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Review

Spectroscopic techniques in the study of membrane solubilization, reconstitution and permeabilization by detergents

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Abstract

This review focuses on the use of spectroscopic techniques for the study of membrane solubilization, reconstitution, and permeabilization by detergents. Turbidity and light scattering,visible and infrared spectroscopic methods, fluorescence, nuclear magnetic resonance, electron spin resonance and X-ray diffraction are examined from the point of view of their applicability to the above detergent-mediated phenomena. A short introduction is provided about each of the techniques, and references are given for further study. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Biomembrane; Detergent; Surfactant; Light scattering; Spectroscopy; Fluorescence; NMR; ESR

1. Introduction and scope

Detergents, like many other biochemical tools, have been extensively used on a semiempirical basis. Simultaneous, parallel rather than converging studies have attempted to establish the foundations for a rational use of surfactants, on the basis of biophysical studies of the molecular interactions of lipids and detergents. Spectroscopic techniques provide a wide variety of enormously powerful methods, that can, when used in wise combination with each other or with non-spectroscopic techniques, supply a uniquely detailed view of the process of detergent-mediated bilayer solubilization and reconstitution.

This brief paper is intended to provide the reader with an overview of methods and results. A detailed explanation of the more technical aspects of the spectroscopic procedures, let alone their physical foundations, would certainly exceed the limits and purpose of the article. Moreover, a comprehensive review of the relevant literature would be almost useless for the sheer volume and repetitiousness of data. Instead, a number of spectroscopic techniques have been selected on the basis of their proven applicability in the study of membrane–surfactant interactions. Each of them will be discussed briefly, particularly with respect to their advantages and disadvantages in the study of membrane solubilization. A few representative references will be cited, some preference being given to the more recent and easily accessible ones, even if this does not always make justice to the pioneers in some areas.

The review is focused on the process of membrane

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; $C_{12}E_8$, octylo-(ethylene oxide) dodecylether; DPH, diphenylhexatriene; DPX, *p*-xylenebis(pyridinium bromide); EPR, electron paramagnetic resonance; IR, infrared; Laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; NMR, nuclear magnetic resonance; NPN, *N*-phenylnaphthylamine; SDS, sodium dodecylsulfate; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; TMA-DPH, 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene; Triton, poly(ethylene oxide)-*t*-octylphenyl ether

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solubilization and reconstitution by detergents, including detergent binding to membranes, and subsequent events leading to the complete transformation of the lipid bilayer into lipid–detergent mixed micelles, and, conversely, the removal of detergent from mixed micelles and eventual reformation of a bilayer in reconstitution experiments. In addition, surfactant-induced permeabilization of membranes will be discussed separately. Phenomena of micellization of pure surfactants will not be considered. Spectroscopic studies on the structure of proteins in micelles or bicelles will not be reviewed either. Focusing on a relatively narrow series of events is expected to increase the usefulness of the present overview. This subject has been (briefly) dealt with previously by Lasch [1], as part of his review on the interaction of detergents with lipid vesicles.

2. Turbidimetry, light scattering

Turbidimetry and light scattering are discussed together because, in spite of the differences in the required instrumentation, they are both based on the same phenomenon, i.e., the scattering of electromagnetic radiation by many particles, and are both used for similar purposes. A good elementary description of the principles involved may be found in the textbook by Van Holde et al. [2]. In practice, an increase in turbidity (or light scattering) is interpreted as an increase in size of the particles in suspension, and the opposite is assumed to occur when turbidity decreases. This is true as far as the wavelength of the incident radiation is larger than the particle size, i.e., when the so-called 'Rayleigh condition' prevails. This is not always the case with certain systems, as will be shown below. However in most cases a decrease in turbidity/light scattering can be safely taken as an indication of membrane solubilization.

2.1. Turbidity measurements

Turbidity is commonly measured in spectrophotometers, using a wavelength that will not be absorbed by the particles (membranes, micelles) in suspension, so that absorbance does not compete with light scattering. A lower wavelength will increase the intensity of the scattered light, but if it becomes equal or smaller than the average particle size then the 'Rayleigh condition' will not hold. A compromise is usually reached with wavelengths in the 400–500 nm range. The simplest instrument will be the best for this purpose, since more sophisticated spectrophotometers are usually designed to prevent scattered light from reaching the detector.

The assessment of membrane solubilization as a decrease in suspension turbidity is perhaps the best known example of a spectroscopic method as applied to the study of membrane–surfactant interactions. It would be impossible to give here even the shortest selection of specific applications, so large is their number. It should be mentioned, however, that turbidimetric studies of membrane solubilization were the basis of the ingenious method developed by Lichtenberg [3] and Schurtenberger et al. [4] for the quantitative evaluation of membrane-bound and free detergent, thus of 'effective' detergent/lipid ratios in membrane solubilization (see also the paper by Lichtenberg et al. [5], in this issue). In those studies, total (i.e., free+membrane-bound) detergent concentrations inducing membrane disruption were measured at varying membrane concentrations, and effective detergent/lipid ratios deduced from the slope of 'total detergent concentration producing solubilization vs. membrane concentration' plots.

Always on the basis of turbidimetric studies, de la Maza, Parra and co-workers have refined the above quantitative measurements to obtain reliable estimates of other parameters of bilayer solubilization by detergents, particularly membrane/water partition coefficients (see, e.g., [6]). Following a similar methodology, the decrease in turbidity that accompanies detergent solubilization of algal (*Phormidium*) membranes was used by Ochoa de Alda et al. [7] to deduce changes in free (non-membrane bound) detergent during solubilization.

Early studies on turbidity changes induced by Triton X-100 in sonicated liposomes [8,9] showed that, at subsolubilizing concentrations, the detergent induced an *increase* in turbidity. The observation, that has been repeatedly confirmed a for a variety of detergents and vesicular systems (see, e.g., [10,11]) was interpreted, with the help of electron microscopy, in terms of vesicle lysis and reassembly into larger, multilamellar structures. The latter, upon increasing detergent concentration, disintegrate into

lipid–detergent mixed micelles, with the expected concomitant decrease in suspension turbidity.

Otherwise, with systems consisting initially of lipid/detergent mixed micelles, surfactant removal leads to an increase in suspension turbidity, due to the reconstitution of bilayered structures. A simple but informative quantitative treatment of these data has been put forward by Almog et al. [12].

2.2. Light scattering

When unqualified, the expression 'light scattering' refers usually to 90°-scattering under steady-state conditions. (The so-called dynamic light scattering is discussed in the next section.) Measurements are carried out most often in conventional spectrofluorometers, with both excitation and emission monochromators set at the same wavelength (in the 400–600 nm range) and slits wide open. Light scattering is far more sensitive than turbidity, thus it can be recommended when the concentration of the sample is small. Also its higher sensitivity allows the use of higher wavelengths, thus ensuring Rayleigh conditions for larger particle sizes. Examples of the application of static light scattering in the study of model and biomembranes can be found in [13,14].

