15

MEMBRANE FLUORESCENT PROBES: INSIGHTS AND PERSPECTIVES

Amitabha Chattopadhyay, Sandeep Shrivastava, and Arunima Chaudhuri

Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, Uppal Road, Hyderabad, 500 007, India

ABBREVIATIONS

2-AS: 2-(9-anthroyloxy)stearic acid

12-AS: 12-(9-anthroyloxy)stearic acid

25-NBD-cholesterol: 25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27- norcholesterol

DOPC: dioleoyl-*sn*-glycero-3-phosphocholine

MβCD: methyl-β-cyclodextrin

NBD: 7-nitrobenz-2-oxa-1,3-diazol-4-yl

NBD-PE: *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine

REES: red edge excitation shift

SDS: sodium dodecyl sulfate

Fluorescent Analogs of Biomolecular Building Blocks: Design and Applications, First Edition.
Edited by Marcus Wilhelmsson and Yitzhak Tor.

15.1 INTRODUCTION

Biological membranes are two-dimensional, anisotropic supramolecular assemblies consisting of lipids, proteins, and carbohydrates. Cellular membranes allow compartmentalization of individual cells and act as the interface necessary for cells to sense their environment and communicate with other cells. Most importantly, cellular membranes provide an appropriate environment for function of membrane proteins.[1] It has been estimated that ~50% of all biological processes occur at the cell membrane.[2]

Membrane lipids represent crucial components of cell membranes since they carry out a variety of cellular functions along with membrane proteins. Monitoring lipid molecules in the crowded membrane constitutes an experimental challenge. In this context, membrane lipid probes assume relevance.[3,4] Fluorescent lipid probes offer advantages in monitoring membrane organization and dynamics due to their high sensitivity, suitable time resolution, and multitude of measurable parameters. Many fluorescent lipid probes have the extrinsic fluorophore covalently attached to the parent lipid molecule. The popularity of these probes arises from the fact that the user has a choice of the fluorescent tag to be used, and, therefore, specific lipid probes with appropriate spectral characteristics can be designed depending on the type of application. Figure 15.1 shows the molecular structures of a few representative membrane fluorescent probes. These probes have sensitive fluorescent groups such as

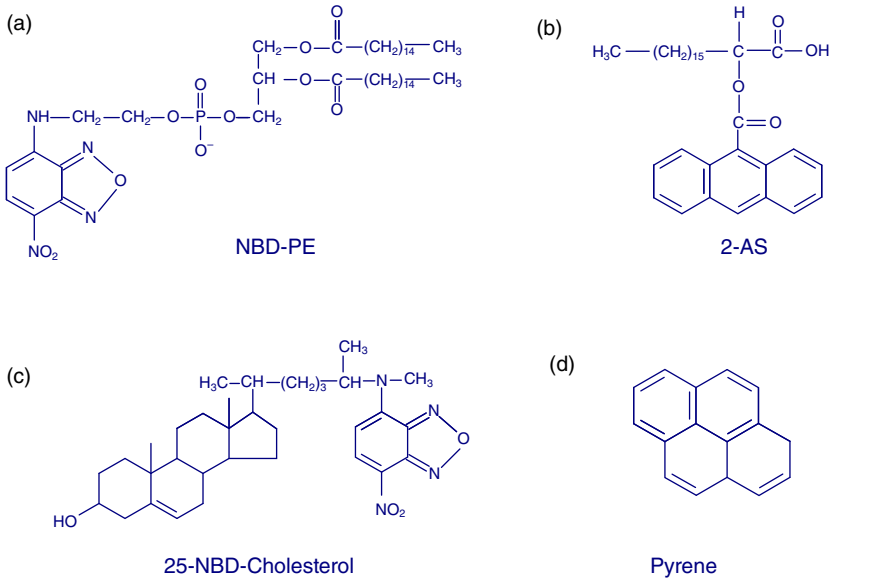

Figure 15.1 Chemical structures of representative membrane fluorescent probes. (a) NBD-PE (the fluorescent NBD group is covalently attached to the polar lipid head group in this molecule); (b) 2-AS (a representative member of anthroyloxy stearic acid probes); (c) 25-NBD-cholesterol (the NBD moiety is attached to the flexible acyl chain of cholesterol); (d) pyrene (a polycyclic aromatic hydrocarbon).

NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) or anthroyloxy moiety attached to various positions of phospholipids, fatty acids, and cholesterol. One of the probes shown in the figure is the polycyclic aromatic hydrocarbon pyrene. It is to be noted that pyrene does not have a fatty acyl chain, characteristic of lipid probes (although it can be conjugated to lipid probes). Pyrene partitions into the membrane bilayer and its use in monitoring membrane environment and dynamics is based on its spectral characteristics (vibronic peaks; see Section 15.4).

In this review, we focus on the application of membrane fluorescent probes to obtain information on environment, organization, and dynamics in membranes (or membrane-mimetic media) with representative examples taken from previous work from our group. Readers interested in a more detailed information about some of these probes are referred to our earlier reviews for such information.[4,5]

15.2 NBD-LABELED LIPIDS: MONITORING SLOW SOLVENT RELAXATION IN MEMBRANES

An extensively used fluorophore in studies of model and cellular membranes is the NBD group.[4,5] The NBD group is very weakly fluorescent in water but displays intense fluorescence in a hydrophobic medium. NBD fluorescence is in the visible range and is characterized by sensitivity to its immediate environment. In addition, fluorescence lifetime of the NBD group is also sensitive to environmental polarity. For these reasons, NBD-labeled lipids are widely used in studies of model and biological membranes.[4,5] We describe below the application of NBD-labeled lipids to monitor slow solvent relaxation in the membrane utilizing red-edge excitation shift (REES).

An interesting consequence of membrane organization is the restriction imposed on the dynamics of the constituent structural components in the membrane. Importantly, this kind of confinement leads to coupling of the motion of solvent molecules with the slow-moving molecules in the membrane.[6] In such a case, REES represents a sensitive approach that can be used to monitor the environment and dynamics around the fluorophore in membranes or membrane-mimetic media such as micelles.[7–11] REES is operationally defined as the shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band. The origin of REES lies in slow (relative to fluorescence lifetime) rates of solvent relaxation around an excited-state fluorophore. As a consequence, REES depends on the environment-induced motional restriction imposed on solvent molecules in the immediate vicinity of the fluorophore. A striking feature of REES is that it allows to assess the rotational mobility of the environment itself (represented by the relaxing solvent molecules) utilizing the fluorophore *merely* as a reporter group (it should be noted here that "solvent" in this context could include the host dipolar matrix such as the peptide backbone in proteins).[12]

The biological membrane, with its viscous interior and characteristic motional gradient along its vertical axis (z-axis), is an ideal molecular assembly for the application of REES.[8,10] The interfacial region in membranes display unique

motional and dielectric characteristics, different from the bulk aqueous phase and the more isotropic hydrocarbon-like deeper regions of the membrane. Since the membrane interface offers slow rates of solvent relaxation, it is most likely to exhibit REES. However, it is important to choose a suitable membrane probe that displays appropriate properties (such as site of localization in the membrane and appreciable change in dipole moment upon excitation).[8,10] The NBD group in membrane-bound NBD-PE (see Fig. 15.2a) fulfills these criteria.[14] The fluorescent NBD group is covalently attached to the head group of phosphatidylethanolamine in NBD-PE. The orientation and location of the NBD group in membrane-bound NBD-PE has previously been well worked out.[15–21] The NBD group in NBD-PE is localized at the membrane interface and is suitable for monitoring REES. Interestingly, the NBD group displays a relatively large change in dipole moment upon excitation (∼4 D),[22] a necessary condition for REES.[10] Fig. 15.2b and c shows REES of NBD-PE in dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) membranes.[14] Since the localization of the fluorescent NBD group in membrane-bound NBD-PE is interfacial,[16–21] REES of NBD-PE implies that the interfacial region of the membrane offers considerable restriction to the reorientational motion of the solvent dipoles around the excited-state NBD group. This property of the membrane interface has huge implications in membrane protein conformation and function. This is due to the fact that tryptophan residues in membrane-spanning proteins and peptides are usually localized at the membrane interface[23] and, therefore, offer the possibility of REES as a novel tool to explore membrane protein conformation.[11,24,25]

