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Laurdan and di-4-ANEPPDHQ do not respond to membrane-inserted peptides and are good probes for lipid packing

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ABSTRACT

Laurdan and di-4-ANEPPDHQ are used as probes for membrane order, with a blue shift in emission for membranes in liquid-ordered (lo) phase relative to membranes in liquid-disordered (ld) phase. Their use as membrane order probes requires that their spectral shifts are unaffected by membrane proteins, which we have examined by using membrane inserting peptides and large unilamellar vesicles (LUVs). The transmembrane polypeptides, mastoparan and bovine prion protein-derived peptide (bPrPp), were added to LUVs of either lo or ld phase, up to 1:10 peptide/total lipid ratio. The excitation and emission spectra of laurdan and di-4-ANEPPDHQ in both lipid phases were unaltered by peptide addition. The integrity and size distribution of the LUVs upon addition of the polypeptides were determined by dynamic light scattering. The insertion efficiency of the polypeptides into LUVs was determined by measuring their secondary structure by circular dichroism. Mastoparan had an α-helical and bPrPp a β-strand conformation compatible with insertion into the lipid bilayer. Our results suggest that the presence of proteins in biological membranes does not influence the spectra of laurdan and di-4-ANEPPDHQ, supporting that the dyes are appropriate probes for assessing lipid order in cells.

1. Introduction

There is a need to study cells and their components by biophysical methods. For biological membranes, the recognition of this need was intensified by the lipid raft hypothesis, which suggests that membrane domains with different packing efficiency exist in the plasma membrane [1]. However, several reports of membrane domains with different lipid compositions in the plasma membrane as well as internal membranes predate the lipid raft hypothesis [2–5]. Probes like laurdan and di-4-ANEPPDHQ, whose spectral properties alter in response to lipid packing, are frequently used to study membrane domains of different lipid phases in biological membranes. Their utility requires a response solely to lipid phases. The concentration of proteins is very likely to differ between different membrane regions [6], and the responses of laurdan and di-4-ANEPPDHQ have until now not been extensively characterized in the presence of membrane proteins.

In model membranes, two liquid phases have been described – the liquid-ordered (lo) phase and liquid-disordered (ld) phase. Cholesterol is an absolute requirement for the formation of the lo phase, in which the acyl chains of the phospholipids are extended and the fluidity is lower [7], but the difference in the cholesterol fraction between co-existing lo and ld phases is rather small [8,9]. In cells, whose plasma membranes are comprised of thousands of different lipids whose phase behavior is largely uncharacterized, it is better to refer to ordered and disordered domains rather than to use the ld and lo terminology.

Laurdan and di-4-ANEPPDHQ display blue shifts of around 50 nm in their emission peaks for model membranes in lo phase relative to membranes in ld phase [10,11]. The probes operate by the same mechanisms, i.e. sensing the reorientation of solvent dipoles, which is related to the level of water penetration into the lipid bilayer [11,12]. However, they report at different depths in the membrane. The chromophore of laurdan probes the interphase region between the lipid head groups and the first C-atoms of the hydrophobic acyl chains, whereas the chromophore of di-4-ANEPPDHQ aligns with the acyl groups deeper in the hydrophobic core. Moreover, laurdan due to its lack of charge should, like cholesterol, easily flip between the two leaflets on a timescale of seconds [13] while di-4-ANEPPDHQ with its two positive charges should have a considerably lower flipping ability comparable to that of the local anaesthetic tetracaine that does not flip at all on an hour timescale [14].

The relative proportion of lo/gel phases and ld phase in a sample can be assessed by generalized polarization (GP) [15]. Importantly, for a membrane with two co-existing phases, a variation in the GP-value

Abbreviations: bPrPp, bovine prion protein-derived peptide; DLS, dynamic light scattering; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol); GP, generalized polarization; GUVs, giant unilamellar vesicles; LUVs, large unilamellar vesicles; laurdan, 6-dodecanoyl-2-dimethyl-aminonaphthalene; ld, liquid disordered; lo, liquid ordered; PBS, phosphate-buffered saline

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does not reflect a global change in overall membrane order but a shift in the relative proportion of the two phases. However, the composition of different ld and lo phases varies and so do their characteristics. That a diverse range of ordered and disordered domains can be formed by plasma membrane lipids was recently demonstrated by measuring the laurdan GP-values of plasma membrane vesicles formed using different methods [16].

The organization of the plasma membrane should be considered from the perspective of both lateral domain formation and cell topography [17,18]. In addition to reporting on the order of the bilayer, laurdan can also report on membrane orientation, when illuminated by polarized light – a characteristic termed photoselectivity [19]. It seems likely that also di-4-ANEPPDHQ can be used to study membrane orientation, although this has not been verified. Confusingly, photoselectivity can also be also used to describe laurdan excitation preferences in membranes of different phases. Laurdan in gel phase is preferentially excited at the red end of the excitation range and laurdan in ld phase is preferentially excited at the blue end of the excitation range [20].

Although laurdan in model membranes undoubtedly reports on the lipid order [21,22], questions have been raised about its behavior in the presence of membrane proteins. In this study, we used two structurally well-characterized polypeptides, the wasp venom protein mastoparan [23] and the 30 N-terminal residues of bovine prion protein [24,25] that insert in large unilamellar vesicles (LUVs) either with α-helix structure or with β-structure. These peptides represent different aspects of membrane-inserted polypeptides and were here used to assess the effects on laurdan and 4-ANEPPDHQ spectral properties.

2. Materials and methods

2.1. Materials

The phospholipids were from Avanti Polar Lipids (Alabaster, Alabama), and cholesterol was from Nu-Chek Prep, Inc. (Elysian, MN). 6-Dodecanoyl-2-dimethyl-aminonaphthalene (laurdan) was from Molecular Probes (Invitrogen, Carlsbad, CA), and di-4-ANEPPDHQ was from Lesley Loew (University of Connecticut Health Center, CT). Mastoparan and custom made bovine prion protein-derived peptide consisting of the 30 N-terminal amino acid residues were obtained from Neosystem Laboratories (Strasbourg, France).

