Chapter 5
Monitoring Membrane Hydration with 2-(Dimethylamino)-6-Acylnaphtalenes Fluorescent Probes

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Abstract A family of polarity sensitive fluorescent probes (2-(dimethylamino)-6-acylnaphtalenes, i.e. LAURDAN, PRODAN, ACDAN) was introduced by Gregorio Weber in 1979, with the aim to monitor solvent relaxation phenomena on protein matrices. In the following years, however, PRODAN and particularly LAURDAN, were used to study membrane lateral structure and associated dynamics. Once incorporated into membranes, the (nanosecond) fluorescent decay of these probes is strongly affected by changes in the local polarity and relaxation dynamics of restricted water molecules existing at the membrane/water interface. For instance, when glycerophospholipid containing membranes undertake a solid ordered (gel) to liquid disordered phase transition the fluorescence emission maximum of these probes shift 50 nm with a significant change in their fluorescence lifetime. Furthermore, the fluorescence parameters of LAURDAN and PRODAN are exquisitely sensitive to cholesterol effects, allowing interpretations that correlate changes in membrane packing with membrane hydration. Different membrane model systems as well as innate biological membranes have been studied with this family of probes allowing interesting comparative studies. This chapter presents a short historical overview about these fluorescent reporters, discusses on different models proposed to explain their sensitivity to membrane hydration, and includes relevant examples from experiments performed in artificial and biological membranes.

Keywords Generalized polarization • Fluorescent probes • Laurdan

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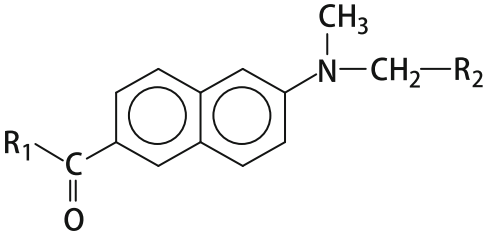
5.1 Brief Historical Overview

Solutions composed of particular substituted aromatic molecules experience a bathochromic (or red) shift on their fluorescence emission spectrum when solvent polarity is increased. This phenomenon, which involves a large increase in the molecule's dipole moment in the electronic excited state over that of the ground state, was the object of intense scrutiny from both theory and experiment since the 1950s. In 1979, Gregorio Weber performed a rational design of environmentally sensitive fluorescent molecules based on a naphthalene structure substituted with an electron acceptor (acyl substituted carbonyl group) and donor (alkylamino group) groups in position 6 and 2 respectively, Fig. 5.1 (Weber and Farris 1979). These molecules hold a maximum distance between the electron acceptor and donor groups, resulting in a lowest electronic excited state with an important charge transfer character. Three compounds with different amphipathic



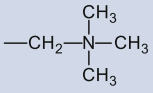
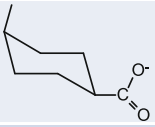
R_1	R_2	Acronymus
$-CH_3$	-H	ACDAN
CH_3-CH_2-	-H	PRODAN
CH_3-$(CH_2)_{10}$-	-H	LAURDAN
CH_2=CH-	-H	ACRYLODAN
CH_3-$(CH_2)_{14}$-		PATMAN
	-H	DANCA
CH_3-$(CH_2)_{10}$-		C-LAURDAN

Fig. 5.1 Chemical structures of different 2,6 substituted naphthalene derivatives

character were introduced in Weber's work, i.e. 6-acetyl-2-dimethylamino naphthalene (ACDAN), 6-propionyl-2-dimethylamino naphthalene (PRODAN), and 6-lauroyl-2-dimethylamino naphthalene (LAURDAN); see Fig. 5.1. Weber's original work also included a careful study of the absorption and fluorescence response of PRODAN to solvents of different polarity, which was interpreted using the Lippert's dipole interaction theory (Lippert 1957). For example, PRODAN exhibits a 130 nm red shift in the fluorescence emission maximum from cyclohexane to water with an important change in the magnitude of its transition dipole (Weber and Farris 1979). Additionally, a significant (larger) Stoke's shift was observed in solvents that can form hydrogen bonds with respect to aprotic solvents, showing an important effect of solvent relaxation on the characteristic nanosecond fluorescence decay of these fluorophores.

The contribution of Gregorio Weber went beyond the rational design and characterization of this family of probes. A significant contribution was their use to study biological material. Specifically, the interaction of PRODAN with bovine serum albumin was also explored in the original Weber paper, proposing the use of this molecule as "... a relaxation probe of various biological environments" (Weber and Farris 1979). In a later work, Weber's group studied nanosecond relaxation phenomenon in a protein matrix (addressed earlier in his laboratory (Lakowicz and Weber 1973)) to determine the polarity of the myoglobin haeme pocket (Macgregor and Weber 1986). Specifically he designed another 2,6 substituted naphthalene derivative, i.e. 2'-(N,N-dimethyl)amino-6-naphtophyl-4-trans-cyclohexanoic acid (DANCA) (Macgregor and Weber 1986), a probe with higher affinity than PRODAN for apo-myoglobin (Fig. 5.1).

Similar strategies were used in the early 1980s to explore solvent relaxation phenomena in membranous systems using different 2-(dimethylamino)-6-acylnaphtalene derivatives, i.e. particularly LAURDAN (Lakowicz and Sheppard 1981; Sumbilla and Lakowicz 1982) and 6-palmitoyl-2-[[[(2-trimethylammonium) ethyl]methyl]amino] naphthalene (PATMAN), another related probe introduced by Lakowicz and collaborators (Lakowicz et al. 1983), Fig. 5.1. During 1986 Parasassi and collaborators first reported a systematic study of LAURDAN sensitivity to membrane phase state in glycerophospholipid vesicles (Parasassi et al. 1986b). Specifically, these authors described a pronounced red shift on the probe's steady state and time resolved emission spectra during (and above) the solid ordered (s_o) to liquid disordered (l_d) membrane phase transition (Parasassi et al. 1986b). As will be discussed in the following sections, this phenomenon is a consequence of a drastic change in the content and dynamics of water associated to the membrane interface during the membrane phase transition. A two state model was proposed to describe LAURDAN response to the $s_o \rightarrow l_d$ phase transition (Parasassi et al. 1986a, b), providing the basis for the well-recognized *Generalized Polarization (GP) function* introduced by these authors in subsequent publications (Parasassi et al. 1990, 1991). The GP function was defined to exploit a simple steady state parameter (i.e. the probe's emission spectra) for studying structural and dynamical aspects of membrane lateral organization. The GP function, which is a generalization of the theory of fluorescence polarization, provides a more rational tool than traditional

ratiometric fluorescence measurements, which are generally empirical and devoid of a defined theoretical framework.