15.3 *n*-AS MEMBRANE PROBES: DEPTH-DEPENDENT SOLVENT RELAXATION AS MEMBRANE DIPSTICK

Membranes display considerable anisotropy along the axis perpendicular to the membrane plane (see Fig. 15.3a).[8,10] The center of the membrane bilayer is nearly isotropic. However, the upper portion of the bilayer, located only a few angstroms away toward the membrane surface, is highly ordered. As a result of such an anisotropic transmembrane environment, the mobility of solvent (water) molecules is differentially retarded at varying membrane depths compared to their mobility in the bulk aqueous phase.[26] In such a scenario, REES can be effectively used to monitor the dynamics of differentially localized reporter fluorophores along the membrane *z*-axis. This has been validated by demonstrating that chemically identical fluorescent probes, differing solely in depths at which they are localized in the membrane, experience different local environments, as monitored by REES.[13,26] This was achieved by the use of anthroyloxy stearic acid (*n*-AS) derivatives in which the anthroyloxy group has earlier been shown to be either located at a shallow [2-(9-anthroyloxy)stearic acid (2-AS)] or a deeper location [12-(9-anthroyloxy)stearic acid (12-AS)] in the bilayer (see Fig. 15.3a). Anthroyloxy fatty acids have been shown to be located at a graded series of depths in the bilayer, depending on the position of attachment of the anthroyloxy group to the fatty acyl chain.[27] Depth analysis using the parallax method has earlier shown