2.2. Large unilamellar vesicle preparation

LUVs were prepared as described previously [26–28]. Briefly, lipids were dissolved in a mixture of chloroform: methanol (13:5 v/v). To produce a film of lipids, the solution was dried to under a stream of nitrogen for 30 min. The lipid solutions were then lyophilized for 30 min. The dry lipids were dissolved in phosphate-buffered saline (PBS), to produce a suspension of large multilamellar vesicles. Samples were vortexed and incubated at room temperature for 1 h. In order to decrease the lamellarity, the solution was then subjected to five fast freeze–thaw cycles, moving the sample between liquid nitrogen and a 40 °C waterbath. To obtain LUVs, the lipid solutions were extruded 21 times through a polycarbonate filter with a pore size of 100 nm (Whatman Plc., UK) in an Avanti manual extruder. The lo phase was made up of sphingomyelin, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) spiked with 5% 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DPPG) and cholesterol at 1:1:2, and the ld phase by DOPC spiked with 5% DPPG. Laurdan, from an ethanol stock, was added to lipid mixture in 1:1000 dye-to-lipid ratio before drying the samples. Di-4-ANEPPDHQ from water stock was added to the lipid mixture at a 1:1000 dye-to-lipid ratio after dissolving the lipids in buffer.

2.3. Giant unilamellar vesicle preparation

Giant unilammelar vesicles (GUVs) were prepared basically according to Akashi et al. [29] from the same lipid mixes as the LUVs. Briefly, lipids from their respective stocks were added to chloroform:methanol (2:1). Samples were subjected to rotary evaporation for 10 min at 50 °C to obtain a dry film of lipids. Subsequently, samples were exposed to high vacuum for 2 h and then left at a water bath at 50 °C under a stream of nitrogen for 30 min. The dry lipid films were dissolved in 5 mM PIPES, 50 mM KCL, and 1 mM EDTA at pH 7.0 and placed on a heating block at 50 °C for 24 h. Samples were then slowly cooled over period of 4 h in a styrofoam box.

2.4. Fluorescence spectroscopy

All fluorescence spectroscopy measurements were carried out using a Fluorolog 3 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ) at 25 °C. The laurdan and the di-4-ANEPPDHQ emission and excitation spectra were measured for both lo and ld phases 1 mM LUVs before and after the addition of polypeptides. The laurdan emission spectrum was measured from 370 to 620 nm with excitation at 350 nm. The di-4-ANEPPDHQ emission spectrum was measured from 500 to 700 nm with excitation at 488 nm. Laurdan excitation spectra were measured between 300 and 405 nm (with the emission measured at 430 and 450 nm for lo and ld phases, respectively), and di-4-ANEPPDHQ excitation spectra were measured between 400 and 520 nm (with the emission measured at 580 and 600 nm for lo and ld phases, respectively). The fluctuations in lamp intensity over the selected wavelength were corrected for in the excitation spectra. The emission and excitation spectra were acquired with a 1- or 2-nm bandwidth and a background subtraction for PBS was performed for all emission and excitation spectra. Where indicated, the spectra were subjected to either rolling average, automated Fourier transform smoothing, or corrected for the wavelength dependence of the monochromators, but most spectra are from raw data. Two membrane interacting peptides, mastoparan (a 14-residue peptide toxin isolated from wasp venom) and the 30 N-terminal residues from bovine prion protein (bPrPp), were added from freshly made stock solutions to LUVs stained with laurdan or di-4-ANEPPDHQ at 1:1000, 1:100, and 1:10 protein-to-total lipid ratio.

2.5. CD spectroscopy

Circular dichroism (CD) measurements for the two peptides in two phospholipid vesicle solvents were made on a Chirascan CD spectropolarimeter (Applied photophysics, Leatherhead, UK) with a 50-μm quartz cuvette. Spectra were recorded for solutions containing 2.5–5 mM phospholipids and 250–500 μM peptide (lipid:protein ratio 10:1) in PBS. Wavelengths between 190 and 250 nm were measured, with a 1-nm step resolution and 100 nm/min scan speed. The response time was 4 s, with 50 mdeg sensitivity and a 0.5- or 1-nm bandwidth. Measurements were conducted at 25 °C, and the temperature was controlled by a TC 125 temperature controller (Quantum Northwest Inc., Seattle, WA). Spectra were collected and averaged over ten scans. The CD spectra were evaluated using Applied Photophysics Pro-Data viewer v 4.0.17. The amount of secondary structure elements was evaluated using the DICHROWEB software [30–32].

2.6. Dynamic light scattering

The integrity and size distribution of the LUVs upon addition of the polypeptides were determined by dynamic light scattering (DLS) experiments at 25 °C on 2.5 mM LUVs dissolved in PBS. DLS measurements were recorded on an ALV/CGS3 goniometer system

(ALV, Langen, Germany) using 5-mm glass tubes. Scattering data were collected over 60 s with three scans per sample and presented as an averaged correlation function. The data were processed with the manufacturer's software to obtain the unweighted radius.

3. Results

Laurdan has been used for decades to characterize phase co-existence in both model membranes and cells, due to its different spectra in ld relative to lo/gel phase [21,33,34]. Di-4-ANEPPDHQ was introduced more recently as an attractive alternative to laurdan, primarily for live cells, mainly due to its solubility in water rather than in organic solvent and its excitability within the visible rather than the UV range [10,12]. Both dyes would be excellent tools for examining order in biological membranes if it can be demonstrated that the change in their emission reflects only membrane order. The crucial characterization of the response of laurdan and di-4-ANEPPDHQ to membrane-inserted peptides has now been performed.