2.3. Dynamic light scattering

This technique is also known as quasielastic light scattering and photon correlation spectroscopy, among other names. In dynamic light scattering, the intensity of scattered light is monitored in the microsecond time range domain, versus the second in static light scattering. The Brownian motion of the particles induces broadening of the spectrum, in a way that is related to their size and shape [15]. Commercially available dynamic light scattering instruments provide direct information on average particle size, thus their applicability to solubilization and reconstitution studies. Specific applications include the characterization of sphingomyelin–Triton X-305 mixed micelles [16], phosphatidylcholine–Triton X-100 mixed micelles [17], myelin basic protein–dodecylphosphocholine complexes [18], and the effect of temperature on the bilayer–micelle transition in the phosphatidylcholine–$C_{12}E_8$ system [19], to mention but a few examples.

2.4. Time-resolved studies

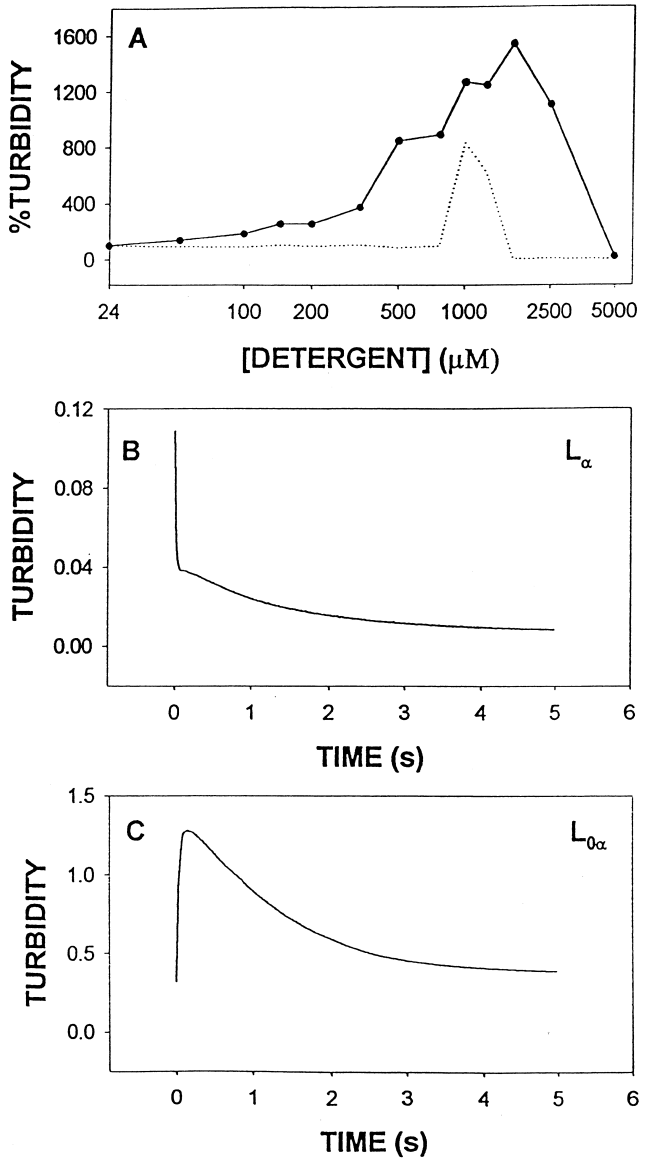
Kinetic studies of the detergent-induced changes in turbidity or light scattering can provide unique clues on the mechanism of detergent effects. This sort of work has been carried out with spectrophotometers equipped with stopped-flow sampling accessories. These devices allow fast mixing of detergent and membrane dispersions, so that events occurring in the millisecond time scale can be conveniently monitored. Elamrani and Blume [20] used this technique to describe the kinetics of lysophosphatidylcholine incorporation into phosphatidylcholine vesicles (half-times on the order of 50–500 ms), and the slower lysophosphatidylcholine-induced vesicle aggregation and/or fusion. With a similar experimental approach, Alonso et al. [21] showed that, upon mixing of liposomes with Triton X-100, a small increase in turbidity occurred in the first 100 ms, that was attributed to detergent insertion in the bilayer. Then, sonicated unilamellar vesicles underwent 'fusion' (or rather lysis and reassembly) in the next 20–40 s, as shown by a large increase in turbidity. Multilamellar vesicles, however, after detergent uptake, gave rise to lipid/detergent mixed micelles in a slow process that could take hours to be completed. Similar results were obtained with $C_{12}E_8$ by Edwards and Almgren [11]. More recently, Sáez-Cirión et al. [22] have used this technique to show the influence of the lipid phase structure on the solubilization abilities of a surfactant. In particular, a fluid-ordered phase formed by dimyristoylphosphatidylcholine and cholesterol (65:35 mole ratio) at 50°C was found to be much more resistant to solubilization than a liquid crystalline phase formed by pure phosphatidylcholine at the same temperatures (Fig. 1). An application of stopped-flow kinetics to the solubilization of a cell membrane (*Halobacterium* purple membrane) can be found in Viguera et al. [23].

Although poly(ethyleneglycol) is not a detergent, some of its effects on membranes are very similar to those of surfactants. In particular, poly(ethyleneglycol) induces aggregation, lipid exchange and fusion of sonicated phospholipid vesicles [24,25]. The kinetics of vesicle aggregation induced by this polymer has been studied by the stopped-flow technique by Viguera et al. [26]. The results include a description of anomalous light scattering due to non-Rayleigh

conditions, when vesicle aggregation leads to particles larger than the incident light wavelength.

3. Absorption spectroscopy: visible

No commonly used detergent absorbs electromagnetic radiation in the visible range, thus the application of absorption spectroscopy is limited to membranes containing either natural or externally added chromophores. The former is the case of light-transducing membranes. In particular, bacteriorhodopsin, the light-absorbing pigment of *Halobacterium* purple membranes has been extensively used as a built-in probe in the study of membrane–surfactant interactions [27–29]. González-Mañas and co-workers [30] established a relationship between binding of Triton X-100, spectral changes and purple membrane solubilization, showing that the detergent has a complex effect on the retinal chromophore, modifying its microenvironment (detected as a blue-shift in the absorption maximum) and promoting hydrolysis of the retinal-bacteriorhodopsin Schiff's base (observed as a decrease in specific absorbance or bleaching). Membrane solubilization is also accompanied by retinal isomerization, from all-*trans* to 13-*cis*.



This isomerization occurs as well in the absence of a surfactant when bacteriorhodopsin is transferred from light to dark environments. González-Mañas et al. [31,32] showed that Triton X-100 influences the kinetics of purple membrane dark adaptation, and that bacteriorhodopsin is more readily solubilized from dark-adapted than from light-adapted membranes. Subsequently, Meyer et al. [33] made good use of visible spectroscopy to describe the solubilization steps of dark-adapted purple membrane by Triton X-100.

