission maxima against pH was found to be 4.4 (Fig. 5A). The plot of ratio of emission intensities at 560 and 465 nm (I_{560}/I_{465}) against pH in SDS however gives an apparent 'pK_a' of 5.8 (Fig. 3B). This shows that the probe follows subtle changes in local pH at the interface of anionic aggregate using a method based on two band emission (Fig. 3). Thus, using this probe it is possible to ratiometrically quantitate the interfacial pH for micellar SDS in a convenient manner using distinct emission bands originating from the neutral and the protonated probe molecules. The result that we get is that the local interfacial pH for SDS micelles is at least one and a half units lower than that of the pH in bulk water.

The response of the probe in micelles and vesicles showed interesting differences. The red-shifts in fluorescence emission in vesicles are smaller as compared to micelles. The probe produces orange/red emission in micelles whereas the emitted light is green in vesicles. There could be different reasons for this variation. One could be related to the differences in the intrinsic acidity of the head groups. In fact a comparison of the emission in DPPA and DCP vesicles themselves shows that probably the interfacial acidity for DPPA vesicles is slightly greater. Another possibility is that the micelles being a dynamic aggregate allows facile interaction of the probe with the head groups unlike the vesicles which form significantly more ordered assemblies.

In conclusion, in the present communication we report a new probe, 4-(2-pyren-1-yl-vinyl) pyridine that is very sensitive to surface charge in various organized aggregates like micelles and phospholipid vesicles. The sensitivity arises mainly due to the protonation of the probe accompanied by related changes in emission properties. Besides, the probe is susceptible to polarity changes in the medium. The probe manifests a large and visually observable wavelength shift in association with surfaces possessing anionic charge and the response is strongly ratiometric in character. Since most of the natural cell surfaces are rich in acidic phospholipids, these probes should be sensitive reporters of charges of cell surfaces and other organized assemblies and may also find useful applications in microscopy and in cytological staining. In addition this may be useful in probing various types of interactions of macromolecules associated with membrane surfaces relevant to different biological processes.

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