3.1. The spectral properties of laurdan and di-4-ANEPPDHQ are not affected by membrane inserting peptides

LUVs of lo phase were prepared from sphingomyelin, DOPC, and cholesterol at 1:1:2 and ld phase LUVs from DOPC [8]. The DOPC contained 5% of the negatively charged lipid DPPG to enable formation of giant unilamellar vesicles (GUVs) by the gentle hydration method for comparative studies [29]. For the purpose of this study, phosphate determination was considered unnecessary. Laurdan was mixed with the lipids prior to LUV formation, whereas di-4-ANEPPDHQ, which is water-soluble, was added directly to LUVs, both at a 1:1000 ratio in relation to the lipids. The laurdan emission spectra in DOPC LUVs had a peak around 483 nm and in lo phase LUVs, around 433 nm (Fig. 1A and B). In ld phase LUVs, there was also a second emission peak at around 452 nm. The variation in intensities of the peaks at 452 and 483 nm between experiments is possibly the result of different batches of DOPC.

The small shoulder around 400 nm in the ld spectra is likely to be the result of insufficient correction for PBS peak at this wavelength, caused by the high sensitivity of cuvette positioning in the spectrophotometer. The emission spectra of laurdan in GUVs were identical to those of laurdan in LUVs of the same lipid compositions (not shown). Mastoparan or bPrPp was sequentially added to the LUVs to achieve a 1:1000, 1:100, and 1:10 peptide-to-lipid ratio. This did not cause any alteration in either in the appearance of the spectra or in the positions of the excitation and emission peaks. The excitation spectra for laurdan peaked at 358 nm in both ld and lo phase LUVs (Fig. 2A and B). The addition of either mastoparan or bPrPp did not affect the laurdan excitation spectra in either ld or lo phase.

The di-4-ANEPPDHQ emission spectra in lo LUVs had a peak maximum around 585 nm and in ld phase LUVs around 597 nm (Fig. 1C and D). The addition of mastoparan or bPrPp to achieve a 1:1000, 1:100, or 1:10 peptide-to-lipid ratio did not affect the position of the emission peaks nor the appearance of the spectra. The excitation spectra for di-4-ANEPPDHQ in both ld and lo phase LUVs peaked at 470 nm (Fig. 2C and D). The addition of either mastoparan or bPrPp did not affect the di-4-ANEPPDHQ excitation spectra in either ld or lo phase. The reason for there only being a modest difference between the emission peaks in LUVs of different phases is

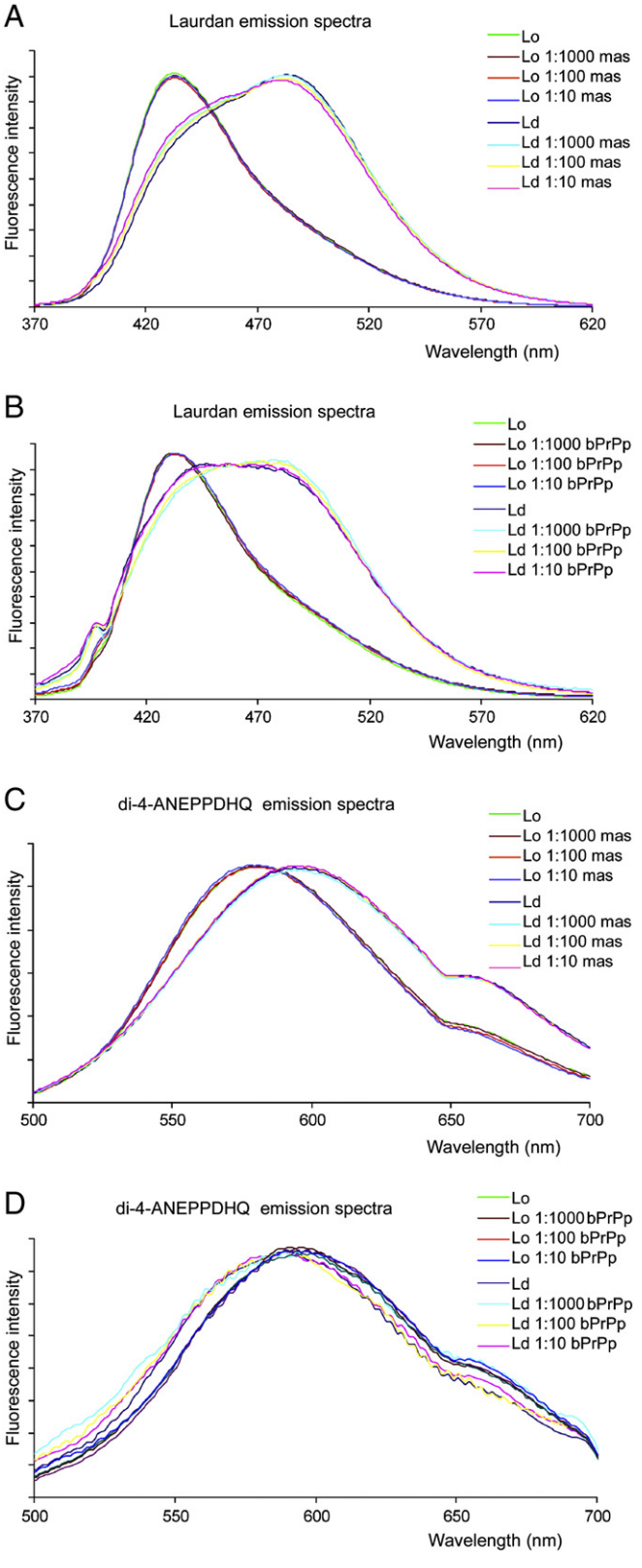

Fig. 1. The effect of membrane-inserted peptides on the emission spectra of laurdan and di-4-ANEPPDHQ. lo phase LUVs were prepared mixing sphingomyelin, DOPC spiked with 5% DPPG and cholesterol in 1:1:2 ratio. ld phase LUVs were made of DOPC spiked with 5% DPPG. Two different transmembrane polypeptides, mastoparan or bPrPp, were added to lo and ld phase LUVs stained with laurdan or di-4-ANEPPDHQ in 1:1000, 1:100 or 1:10 peptide/total lipid ratio, and their emission spectra were measured at 350 and 488 nm excitation wavelength, respectively. Fluorescence intensity values were normalized to the sample with no added peptide. (A) lo and ld emission spectra of laurdan after addition of mastoparan. (B) lo and ld emission spectra of laurdan after addition of bPrPp. (C) lo and ld emission spectra of di-4-ANEPPDHQ after addition of mastoparan. (D) lo and ld emission spectra of di-4-ANEPPDHQ after addition of bPrPp. The spectra in panel B were smoothened using a rolling average and the spectra in panel D were smoothened using Fourier transformation. Spectra shown are representative of five experiments.

