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A convenient and versatile synthesis of Laurdan-like fluorescent membrane probes: characterization of their fluorescence properties.

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It is now well established that biomembranes show a high degree of lateral organization, with the coexistence of lipid domains showing increased order within a fluid phase. Environment-sensitive fluorescent probes such as Laurdan have played an important role in the study of membrane lipids properties. However, it has been shown that, despite its interesting spectroscopic features -including efficient two-photon excitation in the visible range-, Laurdan can be difficult to use for investigating cellular membranes and its capacity to accurately document membrane properties has been challenged. We present here a simple and versatile two-step synthetic scheme that readily leads to Laurdan analogs differing by their polar head. We prepared a small library in order to evaluate the influence of the polar headgroup on the probe's ability to monitor membrane properties. The spectroscopic properties of the probes dissolved in different organic solvents or inserted into liposomes made of synthetic as well as natural phospholipids at different temperatures are described. Comparison of the fluorescence properties of existing Laurdan and C-Laurdan with the newly synthesized fluorescent probes indicates that the structure of the electron-donating amino group is a key parameter for the development of better-performing probes, especially in membranes containing negatively charged phospholipids.

Introduction

The lipids constituents of cell membranes show considerable structural diversity, yet the functional consequences of this diversity are not well understood. It has been shown that lipids are not randomly and passively distributed within the membrane, but that dynamic micro-domains exist within more freely dispersed liquid crystalline molecules [1-3]. The existence of membrane patches, enriched in cholesterol and sphingolipids, which are resistant to solubilization with Triton X100, have led to the concept of lipid rafts [4]. Lipid rafts, or raft-like structures, formed by the lateral organization of lipids, have been shown to be a binding site for many proteins, peptides and drugs [5-8] and are involved in a number of cellular functions, such as signal transduction, lipid trafficking, and membrane protein activity. Membrane microdomains also exist in mitochondrial membranes as well as other organelles [9-11]. However, due to their nanometric size [12], the visualization of such lipid domains in living cells or in mimetic bilayer systems remains a challenge [13-20].

Fluorescent organic probes have long played a prominent role in the study of biological membranes, among them Laurdan (2,2-dimethylamino-6-lauroylnaphthalene). Laurdan is a non-polar fluorescent probe which has been used in a large number of studies because its fluorescence properties are very sensitive to the extent of water penetration into cell membrane[21]. Its spectroscopic properties have been studied extensively[21-27]. On one hand, the presence of a dimethylamino electron-donating group and of a carbonyl electron-withdrawing group separated by a naphthalene nucleus confers an intramolecular charge transfer character to Laurdan as well as a high fluorescence quantum yield. On the other hand, due to its hydrophobic character, the lauroyl acyl chain allows insertion of the probe within the lipid bilayer. Moreover, due to its high cross-section, Laurdan is also a biphotonic probe that can be used in the visible range [22]. Laurdan's spectral features are sensitive to the polarity of its environment and to the packing of lipid molecules, and Laurdan thus provides insight into the membrane's physical state [24]. During the transition from gel to crystal-liquid phase, Laurdan's emission spectrum is red-shifted from 440 nm to 490 nm. Laurdan's position within the membrane has been studied experimentally as well as by numerical simulation, and is reported to be located at the hydrophilic-hydrophobic interface of the bilayer [23, 28, 29]. Its fluorescent properties were shown to be primarily linked to the mobility of water

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molecules within the vicinity of the excited naphthalene moiety [21, 22, 28, 29]. In the specific environment of the interface, water molecule relaxation occurs on a nanosecond time scale clearly different from that in the surrounding solvent [29]. Additionally, probe conformational heterogeneity, which in turn can affect solvent H-bonding, also plays a role [27].

Laurdan's spectroscopic properties have thus made it a common probe for fluorescence microscopy [14]. However, the interpretation of Laurdan's spectroscopic features in reporting membrane state has recently been challenged. Based on images of DMPC and DOPC giant liposomes obtained using two-photon excitation with polarized light, it has been suggested that Laurdan's distribution within the membrane could be more heterogeneous than originally thought [17]. They showed that adding a single carboxyl group improves its position within the membrane as their new probe, called C-Laurdan (6-dodecanoyl-2-[*N*-methyl-*N*-(carboxymethyl)-amino]naphthalene, see structure in scheme 1), remained aligned parallel to the lipid molecules because of favorable hydrophilic interactions with water molecules near the lipid head-groups [17]. Moreover, increasing its water solubility facilitates the use of this new probe as a cellular membranes staining reagent. C-Laurdan has since been used to document plasma membrane heterogeneity [17, 30, 31]

However, C-Laurdan possesses a carboxylic group which dissociates with a pK_a 6.6, it will therefore display an average negative charge that will affect its distribution within a membrane containing charged phospholipids. Organized membrane structures are also likely to exist in cellular organelles such as the Golgi [32] or mitochondria [33, 34]. However, the inner mitochondrial membrane contains a high proportion of anionic phospholipids such as cardiolipin [35], especially near contact sites [36]; a negatively charged probe is therefore likely to present a biased distribution when inserted into such membranes. Moreover, at pH values around neutrality both protonated and unprotonated forms of the molecule are accessible and are likely to probe different microenvironments in the lipid bilayer. C-Laurdan fluorescence yield is strongly dependent on the pH [17], local variations of which will therefore affect fluorescence intensity, thus complicating the analysis of the physical state of the membrane.

New environment-sensitive fluorescent probes are therefore still needed [20]. The published synthesis of C-Laurdan [17] requires the use of amines at high temperatures and pressures on a non-commercial phenol precursor, thus limiting the range of available derivatives, as well as access to C-Laurdan itself. As the substitution on the amine nitrogen is a key parameter in controlling the orientation and distribution of the probe within the biological membrane, we devised a synthetic scheme that can lead to other Laurdan derivatives that retain the improvement brought by C-Laurdan but without the added charge.

We report herein the synthesis of a family of 6-acyl-2-aminonaphthalene-derivatives *via* an improved, two-step sequence including a Buchwald – Hartwig coupling that allows the synthesis of a large variety of aryl amines [37, 38], supplanting the drastic methods required by the previous C-Laurdan synthesis [17] and increasing the range of possible C–N bond formations. Our straightforward and versatile synthesis can be used to produce probes differing by the structure of their polar head groups, in order to test their effect on the insertion of the probe into membranes and on their capacity to monitor variations in lipid state.

A preliminary study of the fluorescence properties of probes has been performed using different solvents as well as liposomes made of synthetic or natural phospholipids, including phosphatidyl choline / phosphatidyl ethanolamine / cardiolipin (PC/PE/CL) liposomes that are commonly used to mimic the mitochondrial inner membrane [33, 39]. As expected based on their similarity to Laurdan, our new probes show a shift in emission wavelength when inserted into lipids that are in the gel vs the liquid-crystalline phase.

Our results indicate that if these molecules maintain the same major features in liposomes made of synthetic phospholipids, the nature of the extremity of the molecule pointing out of the membrane has small but noticeable effects on the fluorescence properties of the molecules. It is difficult at this stage to rationalize, much less predict, the behavior of such fluorescent probes when inserted into membranes. Providing an easy access to libraries of Laurdan derivatives will prove useful in addressing empirically the effect of the polar group on the insertion of the probes into membranes and on their capacity to monitor subtle variations in lipid state.

Our measurements also show for example that C-Laurdan properties are not optimal for vesicles made of natural phospholipids with a large fraction of negatively charged molecules, such as those observed in mitochondrial membranes and that our new probes ECL (Ethyl 2[(6-dodecanoylnaphthalen-2-yl)methyl)amino]acetate) and AADAL (1-(6-((2,2-diethoxyethyl)amino)naphthalen-2-yl)dodecan-1-one) appear to be more useful for these membranes.