An interesting correlation between bacteriorhodopsin solubilization and spectral effects in the visible range was observed by del Río et al. [34]. Observing the effects of a wide variety of detergents, these authors conclude that surfactants containing the cholane ring (cholate, taurocholate, CHAPS) are virtually unable to solubilize native bacteriorhodopsin, and they modify but slightly its absorption spectrum. Conversely, 'linear' detergents (Triton X-

←

Fig. 1. Solubilization of lipid bilayers by the non-ionic detergent $C_{12}E_8$, as seen by (A) steady-state and (B,C) time-resolved changes in suspension turbidity. Two lipid systems are compared, one in the L_α liquid crystalline phase (pure DMPC at 62°C, panel A dotted line, and panel B), and the other one in the $L_{o\alpha}$ liquid-ordered phase (DMPC/cholesterol, 70:30 mole ratio, at 65°C, panel A continuous line, and panel C). Total lipid concentration is 1 mM. In panel A, lipid vesicles and detergent are left to equilibrate for 2 h before measuring turbidity as absorbance at 500 nm. In panels B,C, equal volumes of lipid vesicles and detergent are mixed in a stopped-flow apparatus to give a detergent/lipid molar ratio of 5.15:1. Note that the liquid-ordered phase appears to be more resistant to solubilization under these conditions. From [21].

100, sodium dodecylsulfate, octylglucoside) that solubilize bacteriorhodopsin also produce bleaching, partial or total, of the protein chromophore. These data are probably indicating that a certain flexibility, that cholane-derived amphiphiles do not posses, is required in the surfactant molecule, in order to elicit the double process of protein solubilization and bleaching. Structure–function correlations of this type are not often found in the detergent literature.

The purple membrane of *Halobacterium* is quite unique in that its solubilization by detergents is far from being a rapid phenomenon. It may take hours or even days to be completed, when the corresponding process takes place within seconds in most other membranes. The slow kinetics was noted by Dencher and Heyn [35] and by Casadio et al. [36], and studied in more detail by Viguera et al. [23]. It should be noted that the 'purple membrane' consists actually of patches of a two-dimensional crystalline array of bacteriorhodopsin and lipids interspersed in the bacterium plasma membrane. When purified, the purple membrane exists in aqueous suspension in the form of flat sheets, their rigidity preventing them to form vesicles, so that in their perimeter hydrocarbon–water contacts occur. Viguera et al. [23] suggested, from a combination of equilibrium and stopped-flow measurements, that the tight crystalline organization of the purple membrane prevented the insertion of detergent monomers in the bulk of the lipid bilayer; instead, the surfactant would bind the periphery of the membrane 'patches', i.e., the hydrocarbon–water contact region, and solubilization would take place gradually, from the periphery towards the core of the membrane fragments, at a progressively lower rate as the amounts of free detergent and detergent-binding sites are decreased by the previous solubilization steps. This complex nature of purple membrane interaction with surfactants was also noted by Tan and Birge [37] who studied the solubilization and bleaching of bacteriorhodopsin by a series of alkyl-ammonium surfactants using time-resolved absorption spectroscopy in the time scale of minutes.

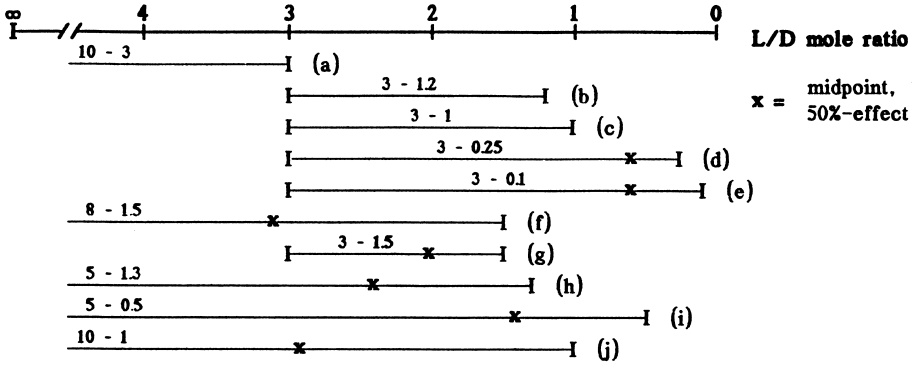
Rhodopsin is an integral membrane protein found in the cells of the eye retina. Like bacteriorhodopsin, it contains retinal as a chromophore. Several studies of rod membrane solubilization and reconstitution have made use of its property of absorbing visible light [38,39].

3.1. Dyes as spectroscopic probes

Dyes have not been commonly used in the study of detergent-membrane interactions, in contrast with the extensive use of fluorescent or spin probes (see below). However, Kaschny and Goñi [40] studied three dyes, namely merocyanine-540, pinacyanol chloride and Nile red, that could be easily incorporated into membranes. Upon addition of surfactants, these optical probes showed characteristic spectral changes, each at specific lipid/detergent ratios, suggesting that they were reporting on different aspects of membrane–surfactant interaction (Fig. 2). The absorption spectrum of merocyanine-540 has a complex shape, and it can be decomposed into bands assigned respectively to dye monomers, dimers, or large aggregates [41]. The spectral parameters (λ_{max} and integrated intensity) of each component can be separately analyzed and their variation studied as a function of detergent/membrane ratios. González-Mañas et al. [42] applied merocyanine-540 to the study of the solubilization of phosphatidylcholine bilayers by Triton X-100, and were able to detect spectral changes signaling a variety of events along a broad range of detergent/lipid ratios, from Triton X-100 incorporation into the bilayer to the formation of mixed micelles. The data in Fig. 2 demonstrate the applicability of dyes as spectroscopic probes in detergent studies.

4. Absorption spectroscopy: infrared

Biological infrared spectroscopy was renovated in the early eighties, when the new generation of Fourier-transform infrared (FT-IR) spectrometers became widely available. Today all commercially available instruments are of the FT-type, thus the technique should better be called again simply infrared (IR), in spite of the charm that the FT letters appear to have for some users. The great advantage of the 'new' IR spectrometers is that spectra can be taken under highly repetitive conditions, have them digitalized and operated with. In turn, this allows the subtraction of the water component from spectra of biological material in aqueous media, thus removing the main difficulty in the application of IR to biological samples. Basic information on the uses of IR in the



	model (MLV)	method	observation	reference
(a)	DMPC	^{2}H-NMR	reduction in quadrupolar splitting, decrease in the static order of acyl chains	(43)
(b)	DMPC	^{31}P-NMR	coexistence of phospholipids in two different states	(43)
(c)	DPPC	DSC	increase of fluidity, decrease of dynamic order	(43)
(d)	egg yolk PC	light scattering	membrane solubilization. decrease of turbidity	(39)
(e)	egg yolk PC	absorbance	blue shifted λ_{max} of MER monomer peak	(39)
(f)	egg yolk PC	absorbance	blue shifted λ_{max} of PIN monomer peak	(39)
(g)	egg yolk PC	absorbance	blue shifted λ_{max} of NIL	(39)
(h)	egg yolk PC	absorbance	increase of MER total absorbance	(39)
(i)	egg yolk PC	absorbance	enhanced MER dimerization	(39)
(j)	egg yolk PC	fluorescence	release of entrapped 6-carboxy-fluorescein	(85)

Fig. 2. Effects of Triton X-100 on various spectral parameters of phosphatidylcholine bilayers (or of spectroscopic probes therein), with an emphasis on the effects on optical probes. The horizontal lines show the range of lipid/detergent mole ratios at which a particular effect is detected, the sign × indicates the approximate lipid/detergent ratio at which a half-maximal effect is observed. MLV, multilamellar vesicles; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; MER, merocyanine 540; PIN, pinacyanol chloride; NIL, Nile red. From [39].

context of lipids and membranes can be found in Arrondo and Goñi [43].