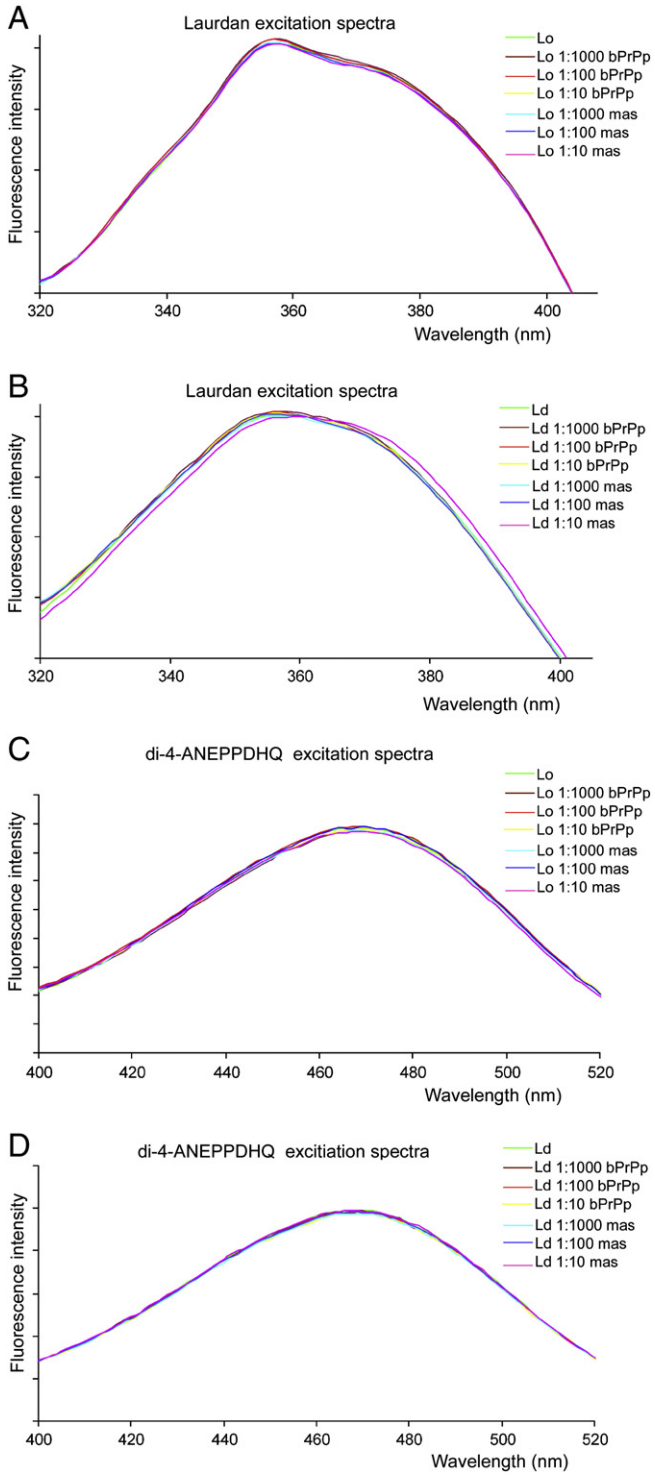

Fig. 2. The effect of membrane-inserted peptides on the excitation spectra of laurdan and di-4-ANEPPDHQ. lo phase LUVs were prepared mixing sphingomyelin, DOPC spiked with 5% DPPG and cholesterol in 1:1:2 ratio. ld phase LUVs were made of DOPC spiked with 5% DPPG. Two different transmembrane polypeptides, mastoparan or bPrPp, were added to lo or ld phase LUVs stained with laurdan or di-4-ANEPPDHQ in 1:1000, 1:100, and 1:10 peptide/total lipid ratio, and their excitation spectra were measured from 320 to 405 nm for laurdan and from 400 to 520 nm for di-4-ANEPPDHQ. Fluorescence intensity values were normalized to the sample with no added peptide. (A) lo phase, laurdan, and mastoparan or bPrPp. (B) ld phase, laurdan, and mastoparan or bPrPp. (C) lo phase, di-4-ANEPPDHQ, and mastoparan or bPrPp. (D) ld phase, di-4-ANEPPDHQ, and mastoparan or bPrPp. Spectra shown are representative of three experiments.

caused by the usage of raw data and is of no importance when studying peptide insertion into the membrane (Supplementary Fig. 1).

Our results suggest that the presence of peptides or proteins in biological membranes does not influence the spectra of laurdan and di-4-ANEPPDHQ and that the dyes thus report exclusively on lipid order.

3.2. Mastoparan and bPrPp insertion does not affect the integrity of the LUVs

The insertion of peptides into LUVs could alter their size and/or stability. The peptides may be fusogenic, cause vesicle fission, or simply increase the LUV size. To examine whether LUVs were affected by mastoparan or bPrPp, their integrity and size distribution were determined by dynamic light scattering. The mean diameter of the ld phase DOPC vesicles varied between 67 and 120 nm, while the diameter for lo phase sphingomyelin:DOPC:cholesterol (1:1:2) vesicles varied between 74 and 204 nm. With the exception of one experiment, the LUVs in lo phase were considerably larger than the LUVs in ld phase. The ld phase LUVs displayed a much more homogenous size distribution than the lo phase LUVs. Insertion of mastoparan did not affect the position of the peak for the hydrodynamic radius of the LUVs at the 1:100 and the 1:1000 peptide-to-lipid ratios in either ld or lo phase, but at the highest concentration, there was a asymmetric shift in both phases, indicating that there were on average larger vesicles as well as a wider spread of vesicle sizes (Fig. 3A–D). Intensity correlation functions are shown for all mastoparan experimental conditions in Supplementary Fig. 2A–D.