Results and discussion

Synthesis of Laurdan-derived probes

The synthesis is presented in Scheme 1 and details on this procedure are given in the experimental section. In the first step, 2-bromo-6-lauroylnaphthalene (compound 2) was obtained by acylation of 6-bromonaphthalene through a Friedel-Crafts reaction [40]. Next, the halogen moiety was substituted with sarcosine ethyl ester (ethyl N-methylglycinate) through a palladium-catalyzed Buchwald-Hartwig reaction under conditions used previously in our group [37, 38, 41]. These reactions lead to formation of ethyl 2-[(6-dodecanoylnaphthalen-2-yl) methyl)amino]acetate (ECL: compound 3) (Scheme 1). ECL also bears a more polar headgroup than Laurdan but without C-Laurdan's negative charge. Saponification of the ethyl ester further provides a convenient access to C-Laurdan. We then turned our attention to the preparation of a small library of other Laurdan

analogues, as a large number of amines of varying size and properties can be used to introduce the electron-donor moiety. We observed that the Buchwald - Hartwig reaction tolerated different functional groups on the amine, such as esters, alkenes or acetals (Table 1, compounds 3 to 6). A range of probes was thus obtained in moderate-to-good yields (52 to 79%).

We compared fluorescence properties of the new probes with those of Laurdan and C-Laurdan, the measurements being performed in liposomes made of pure synthetic or natural phospholipids. The spectral shift is commonly described by way of the Generalized Polarization (GP) parameter which provides a quantitative index of membrane fluidity [22, 24, 42]. This parameter is calculated as the difference between fluorescence emission intensity at a wavelength on the blue edge of the band observed in lipid with gel properties and the intensity at a wavelength on the red side of the band evidenced in lipids with crystal liquid properties divided by the sum of these two intensities. The GP parameter was thus calculated from fluorescence emission intensities according to the following formula:

$$GP = \frac{I_{gel} - I_{LC}}{I_{gel} + I_{LC}}$$

Where I_{gel} and I_{LC} are, respectively, intensities of fluorescence at wavelengths characteristic of probe emission in gel or liquid-crystal phases.

Spectroscopic properties of Laurdan-derived probes in different organic solvents:

We first compared the spectroscopic properties of our new probes to that of Laurdan and C-Laurdan in organic solution, in order to measure the effect of the bulk medium. Laurdan has a large excited state dipole moment, and therefore dipolar relaxation explains the large shift in fluorescence emission wavelength observed when measurements are performed in a range of solvents from apolar to polar-protic. Figure 1A shows the fluorescence emission spectra of Laurdan, C-Laurdan and of our probes, recorded in various solvents at identical 2.6 µM probe concentration with the excitation wavelength set at 360 nm.

For all probes, the maximum emission wavelengths show the same bathochromic effect of solvent polarity. Intensities measured in strongly apolar solvents (cyclohexane and toluene, not shown) are weak but increase notably in going from chloroform to methanol. All probes, except PIPEL, show the same trend indicating that, whatever their polar head, the membrane sensing potential of Laurdan is maintained. Figure 1 also shows that modification of the polar head of these molecules affects fluorescence yield. As already reported by Kim et al [17] C-Laurdan fluorescence intensity is lower than that of Laurdan when measured under the same conditions. When exposed to more polar and protic solvents, most of these probes show a reduced yield, this problem being significant for C-Laurdan. However, in this respect, PIPEL is the most problematic as it shows a very low fluorescence yield in all of the solvents used here that contain oxygen atoms in their structure. As expected for Laurdan and Laurdan-derived probes, the Lippert plots of the probes (Fig 1B) show that the Stokes shift increases with solvent polarity. The similar profiles obtained for the different probes indicate that the nature of their polar extremity does not have major consequences on naphthalene group interaction with surrounding solvent molecules.

GP values calculated for the different probes are presented here (Fig 1C) as a function of solvent orientation polarizability, one of the several scales which have been proposed to quantify solvent effect on spectroscopic properties [43]. All probes show similar profile with a low GP in protic solvents [44], but, due to the differences of the shape of the two emission bands, their GP values will vary within different ranges, suggesting that they will not be strictly equivalent for GP imaging. Molecules that show a high fluorescence yield over a large range of solvent properties, while keeping a polar extremity for a better-defined anchoring at membrane surface [45], will allow GP imaging with greater details thus providing richer information on membrane areas in different states using a similar gain on the different channels. Our results show that the structure of this polar extremity has an impact on the potential imaging properties of the probes.

Fluorescence properties of Laurdan-derived probes in zwitterionic DMPC liposomes at different temperatures:

We then tested the ability of the fluorescent probes to document changes in membrane lipid packing by inserting them into liposomes and measuring the shift in emission wavelength upon temperature-induced phase transition. We first used DMPC liposomes since membranes composed of this synthetic phospholipid, which contains a zwitterionic head group and only fully saturated myristoyl chains, are known to undergo a readily measurable gel-to-liquid phase transition at 23°C [46].

Figure 2 shows the emission spectra measured with these liposomes upon excitation at 360 nm and at temperatures varying from 5 to 55°C. At the lowest temperature used, DMPC membranes are in the gel phase and all probes show emission at a wavelength around 440 nm. When the temperature is increased to values at which DMPC is in a liquid crystalline phase, maximum emission wavelengths are red-shifted by approximately 40 nm. Based on published Laurdan results, [22, 24, 26] one can conclude that these new red-shifted bands corresponds to the fluorescence of probes inserted in liquid-phase lipids. At low temperature, fluorescence is emitted at a wavelength similar to what has been recorded in chloroform or DMSO, while at higher temperatures, at which DMPC is in a liquid crystalline phase, all probes emit fluorescence with an emission wavelength similar to what has been measured with probes dissolved in ethanol (cf figure 1). One can also notice a hypochromic effect on the emission wavelength when probes are inserted into liquid crystalline lipids as opposed to gel

phase. This effect is more pronounced with PIPEL and C-Laurdan than with the other probes, as was to be expected from data obtained in organic solvents.

For each probe, we calculated GP values using fluorescence intensities recorded at wavelengths corresponding to the two maxima observed at 5 and 55°C (see legend of figures 3, 5 and 6). GP values calculated for each probe and plotted as a function of temperature are shown in figure 3. Although actual GP values are dependent on the wavelength chosen to account for gel and liquid phases, and on the fluorescence yield of the two emission bands, one expects higher values for membrane lipids in the gel phase. Upon an increase in temperature, lipids will reach a liquid crystalline state which will be revealed by lower GP values [22]. Each curve shows a sharp transition indicating a Tm of 23°C in accordance with published values [46]. GP variations that can be observed below 20°C and above 30°C could also indicate fluctuations in probe position within lipids within a defined phase. One can also note that, because of the smaller shift of their gel- and liquid-specific fluorescence broad bands, C-Laurdan and AADAL show the smallest difference in their GP variation. It is likely that probes with larger differences, such as ECL and MADAL will allow to extrapolate more detailed information on membrane heterogeneity. PIPEL shows an attractive temperature response of GP, but the much-reduced fluorescence yield in the liquid phase lipids will not be convenient for quantifying changes in the physical state of lipids with low phase transition temperatures such as natural lipids. However, provided its distribution within the membrane is not overly affected by the phase behavior of lipids, it could be useful for staining areas in membranes with gel phase properties, since those areas will emit a strong fluorescence against a darker background for fluid lipids.

Fluorescence properties of Laurdan-derived probes in anionic DMPG liposomes at different temperatures:

Similar measurements were performed with liposomes made from negatively charged phosphatidylglycerol, again bearing saturated myristoyl chains. Such DMPG liposomes are known to show a complex behavior with a phase transition extending over an unusually large temperature range centered around 23°C [47].