Although LAURDAN is presently the most widely used 2-(dimethylamino)-6-acylnaphtalene derivative, PRODAN, PATMAN and ACDAN have been also employed in membrane related studies. The main difference among these probes is their location in the membrane, providing valuable information about trans-bilayer structure and associated dynamics. LAURDAN's fluorescent group was reported to be located 10 Å from the center of the bilayer (Antollini and Barrantes 1998), quite similar to PATMAN which is located 1 Å deeper (Jurkiewicz et al. 2006). PATMAN has been mainly used to study dipolar relaxation processes in membranes composed of different lipids (Hutterer et al. 1996; Jurkiewicz et al. 2006; Olzynska et al. 2007) using a solvent relaxation technique that is based on time-resolved fluorescence measurements (Jurkiewicz et al. 2005). PRODAN on the other hand, is more loosely anchored to the lipid bilayer (it has a shorter hydrophobic tail in position 6 of the naphthalene moiety), and the localization of this probe was shown to be closer to the membrane interface (Chong 1990). The partition of PRODAN into phospholipid membranes was reported to be 2 orders of magnitude lower than LAURDAN (which is virtually insoluble in water). Therefore, a fraction of PRODAN is contributing from the aqueous medium, a situation that is reflected in the characteristic shoulder at 520 nm in its emission spectrum (Zeng and Chong 1995). Experiments exploring dipolar relaxation phenomena in membranes undergoing phase transitions (Krasnowska et al. 1998, 2001) were performed with PRODAN, including effects of pressure on the structure/dynamics of phospholipid containing membranes (Chong 1988, 1990). Finally, ACDAN was also used together with LAURDAN and PRODAN to study lipid interdigitation caused by ethanol in phospholipid bilayers (Zeng and Chong 1995). ACDAN has an even shorter alkyl substitution in the position 6 of the naphthalene ring (i.e. a methyl group) showing a very low partition to the membrane interface, which is approximately three orders of magnitude lower than LAURDAN. Other derivatives were also synthesized in Weber's laboratory, i.e. 2-diisopropylamino-6-lauroylnaphthalene (LAURISAN), 2-methoxy-6-lauroylnaphthalene (LAURMEN) and 2-hydroxy-6-lauroylnaphthalene (LAURNA), and characterized further by Parasassi et al. (1998). Very recently, a new 2,6-naphthalene derivative has been introduced 6-dodecanoyl-2-[N-methyl-N-(carboxymethyl)amino]naphthalene (C-LAURDAN, see Fig. 5.1), to explore membranes mainly using fluorescence microscopy (Kim et al. 2007; Dodes Traian et al. 2012).

5.2 Models for Laurdan Relaxation in Membranes

LAURDAN is a suitable probe to study membrane related phenomena for different reasons: (i) its partition coefficient from aqueous environments to membranes is relatively high; (ii) it displays an exquisite sensitivity to distinct lipid packing in membranes; and (iii) it generally shows an even partition in membranes displaying

phase coexistence (Parasassi et al. 1993b; Bagatolli and Gratton 2000a, b). The last property is not common for many of the amphiphilic fluorescent probes, which show preferential partition to one of the coexisting lipid phases. As I will discuss in the following sections, the even lateral distribution of LAURDAN plus it fluorescence response to membrane packing become a powerful experimental tool to gain information about membrane lateral heterogeneity.

As mentioned above, when LAURDAN is incorporated in membranes composed of single glycerophospholipids an emission red shift of 50 nm was observed when the $s_o \rightarrow l_d$ phase transition takes place, Fig. 5.2 (Parasassi et al. 1986b, 1990, 1991). This emission shift was reported to be independent of the nature of the glycerophospholipid polar head group and also the pH (between 4 and 10) (Parasassi et al. 1991). Since changes in the "static" dielectric constant between the two membrane phases is not sufficient to explain the observed fluorescence emission shift, a model to interpret the changes in LAURDAN's emission properties was originally provided by Parasassi et al. (1991). These authors proposed that the nanosecond relaxation process observed in the l_d phase (Parasassi et al. 1986b, 1990; Parasassi and Gratton 1992) is caused by the presence of water molecules with restricted