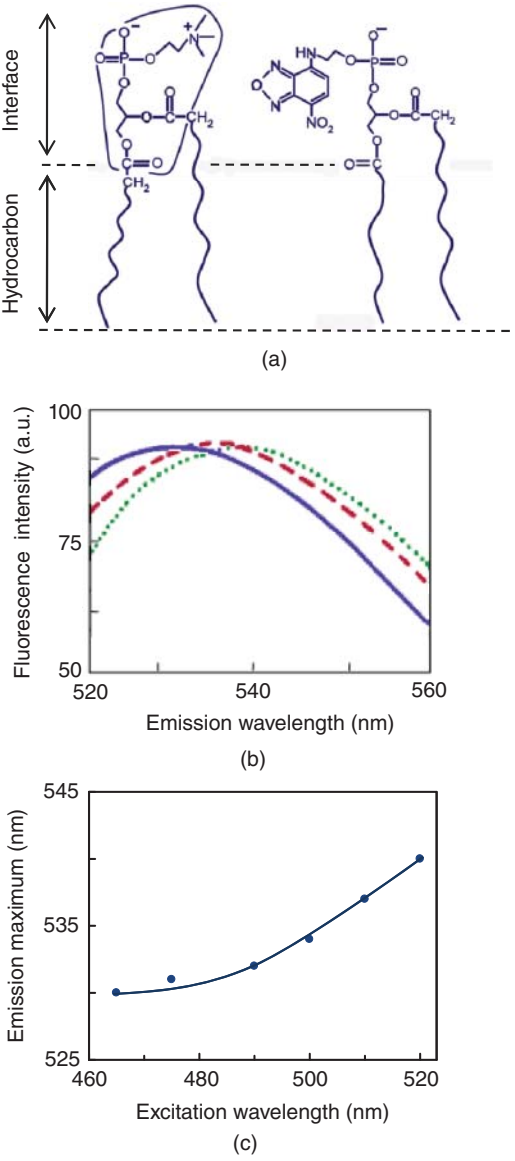

Figure 15.2 (a) A schematic representation of a leaflet of the phosphatidylcholine membrane bilayer showing the localization of the NBD group of NBD-PE. The NBD group of NBD-PE localizes at the membrane interfacial region. The horizontal line at the bottom indicates the center of the bilayer. Chattopadhyay and Mukherjee[13]. Reprinted with permission from American Chemical Society. (b) Intensity-normalized fluorescence emission spectra of NBD-PE in DOPC vesicles at increasing excitation wavelengths: 465 (——), 500 (— —), and 510 (-----) nm. Chattopadhyay and Mukherjee[14]. Reprinted with permission from American Chemical Society. (c) REES of NBD-PE in DOPC membranes. Chattopadhyay and Mukherjee[14]. Reprinted with permission from American Chemical Society. See text for other details.

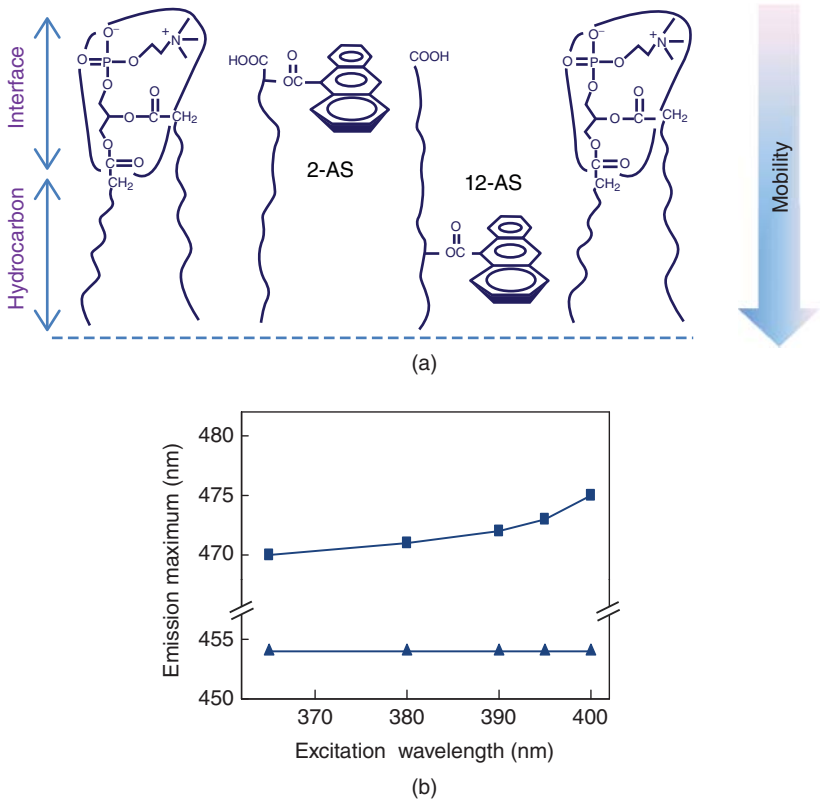

Figure 15.3 (a) A schematic representation of a leaflet of the membrane bilayer showing the localizations of the anthroyloxy groups of 2- and 12-AS in phosphatidylcholine bilayer. The anthroyloxy group of 2-AS localizes at the interfacial region while that of 12-AS resides at the nonpolar hydrocarbon region. A mobility gradient along the bilayer normal is set up (shown in the figure) due to differential dynamics at varying depths in the membrane. The dotted horizontal line at the bottom indicates the center of the bilayer. Chattopadhyay and Mukherjee[26]. Reprinted with permission from American Chemical Society. (b) Depth-dependent REES of 2-AS (■) and 12-AS (▲). Chattopadhyay and Mukherjee[26]. Reprinted with permission from American Chemical Society.