Since PIPEL spectroscopic response is clearly different from the others and since MADAL properties are very similar to Laurdan's, this analysis was performed using Laurdan, C-Laurdan, ECL and AADAL. These probes gave qualitatively similar results, which are shown in figure 4. An increase in temperature translates into a clear hypochromic shift of the emission band, accompanied by the emergence of a new red-shifted band which intensity increases as the proportion of lipids in the liquid phase increases. At low temperature, Laurdan's emission wavelength is identical to what was measured with DMPC liposomes (Figure 2), whereas for the other probes emission wavelength is shorter with DMPG than with DMPC. This could indicate that the modifications of the polar substituent of these probes can affect their interaction with the polar head of phospholipids: with negatively charged phospholipids they are more deeply buried into the bilayer. Probably because of its negative charge, this effect is stronger for C-Laurdan (20 nm blue shift) than for the other probes (10 nm). This is consistent with the fact that for the secondary amine AADAL, the liquid-specific emission band shows an intensity similar to that recorded for gel phase lipids.

As previously shown for Laurdan, GP analysis of emission data allows the study of phase transitions (figure 5). For each of the probes tested here, the GP decreases with temperature with an abrupt change between 20 and 35°C. Each of the probes is thus able to monitor the complex behavior of DMPG [47], though ECL seems to give a response that is less dependent on the chemical nature of the lipids than the others.

Fluorescence properties of Laurdan-derived probes in liposomes prepared using natural phospholipids:

Similar measurements were performed with liposomes made from a mixture of natural phospholipids (phosphatidylcholine : phosphatidylethanolamine : cardiolipine, PC:PE:CL, 2:1:1, which is commonly used to mimic the inner mitochondrial membrane [18, 33, 39]. Figure 6 shows the variations of the GP parameters recorded with such a complex mixture for temperatures ranging from 5 to 55°C. These variations indicate that, although natural lipids containing unsaturated acyl chains have low transition temperatures and are in the liquid phase over the temperature range of this study, Laurdan-derived probes can sense modifications of the arrangement of lipids that occur upon increasing the temperature. The different shape of the profiles, however, may indicate that temperature and lipid movement can also affect the position of the probes within the membrane, which in turn depends on the structure of lipids polar head. Presumably, due to its negative charge C-Laurdan appears not able to sense these changes while its ethyl ester derivatives ECL and the secondary amine AADAL give responses comparable to that of Laurdan and are therefore probably better probes of the characteristics of membrane containing a large fraction of negatively charged phospholipids such as mitochondrial ones. Time-resolved fluorescence measurements will be needed for further characterization of the probes properties [48]. In particular these measurements will allow quantification of the extent of lipid/water and ordered/disordered partition for the different probes[49].

Experimental

General

All reactions were carried out under an argon atmosphere. Solvents were distilled and dried by standard methods. NMR spectra were recorded at 293 K, unless stated otherwise, using a 300 MHz, or a 400 MHz spectrometer. Shifts are referenced relative to deuterated solvent residual peaks. The following abbreviations are used to explain the observed multiplicities: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; t, triplet; td, triplet of doublets; m, multiplet; bs, broad singlet. Low and high resolutions mass spectra were recorded using a Bruker

MicrOTOF-Q II XL spectrometer. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with silica gel 60 F_{254} (Merck). TLC plates were inspected by UV light (λ = 254 nm). Silica gel column chromatography was performed with silica gel Si 60 (40–63 µm).

1-(6-bromonaphthalen-2-yl)dodecan-1-one and 1-(6-chloronaphthalen-2-yl)dodecan-1-one (compound 2):

2-bromonaphthalene (4g, 19.3 mmol) was dissolved in 20 mL of nitrobenzene under an argon atmosphere. The solution was stirred in an ice bath. Aluminium chloride (2.836 g, 21.23 mmol, 1.1 equiv.) was added by portions and lauroyl chloride (5.036 mL, 23.16 mmol, 1.2 equiv.) was added dropwise. The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was quenched with distilled water and extracted three times with ethyl acetate. The organic phases were combined, washed with brine, dried over sodium sulfate and concentrated under reduced pressure. The product was purified by crystallization from cold ethanol to obtain a white powder; yield 4.215 g (56%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J=6.6 Hz, CH_3) ; 1.26-1.37 (m, 16H, CH_2) ; 1.79 (m, 2H, CH_2) ; 3.07 (t, 2H, J=7.5 Hz, CH_2CO) ; 7.62 (d, 1H, J=8.7 Hz, H ar) ; 7.81 (m, 2H, H ar) ; 8.05 (m, 2H, H ar) ; 8.42 (s, 1H, H ar). ^{13}C NMR: δ = 14.1 ; 22.7 ; 24.5 ; 29.4 ; 29.5 ; 29.6 ; 29.7 ; 31.9 ; 38.8 ; 122.7 ; 125.2 ; 127.5 ; 129.4 ; 129.9 ; 130.2 ; 131.0 ; 134.8 ; 136.4 ; 200.2. HRMS: m/z (MH^+) $C_{22}H_{30}BrO$ calculated: 389.1475, found: 389.1471.

2-chloronaphthalene (1g, 6.1 mmol) was dissolved in 6 mL of nitrobenzene under an Argon atmosphere. The solution was stirred in an ice bath. Lauroylchloride (1.7 mL, 7.32 mmol, 1.2 equiv.) was added drop wise. The reaction mixture was treated with aluminium chloride (854 mg, 6.71 mmol, 1.1 equiv.) and stirred at room temperature for at least 18 hours. The reaction mixture was quenched with distilled water and extracted three times with ethylacetate. The organic phases were combined, washed with brine, dried over sodium sulphate and concentrated under reduced pressure. The product was purified by crystallization from cold ethanol to obtain a yellow powder; yield 1.06 g (50%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J=6.9 Hz, CH_3) ; 1.26-1.41 (m, 16H, CH_2) ; 1.79 (m, 2H, CH_2) ; 3.07 (t, 2H, J=7.5 Hz, CH_2CO) ; 7.49 (dd, 1H, J=8.7 Hz, J=2.1 Hz, H ar) ; 7.79-7.91 (m, 3H, H ar) ; 8.05 (dd, 1H, J= 8.7 Hz, J=1.8 Hz, H ar) ; 8.43 (br s, 1H, H ar). ^{13}C NMR: δ = 14.1 ; 22.7 ; 24.5 ; 29.3 ; 29.4 ; 29.5 ; 29.6 ; 31.9 ; 38.7 ; 125.1 ; 126.6 ; 127.6 ; 127.7 ; 129.3 ; 130.8 ; 131.0 ; 134.3 ; 134.6 ; 136.0 ; 200.2. MS: m/z (MNa^+) = 367.2; HRMS: m/z (MH^+) $C_{22}H_{30}ClO$ calculated: 345.1980, measured: 345.1973

Ethyl 2[(6-dodecanoylnaphthalen-2-yl)methyl)amino]acetate (ECL, compound 3):