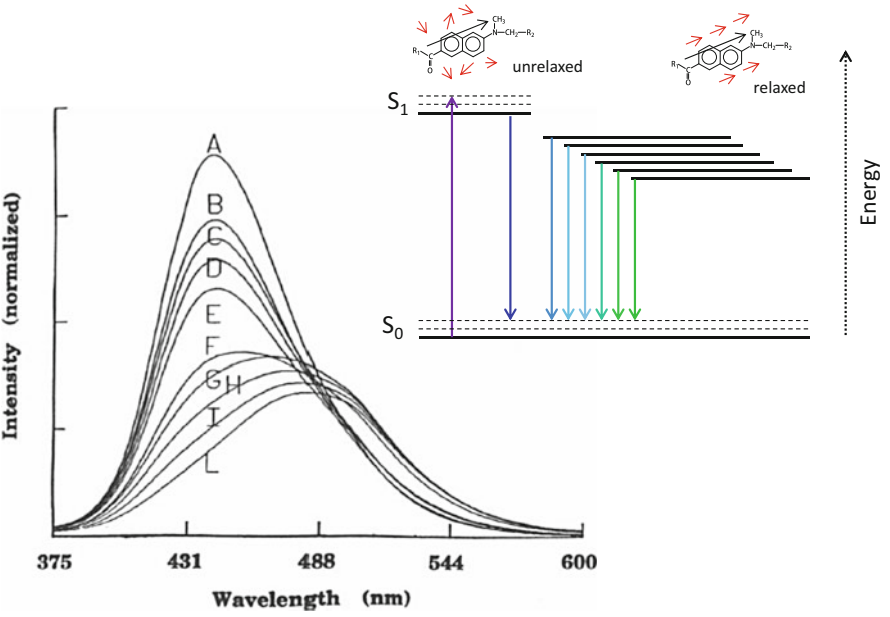


Fig. 5.2 LAURDAN emission spectra in DPPC multilamellar vesicles at different temperatures: (*A*), 30 °C; (*B*), 35 °C; (*C*), 37 °C; (*D*), 38 °C; (*E*), 39 °C; (*F*), 41 °C; (*G*), 42 °C; (*H*), 45 °C; (*I*), 50 °C; (*L*), 60 °C. The $s_o \rightarrow l_d$ main phase transition is 41.5 °C (Adapted from (Parasassi et al. 1991) with permission). The *inset* (*top right*) shows a scheme of the ground (S_0) and excited-state (S_1) energy levels in the presence of the solvent dipolar relaxation. S_1 decreases in energy as the extent of dipolar relaxation of interfacial water increases, *red* shifting the probe's emission spectrum

mobility in the region where LAURDAN is located (nearby the glycerol backbone of the glycerophospholipids) (Parasassi et al. 1991; Antollini and Barrantes 1998; Jurkiewicz et al. 2006). Part of LAURDAN's excited-state energy is utilized for the reorientation of the water dipoles diminishing the singlet excited state (S_1) energy (see Fig. 5.2, inset), consequently shifting the emission spectrum of the probe to longer wavelengths (lower energies). Importantly, the relaxation caused by these water molecules is different to the water molecules existing in bulk that have an orientational relaxation time below one picosecond (Parasassi et al. 1991). The environmental relaxation times have been measured for both the l_d and s_o phases using an equivalent expression of the classical Perrin equation for the GP (for details of the GP definition see below), assuming a two state process (Parasassi and Gratton 1992). These values were reported to be 2.5×10^9 s^{-1} for the l_d phase and 4×10^7 s^{-1} for the s_o phase. Notice that the relaxation time measured in the l_d phase is similar to the lifetime of the probe (3 ns) (Bagatolli et al. 1998).

Taking into account observations obtained by two photon excitation fluorescence microscopy, Parasassi et al. refined the model of water relaxation (Parasassi et al. 1997). This seminal article reported for the first time spatially resolved information of the LAURDAN GP function in micrometer sized (multilamellar) vesicles, showing that the GP images obtained in l_d phase membranes show a much broader GP distribution relative to the s_o phase. This observation indicates the existence of a large dynamical heterogeneity in the l_d phase, unexpected to the authors for a membrane displaying a single l_d phase. To explain these observations Parasassi et al. proposed a distribution of different sites (or cavities) in which the LAURDAN molecule can reside (Parasassi et al. 1997). These sites are characterized by a different number of dynamically restricted water molecules (Parasassi and Gratton 1995). The average number of water molecules at the location of the LAURDAN fluorescent moiety was estimated to be no more than two or three (Parasassi et al. 1997). (*For a further insight see Chaps. 2, 3, and 4). Considering a Poisson distribution of these few water molecules, the authors concluded that there is a distribution of LAURDAN environments with no, one, two, three, etc. molecules of water. For example, for an average of two molecules of water per cavity around the LAURDAN fluorescent moiety, the Poisson distribution of water molecules at the different sites is $0 \rightarrow 0.135$, $1 \rightarrow 0.270$, $2 \rightarrow 0.270$, $3 \rightarrow 0.203$, $4 \rightarrow 0.090$, $5 \rightarrow 0.031$, and more than $5 \rightarrow 0.020$, concluding that the larger the number of water molecules, the lower is the GP, and the larger is the cavity around the probe. This model has been recently supported by scanning-fluctuation correlation spectroscopy measurements of the LAURDAN GP function on the scale of few pixels in single glycerophospholipid containing membranes existing in the l_d phase (Celli and Gratton 2010).

There is considerable experimental evidence that supports the idea that structured water molecules in the vicinity of the probe are the cause of LAURDAN's emission shift in model membranes. One of the most conclusive evidences comes from comparative experiments performed in DMPC membranes prepared in D_2O and H_2O. At and above the $s_o \rightarrow l_d$ membrane phase transition the red shift observed in H_2O is more prominent than in D_2O, indicating a slower relaxation dynamics in heavy

water, i.e. an expectable effect considering the solvent mass difference (Parasassi et al. 1998). Another important experimental proof supporting this model is the consistency on the extent of probe's relaxation in membranes containing different glycerophospholipid polar head groups and pH, i.e. implying that the position of emission spectrum depends on the membrane phase state (Parasassi et al. 1991). The same behavior was also observed in ether derivatives of glycerophospholipids (Bagatolli et al. 1999) and sphingolipid-containing bilayers (Bagatolli et al. 1997, 1998).

Additional experiments were performed to check if structural features of the probe itself can be the cause of the observed fluorescence changes during a membrane phase transition. For example, results obtained using the LAURDAN derivatives 2-hydroxy-6-dimethyl aminonaphthalene (LAURNA) and 2-methoxy-6-dimethyl aminonaphthalene (LAURMEN) suggest that the partial charge separation is necessary to observe the fluorescence emission shift. Also, it was observed that steric hindrance to intramolecular reorientation expected for 6-lauroyl-2-diisopropylamino naphthalene (LAURISAN) has no effect on the probe's red shift, suggesting that the molecular entity responsible for the dipolar relaxation cannot be the fluorophore itself (Parasassi et al. 1998). Additionally, information obtained from classical fluorescence polarization spectra also excludes probe reorientation along its molecular axis to be the cause of the observed emission shift (Parasassi et al. 1998).

5.2.1 Alternative Models

Based on experiments performed in alcohols at very low temperature, Viard et al. (1997) proposed that, depending on the relative orientation of the dimethylamino and carbonyl groups, two states contribute to the emission of LAURDAN: the locally excited state and the charge transfer state. This model was applied with some modifications to explore the nanosecond dynamics of membrane water interface in reverse micelles of AOT-water in isooctane (Vincent et al. 2005). Although the locally excited state can be confirmed in apolar solvents (where the lifetime and quantum yield of the probe are very low), there is no clear evidence that this state contributes to LAURDAN emission in glycerophospholipid membranes. For example, the high quantum yield observed in membranes, the clear lifetime dependence on the membrane phase (3 and 6 ns in the s_o and l_d phases, respectively (Bagatolli et al. 1998)) and the lack of spectral isosbestic point during the phase transition support that the charge transfer state is dominant (Parasassi et al. 1998), i.e. there is one state that relaxes upon the lipid main phase transition causing the emission spectrum shift, not an intramolecular relaxation of the probe. This view is also supported by the above described experiments using LAURNA, LAURMEN and LAURISAN.