that the anthroyloxy probes in 2-AS (the shallow probe) and 12-AS (the deep probe) are localized at 15.8 and 6 Å from the center of the bilayer, respectively (see Fig. 15.3a).[20] REES experiments with these depth-dependent membrane probes show that the anthroyloxy group of 2- and 12-AS experience different local membrane microenvironments, as reflected by depth-dependent variation of REES. The shallow anthroyloxy group in 2-AS displays REES of 5 nm, whereas the deeper anthroyloxy group in 12-AS does not exhibit REES.[26] These results are attributed to differential rates of solvent reorientation in the immediate vicinity of the anthroyloxy group as the membrane penetration depth changes, that is, slower solvent relaxation

at the membrane interface relative to deeper regions. These results show that REES offers a suitable approach to monitor depth-dependent membrane dynamics, that is, as a membrane dipstick. Very recently, we have extended the use of such anthroyloxy membrane probes to monitor depth-dependent heterogeneity in the membrane by analysis of fluorescence lifetime distribution width.[28]

15.4 PYRENE: A MULTIPARAMETER MEMBRANE PROBE

The polycyclic aromatic hydrocarbon pyrene has been widely used as a fluorescent probe in membranes and membrane-mimetic media such as micelles. The emission spectrum of pyrene is sensitive to environmental polarity[29] (see Fig. 15.4a). Pyrene is localized predominantly in the interfacial region in micelles and membranes.[31,32] Interestingly, this is the region of the membrane or micelle that is sensitive to polarity changes due to water penetration. Figure 15.4b and c shows the application of polarity-sensitive vibronic peaks of pyrene to monitor environmental changes in micelles and membranes under various conditions. Structural transition (shape change) can be induced in charged micelles by increasing ionic strength (salt concentration).[33–35] For example, spherical micelles of sodium dodecyl sulfate (SDS) that exist in water at concentrations higher than critical micelle concentration assume an elongated rod-like (prolate) shape in the presence of high salt concentrations. In such a case, utilizing changes in the ratio of polarity-sensitive vibronic peak intensities (I_1/I_3; see Fig. 15.4b), the apparent polarity in spherical- and rod-shaped micelles could be determined.[34] These results showed that the apparent polarity was less in rod-shaped micelles relative to the polarity experienced in spherical micelles. Figure 15.4c shows the change in the ratio of vibronic peak intensities (I_1/I_3) in pyrene emission spectra in neuronal hippocampal membranes with decreasing cholesterol content. Hippocampal membranes represent a convenient natural source for exploring the interaction of neuronal receptors, such as the serotonin$_{1A}$ receptor, with membrane lipids.[30,36,37] Methyl-β-cyclodextrin (MβCD) is a water-soluble compound and has previously been shown to selectively and efficiently extract cholesterol from hippocampal membranes by including it in a central nonpolar cavity.[36] Figure 15.4c shows that hippocampal membranes treated with increasing concentrations of MβCD (i.e., with increasing extents of cholesterol depletion) resulted in an increase in the vibronic peak intensity ratio. This implies an increase in apparent polarity experienced by pyrene in cholesterol-depleted hippocampal membranes, due to an increase in water penetration in the membrane upon cholesterol depletion. This is in agreement with our earlier results using fluorescence lifetime of the hydrophobic probe Nile Red.[38]

15.5 CONCLUSION AND FUTURE PERSPECTIVES

Tracking lipid molecules in a crowded cellular milieu poses considerable challenge. Fluorescent membrane probes offer a sensitive way to achieve this. Lipid probes have proved to be useful in membrane and cell biology due to their ability to monitor

a wide variety of properties such as polarity, rotational dynamics, and diffusion in a depth-dependent fashion. Continuous improvement in instrumentation has allowed detection of fluorescent lipid probes with increasing spatiotemporal resolution.[39] Along with this, there have been new approaches to design membrane probes with specific properties. For example, the use of novel probes to detect membrane

