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Excited state proton transfer based fluorescent molecular probes and their application in studying lipid bilayer membranes

Monalisa Mohapatra [a] and Ashok Kumar Mishra *[b]

Molecules with ionizable protons, with different proton transfer efficiencies in the excited and ground states, show excited state prototropism (ESPT). In suitable proton donating/accepting environments, ESPT of a molecule can result in the observation of emissions from different prototropic species, each characterized by different emission spectra and different emission lifetimes. In condensed media, the immediate environment around the ESPT molecule can significantly influence the emission spectral parameters of different prototropic species. This forms the basis of ESPT based fluorescence sensing. The concept of ESPT has been widely used for probing dynamical and structural information of micro-heterogeneous media like micelles, polymers, lipid bilayer membranes, *etc*. ESPT molecules like naphthol and intra-molecular ESPT (ESIPT) molecules like hydroxyflavones *etc*., are said to be good multi-state fluorescent molecular probes if (i) the partitioning of these molecules to a micro-heterogeneous organized medium is more efficient, and (ii) the molecules possess distinct excitation and emission wavelengths corresponding to their different prototropic forms. The fluorescence of different prototropic forms shows a sensitive response towards the change in the local environment around the micro-heterogeneous organized medium concerning the physical properties, local structure, and dynamics. This review mainly comprises the work carried out on ESPT fluorescence molecular probing of biomimetic liposomes/lipid bilayer membranes from 1990 onwards.

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1. Introduction

1.1. The concept of excited state prototropism (ESPT)

After excitation of a molecule, its properties experience a change owing to the changes in charge density distribution in the electronically excited state compared to the electronic ground state.[1–3] Molecules with enhanced acidity in the excited state are known as excited state acids, *e.g.*, aromatic hydroxy compounds and amines. Molecules which become more basic in the excited state are classified as excited state bases, *e.g.*, carboxylic acids, ketones, and aldehydes. In general, these molecules whether acids or bases are known as ESPT molecules. In the field of chemistry as well as biochemistry, the excited state proton transfer reactions, *i.e.* proton association and dissociation of aromatic molecules, are the elementary processes.[4] In aromatic hydroxy compounds and amines, the lowest singlet excited state is comparatively more acidic than the ground state by a factor of $\sim 10^5$–10^9.[5] This was explained in terms of increased charge transfer from the oxygen or nitrogen center to the aromatic ring in the excited singlet state. The electronic rearrangements associated with the proton transfer, and the electronic structure of the protonated and deprotonated forms play a vital role in the proton transfer from an aromatic molecule to the solvent molecule.[4] The rate of proton transfer is closely associated with the reaction free energy change, bulk solvent properties and microenvironment around an excited acid.

1.2. Thermodynamics and kinetics of the ESPT process

The extensive studies of Förster[6–8] and Weller[1,2,9,10] in the middle of the 20th century had laid the foundation for the concepts and different aspects of ESPT. Förster's study was mostly on the thermodynamics of ESPT, whereas Weller made a significant contribution to the dynamics. Förster[7] constructed a thermodynamic cycle (Fig. 1) for the proton transfer process under certain acceptable approximations, which combines the spectroscopic and thermodynamic data to calculate the excited state chemical equilibrium constants.

From the acidity constant of ESPT molecules in the ground state (pK_a), and electronic transition energies of the protonated form (E_{HA}) and deprotonated form (E_{A^-}), the Förster

[a]*Department of Chemistry, VSS University of Technology, Burla, Odisha 768018, India*
[b]*Department of Chemistry, Indian Institute of Technology Madras, Chennai 600036, India. E-mail: mishra@iitm.ac.in*

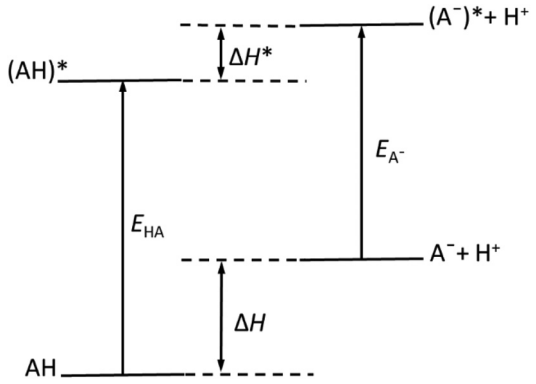


Fig. 1 Förster cycle: representing the electronic ground and excited state energy levels of an acid AH and its conjugate base A^- (adapted from ref. 11).

cycle provides the corresponding acidity constant of ESPT molecules in the excited state (pK_a^*).[11] The energies of the deprotonated form (E_{A^-}) and protonated form (E_{HA}) are estimated from the average of the absorption ν_{acid} and emission ν_{base} maxima of each species. The Förster equation for an equilibrium, $AH^* \rightarrow A^{-*} + H^+$, can be represented as:

$$pK_a - pK_a^* = \left(\frac{N_A h}{2.303RT}\right)(\nu_{acid} - \nu_{base})$$

where h is Planck's constant, N_A is Avogadro's number, T is the absolute temperature, R is the gas constant, and ν_{acid} and ν_{base} are the frequencies of the longest wavelength transitions of the protonated (neutral) and deprotonated (anion) forms, respectively. According to the Förster cycle, if the longest wavelength electronic transition of the deprotonated form is of lower energy compared to that of the protonated form (red-shifted electronic absorption or emission spectrum of the deprotonated form with reference to the protonated form), the molecule shows enhanced excited state acidity (*i.e.*, the pK_a^* of the molecule is lower than pK_a). Some studies on the acidity constant of ESPT molecules show that pK_a^* values are noticeably different from the pK_a values.[11–13] Excited state acids have $pK_a^* < pK_a$, while excited state bases have $pK_a^* > pK_a$. The pK_a^* value calculated from the Förster cycle gives only an indication of the acidity range in which a fluorescence change is expected but does not indicate whether or not proton transfer in the excited state is kinetically feasible. In these cases, fluorimetric titration provides a method for the experimental determination of pK_a^*. However, it has been realized that for a large number of ESPT molecules, there is a frequent mismatch between pK_a^* values obtained from the Förster cycle and fluorimetric titration methods.

Regarding the kinetic aspects of ESPT, Weller[1,2] has brought out the importance of competition between the rate of deactivation of the excited state and the rates of proton transfer. The equilibrium and the possibility of ESPT are illustrated in Fig. 2, where k_{nr} is the non-radiative rate constant, k_f is the fluorescence rate constant, k_{pt} is the proton transfer rate constant, and k_{rpt} is the reverse proton transfer rate constant.

Monalisa Mohapatra

Monalisa Mohapatra received her BSc (2004) and MSc (2006) from Utkal University, and Ph.D. (2011) from IIT Madras under the supervision of Prof. A. K. Mishra. She is the recipient of the best PhD thesis award (Langmuir award) in Physical and Theoretical Chemistry (2012) from IIT Madras. Currently, she is working as an Assistant Professor in the Department of Chemistry, Veer Surendra Sai University of Technology, Burla, India. Her research interests are in Physical Photochemistry, Fluorescence Spectroscopy and Biophysical Chemistry.