The model of LAURDAN relaxation in membranes has been recently revisited by Jurkiewicz et al. (2012). Based on time dependent fluorescence shift measurements

complemented with molecular dynamic simulations, these authors proposed that the nanosecond relaxation times reported by LAURDAN in membranes carry information solely on the mobility of hydrated lipid moieties at the probe's location and not the local dynamic of water molecules theirself, which interchange or even exchange with those from bulk water on a picosecond timescale. The authors' conclusion is based on the fact that at the glycerol backbone level in glycerophospholipid bilayers (i.e. where the probe is located) water molecules are sparse and fully bound to the glycerophospholipid carbonyl groups. However, the aforementioned D_2O experiments together with the fact that similar probe's emission response was observed in bilayers composed of very distinct lipid polar head groups (which coordinate different number of water molecules) undergoing s_o to l_d phase transition, e.g. DPPC and its ether derivative (Bagatolli et al. 1999), sphingomyelin, Gg_3Cer, Phrenosine, sulfatide, and galactosyl-ceramide (Bagatolli et al. 1998), reinforce the idea that the (nanosecond time scale) relaxation of associated water is the main cause of the observed probe's emission shift.

5.3 The Generalized Polarization Function

The generalized polarization (GP) function was proposed by Parasassi et al. in 1990 (Parasassi et al. 1990) as an analytical method to quantitatively determine the relative amount and temporal fluctuations of s_o and l_d phases when they coexist in a model membrane. This function was originally defined as:

$$GP = \frac{I_B - I_R}{I_B + I_R} \tag{5.1}$$

where I_B and I_R are the measured fluorescence intensities under conditions in which a wavelength (or a band of wavelengths) B and R are both observed using a given excitation wavelength. This definition corresponds to the classical fluorescence polarization definition (Jameson et al. 2003) if B and R represent two different orientations of the observation polarizers. The advantage of the GP for the analysis of the spectral properties of the 6-acyl-2-dimethylamino naphthalene probes is related to the well-known properties of the classical polarization function, which contains information on the interconversion between different "states". In the classical polarization definition the "states" correspond to different orientations of the emitting dipole with respect to the laboratory axis. In the GP function the condition of interconversion between two states *a* and *b*, i.e. unrelaxed and relaxed, has been linked respectively to the extent of solvent relaxation observed in the s_o and l_d phases in glycerophospholipid membranes respectively (Parasassi et al. 1990, 1991). Using a general formulation for the decay of *a two state system* the general expression of a steady state GP can be derived ((Parasassi et al. 1990) also reviewed in (Bagatolli 2013)) as:

$$\frac{B-R}{\langle GP\rangle} - (B+R) = \frac{2\left(b_0 + \frac{k_{ab}}{k_a}\right)}{a_0\frac{k_b}{k_a} - b_0 + \frac{k_{ba}}{k_a} - \frac{k_{ab}}{k_a}} \tag{5.2}$$

where GP is the generalized polarization (Eq. 5.1), B and R represent the fractional emission of the unrelaxed state in the blue and red edges of the spectrum, a_0 and b_0 are the relative absorption of the unrelaxed and relaxed states at the wavelength of excitation, k_a and k_b are the intrinsic decay rates of the unrelaxed and relaxed state and k_{ab} and k_{ba} are the forward and backward relaxation rates. It is reasonable to assume that $k_a = k_b$ (the intrinsic decay rates are equal) and that $k_{ba} = 0$, i.e. the decay rate is independent of the relaxation process and the back reaction is slow. After these assumptions Eq. 5.2 reduces to:

$$\frac{B-R}{GP} - (B+R) = \frac{2\left(b_0 + \frac{k_{ab}}{k_a}\right)}{a_0 - b_0 - \frac{k_{ab}}{k_a}} \tag{5.3}$$

From this expression it is interesting to see that it is the ratio k_{ab}/k_a that determines the modalities of the relaxation process and the GP value. Parasassi et al. analyzed three particular limiting cases in the context of this definition (Parasassi et al. 1991):

(i) $k_{ab} >> k_a$, fast relaxation or long lifetime value of the probe. In this case the GP value depends only on the spectral emission properties of the unrelaxed state at the wavelength of excitation, i.e. on B and R and not on the relaxation process of the solvent.

(ii) $k_{ab} << k_a$, slow relaxation or short lifetime value of the probe. The GP depends also on the relative absorption of both the relaxed and unrelaxed state at the wavelength of excitation. If no relaxed state is excited ($b_0 = 0$), then there should be no dependence of the GP value on the excitation wavelength because $a_0 = 1$.

(iii) $k_{ab} = k_a$, the GP value also depends on the dynamic properties of the solvent that relaxes the probe's excited state.

Condition (ii) satisfies what is observed in membranes displaying s_o phase whereas condition (iii) reflects what is observed in a l_d phase (Parasassi et al. 1991).

Although the GP function has been applied to interpreted LAURDAN behavior in diverse type of membranous systems a modification of the GP function has been reported for PRODAN (Krasnowska et al. 1998, 2001). Because of the different lengths of their acyl residues, the partitioning of the two probes between water and membrane differs profoundly. To account for the contribution of PRODAN fluorescence arising from water, Kranowska et al. introduced a three-wavelength generalized polarization method (3wGP) that makes it possible to separate the spectral properties of PRODAN in the lipid phase from water (Krasnowska et al. 1998). This function also allows to determine the probe partition coefficient to membranes as well its distribution between s_o and l_d phases. The major conclusions of this work are that in contrast to LAURDAN, PRODAN preferentially partitions

in the l_d phase with respect to the s_o phase and is sensitive to the membrane pre-transition. Additionally it was reported that PRODAN partition coefficient between the membrane and water depends on the phase state, i.e., PRODAN partitioning is higher in the l_d phase by a factor of 35 respect to the s_o phase. This last observation has been confirmed using two photon excitation fluorescence microscopy in giant unilamellar vesicles labelled with PRODAN (Bagatolli and Gratton 2000b). Last but not least, the 3wGP has been also applied to study the effect of cholesterol in phospholipid containing membranes. Particularly it was noticed that a low amount of cholesterol (3 % mol) causes higher partition of the probe into gel domains of DLPC-DPPC mixtures, reaching a maximum at 15 mol %, a phenomenon associated with the progressive formation of a liquid ordered (l_o) phase (Krasnowska et al. 2001).