An oven-dried Schlenk flask was evacuated and backfilled with argon. The flask was charged with 1-(6-bromonaphthalen-2-yl)dodecan-1-one (1 g, 2.57 mmol), $Pd(OAc)_2$ (46 mg, 206 µmol, 0.08 equiv.), X-Phos (196 mg, 411µmol, 0.16 equiv.), cesium carbonate (3.35 g, 10.28 mmol, 4 equiv.), and sarcosine ethyl ester (790 mg, 5.14 mmol, 2 equiv). The Schlenk flask was capped with a rubber septum, then evacuated and backfilled with argon three times. Anhydrous dioxane (7.5 mL) was added through the septum. The mixture was heated to 100°C in an oil bath and was stirred for 18 hours. The mixture was cooled to room temperature, diluted with ethyl acetate, filtered through Celite, and concentrated under reduced pressure to obtain a yellow solid. The crude product was purified by flash chromatography on silica gel eluting with ethyl acetate/petroleum ether (10/90) to afford a yellow powder (569 mg, yield 52%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J=7.2 Hz, CH_3) ; 1.21-1.36 (m, 16H, CH_2) ; 1.76 (m, 2H, CH_2) ; 3.00 (t, 2H, J=7.5 Hz, CH_2CO) ; 3.16 (s, 3H, CH_3N) ; 4.16 (s, 2H, $COCH_2N$) ; 4.18 (q, 2H, J=7.2 Hz, CH_3CH_2O) ; 6.86 (d, 1H, J=2.4 Hz, H ar) ; 7.06 (dd, 1H, J=9.3 Hz, J=2.7 Hz, H ar) ; 7.62 (d, 1H, J=8.7 Hz, H ar) ; 7.78 (d, 1H, J=9.0 Hz, H ar) ; 7.92 (dd, 1H, J=8.7 Hz, J=1.8 Hz, H ar) ; 8.31 (br s, 1H, H ar). ^{13}C NMR: δ = 14.1 ; 14.2 ; 22.7 ; 24.8 ; 29.3 ; 29.5 ; 29.6 ; 29.7 ; 31.9 ; 38.4 ; 39.8 ; 54.4 ; 61.2 ; 106.0 ; 114.6 ; 115.7 ; 124.7 ; 125.6 ; 126.4 ; 129.6 ; 130.8 ; 131.1 ; 137.4 ; 148.6 ; 170.4 ; 200.2. MS: m/z (MH^+) = 426.3. HRMS: m/z (MH^+) $C_{27}H_{40}NO_3$ calculated: 426.3003, found: 426.2988.

6-dodecanoyl-2-[N-methyl-N-(carboxymethyl)amino]naphthalene (C-Laurdan):

211 mg of ethyl 2[(6-dodecanoylnaphthalen-2-yl)methyl)amino]acetate was dissolved in 7 mL of a freshly prepared solution of potassium hydroxide (0.25M) in ethanol. The reaction mixture was stirred for 2 days at room temperature. The solution was acidified with HCl (1M) until pH 1 and was extracted with ethyl acetate (3×20 mL). The ethyl acetate layers were combined, dried over magnesium sulfate and concentrated under reduced pressure to obtain a brown solid which was purified by crystallization from chloroform/petroleum ether to provide 164 mg (83% yield) of C-Laurdan. ^{1}H and ^{13}C NMR and HRMS data of the product are identical with the published data [17].

1-(6-N-methylpiperazinonaphthalen-2-yl)dodecan-1-one (PIPEL, compound 4):

An oven-dried Schlenk flask was evacuated and backfilled with argon. The flask was charged with 1-(6-chloronaphthalen-2-yl)dodecan-1-one (0.2 g, 0.58 mmol), $Pd(OAc)_2$ (10 mg, 46 µmol, 0.08 equiv.), X-Phos (44 mg, 93µmol, 0.16 equiv.), tBuOK (196 mg, 10.28 mmol, 3 equiv.), and N-methylpiperazine (84 µL, 0.76 mmol, 1.3 equiv). The Schlenk flask was capped with a rubber septum, then evacuated and backfilled with argon three times. Anhydrous dioxane (1.5 mL) was added through the septum. The mixture was heated to 100°C in an oil bath and was stirred for 18 hours. The mixture was cooled to room temperature, diluted with ethyl acetate, filtered through Celite, and concentrated under reduced pressure to obtain a yellow solid. The crude product was purified by flash chromatography on silica gel eluting with ethyl acetate/petroleum ether (50/50) to afford a pale yellow oil (186 mg, yield 78%). ^{1}H RMN (300 MHz, $CDCl_3$): δ = 0.87 (t, 3H, J=6.9 Hz, CH_3) ; 1.22-1.29 (m, 16H, CH_2) ; 1.77 (m, 2H, CH_2) ; 2.31 (s, 3H, NCH_3) ; 2.66 (t, 4H, J=4.8 Hz, CH_2N) ; 3.04 (t, 2H, J=7.5 Hz, CH_2CO) ; 3.41 (t, 4H, J=4.8 Hz, CH_2N) ; 7.09 (d, 1H, J=2.1 Hz, H ar) ; 7.30 (dd, 1H, J=9.0 Hz, J=2.1 Hz, H ar) ; 7.68 (d, 1H, J=8.7 Hz, H ar) ; 7.82 (d, 1H, J=9.0 Hz, H ar) ; 7.95 (dd, 1H, J= 8.7 Hz, J=1.8 Hz, H ar) ; 8.34 (br s, 1H, H ar).. ^{13}C RMN: δ = 14.1 ; 22.6 ; 24.7 ; 29.3 ; 29.4 ; 29.5 ; 29.6 ; 31.9 ; 38.4 ; 46.1 ; 48.5 , 54.9 ; 109.1 ; 119.1 ; 120.2 ; 124.6 ; 126.8 ; 126.9 ; 129.4 ; 130.5 ; 132.0 ; 137.1 ; 150.8 ; 200.2. MS: m/z (MH^+) = 409.3. HRMS: m/z (MH^+) $C_{27}H_{41}N_2O$ calculated: 409.3213, found: 409.3206.

1-(6-((2,2-dimethoxyethyl)(methyl)amino)naphthalen-2-yl)dodecan-1-one (MADAL, compound 5):

An oven-dried Schlenk flask was evacuated and backfilled with argon. The flask was charged with 1-(6-chloronaphthalen-2-yl)dodecan-1-one (200 mg, 0.58 mmol), Pd(OAc)2 (10.4 mg, 46 µmol, 0.08 equiv.), X-Phos (44.3 mg, 93 µmol, 0.16 equiv.), tBuOK (196 mg, 1.75 mmol, 3 equiv.), and 2,2-dimethoxy-N-methylethan-1-amine (97 µL, 0.75 mmol, 1.3 equiv). The Schlenk flask was capped with a rubber septum, then evacuated and backfilled with argon three times. Anhydrous dioxane (1.5 mL) was added

through the septum. The mixture was heated to 100°C in an oil bath and was stirred for 18 hours. The mixture was cooled to room temperature, diluted with ethyl acetate, filtered through Celite, and concentrated under reduced pressure to obtain a yellow solid. The crude product was purified by flash chromatography on silica gel eluting with ethyl acetate/petroleum ether (10/90) to afford an oil (171 mg, yield 69%). ^{1}H RMN (300 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J=6.9 Hz, CH_3) ; 1.21-1.36 (m, 16H, CH_2) ; 1.77 (m, 2H, CH_2) ; 3.03 (t, 2H, J=7.5 Hz, CH_2CO) ; 3.15 (s, 3H, CH_3N) ; 3.42 (s, 6H, CH_3O) ; 3.60 (d, 2H, J=5.1 Hz, CH_2N) ; 4.56 (t, 1H, J=5.1 Hz, CH) ; 6.91 (br s, 1H, H ar) ; 7.20 (dd, 1H, J= 9.0 Hz, J=2.4 Hz, H ar) ; 7.63 (d, 1H, J=8.7 Hz, H ar) ; 7.80 (d, 1H, J=9.0 Hz, H ar) ; 7.93 (dd, 1H, J= 8.7 Hz, J=1.5 Hz, H ar) ; 8.32 (br s, 1H, H ar). ^{13}C RMN: δ = 14.1 ; 22.7 ; 24.9 ; 27.4 ; 27.6 ; 29.7 ; 31.9 ; 38.4 ; 39.6 ; 54.8 , 103.2 ; 105.3 ; 116 ; 124.8 ; 126.2 ; 129.8 ; 130.8 ; 130.9 ; 137.6 ; 200.2. MS: m/z (MH+) = 428.1. HRMS: m/z (MH+) $C_{27}H_{42}NO_3$ calculated: 428.3159, found: 428.3159.