Ashok Kumar Mishra

Ashok Kumar Mishra received his MSc (1980) from the University of Delhi, and Ph.D. (1985) from IIT Kanpur. After a stint in Sambalpur University (India) and in Gunma University (Japan), he joined IIT Madras in 1992. Currently, he works as a Professor in the Department of Chemistry along with the additional responsibility of Dean, Academic Research at IIT Madras, India. Prof. Mishra is the recipient of the CRSI Bronze Medal from the 'Chemical Research Society of India' and a Fellow of the 'National Academy of Sciences, India'. He has also received 'Acharya P C Ray Memorial Award' from Indian Chemical Society and 'Prof W U Malik Memorial Award' from The Council of Chemists. So far he has guided 25 Ph.D. students, 31 M.Sc. students, and 6 M.Phil students and published about 190 research papers, reviews, and monographs. His research interests include Physical Photochemistry, Fluorescence Spectroscopy, development of analytical methods for multifluorophoric systems, and fluorescent probes for micro- and nano-scale organized media and soft matter.

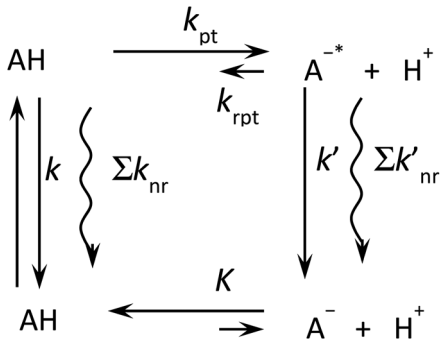


Fig. 2 Kinetic scheme showing the proton transfer processes of a molecule (AH).

If $\tau_0 = \left(\frac{1}{k_f + \sum k_{nr}}\right)$ and $\tau = \left(\frac{1}{k_f + \sum k_{nr} + k_{pt}}\right)$ are the fluorescence lifetimes of the molecule AH in the absence and presence of ESPT, respectively, and if $k_{rpt} \ll k_{pt}$ then the proton transfer rate constant of AH is calculated using the decay times $k_{pt} = \left(\frac{1}{\tau} - \frac{1}{\tau_0}\right)$ as used by Il'ichev *et al.*[14–16]

For excited-state reactions, the two-state model is usually appropriate, where there is the existence of different prototropic forms.[11] In the presence of a suitable base (Fig. 2), if $(k_f + \sum k_{nr}) < k_{pt}$ then complete proton transfer takes place, and emission is observed from only the anion form, A^{-}*. The condition where $(k_f + \sum k_{nr}) > k_{pt}$, incomplete proton transfer takes place, and the emission is observed from both the neutral (AH*) and anionic (A^{-}*) forms. When $(k_f + \sum k_{nr}) \gg k_{pt}$, no proton transfer takes place, and the observed emission is only from the neutral form, AH*. ESPT fluorescent molecules are better probes when the rate of proton transfer (k_{pt}) is faster than the rate of fluorescence.

The introduction of nanosecond time-resolved fluorimetry in the late 1970s and the subsequent development of the picosecond spectroscopic technique have enabled the study of the mechanism and dynamics of proton transfer processes in homogeneous media.[12,17] Dynamic analyses at nanosecond resolution facilitate more consistent estimation of pK_a^* values. From picosecond time-resolved measurements for 1-naphthol in water, Webb *et al.*,[18,19] had estimated the value of the excited state deprotonation rate constant as 2.5×10^{10} s^{-1} and re-protonation rate constant as 6.8×10^{10} s^{-1}. For various ESPT molecules, the proton transfer mechanism and rate constants were determined.[20–23] Lee and coworkers[24–26] suggested that for accepting the dissociated proton a cluster of 4 ± 1 water molecules is needed to act as a base. The ESPT of 1-naphthol to water was studied[27] with a variation of the solvent system size. It was found that ESPT is completely absent in ice, which is evidence of the orientational relaxation of water molecules during the proton transfer process. Under the experimental conditions, a minimum cluster size of 1-naphthol. $(H_2O)_{30}$ was seen to show ESPT. This essentially implies that the 1-naphthol. $(H_2O)_4$ acid–base pair in the Lee model undergoes ESPT only when solvated by bulk water. The results of studies of 1-naphthol-$(H_2O)_n$ gas phase clusters by Zewail and coworkers[28] are also on a similar line. Liu and Chu[29] had also found that the size of the water cluster plays an important role in the proton transfer process in aqueous solution. From these studies, the general conclusion is that the presence of a suitable base is a must for the abstraction of protons irrespective of the mechanism of proton transfer, and the apparent size of the water cluster is related to the base strength of water as a proton acceptor. ESPT of aromatic alcohols and amines in aqueous and mixed aqueous solvents has been extensively studied.[30,31] In this context, the importance of solvent effects in the ESPT process of molecules is being reported in the literature.[20,32–41] Using temperature-dependent picosecond measurement of ESPT of 1-naphthol and solvation dynamics of Coumarin 500, the extent of the intermolecular hydrogen bonding energy in the water cluster associated with the ultrafast ESPT process has been identified.[42] Some light has also been thrown on the importance of geminate recombination in the explanation of pico-second decay dynamics of some ESPT molecules like naphthols and their derivatives.[43–49] It is satisfactory in explaining excited state proton transfer and proton induced quenching of the neutral form of naphthol fluorescence. Their ESPT studies in water–methanol mixtures showed that the solvent effects in the dissociation equilibrium constant and dissociation rate coefficient are equal.[11] Geminate recombination of two separated ions affects the proton dissociation processes. ESIPT has been observed in jet-cooled naphthol derivatives; 2-hydroxy-1-naphthaldehyde and 1-hydroxy-2-naphthaldehyde.[50,51] When excited state acids absorb picosecond pulses of light, they release a transient pulse of protons, causing pH jump. Gutman and coworkers[52–54] have done exhaustive work over 20 years on the development of pH jump methods to monitor proton diffusion dynamics in the microenvironment of the proton emitter. To study the proton hydration dynamics, ESPT has also been employed as a mechanistic tool and in technologist's applications of pH and pOH jump experiments.[55] Regarding ESPT processes of nitrogen, oxygen, and sulfur-containing heterocyclic compounds in homogeneous and micellar media, Dogra and his group[41,56–62] have done extensive work, over three decades. Superphotoacids like naphthols and phenols with electron-withdrawing groups such as cyano, cyano sulfonyl and methanesulfonyl exhibit greatly enhanced photoacidity.[20,45,63] The kinetics and mechanism of ESPT of these superphotoacid molecules are being subjected to extensive investigations.[30,64–70]

For six decades, the work on fundamentals of the ESPT phenomenon and probing of different micro-heterogeneous systems using ESPT fluorophores has been a motivating area of research, and has been reviewed periodically. The review by Ireland and Wyatt[3] based on the ESPT concept contains extensive references available in the literature until 1976. The review by Martynov *et al.*[71] in 1977 discusses the mechanisms of ESPT, excited state double proton transfer (ESDPT) and excited state intramolecular proton transfer (ESIPT) of aromatic mole-

cules in the solid phase and protic and aprotic solvents. To account for the kinetics of proton transfer reactions, various models have been proposed. A review by Shizuka[12] in 1985 covers the essential techniques employed to study the dynamics of intramolecular proton-transfer reactions and certain intermolecular proton-transfer reactions (as in naphthols) in a picosecond time domain. Subsequently, ESPT molecules were reinvestigated using a picosecond spectroscopic technique, and initial work in this area has been summarized by Kosower and Huppert.[72] Furthermore, the review by Gutman and Nachliel[73] discusses the outline of conclusions about the dynamics and mechanism of proton transfer processes. A brief review by Dogra[74] summarizes the mechanism of biprotonic phototautomerism and ESPT in the amino- and hydroxyphenyl-substituted benzoxazoles, benzothiazoles and benzimidazoles. The use of excited state acid–base behavior has been reviewed by Wan and Shukla in 1993.[17] The progress in the areas of fundamentals of intramolecular and intermolecular ESPT up to 1993 has been reviewed and summarized by Arnaut and Förmosinho.[5,75] For molecules with ESIPT and ESDPT, studies in the femtosecond time domain are especially important. In this regard, Elsaesser *et al.*, have[76] summarized early studies in this femtosecond time domain. A review by Tomin *et al.*, discusses the thermodynamic and kinetic control of the ESIPT process upon reviewing an abundant number of ESIPT prototypical systems in 2015.[77]