The IR spectrum provides information on vibrational energy states from all kinds of chemical groupings belonging to lipids, proteins, surfactants, etc. This is why IR is one of the few techniques that can provide comprehensive information on all the components of a membranous system. When applied to membrane solubilization and reconstitution, the various authors have indeed taken advantage of this fact, focusing on one or the other component as required. Goñi et al. [44] provided some of the early data in this field, analyzing the interaction of saturated phosphatidylcholines with Triton X-100: The IR spectral position of the CH_2 stretching bands is a good indicator of hydrocarbon chain order, the band being shifted to higher wavenumbers when 'disorder' (i.e., proportion of *gauche* conformers) increases. Typically, a large increase is observed when saturated phospholipid bilayers undergo the gel-to-

fluid transition. Goñi et al. [44] showed that Triton X-100 increased the disorder of the acyl chains thus facilitating the gel-fluid transitions. A similar effect was observed by Bayerl et al. [45] with sodium deoxycholate as surfactant. Moreover, at high surfactant/lipid ratios (S:L = 2) deoxycholate was found to broaden the transition temperature range, and, interestingly, increase the proportion of *gauche* conformers in the gel state, while decreasing them in the fluid state. The latter phenomenon, not seen with Triton X-100, is reminiscent of the effect of cholesterol [46] and must be due to the rigid cholane ring of deoxycholate.

Using a more complex system, namely mitochondrial complex III–phospholipid–Triton X-100 mixed micelles, and the reconstituted membranes obtained after detergent elimination from the mixed micelles, Valpuesta et al. [47] were able to show that surfactant removal led to an important rearrangement in the methylene stretching vibrations of protein and phospholipids. In the same system, those authors detected as well an influence of detergent on the polar regions of membrane phospholipids, in the form of changes in the 1000–1300 cm^{-1} spectral region, corresponding to phosphate stretching vibration.

In the studies mentioned above, CH_2 vibration signals arising from lipid, detergent, and eventually protein could not be distinguished from each other. This problem can be circumvented by using isotopic derivatives of some of the components in the mixture. In particular, the use of deuterated lipids has been helpful in this context, because the C–D stretching vibrations appear in a spectral region (2050–2250 cm^{-1}) that is both separate from the one corresponding to C–H vibrations (2800–3000 cm^{-1}) and unencumbered by other IR signals. Echabe et al. [48] studied mixtures of perdeuterated-chain saturated phosphatidylcholine with natural palmitoylcarnitine or palmitoyl-coenzyme A, two well known metabolic intermediates, the former possessing surfactant properties. The IR data show that palmitoylcarnitine mixes well with the bilayer, and undergoes an order-disorder transition together with the main transition of the pure phospholipid, an effect that reminds of surfactants of the lysophospholipid type [49]. In contrast, under the same conditions palmitoyl-coenzyme A smears out the phospholipid transition, a phenomenon attributed by Echabe et al. [48] to bilayer interactions with the bulky coenzyme A polar moiety. More recently, Mädler et al. [50] have made use of perdeuterated-chain dimyristoylphosphatidylcholine in mixtures with $C_{12}E_4$ to construct, with the combined use of IR and differential scanning calorimetry, a detailed phase diagram of the lipid–detergent system in excess water.

Detergent and membrane components can also be separately studied by IR picking up vibrational modes that correspond only to one class of molecules. This was the approach of Pistorius et al. [51] who detected detergent-specific bands in cholate and dodecylmaltoside and used them to quantify the residual amount of detergent left in reconstituted proteoliposomes by ratioing the detergent-specific band (carboxylate band of cholate at 1397 cm^{-1}, carbohydrate C–O stretching band of dodecylmaltoside at 1150 cm^{-1}) to the lipid-specific phosphate (asymmetric P–O) vibration band at 1235 cm^{-1}. Detergent/phospholipid ratios as low as 1:10 could be estimated by this method.

The perturbation of proteins, the other main membrane component, by detergents has also been studied by infrared spectroscopy. For the application of IR to membrane proteins see Arrondo and Goñi [52]. Arrondo and co-workers, in two different membraneous systems, namely natural sarcoplasmic reticulum membranes [53] and reconstituted mitochondrial complex III [47], have observed that addition of detergent (SDS or Triton X-100) is accompanied by a decrease in the proportion of protein β-sheet structure, and the opposite happens when the detergent is removed.

5. Fluorescence spectroscopy

The term fluorescence spectroscopy encompasses a wide variety of techniques having in common their being based on the phenomenon of fluorescence. Fluorescence techniques are extremely versatile and, like most luminescence techniques, highly sensitive. There is hardly an area of molecular biology that has not benefited from the use of fluorescence in one or another form, and membrane-detergent interactions are not an exception. The book by Lakowicz [54] is an excellent source of information on fluorescence spectroscopy at an introductory/intermediate

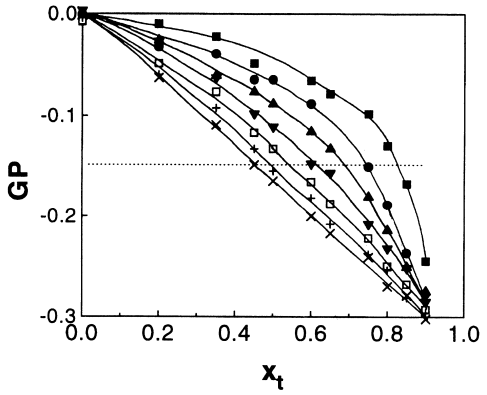


Fig. 3. An example of the use of the fluorescence probe Laurdan in the study of lipid–detergent interactions. The fluorescence of Laurdan is usually expressed in terms of a 'generalized polarization' (GP) parameter, that is computed on the basis of the fluorescence intensities at two predetermined wavelengths [55]. The figure shows the Laurdan generalized polarization in 1-palmitoyl-2-oleoylphosphatidylcholine/$C_{12}E_4$ mixtures versus the total mole fraction (x_t) of the detergent. Lipid concentrations are 5 μM (■), 10 μM (●), 15 μM (▲), 25 μM (▼), 45 μM (□), 100 μM (+), 200 μM (×). From [56].

level. From a more practical point of view, the Molecular Probes web site (www.probes.com) offers a wealth of useful information.