Insertion of bPrPp at the ratios 1:1000 or 1:100 did not affect the position of the peak for the hydrodynamic radius of either ld or lo phase LUVs, but at the 1:10 ratio, a slight shift of the peak to a higher value occurred in most experiments (Fig. 3E–H). The peak shift was accompanied by an asymmetric shift of the mean size of the radius, consistent with a wider distribution of vesicle sizes and somewhat larger vesicles after peptide addition. Intensity correlation functions are shown for all bPrPp experimental conditions in Supplementary Fig. 2E–H.

It is clear from all curves that the addition of the peptides caused either disintegration nor fusion of the LUVs. The increase in vesicles size is compatible with peptide insertion into the membrane.

3.3. Mastoparan and bPrPp are inserted into the LUVs with different secondary structures

The increase in vesicular size in the presence of peptides clearly suggested that mastoparan and bPrPp inserted into the vesicles, but to confirm the membrane insertion further, we examined the structural conversion of the peptides in the presence of LUVs. In water solution, both peptides have a random coil conformation. Mere membrane association may not induce changes in their conformation, whereas distinct structural features should be observable upon membrane insertion.

To ensure that the two peptides were inserted into the vesicles in the presence of the two dyes, the structure induction in the two peptides was measured by far-UV CD spectra. First, note that for both peptides and both dyes, the results were very similar (Fig. 4). Mastoparan clearly becomes mainly α-helical when inserted into vesicles of either ld or lo phase mixture. It also appears that the peptide is more structured in the vesicles with the lipids in the ld phase (Fig. 4A and C). On the other hand, the bPrPp CD spectrum has a different appearance, with a large proportion of β-strand structure (Fig. 4B and D). This is in line with a previous study demonstrating that bPrPp has β-strand structure in the presence of negatively charged lipids [25], which was an additional reason for the inclusion of the negatively charged DPPG in the LUVs.