In the water-deficient environment of a variety of organized media, the rates of forward and reverse ESPT get affected.[5,16,78] The changes in ESPT rates are often reflected in the steady state and time-dependent fluorescence spectral properties of ESPT molecules. It is generally accepted that in the hydrophilic–hydrophobic interfaces of organized media, the deprotonation rate of ESPT molecules is lower than that in water. ESPT based fluorescent molecular probes are known to be efficient in probing structural and dynamical information on a variety of aggregated and organized systems.[79–92] A review by Mishra[85] provides an extensive collection of literature up to 2001, based on the fundamental concept of ESPT, ESIPT and ESDPT, and their uses in studying different organized media like liposomes, cyclodextrins, proteins, sol–gel glasses, polymers, LB films, and solids. A review by Mallick *et al.*[81] in 2007 gives a report on the study of various photoprocesses including ESPT, in different confined micro-heterogeneous environments like reverse micelles, micelles, cyclodextrins, proteins, *etc*.

Reviews exclusively based on ESPT probing of lipid bilayer membranes are not yet reported, although there are a large number of literature studies available. There are certain unique advantages of ESPT probing in the lipid bilayer membrane context, because of the rapid changes in the environment across the lipid bilayer from aqueous to the interface to the nonpolar core region. It is felt necessary to write this perspective by capturing all possible recent literature studies and discussing the unique advantages of ESPT fluorescent probes in studying lipid bilayer membranes.

2. ESPT in liposomes

2.1. Liposomes

Amphiphilic phospholipid molecules are the foundation of cells and cell organelles. In aqueous solution, phospholipid molecules spontaneously aggregate and form three dimensional closed bilayer membrane structures known as liposomes or vesicles (Fig. 3).[93] Liposomes have been widely used as model systems for biological membranes.[94,95] They have an extensive application in drug loading, food processing, diagnosis, cosmetics, and biosensors.[96–98] Liposomes of different sizes, charges, and compositions can be formulated and processed. A variety of hydrophobic and hydrophilic compounds can be incorporated into either the lipid bilayer or aqueous part of the liposome. This flexibility has provided a different path of research towards several potential applications of liposomes.[99,100] Due to biodegradability and non-toxic nature of phospholipid molecules, liposomes can be administered without severe side effects.[101] For pharmaceutical applications, some commercial liposome-based drugs have already been discovered, registered and introduced with immense success.[102]

2.2. Chemical constituents of the liposomes

Phospholipid molecules are the major constituents of liposomes. Two classes of phospholipids exist, glycerophospholipids and sphingophospholipids. The most common and extensively studied phospholipids are phosphatidylcholine molecules, in which a pair of hydrophobic chains are linked with a hydrophilic polar head group *via* glycerol bridges.[101] The hydrophobic chains with the same or different length can either be saturated or unsaturated, with the same or different length. The properties of the liposomes are decided from the length and saturation properties of the fatty acids. The head group can be either choline, ethanolamine, serine, acid or alcohol. The charge on the lipid is the same as the charge on the head group *e.g.*, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are neutral; phosphatidylglycerol (PG),

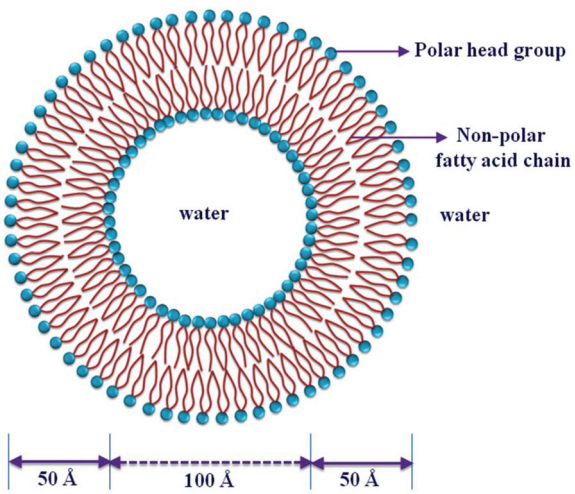


Fig. 3 Schematic representation of liposomes.

phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidyl acid (PA) are negatively charged.

2.3. Size of liposomes

Apart from chemical constituents, liposomes can be characterized by their shape and size. To have liposomes of varying sizes, different methods of preparation are required. Different applications often require the use of liposomes of a particular size range. Classification of liposomes according to size is the most common index of characterization.[101] Liposomes are broadly classified into three categories (Fig. 4): SUVs (small unilamellar vesicles; 15–25 nm), MLVs (multilamellar vesicles; 100 nm to few μm), and LUVs (large unilamellar vesicles; ~1000 nm). MLVs are mostly prepared by the hydration of dried phospholipids deposited on a glass surface by evaporation of a suitable solvent and vortexing it with an appropriate amount of distilled water or buffer in the presence of small glass beads.[93] By the extrusion method, MLVs of definite size and homogeneity have been prepared.[102] Sonication of MLVs, using either a bath type[103] or a probe type sonicator,[104] results in the formation of SUVs. SUVs can also be prepared by the ethanol injection method.[101] SUVs of more uniform sizes with diameters of 25 ± 1 nm can be obtained by gel filtration on Sepharose 4B.[103] By using the reversed-phase evaporation method, LUVs (~460 nm) with larger compartments can be prepared.[105]

2.4. Phase transitions in lipid bilayers

Phospholipid bilayers exist in different phases depending on temperature, pressure, hydration, *etc*. The phase behavior of a bilayer mainly depends on the composition of the polar head group and the length of the hydrocarbon acyl chains. The types of thermotropic phase transitions observed are sub-transition (T_s), pre-transition (T_p) and main-transition (T_m). These three transition temperatures separate the lipid bilayer into four distinct phases: lamellar crystalline orthorhombic ($L_{c'}$), lamellar orthorhombic (L_β or $L_{\beta'}$), hexagonal periodical ($P_{\beta'}$) and disordered liquid crystalline (L_α).[106] At the main transition temperature (T_m), phospholipid bilayers transit from a highly ordered solid gel (SG) phase to a more fluid and disordered liquid crystalline (LC) phase. In the case of bilayers constructed of pure phospholipids, phase transitions occur over a narrow range of temperature which is highly cooperative and endothermic.[107] With an increase in the chain length of the acyl chain, the transition temperature in disaturated phosphatidylcholines increases.[108,109] The main phase transition temperature for DMPC (14 carbon) and DPPC (16 carbon) is 23 °C and 42 °C, respectively.[110] The phase transition temperature decreases with unsaturation within the acyl chain. In determining the phase transition temperature and enthalpy of liposomes, the number and position of the double bonds play vital roles.[111]

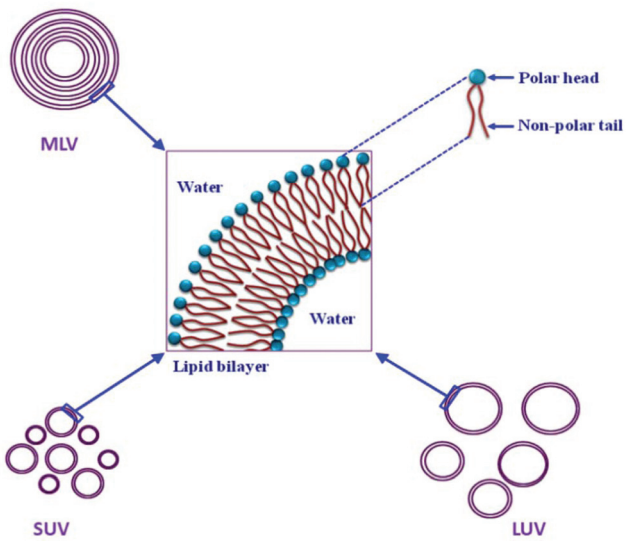


Fig. 4 Schematic representation of three types of vesicles (structures not to scale). (Adapted from ref. 101).