Usually each fluorescence probe, or family of them, provides a particular kind of information. Therefore, it is not inadequate to discuss the various forms and applications of fluorescence by looking at the main probes used in the field of membranes. ANS (8-anilino-1-naphthalenesulfonate) is commonly used as a fluorescent probe in membrane studies because, when transferred from a polar (e.g., aqueous) to non-polar (e.g., membrane or micellar) environment, its fluorescence emission increases notoriously in intensity, while being shifted to lower wavelengths. This makes ANS suitable, for instance, in the determination of critical micellar concentrations of detergents. DeGrip et al. [39] have used ANS to measure the removal of surfactant from detergent/lipid/protein mixed micelles using cyclodextrins, in a novel procedure for the preparation of reconstituted proteoliposomes. Alonso et al. [55] performed a parallel study of membrane–detergent interactions using ANS, believed to be located near the lipid–water or detergent–water interface, and *N*-phenyl-1-naphthylamine (NPN), that partitions deeply into the hydrophobic matrix of bilayers and micelles. ANS, but not NPN, reported on a release of environmental constraints, i.e., a fluidization, upon addition of surfactant to preformed bilayers.

Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) is a relatively new addition to the collection of fluorescence probes [56]. This molecule is particularly sensitive to environmental polarity, with a very large red shift in the emission maximum when transferred to polar solvents, and virtually no fluorescence in water. In membranes, Laurdan is only found in the bilayer interior. These properties make it appropriate for detecting phase changes, as well as formation of microdomains in membranes [56]. Heerklotz et al. [57] have used Laurdan to measure the partition of oligo(ethylene oxide) dodecylethers ($C_{12}E_{2-8}$) between phospholipid membranes and the aqueous phase (Fig. 3). The experimental data so obtained allowed these authors to analyze solubilization in terms of several useful solubilization parameters, namely the limiting detergent fraction in the membranes, the minimal detergent fraction in mixed micelles, and the critical detergent concentration in water.

Pyrene derivatives are used in membrane studies because, under favorable conditions, they can give rise to non-covalent dimers ('excimers') within the lipid matrix. Excimers have different fluorescent properties than monomers (their emission spectra is red-shifted), so that they can be easily detected. Excimer formation is highly dependent on membrane fluidity. Yegutkin [58], using a pyrene probe, observed an increased fluidity in rat liver and adipose plasma membrane upon addition of Triton X-100 at concentrations above the 'critical micellar concentration'. In a different context, Zhou and Roberts [59] used pyrene-labeled diacylglycerol molecules to study their solubilization by a variety of detergents, with the aim of understanding certain kinetic aspects of enzymes that utilize or produce diacylglycerols.

With some probes, the anisotropy of fluorescence emission is used to monitor membrane fluidity. For this purpose, polarized excitation light is used, and emission is measured at both parallel and perpendicular planes with respect to excitation. The 'depolarization' of fluorescence emission can be related to the motion of the probe in its specific environment. Diphenylhexatriene (DPH) is frequently used as a hydrophobic environment probe in polarization studies. The trimethylammonium derivative (TMA-DPH)

provides information on the lipid–water interfaces, where it resides. Koga et al. [60] provided an interesting example of the use of DPH and TMA-DPH in membrane–detergent studies. With a variety of surfactants of the Span and Tween families applied at subsolubilizing concentrations on rat intestinal brush-border membrane vesicles, DPH reported a decreased anisotropy (increased fluidity) for all detergents used. In contrast, the anisotropy of the interfacial probe TMA-DPH decreased with those detergents with a lower hydrophile/lipophile balance (e.g., Tween-81), but increased with the more hydrophilic ones (e.g., Tween-20), probably reflecting the location of these surfactants within the bilayer.

Advanced fluorescence instrumentation allows the measurement of parameters such as time-resolved fluorescence anisotropy, and lifetimes of fluorescence emission. Das [61] applied these powerful methods to the study of cholate/phospholipid mixtures in the presence of the probe 3,3′-diethyloxadicarbocyanine iodide, to observe that, in lipid–detergent mixed micelles, gradual removal of cholate from the medium leads to a large increase in rotational correlation time, corresponding to the formation of vesicles.

Fluorescence by resonance energy transfer is very sensitive to changes in relative orientation or distance between two molecules. In this relatively simple technique, two separate fluorescence probes are used, a donor and an acceptor. The system is excited at a frequency that will be absorbed by the donor, but not by the acceptor. Then, under certain circumstances, requiring short distance and adequate relative orientation, energy transfer occurs between donor and acceptor, and, when the former is excited, the latter emits fluorescence at its own characteristic wavelength. Naturally many surfactant-dependent events in membranes are reflected by the efficiency of energy transfer between the appropriate molecules. Ollivon et al. [62] made good use of this phenomenon to describe in detail both the solubilization and reconstitution of phosphatidylcholine vesicles in the presence of octylglucoside. Two fluorescent derivatives, respectively *N*-(7-nitro-1,2,3,-benzoxadiazol-4-yl) and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine were the acceptor and donor molecules.

In the example above, the fluorophore was always an externally added molecule, or probe. This is not always required since some biological molecules exhibit fluorescence by themselves. Tryptophanyl residues in proteins are particularly useful in this respect, their fluorescence being referred to as 'intrinsic' protein fluorescence. The following studies are based on Trp fluorescence and its quenching, or attenuation, by specific reagents. In the above-mentioned investigation on mitochondrial complex III–Triton X-100–phospholipid mixed micelles [47] the authors detected changes in Trp fluorescence emission as the surfactant was added or removed, suggestive of changes in the protein environment. Moreover, the fluorescent centers accessible to the water-soluble quencher iodide, probably located in the outermost protein region, did not experience, on the average, an important change in microenvironment upon removal of detergent. However a significant proportion of the Trp residues 'sensing' the less polar quencher acrylamide became less readily available to this reagent in the absence of Triton X-100.

The phenomenon of fluorescence quenching was given a novel use in the field of membrane–surfactant interactions by de Foresta et al. [63,64] who used brominated lipids and detergents with that purpose. Bromine, either pure or in combination, is a powerful fluorescence quencher. Brominated lipids can be easily prepared by the addition of Br_2 to unsaturated fatty acyl chains. Starting with lipids containing double bonds at various positions along the chain, a collection of molecules may be obtained that, when incorporated into a bilayer, will contain a quenching agent located at specific depths. De Foresta et al. [63] applied this procedure to study the delipidation caused by $C_{12}E_8$ or dodecylmaltoside on sarcoplasmic reticulum Ca^{2+}-ATPase. In the absence of surfactant, the intrinsic fluorescence of the ATPase is low, because it is quenched by the bromine atoms in the lipids. Delipidation is accompanied by an increase of Trp fluorescence. Parallel studies of enzyme function showed that full activity required that the ATPase hydrophobic surface be occupied by phospholipids; binding of only a few detergent molecules on that hydrophobic surface appeared to be sufficient to inhibit the enzyme. In a more recent study, de Foresta et al. [64] used brominated analogs of dodecylmaltoside and of 2-*O*-laurylsucrose. With this system detergents caused, even at subsolubilizing concentrations, quenching of ATPase intrinsic fluo-

rescence, a phenomenon that could be reverted by addition of excess non-brominated surfactant. It will be interesting to see further developments using this experimental approach.