In summary, the GP function applied to LAURDAN (or PRODAN) is responsive to membrane hydration, which in turn is related to membrane lateral structure. This function exhibits different values depending of the membrane lateral packing. Figure 5.3 shows a graphical example for computation of the GP function in model membranes displaying s_o and l_d phases. The function values scale from 0.6 ± 0.1, characteristic of the s_o phase, to below -0.1 for the l_d phase. In the latter case the GP function shows an additional decrease (although minor compared with the change observed at the membrane main phase transition temperature) when the temperature is increased associated with an augmentation in the extent of solvent relaxation upon increasing the temperature of the system. Finally, and importantly, membranes

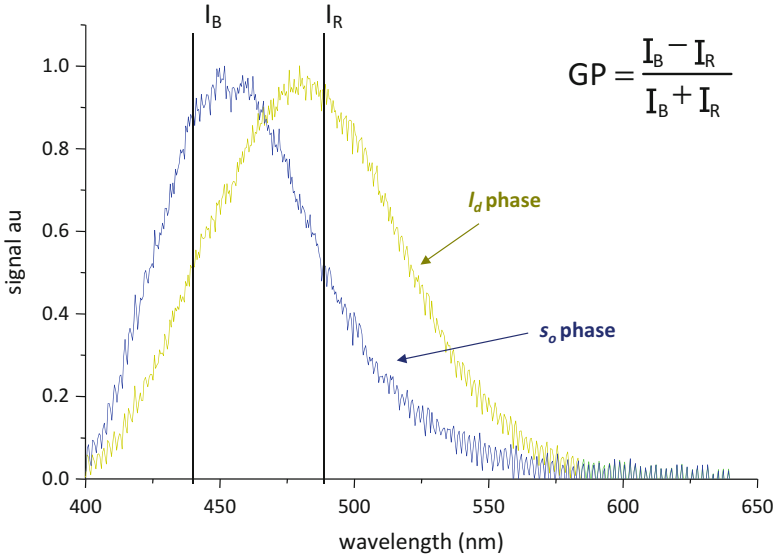


Fig. 5.3 LAURDAN emission spectra in the solid ordered (*gel; blue*) and liquid disordered (*green*) phases. The emission spectrum shift is 50 nm. The Generalized Polarization parameter, which depends on the position of the LAURDAN emission spectrum, contains information about water content and dynamics existing at the membrane interface

displaying l_o phase show intermediate GP values that depend on the amount of cholesterol and the temperature ((Parasassi et al. 1994a, b; Dietrich et al. 2001), see also Fig. 5.6 in Sect. 5.4.2). Cholesterol effects are briefly addressed in this chapter (see Sect. 5.4) as they have been widely reviewed elsewhere (Bagatolli 2013).

5.3.1 Generalized Polarization Spectra

By measurement of LAURDAN time resolved emission spectra, Parasassi et al. showed that s_o and l_d domains co-exist in multilamellar vesicles composed of DLPC/DPPC (Parasassi et al. 1993b). The authors estimated membrane domain dimensions of 20 to 50 Å, and measured domain fluctuations of 25 ns (Parasassi and Gratton 1995). By applying the additive property of the GP function (Parasassi et al. 1991, 1993b) the fractions of the coexisting phases were also obtained. These authors also concluded that the intrinsic characteristics of the s_o and l_d phases in the single lipid containing membranes is somehow modified when these phases coexist in the lipid mixture, i.e., it reflects partial lipid miscibility in the DLPC/DPPC mixture (Parasassi et al. 1993b). Based on these findings, the authors presented a simple strategy to ascertain phase coexistence in membranes using steady state "bulk" fluorescent measurements (Parasassi et al. 1991) built on the particular response of LAURDAN emission (and excitation) spectra to the phase state (s_o, l_d or s_o/l_d phase coexistence) in glycerophospholipid containing membranes. Specifically, they showed that the excitation or emission wavelength response of the GP function (i.e. called GP_{ex} and GP_{em}), provided a fingerprint to discriminate among different membrane phase state scenarios (Parasassi et al. 1991, 1994b; Parasassi and Gratton 1995). I will only discuss below the characteristics of the GP_{ex} spectrum. A complete description of the GP_{em} can be found in (Parasassi and Gratton 1995); also recently reviewed in (Bagatolli 2013).

In order to obtain a GP_{ex} spectrum the excitation spectra of LAURDAN are measured at two fixed emission wavelengths (440 and 490 nm) and the GP calculated at each excitation wavelength according to Eq. 5.1 (I_B is the intensity at 440 nm and I_R the intensity at 490 nm). In glycerophospholipid membranes in the s_o phase the GP_{ex} spectrum is independent of the excitation wavelength, since no relaxation occurs (see above condition (ii) in Sect. 5.3). However in the l_d phase the response is different. One of the characteristics of the dipolar relaxation process is that by exciting in the blue part of the excitation spectrum, molecules with an energetically unfavorable ground state (surrounded by randomly oriented dipoles) are photoselected. As a consequence, by moving the excitation toward the blue, more blue emitting molecules are excited. Since the GP_{ex} value depends on the difference between the emission intensities at 440 and 490 nm, when relaxation occurs (as it happens in the l_d phase), higher GP_{ex} values are expected at shorter excitation values, i.e. the slope of the GP_{ex} spectrum is negative, Fig. 5.4 (Parasassi et al. 1990, 1991, 1994b). When the membrane displays s_o/l_d phase coexistence the GP_{ex} spectrum shows a positive slope (Parasassi et al. 1991), since there are relaxed and unrelaxed states coexisting in the membrane. Particularly, the unrelaxed