2.5. ESPT probing in the lipid bilayer membrane of the liposomes

The use of two state ESPT fluorescent molecular probes in the studies of lipid bilayer membranes is an ongoing era of research. ESPT molecules like aromatic hydroxy compounds, *i.e.* 1-naphthol in the aqueous medium, are in their neutral form (NpOH) in the ground state (pK_a 9.2).[13] Upon excitation, the anion (NpO^{-*}) becomes the predominant light emitting form as rapid proton transfer occurs in the excited state (pK_a^* 0.4).[13] In the lipid bilayer membrane, although the light absorbing species maintain the neutral form (NpOH), there is emission from both neutral (NpOH*) and anion (NpO^{-*}) forms. 1-Naphthol is strongly fluorescent both in its neutral and anionic forms. It is reasonably hydrophobic, flat and small, and thus partitions efficiently into the lipid bilayer membrane. A similar effect is also observed for 2-naphthol with its incorporation into the lipid bilayer membrane. Kuzmin's group[14,15,112,113] had performed detailed studies on the dynamics of naphthols, the prototype ESPT molecules, and some of their substituents in synthetic as well as natural membranes. The biexponential fluorescence decay of the neutral form of naphthol suggests the presence of two sites of its localization in lipid bilayer membranes.[14] In this regard, for the neutral form of 1-naphthol, a two-state model of probe distribution in the lipid bilayer membrane was proposed by Sujatha and Mishra.[114] According to the model, the neutral form of 1-naphthol molecules distributes itself in two types of regions in the lipid bilayer membranes: near the interface region and inner hydrocarbon chain core region. The scheme for the distribution and prototropic equilibrium of 1-naphthol incorporated into the lipid bilayer membrane is given in Fig. 5.

There are some important features of excited state prototropism of 1-naphthol that makes it an excellent ESPT fluorescent probe. (i) It is essentially a two-state emitting (neutral NpOH* and anion NpO^{-*}) probe with a very large difference in pK_a 9.2 and pK_a^* 0.4.[13,16] In water, in the mid-pH range exclu-

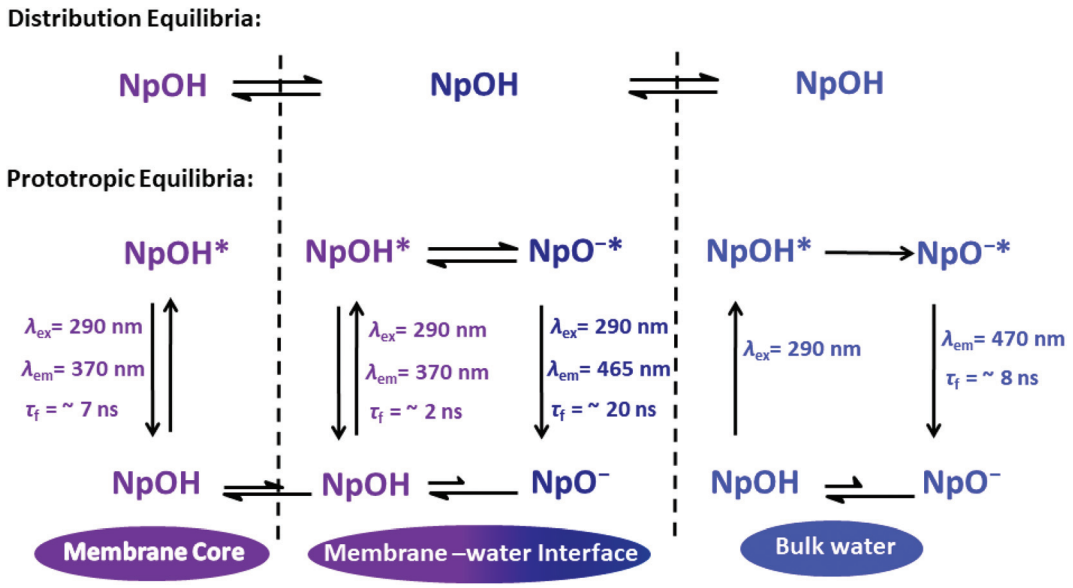


Fig. 5 Scheme for distribution and prototropic equilibria of 1-naphthol (NpOH) in the lipid bilayer membrane.

sively there is emission from the excited state anion.[85] The ground state is the neutral (NpOH) form; hence, the excitation wavelength (λ_{ex} = 290 nm) need not be changed. The equilibrium in the excited state is independent of excitation wavelength. The system of study can be adequately monitored at distinct wavelengths as fluorescence intensity maxima of different prototropic forms are widely separated (NpOH* λ_{em} = 370 nm; NpO^{-}* λ_{em} = 465 nm).

(ii) Usually, the prototropic forms show good fits for biexponential decay functions. The observed biexponential fluorescence decay of NpOH* in the lipid bilayer membrane is as expected according to the two-state distribution model involving the naphthol molecules located at the membrane interface and hydrocarbon core.[114] The dissociation of NpOH* at the hydrocarbon core gets suppressed due to the absence of water. This results in the emission of NpOH* fluorescence with a long lifetime of *ca.* 7 ns. At the membrane–water interface, its dissociation rate constant decreased due to the non-availability of free water and NpOH* emission has a short lifetime of *ca.* 2 ns.[112,114] The decay of NpO^{-}* fluorescence in the lipid bilayer membrane is biexponential in nature with a short lifetime of *ca.* 8 ns and a long lifetime of *ca.* 20 ns. Clear, distinguishable lifetime values of prototropic forms make 1-naphtol a suitable fluorescent probe to study heterogeneous systems more finely.

(iii) As per the distribution model (Fig. 5), the neutral (NpOH*) form of 1-naphthol originates purely from the naphthols present in the lipid bilayer membrane; near the interface and inner hydrocarbon core region. However, the anion (NpO^{-}*) form of 1-naphthol originates from the naphthols present in the aqueous bulk phase and interface region of the lipid bilayer membrane. Anions originating from these two regions can be differentiated from their fluorescence emission wavelengths and lifetime. (a) The emission maximum of NpO^{-}* present in the bulk water phase is found to be at 470 nm; however, the NpO^{-}* emission in lipid medium shows a small but significant blue shift of *ca.* 5 nm in the emission maximum at 465 nm.[16] (b) From the fluorescence lifetime data of NpO^{-}*, the shorter lifetime component of *ca.* 8 ns in the lipid membrane matches very closely with the fluorescence lifetime of NpO^{-}* originating from the bulk water. Thus, this component of NpO^{-}* appears to originate from the unpartitioned 1-naphthol present in bulk water rather from the NpO^{-}* in the membrane–water interface.[16] Consequently, the longer lifetime component of NpO^{-}* *ca.* 20 ns can be assigned to the NpO^{-}* present in the membrane–water interface environment in which the non-radiative decay processes are expected to be slower than that in water.