1-(6-((2,2-diethoxyethyl)amino)naphthalen-2-yl)dodecan-1-one (AADAL, compound 6):

An oven-dried Schlenk flask was evacuated and backfilled with argon. The flask was charged with 1-(6-chloronaphthalen-2-yl)dodecan-1-one (200 mg, 0.58 mmol), $Pd(OAc)_2$ (10.4 mg, 46 µmol, 0.08 equiv.), X-Phos (44.3 mg, 93 µmol, 0.16 equiv.), tBuOK (196 mg, 1.75 mmol, 3 equiv.), and 2,2-diethoxyethan-1-amine (110 µL, 0.75 mmol, 1.3 equiv). The Schlenk flask was capped with a rubber septum, then evacuated and backfilled with argon three times. Anhydrous dioxane (1.5 mL) was added through the septum. The mixture was heated to 100°C in an oil bath and was stirred for 18 hours. The mixture was cooled to room temperature, diluted with ethyl acetate, filtered through Celite, and concentrated under reduced pressure to obtain a yellow solid. The crude product was purified by flash chromatography on silica gel eluting with ethyl acetate/petroleum ether (15/85) to afford a yellow solid (193 mg, yield 75%). ^{1}H RMN (300 MHz, $CDCl_3$): δ = 0.87 (t, 3H, J=6.9 Hz, CH_3) ; 1.23-1.36 (m, 22H, CH_2 + 2 CH_3) ; 1.77 (m, 2H, CH_2) ; 3.02 (t, 2H, J=7.5 Hz, CH_2CO) ; 3.38 (d, 2H, J=5.4 Hz, CH_2N) ; 3.58 (m, 2H, CH_2O) ; 3.76 (m, 2H, CH_2O) ; 4.20 (br s, 1H, NH) ; 4.75 (t, 1H, J=5.4 Hz, CH) ; 6.81 (d, 1H, J=1.8 Hz, H ar) ; 6.94 (dd, 1H, J= 9 Hz, J=2.4 Hz, H ar); 7.60 (d, 1H, J=8.7 Hz, H ar) ; 7.71 (d, 1H, J=8.7 Hz, H ar) ; 7.93 (dd, 1H, J= 8.7 Hz, J=1.8 Hz, H ar) ; 8.30 (br s, 1H, H ar). ^{13}C RMN: δ = 14.1 ; 15.3 ; 22.6 ; 24.8 ; 29.3 ; 29.4 ; 29.5 ; 29.6 ; 31.9 ; 38.3 ; 46.0 ; 62.4 , 100.5 ; 104.1 ; 118.6 ; 124.8 ; 125.9 ; 126.2 ; 129.7 ; 130.7 ; 130.9 ; 137.7 ; 147.6 ; 200.1. HRMS: m/z (MH+) $C_{28}H_{44}NO_3$ calculated: 442.3316, found: 442.3317.

Preparation of LUVs

Different liposomes were prepared from DMPC, DMPG, DMPC-DMPG (3:2 molar ratio), PC, and PC-PE-CL (2:1:1 molar ratio) as described in [50].

Spectroscopic measurements

Probes features in organic solvents

Absorption spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer.

Fluorescence measurements (excitation and emission spectra) were performed with a Hitachi F4500 fluorometer. Excitation and emission slits were fixed to 5 nm and spectra were recorded at 25°C using a 1 cm path length thermostated quartz micro-cuvette.

0.26 mM solutions of the probes were prepared in a dichloromethane: methanol mixture (3:1 volume ratio). Measurements were performed with 2.6 µM probes diluted in the different solvents. The initial solvent of the stock solution thus represents 1% of the final volume. The GP parameter [22] being the ratio of emission intensities recorded at 2 wavelengths from the same sample, it corrects for variations due, for instance, to probe concentration. GP values calculated from emission spectra recorded at different temperatures in a specific solvent are independent on temperature (not shown).

Probe features in LUVs at different temperatures

Fluorescence data were obtained using a Hitachi F4500 fluorometer. The excitation and emission slits were fixed to 5 nm. Emission spectra were recorded between 420 and 600 nm with excitation wavelength varying between 310 and 420 nm and at several temperatures ranging from 5 to 65°C. Ethanol solutions of fluorescent probes were prepared and added to liposomes to reach a 3 µM final concentration, corresponding to a 1:400 (probe:phospholipid) molar ratio.

For generalized polarization calculations (GP), fluorescence emission intensities recorded and for the gel and liquid phase are given in figures legend.

Conclusions

We present here an original, short, easy to implement, and versatile synthetic approach to Laurdan analogues. A two-step sequence using a Buchwald – Hartwig amination reaction allows us to prepare different 6-acyl-2-aminonaphthalene-derivatives in good yields. The drastic conditions required for the reported C-Laurdan synthesis [17] are not amenable to introducing diversity on the nitrogen, and limit the availability of C-Laurdan itself. thus, we also provide an alternative preparation of C-Laurdan from ECL that can be performed in most laboratories.

We compared fluorescence properties of the new probes with those of Laurdan and C-Laurdan, the measurements being performed in liposomes made of pure synthetic or natural phospholipids. They show that the modifications to the amine group provide molecules that remain sensitive to changes in the lipid physical state. However, their performance under the different conditions tested are not identical, indicating that the structure of the electron-donating amino group is a key parameter for the development of better-performing probes.

Although it is difficult at this stage to rationalize the spectroscopic properties of Laurdan-like probes, the newly synthesized ECL and AADAL probes are able to discriminate subtle modifications of fluidity within a membrane in a disordered state, indicating that engineering the probe interface-exposed moiety will allow for better tools for biological membranes analysis compared to existing fluorescent probes.

In this regard, the versatility of our synthetic scheme allows the synthesis of additional probes to make a wider range of solutions accessible to the scientific community. The spectroscopic characterization of new probes in liposomes of defined composition, coupled with recent improvements in

membrane computer simulation [29, 51], will eventually permit the elaboration of clear rules for the rational design of new environment-sensitive fluorescent molecules that can be used to probe, at a range of depths, the interface between the hydrophobic core and the hydrophilic surface of the membrane. For instance, a bulky polar head may limit distribution of the probe to the external leaflet of the membrane, or the insertion of weakly acidic groups of varying pK_a could allow measurement of local pH near the membrane surface. Since Laurdan and its derivatives are good two-photon fluorescence probes, the development of new molecules which insert with better defined positions within the membrane will be very useful for staining cell membranes in studies of the biological role of membrane structures, and of the changes in their properties that can occur in pathophysiological conditions. Convenient access to Laurdan-like probes is a key step in this process.

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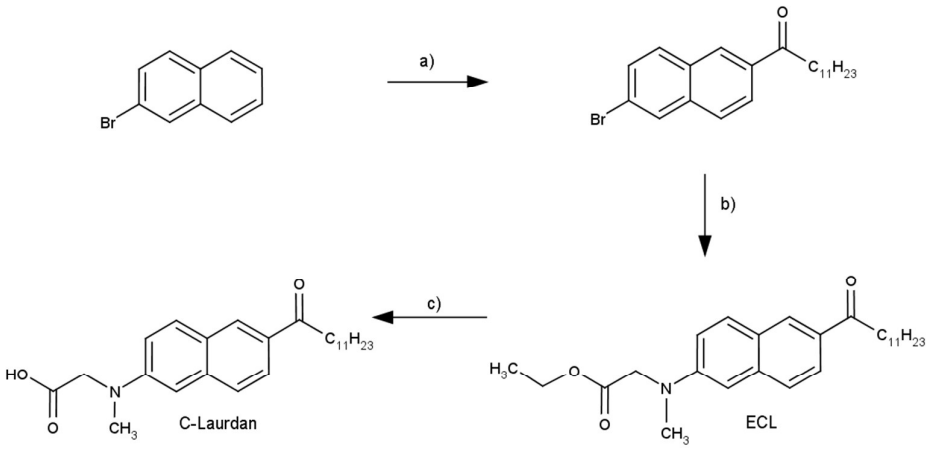