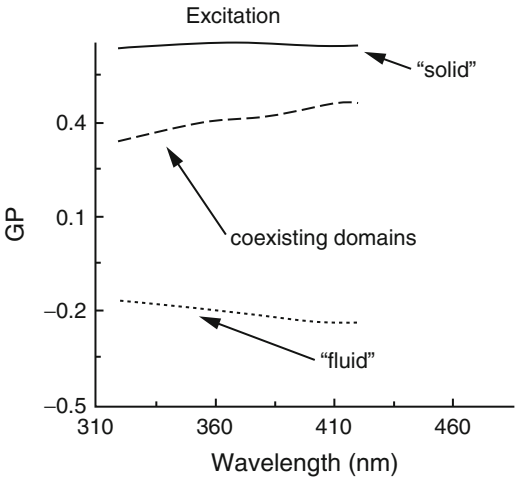


Fig. 5.4 LAURDAN GP_{ex} spectra obtained in phospholipids membranes in different phase scenarios. GP_{ex} spectra were calculated by $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$, using two excitation spectra obtained at 440 and 490 nm emission wavelenghts (Adapted from (Parasassi and Gratton 1995) with permission)

state contributes in the red region of the excitation spectrum (i.e., the red band centered at 390 nm of the LAURDAN excitation spectrum is prominent in the s_o phase but not in the l_d phase (Parasassi and Gratton 1995; Bagatolli et al. 1999); also recently reviewed in (Bagatolli 2013)) and in the blue side of the emission spectrum, increasing the GP value at longer excitation wavelengths, Fig. 5.4. This strategy has been used to ascertain phase coexistence in model membranes and also in natural membranes (Parasassi et al. 1993a; Parasassi and Gratton 1995). However, it is important to remark that in membranes composed of sphingolipids, the changes observed in the LAURDAN excitation spectrum (particularly the red excitation band) between the s_o phase to l_d phase are not the same than in glycerophospholipid membranes (Bagatolli et al. 1998, 1999). This divergent response, attributed to specific interaction in the ground state of the probe (Bagatolli et al. 1999), has important consequences on the sensitivity of the GP_{ex} spectrum to s_o/l_d phase coexistence mentioned above, i.e. the GP_{ex} (and also the GP_{em}) spectrum in membranes composed of sphingolipids becomes insensitive to phase coexistence. Taking into account these facts, caution is recommended if the LAURDAN GP spectra are used to ascertain phase coexistence in membranes composed of lipids other than glycerophospholipids or membranes displaying complex compositions (e.g. natural membranes) (Bagatolli et al. 1999; Bagatolli 2013).

5.4 Relevant Examples

The GP function has been extensively applied to study different membrane related phenomena. For example, many studies have made use of the LAURDAN GP approach in a fluorometer (cuvette studies) using suspensions of liposomes (or cells) to dissect membrane lateral heterogeneity, membrane interactions with peptides, proteins, or other ligands (Parasassi et al. 1993a, 1998, Parasassi and Gratton 1995; Antollini and Barrantes 1998; Henshaw et al. 1998; Vanounou et al. 2002; Nielsen

and Otzen 2010). Additionally, studies involving *spatially resolved* GP experiments (performed in a microscope) have been also reported using giant unilamellar vesicles (Bagatolli and Gratton 2001; Bagatolli 2006). Since space restrictions do not allow a review of the over 440 papers reported using these two experimental strategies, this section will focus on a few representative examples to provide a general idea of the potentialities and limitations of the technique.

5.4.1 Phase Transitions and the Effect of Membrane Hydration in Liposome Suspensions

Seminal LAURDAN GP studies on suspensions of liposomes of diverse composition were performed by Parasassi and collaborators (Parasassi et al. 1990, 1991, 1993a, b, 1994a, b, 1995; Parasassi and Gratton 1992, 1995). Typical examples are the detection of the s_o to l_d lipid main phase transition temperature in liposomes composed of single glycerophospholipids (Parasassi et al. 1990), including studies on the effect of cholesterol, which decreases the cooperativity and gradually abolishes the glycerophospholipid main phase transition (Parasassi et al. 1994b). However, instead of performing a systematic review on the extensive literature reporting on LAURDAN GP and membrane phase state/phase transitions, I will discuss the correlation of the GP function with the water content and extent of solvent relaxation in membranes composed of a series of sphingolipids and sphingolipid-glycerophospholipid mixtures (Bagatolli et al. 1997, 1998). These studies showed that the extent of solvent relaxation detected by the probe was strongly influenced by the chemical nature of the studied lipids, which in turn affects the overall structure of the membranous system. For example, for bilayer-forming sphingolipids such as Gg_4Cer, Gg_3Cer, Phrenosine, sulfatide, GalCer, and sphingomyelin, the LAURDAN GP function showed the characteristic values and sensitivity to the $s_o \rightarrow l_d$ phase transition observed in glycerophospholipid bilayers (Bagatolli et al. 1997, 1998). However, in glycosphingolipids with bulky sugar-based polar head groups (i.e. gangliosides G_{M2}, G_{M1}, G_{D1a}, G_{T1b}) the response of LAURDAN was different. Gangliosides form micellar structures that have a much higher curvature compared to bilayers. Consequently the measured LAURDAN GP values were low and relatively constant both below and above the lipid main phase transition temperatures detected by differential scanning calorimetry (Bagatolli et al. 1997, 1998). This lack of sensitivity to the micelle phase transition is consistent with the probe experiencing a highly relaxed environment, i.e. a highly curved and hydrated interface (Arnulphi et al. 1997), compared to that observed in bilayers. Remarkably, a close correlation between the GP function and the intermolecular spacing among these lipids measured at 30 mN/m in monolayers, which can be related to different hydration states, was reported for both neutral and anionic lipid species, Fig. 5.5 (Bagatolli et al. 1998). This finding strongly supports the water dipolar relaxation model discussed above for LAURDAN, i.e. LAURDAN resides in