(iv) The fluorescence intensity of the neutral and the anionic form varies depending on the polarity of the surrounding microenvironment. The prototropic equilibrium is sensitive to the microenvironment of the system of study. Any small change in the microenvironment affects the prototropic equilibrium and, hence, leads to a change in the ratio of anionic to neutral peak intensities. If a clear isoemissive point is present in the steady-state fluorescence spectrum, then the relative intensities of emission at the corresponding two wavelengths can be related to the population of the prototropic species in a particular microenvironment. The variations of fluorescence lifetime components of the corresponding prototropic forms and their relative amplitudes signify lot more information about their population distribution within the lipid bilayer membrane and bulk water phase.

1-Naphthol acts as a suitable fluorescent ESPT probe to study the membrane-related changes induced by temperature, as well as additives like cholesterol,[114] surfactants,[16,115,116] ethanol-induced interdigitation,[117] *etc*. The ESPT process of 1-naphthol was used to observe the phase transition behavior

of DMPC, DPPC liposomes,[114] and liposomes made from natural lipids like human erythrocyte membranes[118] through its fluorescence parameters. At the phase transition temperature, the partitioning of 1-naphthol is maximum, and that was reflected from the maximum partition coefficient and molar fraction value.[114] A shift in the phase transition temperature was observed for the mixed lipid systems of DMPC and DPPC.[114] The model could satisfactorily explain the blurring of phase transition with the addition of higher mole% of cholesterol.[117] The response of the neutral form of 1-naphthol towards the interdigitation of the lipid bilayer membrane in the presence of ethanol was also studied.[117] The critical ethanol concentration, which induces a complete interdigitated phase in DPPC lipid bilayer membranes, was determined to be 0.9 M. The ESPT dynamics of 1-naphthol was studied in the bilayer membrane of cationic vesicles of di-octadecyldimethylammonium bromide and di-dodecyl dimethyl ammonium bromide.[14] It was observed that for both the forms in bilayer membranes of cationic lipids, the rate constants of ESPT were higher than those of zwitterionic lipids (DPPC and egg lecithin). Using 1-naphthol and conceptually different fluorescent probes like ANS and DPH, the effect of different detergents (Triton X-100, SDS, and CTAB) on the physical properties of the DMPC bilayer membrane was studied.[115] Because of the variation in the water of hydration of the lipid molecules, the phase transitions of lipid bilayers get affected moderately by the neutral surfactants and substantially by charged surfactants. The effect of hydrophobic tripeptides like leucinyl-phenylalanyl-valine on the DMPC bilayer membrane was studied by using 1-naphthol in addition to some other fluorescent probes.[119] It was observed that leucinyl-phenylalanyl-valine increases the compactness of the membrane to a considerable extent. Perturbative interaction of lower concentrations (0.05 to 1 mM) of various physiologically important unconjugated (NaC, NaDC) and conjugated (NaTC, NaTDC, NaGC, NaGDC) bile salts with vesicles composed of DMPC and DPPC lipids was studied using the excited state prototropic equilibrium of 1-naphthol.[16,116,120]

From the fluorescence intensity, decay parameters and proton transfer rate constants of 1-naphthol, it has been observed that the presence of a lower concentration of bile salts results in noticeable hydration of the lipid bilayer membrane up to the hydrocarbon core region and a shift in the phase transition temperature of the bilayer (Fig. 6). In DPPC and DMPC vesicles in both SG and LC phases of the membrane, the lipid bilayer hydration efficiency in the presence of bile salts follows the order NaDC > NaC > NaGDC > NaTDC > NaGC > NaTC. The interaction of FDA approved drug molecule FTY720 (fingolimod hydrochloride) with the DMPC lipid bilayer membrane was studied using fluorescence intensity and lifetime of 1-naphthol.[121] It was observed that there is a decrease in the neutral form fluorescence intensity of 1-naphthol with the increase in the concentration of FTY720. Also, there is a decrease in the 1-naphthol partition coefficient value in the presence of more FTY720. This signifies that the presence of FTY720 slows down the partitioning of these small molecules into the lipid bilayer membrane. In the DMPC bilayer membrane, the steady-state fluorescence response of a series of substituted naphthols was studied.[122] Prototype molecules like *N*,*N*-dimethylaminomethyl-2-naphthol, and morpholinomethyl-2-naphthol, which are ESIPT systems, were found to show a less sensitive response towards the phase transition temperature-related changes.[122] Using fluorescence of 1-naphthol, the interaction of kojic acid esters with the DPPC lipid bilayer membrane was studied.[123] It was observed that the kojic acid esters interact efficiently with the lipid bilayer membrane. The hydrophilic part of the ester localizes near the bilayer–water interface.

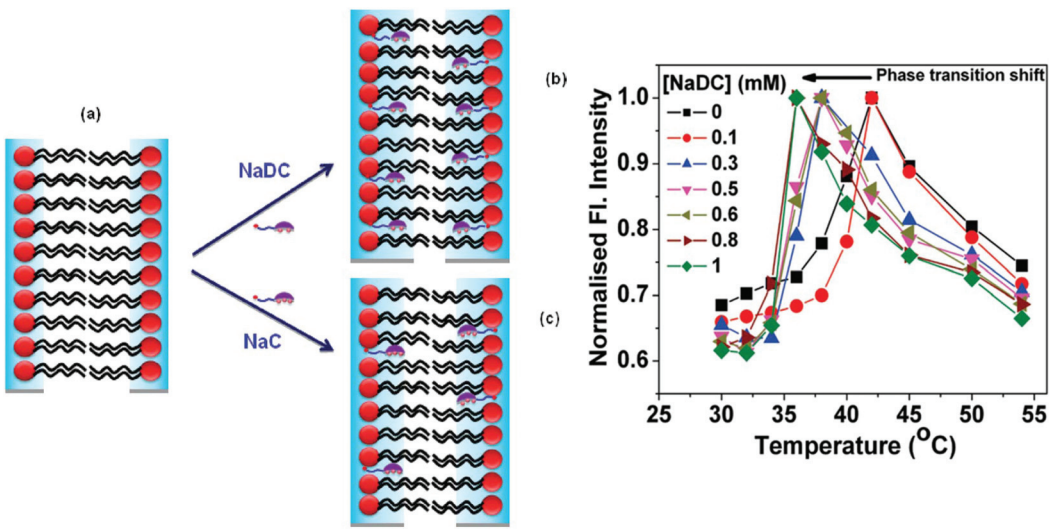


Fig. 6 (A) Model indicating the extent of hydration (in light blue) in DPPC bilayer membrane (a) without bile salt, (b) with NaDC and (c) with NaC. (B) Response of neutral form of 1-naphthol fluorescence intensity to the changes induced by NaDC (0–1 mM) with temperature in DPPC vesicles (λ_{ex} = 290 nm, λ_{em} = 370 nm). [1-naphthol] = 4 μM, [DPPC] = 0.4 mM (reproduced from ref. 16 with permission from The American Chemical Society).