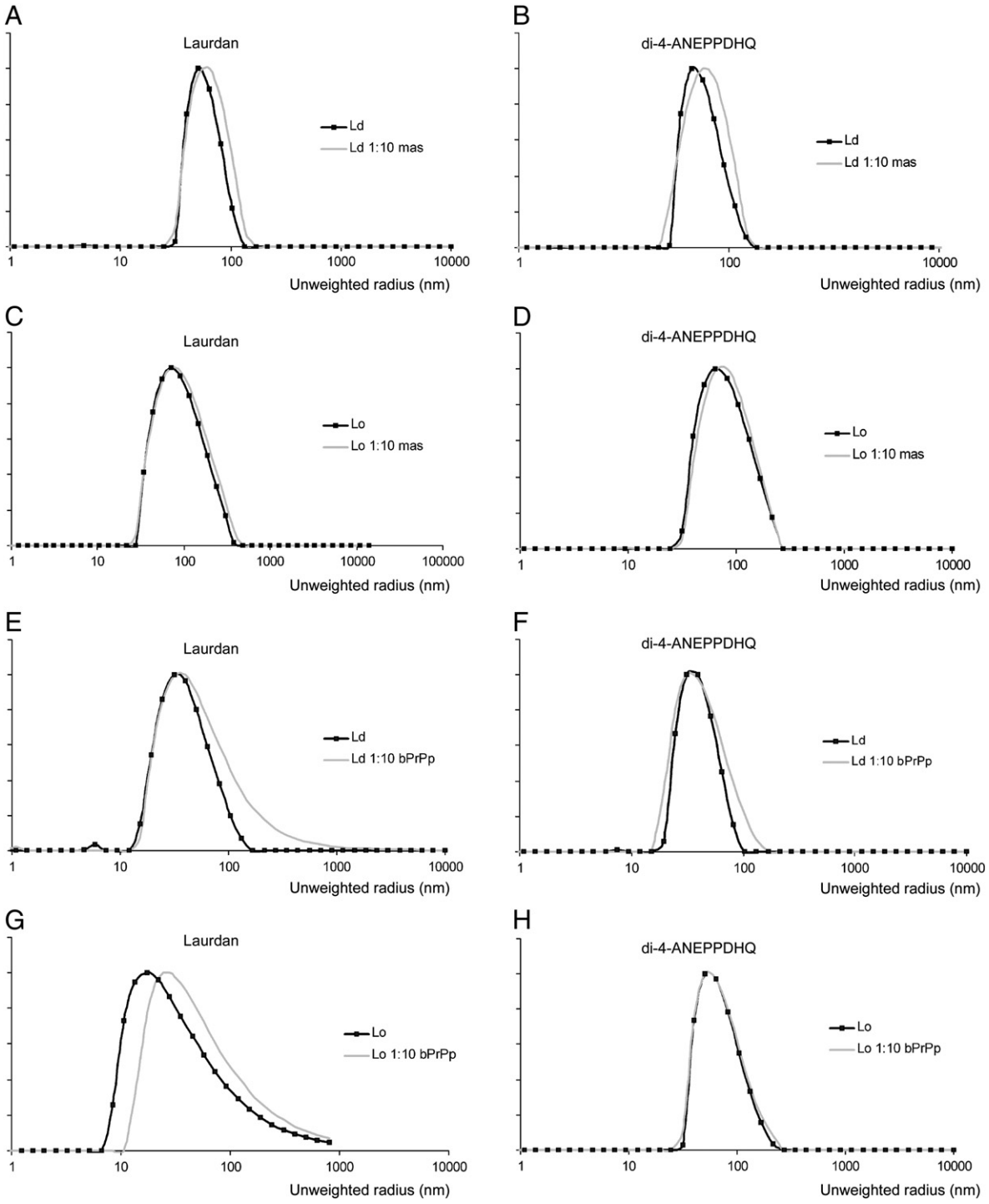

Fig. 3. Hydrodynamic radius of LUVs increases after addition of membrane inserting peptide. The integrity and size distribution of the LUVs upon addition of the transmembrane polypeptides were determined by dynamic light scattering. The measurements for both laurdan- and di-4-ANEPPDHQ-stained vesicles were repeated with the addition of mastoparan bPrPp in 1:1000, 1:100, and 1:10 peptide/total lipid ratio. Figure shows hydrodynamic radius distribution of lo and ld LUVs before and after addition of transmembrane peptide. Addition of transmembrane peptides increases the mean radius and size range of LUVs. (A) Laurdan, ld phase and mastoparan. (B) Di-4-ANEPPDHQ, ld phase and mastoparan. (C) Laurdan, lo phase and mastoparan. (D). Di-4-ANEPPDHQ, lo phase and mastoparan. (E) Laurdan, ld phase and bPrPp. (F) Di-4-ANEPPDHQ, ld phase and bPrPp. (G) Laurdan, lo phase and bPrPp. (H). Di-4-ANEPPDHQ, lo phase and bPrPp. The experiments were repeated between two and four times.

The secondary structure was estimated using DICHROWEB [30–32]. For mastoparan, we found that the peptide structure is around 80% helical in the ld phase and around 60–70% helical in the lo phase, with the rest being in a random coil conformation. This is in agreement with what is known of mastoparan's interactions with model membranes [23,35]. The results for the bovine prion protein-derived peptide, in both ld and lo lipid mixtures, indicate the presence of around 15–20% helix and around 50–60% β-sheet structure, with the rest being random coil. It has previously been observed that the prion-derived peptide interacts with phospholipid vesicles to obtain either α-helical or β-sheet structure, depending on the lipid composition [25]. The CD analysis clearly demonstrates that the peptides insert into the bilayers of the

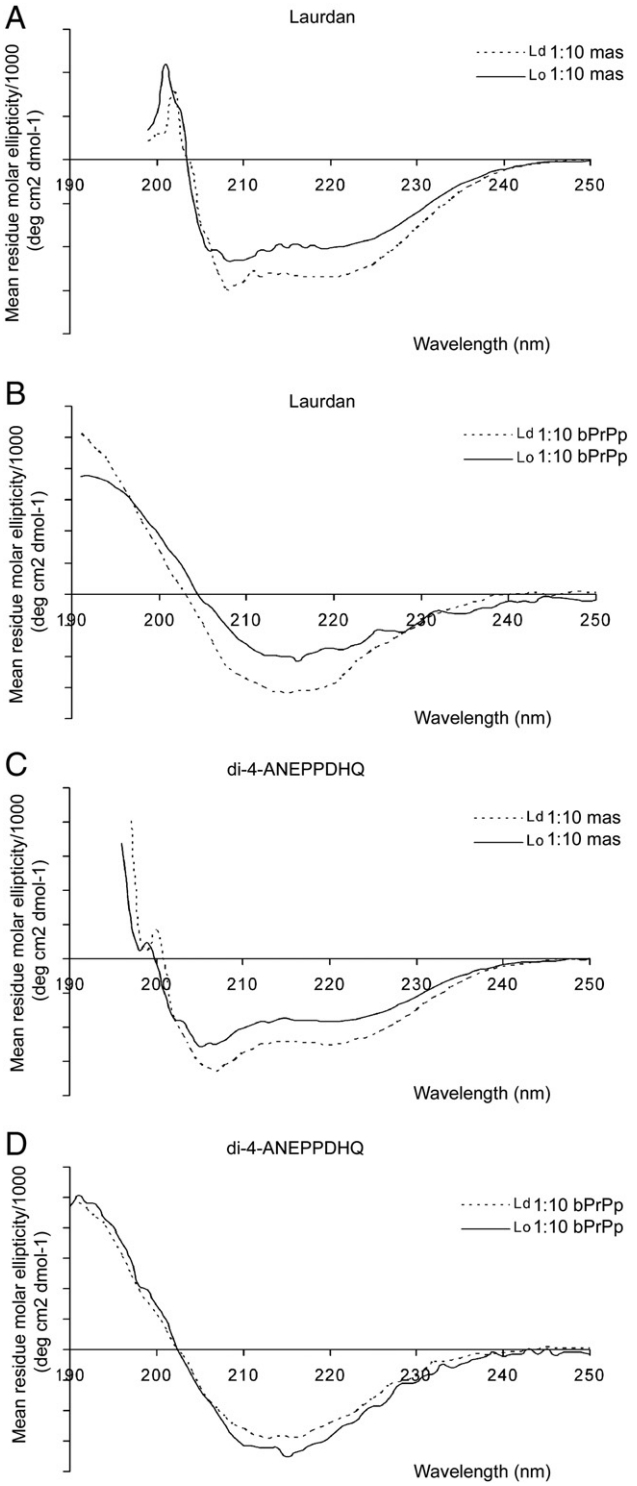

Fig. 4. Insertion efficiency of transmembrane polypeptides into LUVs. The insertion efficiency of the polypeptides into LUVs was determined by measuring their 3D polypeptide structure by circular dichroism. Spectra were recorded for solutions containing 2.5–5 mM phospholipids and 250–500 μM peptide (lipid:protein ratio 10:1) in PBS buffer. Wavelengths between 190 and 250 nm were measured, with a 1-nm step resolution and 100 nm/min. Measurements were conducted at 25 °C. (A) Mastoparan spectra in lo and ld phase LUVs stained with laurdan. (B) Mastoparan spectra in lo and ld phase LUVs stained with di-4-ANEPPDHQ. (C) bPrPp spectra in lo and ld phase LUVs stained with laurdan. (D) bPrPp spectra in lo and ld phase LUVs stained with di-4-ANEPPDHQ. Spectra shown are representative of three experiments.