One important point, before leaving this section on fluorescence techniques, is the problem presented in this kind of studies by contaminant fluorescence arising from reagents or labware. The sensitivity of the technique is such that otherwise 'pure' reagents may be inadequate for this purpose. Ironically, one of the main sources of fluorescence contaminations are the detergents themselves. The aromatic ring of Triton X-100 may give rise to some fluorescence, and a hydrogenated version of the detergent, otherwise very similar in its solubilizing properties, is commercially available (see [23] for an application). In other occasions, fluorescent impurities accompany the surfactant preparations. Dijkstra et al. [65] have published a method for the removal of fluorescent impurities from surfactants, in order to use these molecules in conjuction with studies of protein intrinsic fluorescence.

6. Nuclear magnetic resonance (NMR)

Like fluorescence, nuclear magnetic resonance is extremely versatile, and has found applications in almost every field of biology, chemistry, and even medicine. The technique reports essentially on the mobility of certain nuclei (^{1}H, ^{2}H, ^{13}C, ^{31}P, etc.) that have the property of becoming oriented with respect to an externally applied magnetic field. Inter and intramolecular interactions modify the local effects of the magnetic field on a given nucleus, thus contributing to the richness and complexity of the spectra. NMR can provide very detailed structural and kinetic information, its main drawback being probably its inherent low sensitivity, that makes mandatory the use of large (or very large) amounts of sample for compensation. For an introduction to the principles and applications of NMR the reader may refer to, e.g., Campbell and Dwek [66], or Sanders and Hunter [67].

After the pioneering work by Ribeiro and Dennis [68,69] describing the structure and dynamics of Triton X-100–phospholipid mixed micelles by ^{1}H-NMR and ^{13}C-NMR, two nuclei have been most often used in the study of membrane–surfactant interactions, namely ^{31}P and ^{2}H. The former occurs naturally in lipids, while deuterium has to be introduced by organic or biological synthesis in the molecules to be studied, its natural abundance and NMR sensitivity being quite low. ^{31}P is particularly useful because it is naturally present in phospholipids and membranes. The correlation time of ^{31}P nuclei is such that in cell membranes or large vesicles, the nuclei appear immobile, and a broad powder spectrum is produced. Conversely, in micelles, or in sonicated vesicles, the rapid tumbling of the particles averages out the spectral anisotropy, and ^{31}P nuclei give off a narrow, symmetric, isotropic signal. Thus vesicle–micelle transitions are very easily detected by ^{31}P-NMR. In addition, the technique allows quantitation of the isotropic and anisotropic components, so that solubilization and reconstitution can be accurately monitored in this way.

^{31}P-NMR was used by Jackson et al. [70] to describe the solubilization of large unilamellar phospholipid vesicles by octylglucoside, and later in similar studies with other surfactants by Goñi et al. [44], Paternostre et al. [71], and Otten et al. [19]. These studies show, in addition, a very good correlation between ^{31}P-NMR and other methods for detecting vesicle–micelle transitions. ^{31}P-NMR and turbidity were used in conjunction by King and Marsh [72] to describe the polymorphic behavior of lysophosphatidylcholine in poly(ethylene glycol)–water mixtures. Valpuesta et al. [47] took advantage of this technique to observe the conversion of mitochondrial complex III–phospholipid–Triton X-100 mixed micelles into membranous structures upon removal of detergent.

^{2}H-NMR was probably introduced in the field of membrane solubilization by detergents in a study by Goñi et al. [44], using saturated phosphatidylcholines containing perdeuterated acyl chains in the form of multilamellar vesicles, that were solubilized by Triton X-100. ^{2}H-NMR provides a direct and convenient measure of static order in the alkyl chains, since the order parameter is related to the quadrupolar splitting in the spectrum. Subsolubilizing concentrations of Triton X-100 (detergent/lipid ratios below 1:3) produced a decrease in quadrupolar splitting, thus in acyl chain order. This parameter did not change at detergent/lipid ratios between 1:3 and

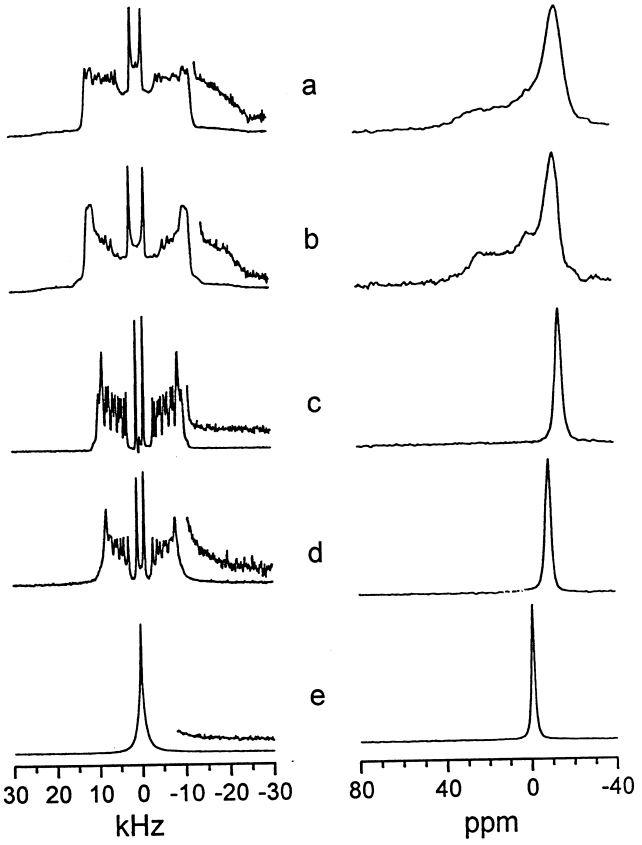


Fig. 4. ^{2}H-NMR spectra (left column) and ^{31}P-NMR spectra (right column) of pure alkyl-chain perdeuterated DMPC (DMPC-d_{54}) (a), and of DMPC-d_{54}/$C_{12}E_8$ mixtures at molar ratios 3:1 (b), 2:1 (c), 1.5:1 (d), 1:1 (e). Note that the solubilization is marked by the abolition of spectral anisotropy and the appearance of narrow symmetric signals in both NMR techniques. From [18].

1:1, while, at higher detergent concentrations, the quadrupolar splitting collapsed, and an isotropic signal appeared instead, marking the vesicle-to-micelle transition. A good agreement was found between ^{2}H-NMR, ^{31}P-NMR, turbidimetric and calorimetric observations of this phenomenon.

A similar combination of techniques was applied by Otten et al. [19] to study the phosphatidylcholine–$C_{12}E_8$–water system (Fig. 4). Interestingly, these authors incorporated temperature changes in their solubilization studies, including conditions above and below the gel–fluid transition temperatures of the pure phospholipids. At a phospholipid/detergent molar ratio of 2:1, membranes oriented in the magnetic field could be observed above the transition temperature, while cooling the mixture below T_c led to micelle formation. The temperature dependence of ^{2}H-NMR segmental order parameters of the acyl chains allowed these authors to conclude that the bilayer–micelle transition is the result of an imbalance between the chain and head group repulsion forces.