It was observed that the ESPT process for natural oestrogenic steroids like d-equilenin and dihydroequilenin depends on both pH and the concentration of the proton acceptor in the dimyristoyllecithin membrane.[124] To synthesize a fluorescent phospholipid for ESPT studies, Neyroz *et al.*,[125] covalently linked 2-naphthol to the phosphatidylethanolamine moiety. This fluorescent phospholipid gets into the dimyristoyl lecithin bilayer and exhibits the behavior of ESPT molecular probes. The photophysics of 1-hydroxy-2-naphthaldehyde was studied in liposome membranes of varying surface charges.[126,127] To study the proton dissociation and diffusion dynamics in the aqueous layer of MLVs, pyranine was used.[128] The binding of pyranine to phosphatidylcholine vesicles as a function of the probe and electrolyte concentration was investigated.[129] The binding of this probe was found to be more significant to the internal leaflet of lecithin SUVs than that to the external leaflet. pH jump methods measured the dynamics of proton binding to the cytoplasmic surfaces and the extra cellular part of the purple membranes.[130] In the DMPC : DMPG bilayer membrane (9 : 1 ratio), the ESPT behavior of various heterocyclic molecules like carvedilol, carbazole and 4-hydroxycarbazole was also studied.[131] In DMPC and DPPC liposomes, the simultaneous presence of both the neutral and anion form fluorescence of carbazole could be observed in a pH interval of 11–13 with maximum fluorescence sensitivity at pH = 12.[132]

For the ESPT and ESIPT processes, hydroxyflavone molecules are the eminent exhibitors. In the excited state, the ESIPT of 3-hydroxyflavone (3HF) leads to the formation of the phototautomeric form (PT), whose fluorescence shows a significant redshift as compared to the fluorescence of the neutral form. At neutral pH, the existence of the ground state anion (A^-) of 3HF in DMPC[133] and egg yolk phosphatidylcholine (EYPC)[134] bilayer membranes was observed. Sengupta's group studied the interaction of 3HF and 4-*N*,*N*′-dimethylamino-3HF with the bilayer membranes made up of synthetic lipids.[135,136] These studies show that the molecules stay near the polar, aprotic environment in the bilayer membrane probably near the carbonyl group of the acyl chains in the interfacial region. However, under various lipid bilayer membrane conditions, a thorough elucidation of the photophysical behavior of the phototautomer and ground state anion of 3HF was done by Mohapatra *et al.*[137] Here, a quenching study using a hydrophilic quencher Ag^+ provides a strong confirmation about the preferential location of the phototautomer and ground state anion forms of 3HF near the water inaccessible hydrocarbon core and water accessible interface regions of the bilayer, respectively (Fig. 7). A redistribution of 3HF between these two locations was observed, from the temperature dependence fluorescence studies and effects of ethanol experiments.[137] For the bilayer membranes composed of a different ratio of DMPC and DPPC lipids, with an increase in the DPPC molar percentage, the sigmoidal plots were found to shift towards the higher temperature suggesting an increase in the phase transition temperature.

3HF and its derivatives were used to study the polarity and hydration in the lipid bilayer membrane.[138–142] A few derivatives of 3HF were synthesized by Duportail's group and were found to have numerous applications in biomembrane studies. They were found to be used in monitoring the biophysical properties, *i.e.* electrostatic potentials at the sites of their location, environmental relaxation, molecular order, *etc.* of lipid bilayer membranes.[142–147]

The same group developed the first ratiometric fluorescent probe F2N12S for apoptosis detection.[148] The fluorescence reporter (4′-(diethylamino)-3-hydroxyflavone) of F2N12S exhibits ESIPT. Both normal (Fig. 8A) and apoptotic cells (Fig. 8B) were stained with F2N12S. The normal cells which were stained with F2N12S show an intensity ratio of T*/N* (excited tautomer/excited normal) between 4 and 6. The cells which were treated with actinomycin D (a chemotherapy drug) exhibit heterogeneous intensity ratios (Fig. 8B). These cells exhibit T*/N* intensity ratios close to 5 as well as between 1 and 2. It was observed that the latter cells are most likely apoptotic. The cells with intermediate ratios between 2 and 4 were

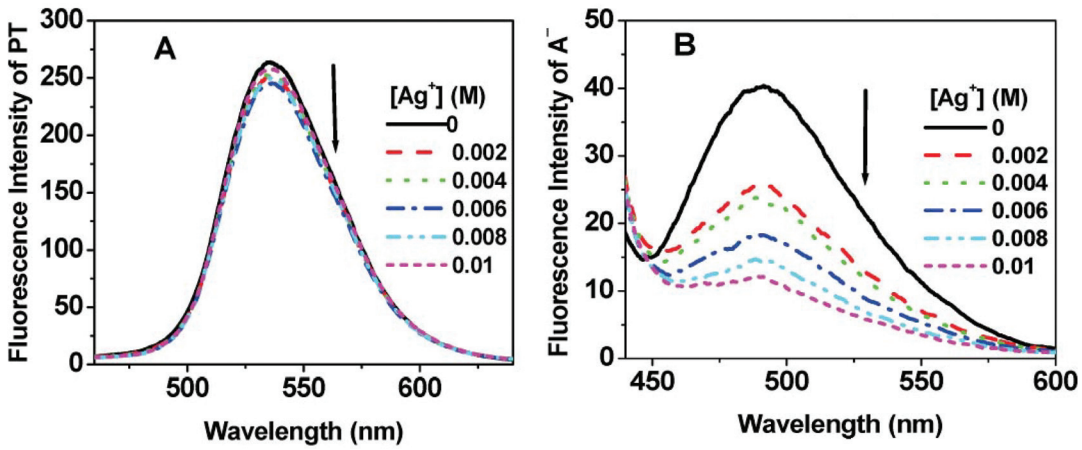


Fig. 7 Emission spectra of (A) PT (λ_{ex} = 345 nm), (B) A^- (λ_{ex} = 417 nm) with increasing Ag^+ concentration (0 M to 0.01 M) at 35 °C. [3HF] = 5 mM, [DMPC] = 0.6 mM (reproduced from ref. 137 with permission from The Royal Society of Chemistry).

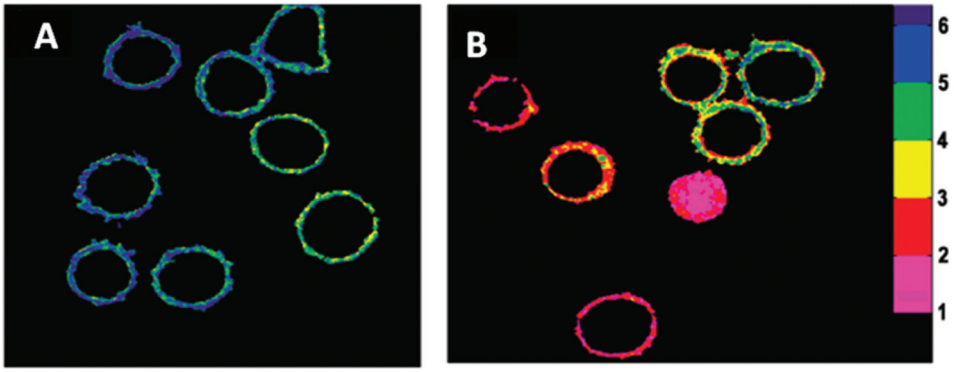


Fig. 8 Ratiometric fluorescence images of (A) normal and (B) actinomycin D-treated cells stained with 0.2 μM F2N12S. The displayed color code on the right scale indicates the ratios of intensities of the two bands T*/N*. The size of the images is 60 × 73.1 μm (reproduced from ref. 148 with permission from The American Chemical Society).

also observed and expected to be on the initial steps of apoptosis. F2N12S identifies the apoptotic cells and even quantifies the level of their apoptotic transformation.