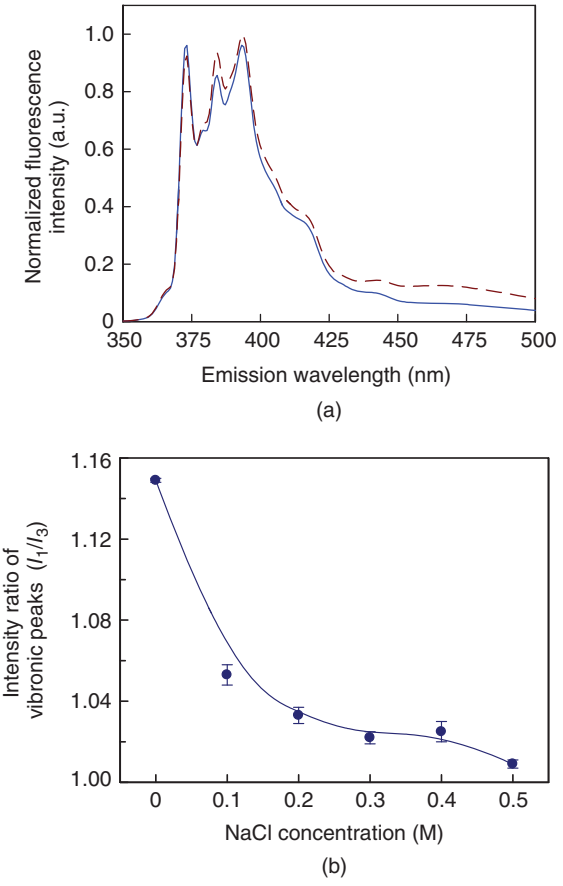

Figure 15.4 Change in environmental polarity monitored using pyrene fluorescence. (a) Intensity-normalized fluorescence emission spectra of pyrene in SDS micelles in the absence (——) and presence (— —) of NaCl. The polarity-sensitive vibronic peaks are clearly seen in the spectra. Chaudhuri *et al.* [34]. Reprinted with permission from Elsevier. (b) Change in fluorescence intensity ratio of the first (373 nm) and third (384 nm) vibronic peaks of pyrene (I_1/I_3) in SDS micelles as a function of increasing NaCl concentration. Chaudhuri *et al.* [34]. Reprinted with permission from Elsevier. (c) Change in fluorescence intensity ratio of the first (373 nm) and third (384 nm) vibronic peaks of pyrene (I_1/I_3) in hippocampal membranes with decreasing membrane cholesterol content (i.e., increasing MβCD concentration). Chaudhuri *et al.* [34]. Reprinted with permission from Elsevier. See text for more details.

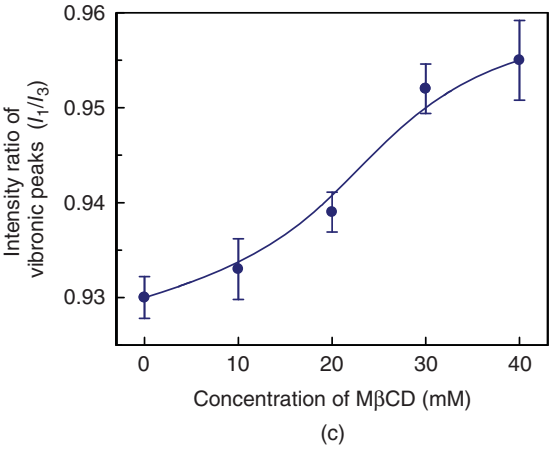

Figure 15.4 (*Continued*)

phases could be useful.[40] Future exciting applications include monitoring amyloid fibril formation utilizing NBD-labeled lipids and mapping cellular viscosity using fluorescent "molecular rotors".[41–43] Another important and exciting area is the determination of membrane dipole potential in which electrochromic membrane probes are used.[44–47] We envision that with novel and intelligently designed probes, and increasing instrument resolution, membrane probes will provide insightful information in the context of organization and dynamics of cellular membranes.