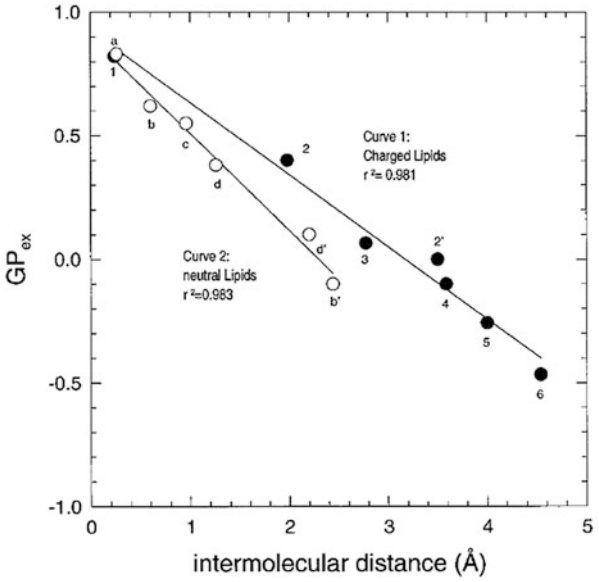


Fig. 5.5 Variation of LAURDAN GP_{ex} obtained in membranous suspensions with the calculated intermolecular distance (measured at 30 mN/m in monolayers at the air water interface) of anionic GSLs (*filled symbols*) and neutral GSLs and phospholipids (*empty symbols*). Neutral lipids: GalCer (*a*); DPPC below (*b*), and above (*b′*) the transition temperature; Gg_3Cer (*c*); Gg_4Cer below (*d*) and at (*d′*) the transition temperature. Anionic lipids: Sulf (*1*); G_{M3} below (*2*), and above (*2′*) the transition temperature, G_{M2} (*3*); G_{M1} (*4*); G_{D1a} (*5*); G_{T1b} (*6*) (Adapted from (Bagatolli et al. 1998) with permission)

cavities containing different amounts of associated water in the membrane which is regulated by curvature and the capability of the lipid polar head group to coordinate water (Bagatolli et al. 1997, 1998).

5.4.2 Membrane Phase Coexistence Study by Fluorescence Microscopy

Fluorescence microscopy experiments using giant unilamellar vesicles (GUVs) composed of distinct lipid mixtures further advanced the understanding of lateral phase separation in membranes. Using LAURDAN labeled GUVs and two photon excitation (TPE) fluorescence microscopy, direct information on local membrane packing can be obtained from membranes displaying coexisting domains at the level of single vesicles (Bagatolli and Gratton 2001; Bagatolli 2006). The LAURDAN GP imaging approach was shown to be superior to other confocal fluorescence microscopy strategies, which use pairs of lipophilic probes that preferentially partition to each of the coexisting membrane regions. Particularly, it has been

demonstrated that the partition of those probes depends on the chemical nature of the domains, i.e. probe partition *is not* necessarily "phase" specific (Bagatolli and Gratton 2001; Bagatolli 2006; Juhasz et al. 2010). In the case of confocal fluorescence microscopy experiments, additional diffusion experiments using fluorescence correlation spectroscopy (FCS) must be performed to obtain local information on domain packing (Korlach et al. 1999). This is not needed for LAURDAN, since the probe is evenly distributed and responsive to membrane packing (Bagatolli 2006).

The effects of temperature on the lateral structure of LAURDAN labelled GUVs composed of pure phospholipids and different mixtures thereof were first reported in 1999 and 2000 (Bagatolli and Gratton 1999, 2000a, b). Micrometer-sized domains with s_o character, surrounded by membrane regions corresponding to a l_d phase, where observed at particular temperatures depending on the lipid mixture. These domains spanned the bilayer, demonstrating inter leaflet coupling in the membrane (Bagatolli and Gratton 2000a, b). Also, these experiments first demonstrated the generation of micrometer sized domains in bilayers, disproving the general assumption that nanometer-sized domains would exist in membranes composed of those lipid mixtures. This result also linked the observation of micrometer sized domains in bilayers with that previously observed in Langmuir lipid films (Nag et al. 2002; Brewer et al. 2010; Bernardino de la Serna et al. 2013). In one of these studies, for example, a correlation between domain shape and lipid miscibility was reported for binary mixtures of different phospholipids displaying s_o/l_d phase coexistence (Bagatolli and Gratton 2000a). The same strategy was implemented to explore the effect of cholesterol in lipid mixtures. Particularly, the lateral structure of canonical "raft" mixtures (DOPC/sphingomyelin/cholesterol) was first visualized in free standing bilayers using LAURDAN and TPE fluorescence microscopy (Dietrich et al. 2001). For this mixture, the coexistence of two liquid phases (l_o/l_d), characterized by the presence of round micrometer sized domains and intermediate LAURDAN GP values to those observed for the s_o/l_d case was described, Fig. 5.6. In this last case it is clear that cholesterol substantially modifies the extent of water relaxation in the membrane when l_o/l_d phases coexist. This is reflected for example in the slower water dipolar relaxation observed in the l_o phase respect to the l_d phase, but also in the different GP values observed for the l_d phase itself in presence and absence of cholesterol (Fig. 5.6). The GP function has been also applied to planar supported bilayers and Langmuir films, providing the possibility to perform texture analysis on s_o (gel) domains, to characterize membrane hydration in monolayers upon compression and also to correlate membrane packing information among the different planar membrane systems (Bernchou et al. 2009; Brewer et al. 2010; Bernardino de la Serna et al. 2013).

The information gathered using LAURDAN and TPE fluorescence microscopy has also aided in the characterization of other specialized phase separated membranes, such as those containing lipopolysaccharides, cerebrosides and their mixtures with glycerophospholipids and cholesterol (Fidorra et al. 2006, 2009; Sot et al. 2006; Kubiak et al. 2011). Additionally, the LAURDAN GP imaging approach has been also successfully applied to the study lateral heterogeneity in model systems composed of specialized innate membranes such as skin stratum