Scheme. 1. Synthetic scheme and conditions for an alternative synthesis of C-Laurdan.
Reagents and conditions of the different steps: a) Lauroyl chloride, aluminium chloride, nitrobenzene, r.t., 18h, 56%. b) $Pd(OAc)_2$, X-Phos, Cs_2CO_3, sarcosine ethyl ester, dioxane, 100°C, 15h, 52%. c) KOH, EtOH, 5h, r.t., 83%

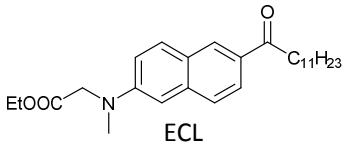
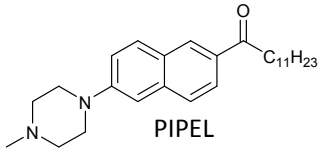
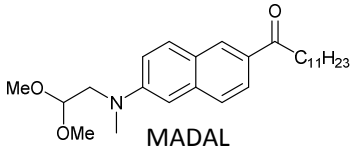
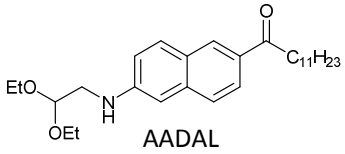
Compound	Structure	Yield
3	 ECL	52
4	 PIPEL	79
5	 MADAL	69
6	 AADAL	75

Table. 1. Synthesis of 6-acyl-2-aminonaphthalene-derivatives. Compound **3** is ECL (Ethyl 2[(6-dodecanoylnaphthalen-2-yl)methyl)amino]acetate); compound **4** is PIPEL (1-(6-N-methylpiperazinonaphthalen-2-yl)dodecan-1-one); compound **5** is MADAL (1-(6-((2,2-dimethoxyethyl)(methyl)amino)naphthalen-2-yl)dodecan-1-one and compound **6** is AADAL (1-(6-((2,2-diethoxyethyl)amino)naphthalen-2-yl)dodecan-1-one.

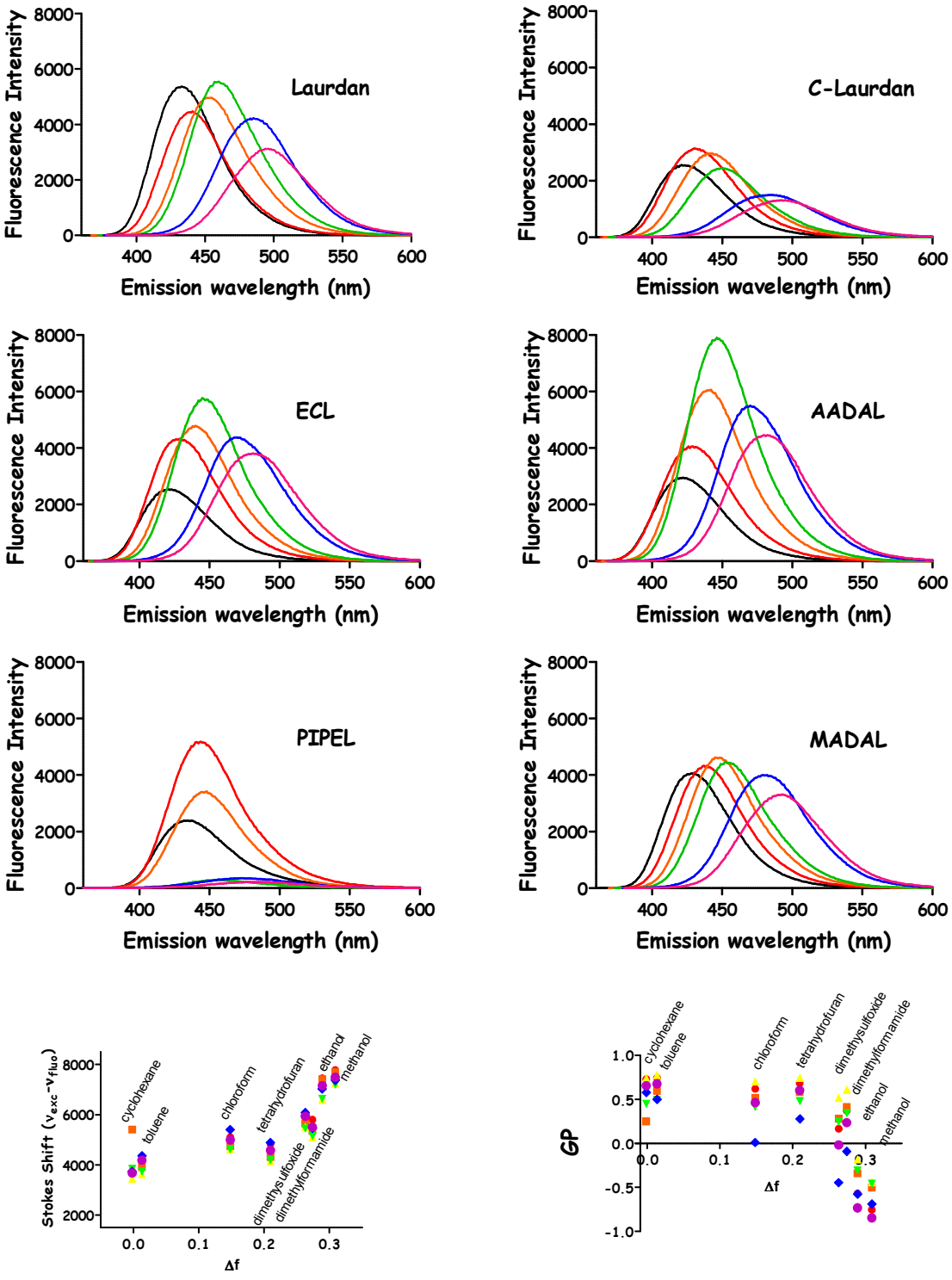


Figure 1. A- Emission spectra recorded for the different probes dissolved in organic solvent with excitation at 360 nm. Color code for traces is as follows: Black: tetrahydrofurane,; red chloroforme; orange: dimethylformamide; green dimethyl sulfoxide; blue: ethanol and pink: methanol. B- Lippert plot (Stokes shift (ν_{exc} - ν_{em}) variations versus orientation polarizability of the solvents) of the diferent dyes. C- GP (Generalized Polarization) versus solvent orientation polarizability.For panels B and C, color codes are: Red: Laurdan, orange: C-Laurdan, yellow: ECL, green: AADAL, blue: PIPEL and violet: MAADAL