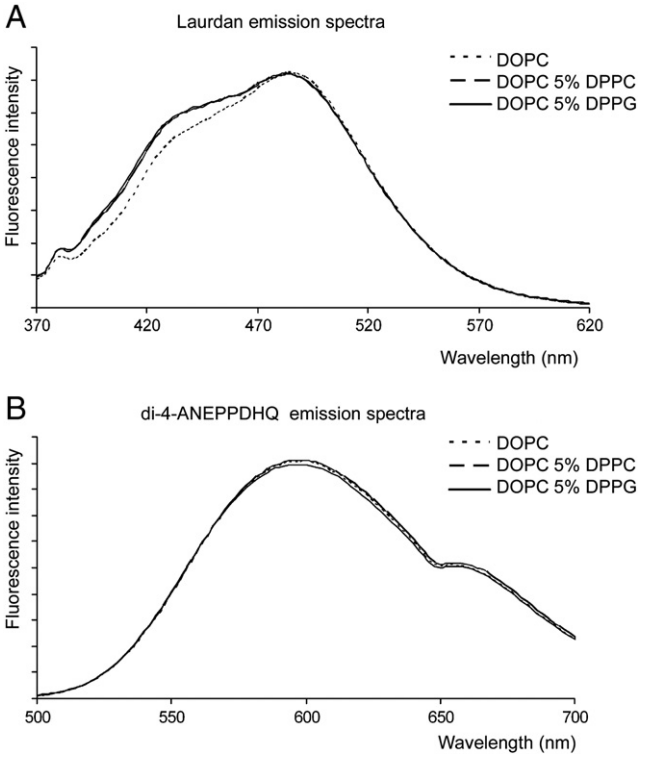

Fig. 5. The effect of negative charge and hydrophobic mismatch on the emission spectra of laurdan and di-4-ANEPPDHQ. Liposomes were prepared from DOPC, DOPC plus 5% DPPC, or DOPC plus 5% DPPG and stained with laurdan or di-4-ANEPPDHQ. The emission spectra were measured at 350- and 488-nm excitation wavelength, respectively. Fluorescence intensity values were normalized to the DOPC samples. Spectra shown are representative of two experiments. (A) Emission spectra of laurdan. (B) Emission spectra of di-4-ANEPPDHQ.

different vesicles and, furthermore, in a fashion that is independent of the added dyes.

4. Discussion

There is a heated debate about whether lipid domains exist in biological membranes. It mainly reflects a lack of methods for resolving membrane regions that may be smaller than 20 nm in diameter [36,37]. Laurdan and di-4-ANEPPDHQ are well suited to address lipid domain questions, since they report on the relative proportions of ordered and disordered domains, regardless of the size of the individual domains. Their use is therefore increasingly popular, but objections are frequently raised that the dyes may not report exclusively on lipid packing but that the spectra may be influenced by interactions with proteins. To address to this caveat, we have performed a careful study on the effect of membrane-inserted peptides of both α-helical and β-strand structure on the spectra of the two dyes in different membrane phases. We found no changes in their spectra.

Membrane curvature and lateral membrane tension are other features that may affect the spectral properties of probes assigned to report on membrane order. The higher the curvature, the lower the lateral membrane tension, which means that more water molecules can penetrate into the bilayer and increase its fluidity. This implies that lower laurdan excitation GP values indicate a higher curvature [38]. However, GP reports on the mobility of the water molecules rather than their number and low GP values do not necessarily mean that more water molecules enter the bilayer. In a study of Prodan, a

laurdan relative, it was concluded that although the membrane fluidity and solvent relaxation rates increase with membrane curvature, the Stokes shift was not affected [39]. This is compatible with an increase in the mobility of the water molecules, an increase in the number of water molecules in the bilayer, or an increase in both.

It has been reported that high concentrations of laurdan in cells enable FRET between the donor tryptophan (Trp) residues in membrane proteins and the acceptor laurdan [40]. bPrPp contains two Trp residues at positions 9 and 18 that are in its membrane-inserted region [25]. FRET between these Trps and laurdan would effectively cause a new peak to appear in the laurdan excitation spectra below 310 nm, but no difference in the laurdan excitation spectra in the UV region was observed upon bPrPp addition (not shown). The absence of spectral changes when bPrPp was used (at a peptide-to-lipid ratio of 1 to 10) in the presence of laurdan (at a dye-to-lipid ratio of 1 to 1000) suggests both that laurdan is not found in the vicinity of Trps and the fluorescence of Trp itself is weak in comparison to that of laurdan. This is a likely result of bPrPp having β-structure rather than α-helical structure since the Trps are then not expected to be as close to the membrane interphase where the fluorophore moiety, the naphthalene ring, of laurdan resides [41,42]. It is also possible that the membrane-inserted peptides aggregate and that laurdan prefers a pure lipid environment and therefore avoids the aggregated areas. Moreover, most wavelengths and filters used for laurdan excitation would not excite Trp, making any Trp–laurdan FRET irrelevant. For instance, in a study where the laurdan excitation GP values were reported to decrease as the laurdan-to-cell ratio increased, the excitation wavelength employed precludes FRET between Trp and laurdan [43]. Since laurdan partitions equally to ordered and disordered domains [44], self-quenching also seems an unlikely cause of the reported decrease in GP values [43], but the distribution of phases may cause preferential self-quenching in the ordered phase.

Laurdan in DOPC spiked with 5% DPPG emits more in the blue part of the spectra than laurdan in pure DOPC [19], with the peaks at 452 and 483 nm being of more equal height. However, this was the result of hydrophobic mismatch (Appendix A) supporting a study claiming that the head groups of phospholipids are not responsible for the spectral properties of laurdan [45]. We have chosen the emission peak at 483 nm for comparisons in this study since it gives the shift of 50 nm between lo and ld phases anticipated for laurdan. However, the spectra were not affected by peptide insertion into the LUVs so our conclusions would have been the same regardless of the peak selected for analysis.