A related study was performed by Wenk et al. [73], combining ^{2}H-NMR and calorimetry in exploring the phosphatidylcholine-octylglucoside system. 1-Palmitoyl-2-oleoyl-phosphatidylcholine was selectively deuterated at the headgroup segments and at different positions of the fatty acyl chains. Measurements of the quadrupolar splittings indicated that octylglucoside had almost no influence on the lipid headgroup region, even at near solubilizing concentrations. In contrast, the fluctuations of fatty acyl chain segments located in the inner part of the bilayer, increased strongly with increasing surfactant concentrations. It is noteworthy that, while Otten et al. [19] found that $C_{12}E_8$ significantly disordered the headgroup region of the phospholipid at a 2:1 lipid/detergent ratio, an equivalent proportion of octylglucoside leaves the headgroup unchanged [73], which could suggest a different orientation of these two surfactants relative to the phospholipid bilayer, or perhaps be due to the different size of their polar head groups.

7. Electron spin resonance (ESR)

This resonance technique, also known as electron paramagnetic resonance (EPR), is based on the orientation of unpaired electrons, rather than nuclei, in a magnetic field. Since the electron magnetic moment is much higher than that of any nuclei, the inherent sensitivity of ESR is correspondingly higher than that of NMR. The difficulty here is that neither biomolecules (apart from some metalloproteins), nor detergents contain unpaired electrons, thus the use of spin label probes is necessary. 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), *N*-oxyl-4′,4′-dimethyloxazolidine (Doxyl) an their derivatives are most often used in membrane studies. Both TEMPO and Doxyl probes can be chemically linked to lipid or detergent molecules at a variety of positions, so that the membrane bilayer may be explored at various depths. The applications of ESR to biomembranes have been reviewed by Marsh [74].

Spin labels are highly sensitive to molecular motion, including the changes in molecular order that accompany phase transitions, hence their applicability to studies on membrane–detergent interactions. In fact, a large number of ESR studies have been carried out using a variety of membranes and surfactants. The invariable result is that detergent incorporation into the bilayer increases the molecular disorder and mobility of the hydrocarbon chains. To mention but a few specific examples, Sersen et al. [75] analyzed the interactions of bactericidal surfactants with liposomes prepared from *Escherichia coli* isolated lipids. A correlation was observed between the extent of membrane perturbation and the bactericidal potency of *N*-(1-methyldodecyl)-*N*,*N*,*N*-trimethylammonium bromide and *N*-(1-methyldodecyl)-*N*,*N*-dimethylamine oxide. The authors proposed a model on the location of surfactant molecules in the bilayer, according to which at low concentrations detergent molecules occupy structural defects in the bilayer, and only when these are filled up do the surfactants penetrate the bilayer core, expand it laterally, and increase the proportion of gauche conformations in the hydrocarbon chains.

Glover et al. [76] have also explored by ESR the surfactant action on bacterial membranes, in this case using whole bacterial cells, and a variety of detergents. While observing in all cases the usual detergent-induced increase in fluidity, no correlation was seen in this case between enhanced membrane fluidity and biocidal activity. It should be noted, however, that Glover et al. studied surfactants belonging to different chemical families (quaternary ammonium compounds, alkylsulfates, etc.) so that a strict physico-biological correlation is perhaps not to be expected. It is interesting in this respect that Galembeck et al. [77], studying the effects of a series of poly(oxyethylene)$_n$ nonylphenols, with $n = 9.5$–100, on erythrocyte membranes, observed in all cases an increased mobility of spin-labeled stearic acid upon addition of the amphiphiles, although some of these molecules were hemolytic, while others actually protected erythrocytes against hypotonic hemolysis. It appears that, while detergents increase membrane fluidity, this is not enough by itself to cause membrane disruption, instead an imbalance between interactions at the hydrocarbon chain and polar headgroup levels is required for bilayer disruption to occur.

The importance of the stratum corneum in skin care and dermatology treatments has been put forward repeatedly in the recent years (see paper by López et al. in this issue). Kawasaki et al. [78] examined the response of the stratum corneum lipid bilayer to anionic surfactants (sodium dodecylsulfate and sodium dodecylglutamate) by ESR. The expected increase in mobility was observed, and, in this case, it appears to be correlated to a decrease in the skin barrier function.

A particularly detailed study of membrane–surfactant interactions by ESR was carried out by Gallová et al. [79]. These authors prepared oriented phosphatidylcholine bilayers and could calculate various order parameters from the angular dependence of the spectral parameters. Hexyltrimethylammonium was estimated to increase the probability of *gauche* conformations of the lipid chains by about 13–14%, and decrease the effective energy difference between the *trans* and *gauche* conformations by about 420–480 J/mol, at a lipid/detergent 2:1 molar ratio. The results are in special agreement with the 'free volume model', according to which the surfactant orients itself in the membrane, parallel to the phospholipids. Insertion of the detergent increases the packing density of lipids at the headgroup level, while a free volume is formed at the alkyl chain level. The free volume is filled in, due to increased *trans–gauche* isomerization (see also [75]).

The sensitivity of spin label probes to molecular motion has also been applied to the study of protein–surfactant interaction, and particularly to the incorporation of membrane or amphipathic proteins to detergent micelles. This is the case of studies of binding of myelin basic protein to dodecylphosphocholine micelles [18], binding of human growth hormone or human interferon-γ to Brij or Tween [80], or binding of human tissue factor (rhTF) to $C_{12}E_8$ micelles [81]. The early study by Holladay and Wilder [82] on somatostatin-detergent interactions is singular in that the protein itself was spin-labeled. In all of these cases, ESR was shown to be an excellent method for measuring detergent–protein stoichiometries and, in some occasions, also for exploring the topology of the resulting complexes.

As mentioned above, some metalloproteins contain unpaired electrons that may give rise to ESR effects. One representative example of how this prop-