ACKNOWLEDGMENTS

Work in A.C.'s laboratory was supported by the Council of Scientific and Industrial Research, and Department of Science and Technology, Government of India. Some of the work described in this chapter was carried out by former members of A.C.'s research group whose contributions are gratefully acknowledged. A.C. thanks the Council of Scientific and Industrial Research for the award of a Senior Research Fellowship. A.C. is an Adjunct Professor at the Special Centre for Molecular Medicine of Jawaharlal Nehru University (New Delhi, India) and Indian Institute of Science Education and Research (Mohali, India), and Honorary Professor of the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India). A.C. gratefully acknowledges J.C. Bose Fellowship (Department of Science and Technology, Government of India). We thank members of our laboratory for critically reading the manuscript.

REFERENCES

1. Jafurulla, M.; Chattopadhyay, A. *Curr. Med. Chem.* **2013**, *20*, 47.
2. Zimmerberg, J. *Curr. Biol.* **2006**, *16*, R272.

3. Chattopadhyay, A. (Ed.) *Chem. Phys. Lipids* **2002**, *116*, 1.
4. Haldar, S.; Chattopadhyay, A. In Fluorescent Methods to Study Biological Membranes; Mely, Y. and Duportail, G., Eds.; Springer, Heidelberg, **2013**; pp 37.
5. Chattopadhyay, A. *Chem. Phys. Lipids* **1990**, *53*, 1.
6. Bhattacharyya, K.; Bagchi, B. *J. Phys. Chem. A* **2000**, *104*, 10603.
7. Mukherjee, S.; Chattopadhyay, A. *J. Fluoresc.* **1995**, *5*, 237.
8. Chattopadhyay, A. *Chem. Phys. Lipids* **2003**, *122*, 3.
9. Demchenko, A. P. *Methods Enzymol.* **2008**, *450*, 59.
10. Haldar, S.; Chaudhuri, A.; Chattopadhyay, A. *J. Phys. Chem. B* **2011**, *115*, 5693.
11. Chattopadhyay, A.; Haldar, S. *Acc. Chem. Res.* **2014**, *47*, 12.
12. Haldar, S.; Chattopadhyay, A. *J. Phys. Chem. B* **2007**, *111*, 14436.
13. Chattopadhyay, A.; Mukherjee, S. *J. Phys. Chem. B* **1999**, *103*, 8180.
14. Chattopadhyay, A.; Mukherjee, S. *Biochemistry* **1993**, *32*, 3804.
15. Haldar, S.; Chattopadhyay, A. In Reviews in Fluorescence 2010; Geddes, C. D., Ed.; Springer, New York, **2012**; pp 155.
16. Chattopadhyay, A.; London, E. *Biochemistry* **1987**, *26*, 39.
17. Chattopadhyay, A.; London, E. *Biochim. Biophys. Acta* **1988**, *938*, 24.
18. Mitra, B.; Hammes, G. G. *Biochemistry* **1990**, *29*, 9879.
19. Wolf, D. E., Winiski, A. P.; Ting, A. E.; Bocian, K. M.; Pagano, R. E. *Biochemistry* **1992**, *31*, 2865.
20. Abrams, F. S.; London, E. *Biochemistry* **1993**, *32*, 10826.
21. Mukherjee, S.; Raghuraman, H.; Dasgupta, S.; Chattopadhyay, A. *Chem. Phys. Lipids* **2004**, *127*, 91.
22. Mukherjee, S.; Chattopadhyay, A.; Samanta, A.; Soujanya, T. *J. Phys. Chem.* **1994**, *98*, 2809.
23. Kelkar, D. A.; Chattopadhyay, A. *J. Biosci.* **2006**, *31*, 297.
24. Rawat, S. S.; Kelkar, D. A.; Chattopadhyay, A. *Biophys. J.* **2004**, *87*, 831.
25. Raghuraman, H.; Kelkar, D. A.; Chattopadhyay, A.. In Reviews in Fluorescence 2005; Geddes, C. D., Lakowicz, J. R. Eds.; Springer, New York, **2005**; pp. 199.
26. Chattopadhyay, A.; Mukherjee, S. *Langmuir* **1999**, *15*, 2142.
27. Abrams, F. S.; Chattopadhyay, A.; London, E. *Biochemistry* **1992**, *31*, 5322.
28. Haldar, S.; Kombrabail, M.; Krishnamoorthy, G.; Chattopadhyay, A. *J. Phys. Chem. Lett.* **2012**, *3*, 2676.
29. Dong, D. C.; Winnik, M. A. *Photochem. Photobiol.* **1982**, *35*, 17.
30. Saxena, R.; Shrivastava, S.; Chattopadhyay, A. *J. Phys. Chem. B* **2008**, *112*, 12134.
31. Shobha, J.; Srinivas, V.; Balasubramanian, D. *J. Phys. Chem.* **1989**, *93*, 17.
32. Hoff, B.; Strandberg, E.; Ulrich, A. S.; Tieleman, D. P.; Posten, C. *Biophys. J.* **2005**, *88*, 1818.
33. Rawat, S. S.; Chattopadhyay, A. *J. Fluoresc.* **1999**, *9*, 233.
34. Chaudhuri, A.; Haldar, S.; Chattopadhyay, A. *Biochem. Biophys. Res. Commun.* **2009**, *390*, 728.
35. Chaudhuri, A.; Haldar, S.; Chattopadhyay, A. *Chem. Phys. Lipids* **2012**, *165*, 497.
36. Pucadyil, T. J.; Chattopadhyay, A. *Biochim. Biophys. Acta* **2004**, *1663*, 188.