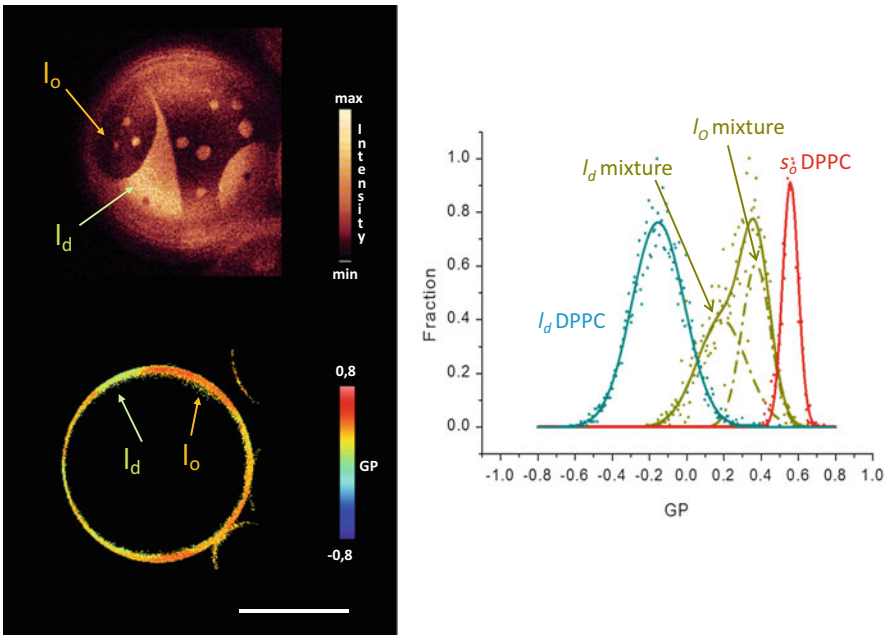


Fig. 5.6 *Left panel*: LAURDAN two photon excitation fluorescence intensity image (taken at the polar region of the vesicle) and LAURDAN GP image (taken at the equatorial region of the same vesicle) of a GUV composed of DOPC/Cholesterol/Sphingomyelin 1:1:1 mol displaying l_o/l_d phase coexistence. The *bar* corresponds to 20 μm. *Right panel*: GP distribution measured from the DOPC/Cholesterol/Sphingomyelin GP image described above. Notice that the GP distribution for this mixture is bimodal (two phases are present) and the GP center value for the l_o phase (0.4) is higher compared to the l_d phase (0.2). Additionally, the GP center values for the coexisting l_o/l_d phases are intermediate between those observed for DPPC GUVs displaying s_o and l_d phases (Adapted from (Dietrich et al. 2001) with permission)

corneum lipid membranes (Plasencia et al. 2007; Norlen et al. 2008), lung surfactant (Nag et al. 2002; Bernardino de la Serna et al. 2009, 2013) and red blood cells (Montes et al. 2007), or to study non-equilibrium phenomena in membranes upon the action of lipases (Sanchez et al. 2002; Stock et al. 2012).

5.4.3 Natural Membranes

In the pioneering work of Yu et al. and Parasassi et al. (Yu et al. 1996; Parasassi et al. 1997), LAURDAN was proposed to be a promising tool in exploring cell membranes. In those studies, domains of sizes below, in the same range and above the microscope resolution limit (0.3 μm radial) were observed in OK cells, red blood cells and brush border native membranes respectively (Yu et al. 1996; Parasassi et al. 1997). The LAURDAN GP differences observed in

compositionally complex mixtures and artificial lipid ternary mixtures containing phospholipids, sphingomyelin and cholesterol (Dietrich et al. 2001) were used to interpret LAURDAN GP images in cellular membranes (Gaus et al. 2003). In this work the LAURDAN GP function was used to directly observe in living macrophages transient micron sized-high GP regions surrounded by low GP areas, supporting the presence of lateral heterogeneity in cellular membranes under *in vivo* conditions. Lately, temporal fluctuations of the GP function have been exploited using LAURDAN to ascertain membrane heterogeneity in rabbit erythrocytes and Chinese hamster ovary cells (Sanchez et al. 2012). This approach that takes into account the amplitude and diffusion relaxation time obtained from autocorrelation analysis of LAURDAN GP fluctuations constitutes a very interesting method to measure transient membrane domains with sizes below the resolution of classical fluorescence microscopy.

Very recently, it was reported that the fluorescence lifetime of LAURDAN (instead of the emission spectra) measured at two different emission wavelengths provided the ability to resolve *in vivo* cellular membranes with different properties such as water and cholesterol content (Golfetto et al. 2013). This last study provided a comprehensive analysis of cell membrane heterogeneity by isolating and analyzing the probe's emission decay during dipolar relaxation. Finally, LAURDAN GP imaging has been also applied to tissues. Sun et al. (2004) have demonstrated that both LAURDAN multiphoton polarization and GP can be combined using a TPE fluorescence microscopy to characterize the structural changes of intercellular lipids in skin tissue. This work showed how treatment with oleic acid increases water dipolar relaxation in skin stratum corneum membranes (Sun et al. 2004). A similar strategy using LAURDAN GP has been reported in order to characterize membranous structures in pig skin (Carrer et al. 2008), perform a comparative evaluation between lipid stratum corneum membranes of normal skin and acquired cholesteatoma (Bloksgaard et al. 2012b) and evaluate the effect of detergents in excised skin (Bloksgaard et al. 2014).

5.5 Conclusions

The use of different 2,6 substituted naphtalenes derivatives (e.g. LAURDAN, PRODAN, PATMAN) allow the study of important structural and dynamical aspects of membranes. The accessibility of bulk information from fluorescence "cuvette" trials together with spatially resolved information from fluorescence microscopy experiments have been important to better describe membrane lateral heterogeneity. For example using LAURDAN, the combination of these two strategies allowed to link membrane related phenomena from simple compositionally situations (observed in distinct model membranes) to those occurring in more complex compositional scenarios such as biological membranes existing in cells and tissues. Particularly, spatially resolved information has been essential to disentangle different supramolecular processes occurring in these systems. Although most of the

reported applications focus on membrane lateral packing information, the exquisite sensitivity of these probes to membrane hydration should be exploited in more detail. For example, heterogeneity reported for these probes when inserted in natural membrane systems could be interpreted in terms of distinct "structured water domains" (Bloksgaard et al. 2012a; Almaleck et al. 2013), not exclusively related to lipid packing, but to the combined ability of different membrane constituents to generate areas of different water content and dynamics.(* N.of E: see Chap. 4 by Pfeiffer, Chap. 6 by Arzov and Chap. 7 by Alarcon et al. Appignanessi)

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