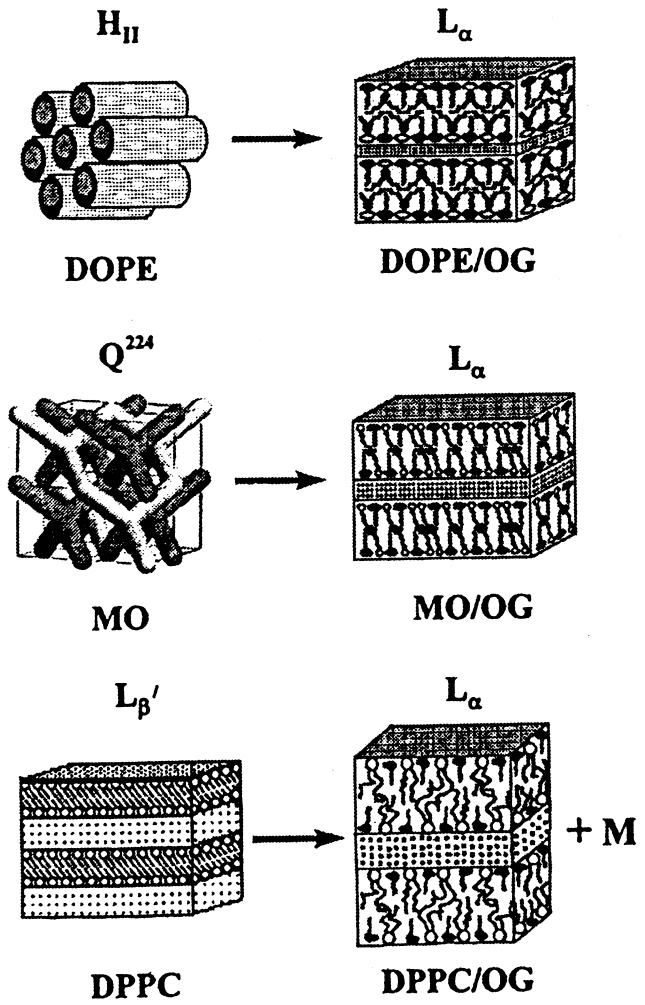


Fig. 5. Scheme of the structural phase changes of lyotropic phases of dioleoylphosphatidylethanolamine (DOPE), monoolein (MO), and DPPC upon mixing of these lipids with the detergent octylglucoside (OG) at a temperature of 30°C. The polar groups of OG are drawn in black. H_{II}, inverted hexagonal phase; Q^{224}, inverted bicontinuous cubic phase; $L_{\beta'}$, gel lamellar phase; L_α, liquid crystalline lamellar phase; M, micellar phase. From [84].

erty may be used to explore membrane–detergent interactions is given in the work by Montoya et al. [83], showing that the photosystem II reaction center undergoes a conformational change in the presence of Triton X-100, so that the light-induced spin-polarized triplet ESR signal is reduced. Interestingly, substitution of dodecylmaltoside for Triton X-100 reverses the situation to the native state including recovery of the triplet ESR signal.

8. X-ray diffraction

There is a distinguished tradition of surfactant studies using X-ray diffraction. In fact, the classical studies by Luzzati on soaps (reviewed in [84]) were the basis for establishing fundamental concepts in membrane structure, now widely accepted, the very concept of lipid bilayer among them. X-ray scattering and diffraction techniques have the notorious advantage of being able to provide information on molecular structure, sometimes at the atomic level. However they are highly specialized techniques, requiring lengthy and complex procedures for data analysis, that preclude their use in most routine work.

A recent study by Angelov et al. [85] uses X-ray diffraction for examining the effects of octylglucoside on three different lipids in excess water, namely dioleoylphosphatidylethanolamine, monoolein and dipalmitoylphosphatidylcholine. When dispersed in water, these lipids give rise respectively to inverted hexagonal (H_{II}), bicontinuous cubic (Q^{224}), and lamellar gel ($L_{\beta'}$) phases. At lipid/detergent ratios approaching 1:1, octylglucoside destabilizes the H_{II} phase of the phosphatidylethanolamine, and the Q^{224} phase of monoolein, converting them into a lamellar liquid crystalline (L_α) phase. The gel phase of dipalmitoylphosphatidylcholine is transformed into a mixture of L_α and micellar phases, both containing phospholipid and detergent (Fig. 5). The results with dioleoylphosphatidylethanolamine and monoolein are particularly interesting, because octylglucoside appears to form lamellar structures out of these essentially non-lamellar lipids, and this may be relevant in relation to membrane protein reconstitution protocols using both non-lamellar lipids and octylglucoside.

9. Assessing membrane permeabilization by detergents

In the previous sections, spectroscopic techniques have been described that allow the study of membrane solubilization and reconstitution. However surfactants exert an additional effect on bilayers, often at subsolubilizing concentrations, namely they cause the breakdown of the hydrophobic barrier and its permeabilization to solutes. This is a process that

has not been studied in great detail, and several important questions, e.g., the relationship between detergent/lipid ratios and size of the diffusing solutes, have not been addressed to date. We have published accounts of the main procedures used to assess membrane leakage induced by soluble amphiphiles [86,87]. Three main spectroscopic methods have been applied in these kinds of studies, namely visible, fluorescence and ESR spectroscopy.

9.1. Visible spectroscopy

In a representative study [88], dry phospholipids are swollen in buffer containing $FeSCN^{2+}$. The resulting vesicles are made to react with a 20-fold excess F^- in a stopped-flow apparatus. External iron complexes react with fluoride during the mixing and dead time of the stopped-flow experiment, thus the observed absorption changes (in the visible range) reflect ion diffusion through the membranes. Addition of the poly(ethylene oxide) detergent OP-10 increases membrane permeability to ions, as seen by a dramatic decrease of the relaxation times of the reaction between $FeSCN^{2+}$ and F^- [88].

Similarly, glucose may be entrapped in liposomes and its release followed by a colorimetric method, e.g., glucose oxidase/peroxidase [89,90]. When detergents are tested on erythrocytes, release of hemoglobin (hemolysis) has been assayed by visible spectroscopy, after sedimentation of the detergent-treated red blood cells, in order to test the surfactant effects on membrane permeability [77].

9.2. Fluorescence techniques

Once again the high sensitivity of fluorescence detection, together with relatively accessible instrumentation, make this the technique of choice for detection of vesicle leakage in most cases. Water-soluble fluorescence probes are entrapped in liposomes and the non-entrapped fraction is removed by gel filtration. Probes used for this purpose include 6-carboxyfluorescein [6,91], terbium/dipicolinic acid [25], and ANTS/DPX [92]. It should be noted that these probes, and others that could be used, are not equally applicable to any problem, rather a careful choice has to be done in each case [87]. De la Maza, Parra and coworkers (see, e.g., [6,91]) have developed a procedure, following the ideas of Lichtenberg [3] for solubilization, for the quantitative study of detergent-induced permeability. As a result, by measuring permeabilization at varying membrane concentrations they were able to compute 'effective' detergent/lipid ratios producing permeability alterations at detergent concentrations well below those causing solubilization. Fluorescence probe methods are commonly used in connection with liposomes, but cell membrane-derived vesicles have also been loaded with water-soluble probes [93].

9.3. ESR spectroscopy

The high sensitivity of ESR also finds a natural application in this kind of study. In a typical application, Chan et al. [94] entrapped a water-soluble TEMPO derivative in liposomes, and reduced the non-entrapped spin label with the membrane impermeable reagent ascorbate. Reduced TEMPO does not give off an ESR signal. Then these liposomes were used to detect leakage caused by surface-active components of the blood. In an earlier interesting work, Miller and Barran [95] loaded macroconidia of the fungus *Fusarium sulphureum* with Tempone, then observed the effects of a cationic and an anionic surfactant (respectively dodecylguanidinium acetate and sodium dodecylsulfate) on membrane permeability. These authors were able to distinguish between detergent-induced water uptake by the cells, membrane permeabilization to divalent cations, and inhibition of active transport function, depending on the species of detergent, and on its concentration.

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