37. Singh, P.; Tarafdar, P. K.; Swamy, M. J.; Chattopadhyay, A. *J. Phys. Chem. B* **2012**, *116*, 2999.
38. Mukherjee, S.; Kombrabail, M.; Krishnamoorthy, G.; Chattopadhyay, A. *Biochim. Biophys. Acta* **2007**, *1768*, 2130.
39. Eggeling, C.; Ringemann, C.; Medda, R.; Schwarzmann, G.; Sandhoff, K.; Polyakova, S.; Belov, V. N.; Hein, B.; von Middendorff, C.; Schönle, A.; Hell, S. W. *Nature* **2009**, *457*, 1159.
40. Kucherak, O. A.; Oncul, S.; Darwich, Z.; Yushchenko, D. A.; Arntz, Y.; Didier, P.; Mély, Y.; Klymchenko A. S. *J. Am. Chem. Soc.* **2010**, *132*, 4907.
41. Ryan, T. M.; Griffin, M. D. W. *Biochemistry* **2011**, *50*, 9579.
42. Kuimova, M. K.; Yahioglu, G.; Levitt, J. A.; Suhling, K. *J. Am. Chem. Soc.* **2008**, *130*, 6672.
43. Hosny, N. A.; Mohamedi, G.; Rademeyer, P.; Owen, J.; Wu, Y.; Tang, M.-X.; Eckersley, R. J.; Stride, E.; Kuimova, M. K. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 9225.
44. Clarke, R. J. *Adv. Colloid Interface Sci.* **2001**, *89–90*, 263.
45. Wang, L. *Annu. Rev. Biochem.* **2012**, *81*, 615.
46. Haldar, S.; Kanaparthi, R. K.; Samanta, A.; Chattopadhyay, A. *Biophys. J.* **2012**, *102*, 1561.
47. Singh, P.; Haldar, S.; Chattopadhyay, A. *Biochim. Biophys. Acta* **2013**, *1828*, 917.