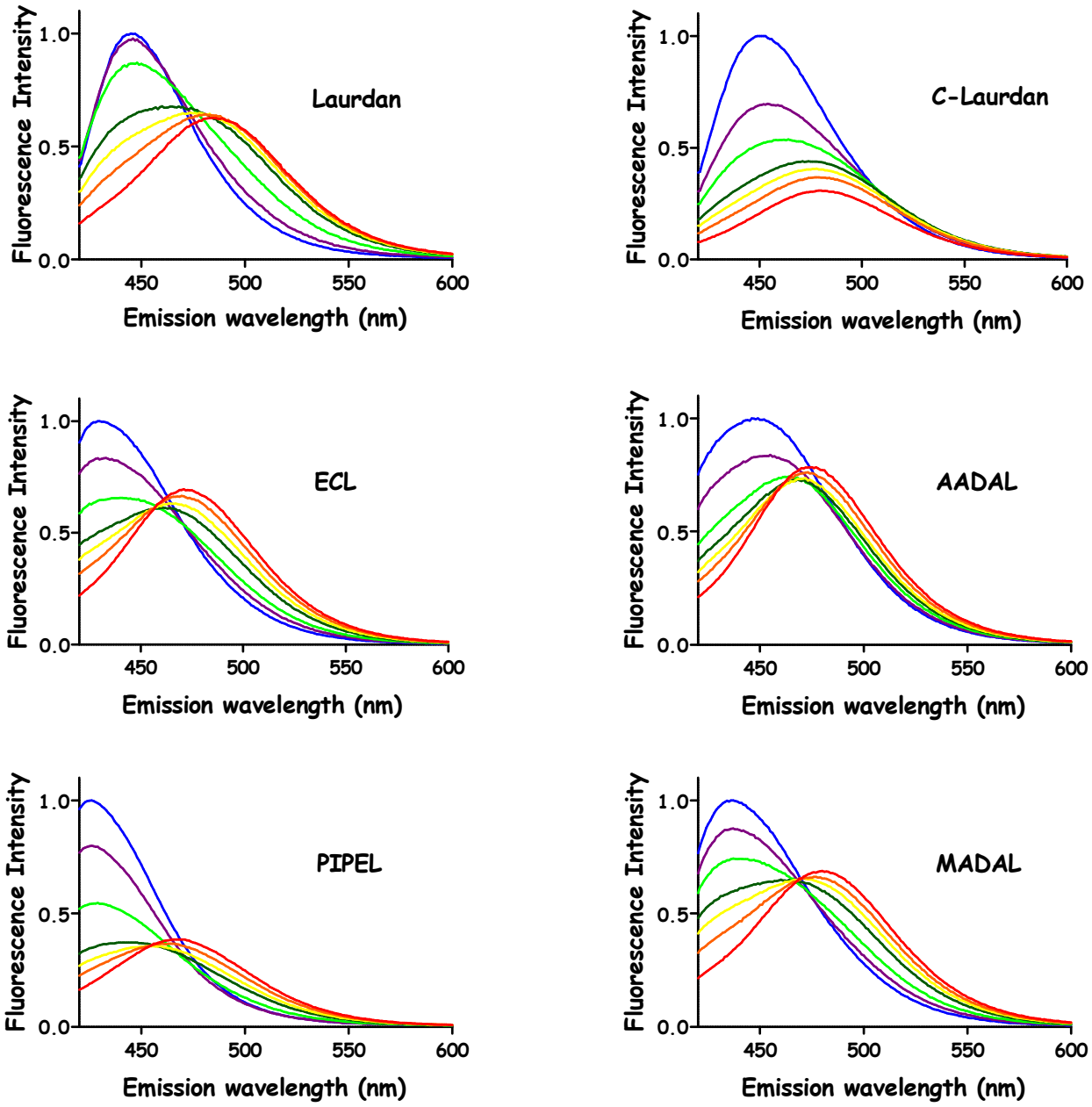


Figure 2. Effect of temperature on fluorescence emission by the different probes inserted into DMPC liposomes. Normalized emission spectra recorded with labeled DMPC liposomes incubated at temperatures varying from 5 to 65°C. Excitation was set at 360 nm. Colour code of the different traces is as follows: blue: 5°C; violet 18°C; green: 23°C; dark green: 28°C; yellow: 33°C; orange: 40°C and red: 55°C.

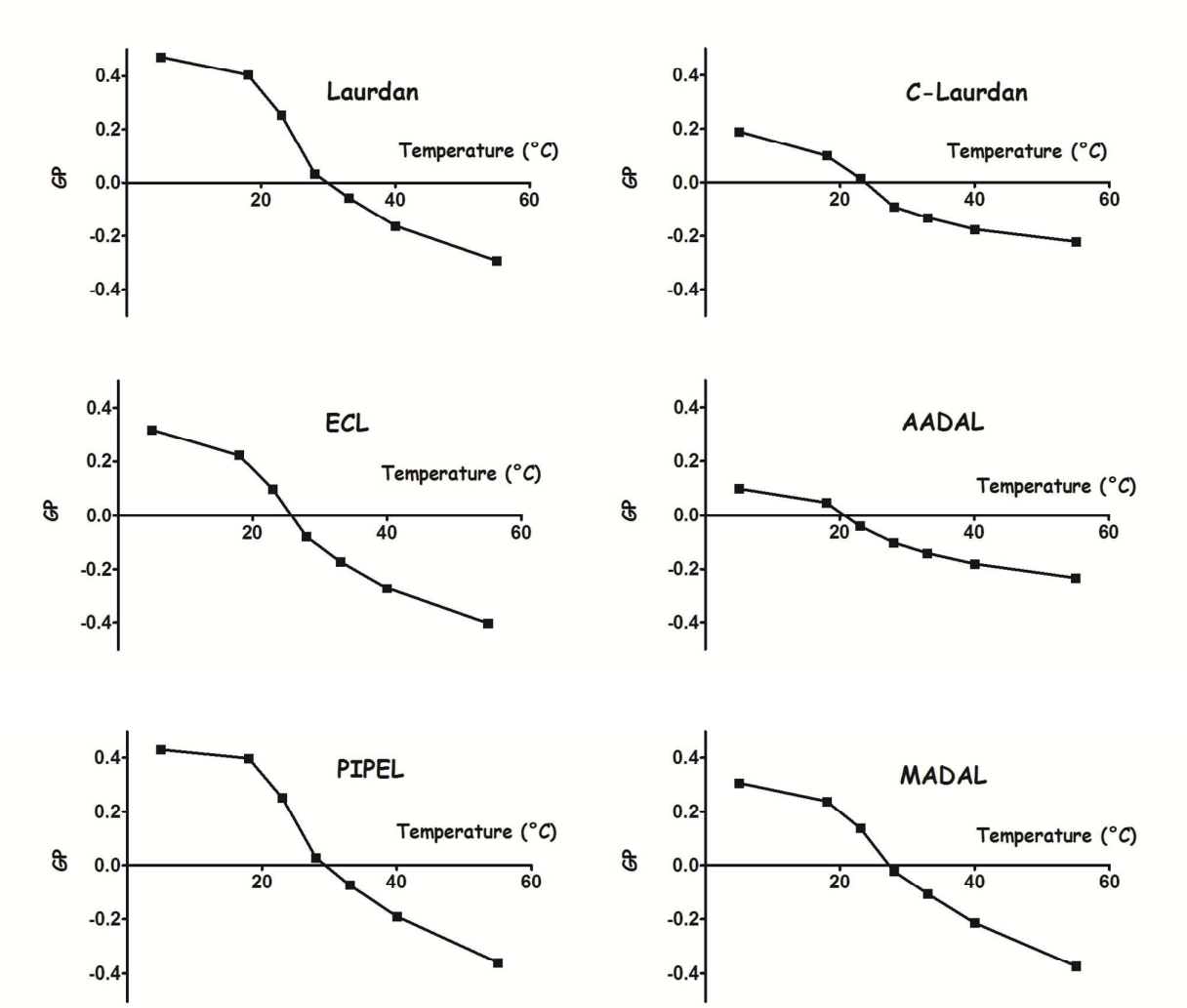


Figure 3 Generalized Polarization of probes inserted in DMPC liposomes as a function of temperature. GP are calculated using emission intensities at the following wavelength: Laurdan 445 and 490 nm; C-Laurdan 448 and 480 nm; ECL: 429 and 472 nm; AADAL: 445 and 472 nm; PIPEL: 424 and 468 nm and MADAL: 434 and 478 nm.