The fluorescent molecular probes based on 3HF were also used for the visualization of lipid domains in giant unilamellar vesicles.[149,150] The specificity and kinetics of a neuronal protein α-synuclein binding to model membranes were determined with fluorescent ESIPT probes diethylamino-3-hydroxyflavone and *N*-(2-[4-(diethylamino)phenyl]-3-hydroxy-4-oxo-4*H*-chromen-6-yl)-4-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl) propanamide.[151] The intrinsic fluorescence feature of the plant flavonoid fisetin was also used to explore its binding sites in egg lecithin bilayer membranes[152] as well as in red blood cell membranes.[153] Its photophysical study was carried out in detail in the DMPC bilayer membrane.[154] It was found to partition well into the lipid bilayer membrane and is located near the interfacial head group region. Fisetin proved itself as a good fluorescent probe for studying the temperature dependent phase change behavior of single as well as mixed lipid systems (DPPC and DMPC) and the effect of cholesterol on membrane properties. As far as antioxidant activity of fisetin is concerned, its location in the interfacial polar head group region of the bilayer membrane and the observation that it is getting expelled from the membrane region by cholesterol can have an important implication.[154] In aqueous media, there is the absence of fisetin phototautomer fluorescence; however, there is the existence of very weak photoanion fluorescence (λ_{ex} = 370 nm, λ_{em} = 500 nm). In the presence of the lipid bilayer membrane, there is the formation of intense green fluorescence of the phototautomer, and the large Stoke's shift of *ca.* 175 nm (λ_{ex} = 370 nm, λ_{em} = 545 nm), the large partition coefficient (*ca.* $(5.1 \pm 0.5) \times 10^5$), and the high steady-state fluorescence anisotropy make fisetin a good fluorescent molecular probe to be used as a marker, and an imaging dye (Fig. 9).

The intrinsic florescence parameters of 7-hydroxyflavone (7HF) were also used to probe the microenvironments around the bilayer of model membranes.[155] Furthermore, using ESIPT of robinetin, the binding of robinetin with EYPC liposomes and normal human hemoglobin was studied.[156] The effects of lecithin liposomes on the binding parameter of plant flavinoids like quercetin and rutin to Hb were also considered.[157] Mateo and Douhal established the ESIPT behavior of a responsive fluorescent molecular probe, 2-(2′-hydroxyphenyl) imidazo [1,2-*a*] pyridine (HPIP).[158] It was used to monitor the structural

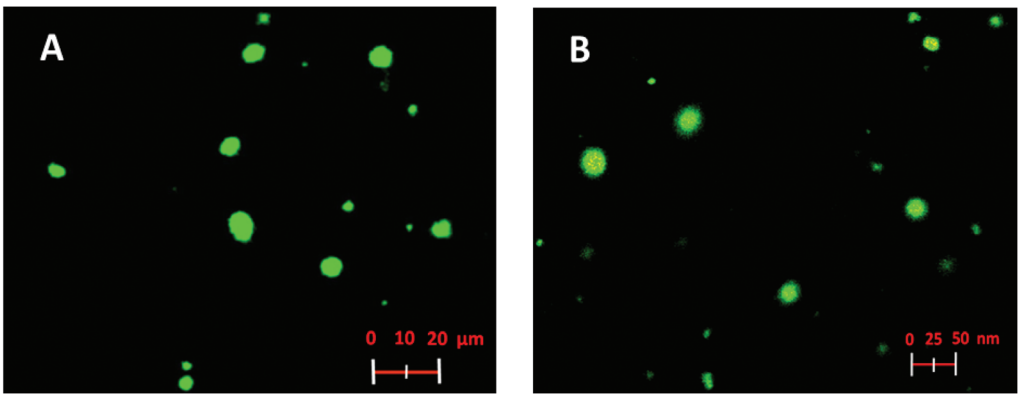


Fig. 9 (A) Fluorescence microscopic image of fisetin in DMPC MLVs; (B) fluorescence confocal image of fisetin in DMPC SUVs. [Fisetin] = 5×10^{-5} M, [DMPC] = 0.6 mM (reproduced from ref. 154 with permission from The American Chemical Society).

Table 1 Certain lipid bilayer properties that can be studied using ESPT dynamics

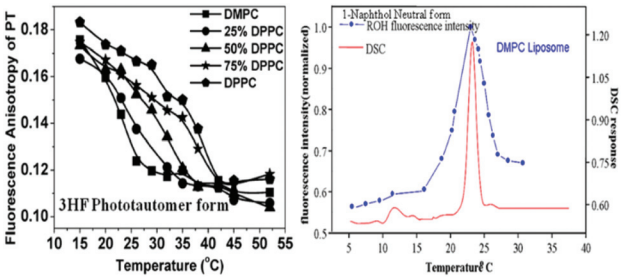
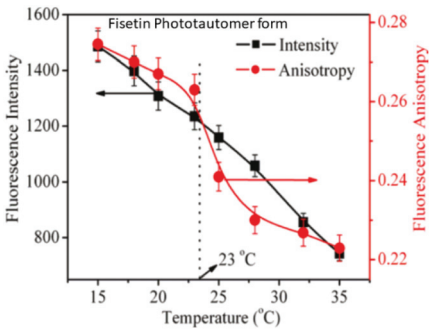
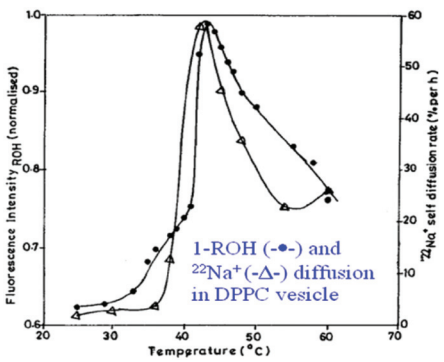
Liposome/lipid bilayer membrane property	Fluorescence parameters that can be used for the study	A brief description/example of such study	Ref.
Phase transition	Fluorescence intensity, fluorescence anisotropy	 (Reproduced from ref. 137 with permission from The Royal Society of Chemistry) ESPT dynamics of 1-naphthol, 3-hydroxyflavone, *etc*. are sensitive to the phase change of lipid bilayer membrane (composed of single or mixed lipid), from a highly ordered solid gel phase to a fluidic liquid crystalline phase with an increase in temperature[137,162]	16, 83, 85, 114, 115, 118–121, 125, 126, 133–137, 154, 155, 158 and 162
Bilayer fluidity	Fluorescence anisotropy	 (Reproduced from ref. 154 with permission from The American Chemical Society) Steady state fluorescence anisotropy of 3-hydroxyflavone, fisetin *etc*. are observed to be sensitive to the lipid bilayer fluidity[137,154]	114, 121, 122, 137 and 154
Permeability	Fluorescence intensity	 (Reproduced from ref. 114 with permission from The American Chemical Society) In DPPC liposomes, the change in permeability with temperature has been studied using the ESPT-induced changes in the fluorescence intensity of the neutral form of 1-naphthol and variation of the self-diffusion rate of $^{22}Na^{+}$. A good correlation suggests the sensitivity of the ESPT probe to the permeability changes with temperature[114,163]	16, 114, 121, 122 and 163