Several studies have indicated that laurdan's emission spectrum is not affected by the presence of membrane proteins. When comparing laurdan emission in electric tissue from *Torpedo marmorata* with that in liposomes made from lipids extracted from the same tissue, no differences were detected [46]. Recently, it was shown that a synthetic peptide did not affect laurdan GP values in LUVs when mixed with lipids up to a 1-in-50 ratio [16]. However, it was not shown that the peptide was inserted in the LUVs and a 1-to-50 ratio is likely to be an underestimate of the *in vivo* situation [47]. In the present study, a level of insertion of at least 60% was confirmed, based on the structure induced in the peptides, and peptide-to-lipid ratios of up to 1 to 10 were used. This degree of peptide prevalence is comparable with the *in vivo* situation since estimations predict that the plasma membrane is crowded with proteins [47].

While laurdan can interact with proteins in water solution [3], there is, to the best of our knowledge, no evidence suggesting that laurdan interacts with proteins in membranes. By measuring FRET between tryptophans in acetylcholine receptors and laurdan, it was concluded that the dye was randomly distributed in the membranes, showing no affinity for the protein [46]. Nor did laurdan avoid the acetylcholine receptors. Interestingly, it was demonstrated that laurdan molecules in close proximity to the acetylcholine receptors were in a more ordered environment than laurdan molecules found in the bulk membrane [46], suggesting that membrane proteins need not have global effects on membrane order. This is also the case for peptides that insert parallel to the membrane surface [48]. The dyes in our lipid mixes may avoid the areas close to the peptides, and if the peptides aggregate, there should be plenty of regions devoid of peptides. This would be reminiscent of the situation where the laurdan relative Patman only inserts in the lipid part of phospholipid-poly(diacetylene) vesicles [49]. The lack of FRET between Trps in bPrPp and laurdan supports this interpretation. On the other hand, the lack of effect on the emission spectra of both laurdan and di-4-ANEPPDHQ strongly suggests that the membrane order in the lipid mixes used, DOPC and SM:DOCP:chol (1:1.:2) with 5 mol% DPPG in the DOPC, is not affected by mastoparan or bPrPp insertion as is the case for a range of other membrane inserting peptides [48]. It is also reassuring that laurdan itself does not alter the membrane order [50].

It has previously been reported that bPrPp has a significant effect on membrane dynamics and order [51] and that it can cause leakage in both cells and model membranes [25]. However, the leakage occurs at far lower concentrations than those that alter diphenylhexatriene fluorescence polarization (membrane order at the hydrophobic core of the bilayer) and the two can therefore not be equated [25]. Prothrombin, which interacts with membranes, has been reported to have an effect on the solvent relaxation rate of Patman, a probe that inserts in membranes similarly to laurdan [52]. While mastoparan and bPrPp insert into the hydrophobic core of the bilayer, prothrombin forms a complex with negatively charged head groups and calcium ions in the hydrophilic part of the membrane, which may account for the difference. Nonetheless, the membrane inserting peptides magainin, melittin, and KAL have also been reported to affect the solvent relaxation rate of Patman, especially in the presence of negatively charged lipids [49]. An explanation for the difference between our results with laurdan and the peptides mastoparan and bPrPp and those with Patman and other peptides is that a change in the solvent relaxation rate does not of necessity alter the Stokes shift [39]. We would also like to emphasize that although our data support the interpretation that the membrane order is not affected by mastoparan and bPrPp insertion in the lipid mixes we used, time-resolved solvent relaxation studies may produce a more nuanced answer. Nevertheless, our data clearly demonstrate that the spectral properties of laurdan and di-4-ANEPPDHQ are not affected by the insertion of membrane peptides.

The secondary structure of bPrPp has previously been found to depend on factors such as relative peptide concentration, salt concentration, and lipid head group properties and can vary between α-helix and β-sheet [25,51]. Here we find that the peptide adopts a combination of mainly β-sheet with some α-helical structure, which is in agreement with the previous results in bilayers that contain negatively charged phospholipids [25]. The wasp venom mastoparan, on the other hand, adopts a clear α-helical structure when interacting with the lipid bilayers in the LUVs, which is consistent with previous work [23,53,54]. Mastoparan has the ability to form transient pores [55] and can switch between a transmembrane and in-plane orientation, which is believed to contribute to membrane leakage [56]. The different properties of the two peptides make them representative of membrane-inserted proteins.

5. Conclusions

Our results demonstrate that, despite variations in secondary structure and the known membrane-perturbing effects of mastoparan and bPrPp, neither peptide has any significant effect on either laurdan or di-4-ANEPPDHQ. Hence, our findings suggest that the dyes report exclusively on changes in the membrane phase properties and are not influenced by the presence of peptides or proteins. Laurdan and di-4-ANEPPDHQ are well suited for assessing lipid order in cells.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamem.2010.10.002.

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Appendix A

We are intrigued by the blue shift of laurdan in DOPC LUVs containing 5% DPPG and have examined this phenomenon in great detail. To distinguish an effect of charge, present in DPPG but not in DOPC, from that of a hydrophobic mismatch caused by DPPG having longer acyl chains than DOPC, we compared the spectra of both dyes in pure DOPC, DOPC + 5 mol% DPPG and DOPC + 5 mol% DPPC. The increase in the laurdan emission peak at 452 nm was due to the presence of longer acyl chains, not the charge (Fig. 5A). The emission spectra of di-4-ANEPPDHQ were to our surprise not sensitive to the presence of either 5 mol% DPPG or 5 mol% DPPC. In pure DOPC, di-4-ANEPPDHQ also had an emission peak at 590 nm (Fig. 5B). Since di-4-ANEPPDHQ has two positive charges, it is possible that its spectral properties are sensitive to pH. We therefore compared the emission spectra of di-4-ANEPPDHQ in DOPC LUVs in PBS and buffers at pH 3.8 or 8.8. The spectra were identical in all three solvents strongly suggesting that the emission spectra of di-4-ANEPPDHQ are not sensitive to alternative protonation of the dye.

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