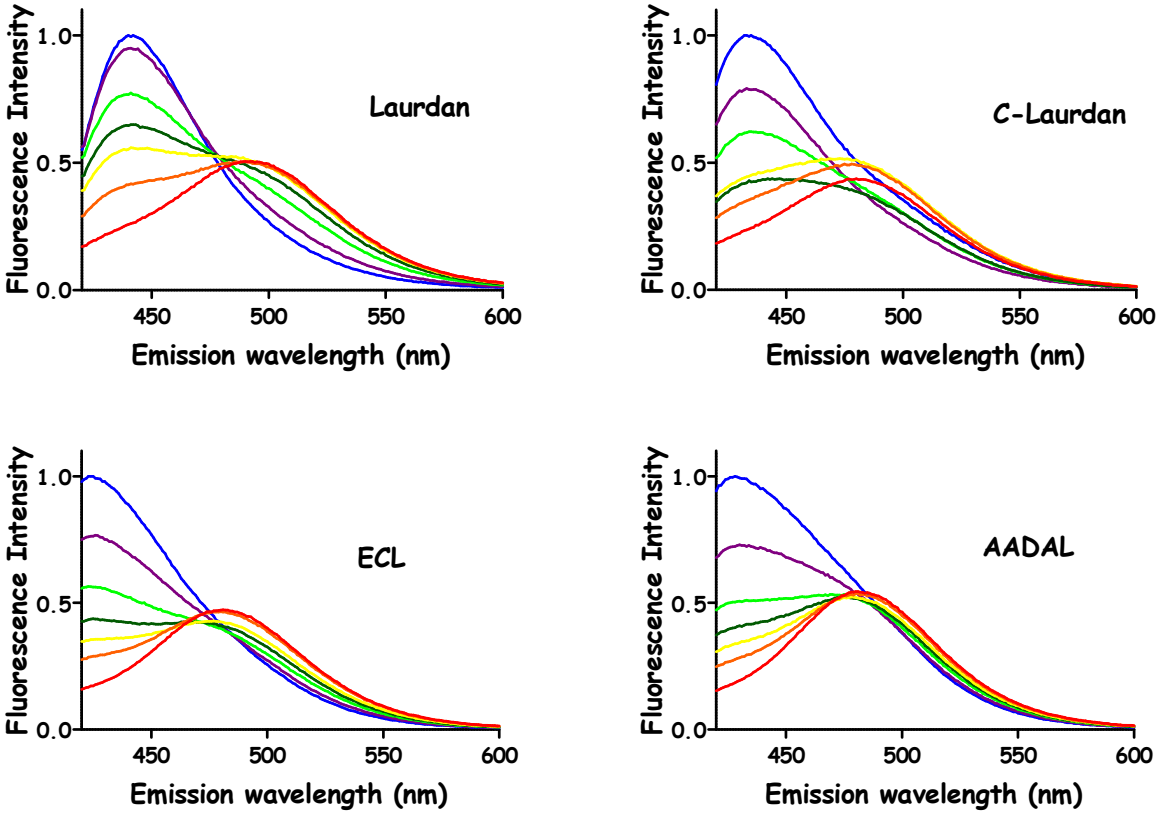


Figure 4. Emission spectra recorded with labeled DMPG liposomes at different temperature.
Normalized emission spectra recorded with Laurdan (a), C-laurdan (b) ECL (c) and AADAL inserted into DMPG liposomes incubated at temperatures varying from 5 to 65°C. Excitation was set at 360 nm. Color code of the different traces is as follows: blue: 5°C; violet 18°C; green: 23°C; dark green: 28°C; yellow: 33°C; orange: 40°C and red: 55°C

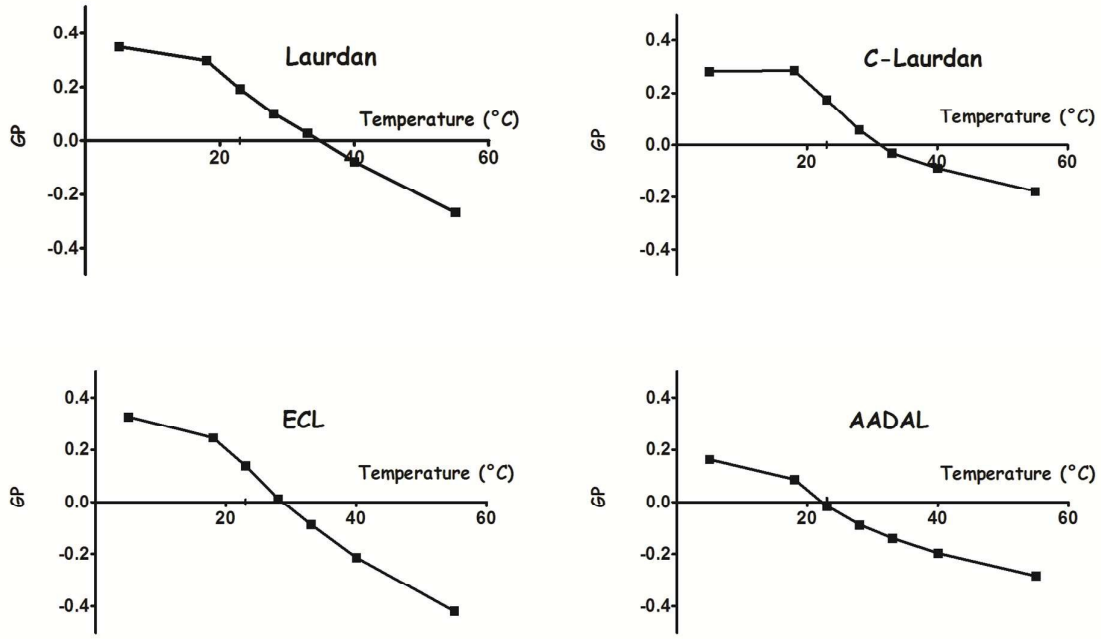


Figure 5 Generalized Polarization of probes inserted in DMPG liposomes as a function of temperature.
GP are calculated using emission intensities at the following wavelength: Laurdan 445 and 490 nm; C-Laurdan 448 and 480 nm; ECL: 429 and 472 nm; AADAL: 445 and 472 nm

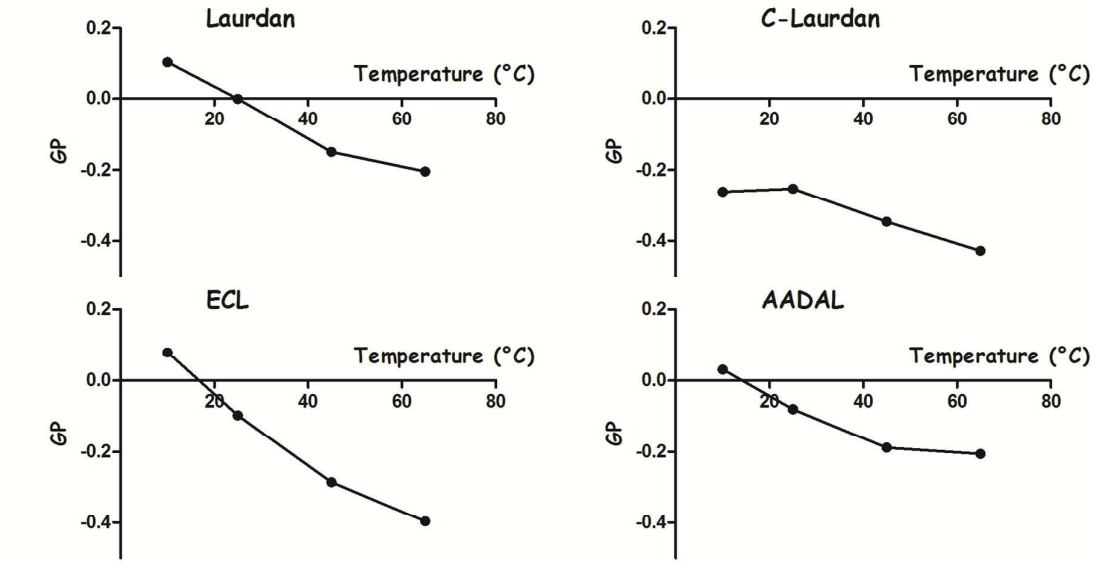


Figure 6 Generalized Polarization of probes inserted in PC PE CL liposomes as a function of temperature. GP are calculated using emission intensities at the following wavelength: Laurdan 445 and 490 nm; C-Laurdan 448 and 480 nm; ECL: 429 and 472 nm; AADAL: 445 and 472 nm