Table 1 *(Contd.)*

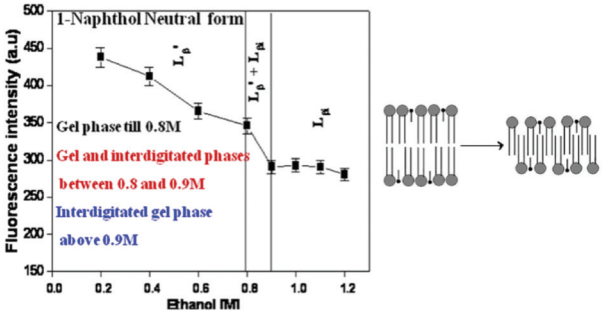
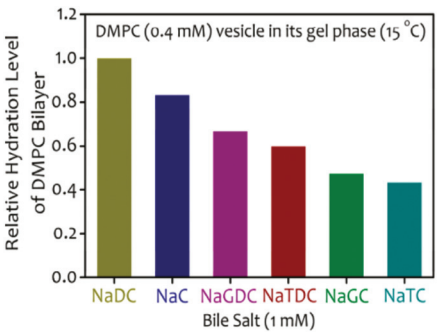
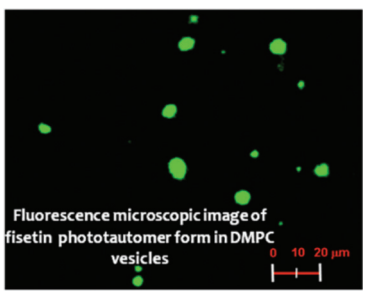
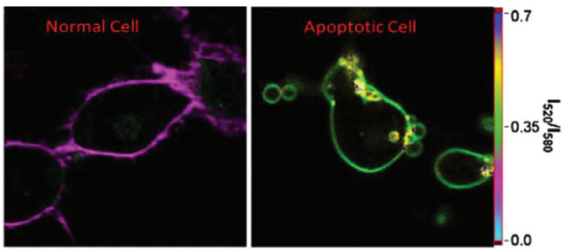
Liposome/lipid bilayer membrane property	Fluorescence parameters that can be used for the study	A brief description/example of such study	Ref.
Lipid bilayer interdigitation	Fluorescence intensity and lifetime	 (Reproduced from ref. 117 with permission from The John Wiley and Sons) The fully interdigitated structures have a high molecular order in lipid acyl chains and shorter membrane thickness as compared to non-interdigitated lipid bilayers.[164,165] ESPT dynamics of 1-naphthol can also successfully sense the transition from non-interdigitated to interdigitated gel phase in DPPC lipid bilayers in the presence of ethanol[117]	117, 137, 164 and 165
Bilayer hydration	Fluorescence lifetime	 (Reproduced from ref. 116 with permission from The American Chemical Society) Proton transfer rate constant k_{pt} of 1-naphthol is found to be sensitive to the relative hydration level of the lipid bilayer membrane in the presence of a sub-micellar concentration of unconjugated as well as conjugated bile salts[16,116]	16, 116, 139, 140 and 166
Budding, lipid rafts, a fusion of vesicles	Fluorescence microscopy	 (Reproduced from ref. 154 with permission from The American Chemical Society) The domain induced budding and fusion of giant vesicles has been experimentally confirmed by fluorescence microscopy and differential interference contrast microscopy using non-ESPT dye molecules.[167,168] However, the phototautomer form of fisetin and its derivatives can be used as a marker and dye molecule for fluorescence imaging applications[80,154]	80, 148, 150, 154, 167 and 168

Table 1 *(Contd.)*

Liposome/lipid bilayer membrane property	Fluorescence parameters that can be used for the study	A brief description/example of such study	Ref.
Apoptosis	Steady-state fluorescence intensity, fluorescence microscopy	 (Reproduced from ref. 160 with permission from The Elsevier) Using fluorescence spectroscopy and microscopy measurements, the emission parameters of a derivative of 3-hydroxyflavone, an ESIPT probe, with two emission bands corresponding to the normal (N*) and phototautomer (T*) form, is found to change dramatically in response to apoptosis[160]	80, 148 and 160

changes in lipid bilayer membranes. In the ground and the excited states, the rotation around the C (2)–C (1′) single bond makes HPIP have four different structures. This molecular probe also responds to the phase transition temperature of DMPC and DPPC bilayer membranes, as well as cholesterol-induced membrane changes. As far as the location of ESPT molecules in the lipid bilayer membrane is concerned, until now it has been a prominent area of research. The ratiometric ESIPT fluorescent probes 2′-hydroxy derivatives of 2,5-diaryl-1,3-oxazole are widely used to monitor the physicochemical properties of lipid membranes. Computational and experimental studies have been carried out to examine their location in the lipid membrane.[159]

The choice of a fluorophore is essential for obtaining clear interpretations. An important criterion is its sensitivity to a particular property of the micro-heterogeneous environment in which it is located. The efficiency of ESPT is an active function of the local microenvironment in which it occurs; hence, information regarding a microenvironment in an organized heterogeneous medium is obtainable. The ESPT molecules are unique and suitable for their certain advantages over conventional fluorescent probes that are listed below;

(a) They are mostly multi-emitting state probes. They exhibit different bands representing emissions from the corresponding prototropic form.

(b) The system of interest can be monitored appropriately at distinct emission wavelengths, as the fluorescence maxima of different prototropic forms are often widely separated.

(c) If a clear emissive point is present in the steady-state fluorescence spectrum, the relative intensities of emission can be correlated with the population of the prototropic forms in a specific microenvironment.

(d) The prototropic equilibrium is sensitive to the microenvironment; a small change in the environment can affect the ratio of intensities of prototropic forms.

(e) If the fluorescence decay of a particular prototropic form is multicomponent, the lifetime and corresponding amplitudes provide sensitive information about the micro (or nano) heterogeneity of a specific domain in the organized system.

(f) These molecules are relatively small and cause less perturbation to the system of study.

Excited-state intermolecular proton transfer is very sensitive to the presence of receptor water molecules (or other acceptors). The introduction of 1-naphthol as a sensitive indicator of the extent of membrane hydration (Fig. 6A and Table 1)[16,116] opens up the possibility of introducing more 1-naphthol conjugated probes with different lipophilicity and emissive properties. It is possible that 1-naphthol can be used as a standard fluorophore for studying the hydration property of organized heterogeneous assemblies, taking it to a status similar to DPH as a standard fluorescence anisotropy probe for membranes,[11] and pyrene (I_1/I_3 fluorescence intensity ratio) as a polarity probe.[11]

There is a constant demand for newer molecular probes which can sense the physical properties of the lipid bilayer membrane induced by membrane perturbing agents. Few important lipid bilayer membrane properties that can be studied using ESPT dynamics are listed in Table 1. These molecules can be further used to study various properties of other organized systems that are not explored before.

As far as excited-state intramolecular proton transfer (ESIPT) is concerned, in the absence of water, the phototautomer is the prominent emitting proton-transferred species and in a protic environment both intra and inter molecular hydrogen bonding takes place resulting in dual emission from the neutral and phototautomer species. A few fluorophores showing excited state ESIPT have proved themselves to be the better candidate for the fluorescence imaging study (Fig. 8, 9 and Table 1).[148,154,160] However, many ESPT molecules do not emit in the visible region. Possibly, new ESPT molecules can be designed in such a way that they emit in the visible region and can be used as a marker in biomedical imaging applications.

In addition to fluorescence lifetime and intensity, the fluorescence anisotropy parameter of ESPT molecules is of great interest. The fluorescence anisotropy parameter of few ESPT fluorophores like 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) has been used to study the effect of co-surfactants on CTAB quaternary reverse micelles.[161] Furthermore, they can be used for studying lipid bilayer membranes. There are few ESIPT molecules like 3-HF and fisetin, whose fluorescence anisotropy parameter has been used to study the phase behavior and bilayer fluidity of lipid bilayer membranes.[137,154] 1-Naphthol, however, does not show sensitivity to the fluorescence anisotropy parameter.

3. Conclusion

This perspective on ESPT fluorescent molecular probes towards the study of lipid bilayer membranes is aimed at bringing out the sensitivity of multiple fluorescent parameters of different prototropic forms: emission maxima, emission intensity, emission lifetime, and the amplitudes of different lifetime components, and the fluorescence anisotropy of different prototropic forms. It also showcases the ease of employing such small distributive fluorescent probes in unraveling a variety of lipid bilayer membrane properties related to the organization of the membranes and membrane hydration properties, as well as the dynamics of small molecules in the membrane environments.

Conflicts of interest

There are no conflicts to declare.

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