Peptide insertion into membranes does not alter the spectral properties of laurdan and di-4-ANEPPDHQ

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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 or bovine prion protein N-terminal residues 1–30 (bPrPp), were added to LUVs of either lo or ld phase, up to 1:10 peptide/total lipid ratio. Neither excitation nor emission spectra of laurdan and di-4-ANEPPDHQ in either lipid phase were altered 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, showing that the dyes are excellent probes for lipid order in cells.

INTRODUCTION

There is a need to study cells and their components by biophysical approaches in order to increase our understanding of their functions. For biological membranes, this was brought up to date by the lipid raft hypothesis suggesting that membrane domains of different packing efficiency exist in the plasma membrane (1), although multiple reports on membrane domains of 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, that change their spectral properties in response to lipid packing, are frequently used to study membrane domains of different lipid phases in biological membranes. Their utility is dependent on a response solely to lipid phases and not to other features of biological membranes. The concentration of proteins is very likely to differ between different membrane regions (6) and the responses of laurdan or di-4-ANEPPDHQ have until now not been extensively characterised 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 lo phase in which the acyl chains of the phospholipids are extended and the fluidity is lower (7), but the difference in cholesterol concentration 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 behaviour is largely uncharacterised, it is better to refer to ordered and disordered domains rather than to use the ld and lo terminology.

Laurdan and di-4-ANEPPDHQ have blue shifts of around 50 nm in their emission peaks for 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 do so at different positions in the membrane. The chromophore of laurdan probes the interphase region whereas the chromophore of di-4-ANEPPDHQ aligns with the acyl groups deeper in the hydrophobic core. Moreover, laurdan easily flip-flops between the two leaflets while di-4-ANEPPDHQ with its two positive charges has considerably lower flip-flop ability.

The relative proportion of lo/gel phases and ld phase in a sample can be assessed by generalised polarisation (GP) (13). In a membrane with two co-existing phases, a variation in the GP-value to either a higher or a lower number does thus 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 vary and so do their characteristics. That a range of ordered and disordered domains can be formed by plasma membrane lipids was recently

demonstrated by measuring the laurdan GP-values plasma membrane vesicles formed using different methods (14).

The organisation of the plasma membrane should be considered from the perspective of both lateral domain formation and cell topography (15, 16). In addition to reporting on the order of the bilayer, laurdan can also report on membrane orientation, when illuminated by polarised light – a characteristic termed photoselectivity (17). It seems likely that also di-4-ANEPPDHQ can be used to study membrane orientation, although this has not been verified. Confusingly, the term photoselectivity is sometimes 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 interval and laurdan in ld phase is preferentially excited at the blue end of the excitation interval (18).

Although laurdan in model membranes undoubtedly reports on the lipid order (19, 20), its behaviour in the presence of membrane proteins is less well characterised. In this study, we used two structurally well-characterised polypeptides, the wasp venom protein mastoparan (21) and the 30 N-terminal residues of bovine prion protein (22, 23) 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 of on laurdan and 4-ANEPPDHQ spectral properties.

MATERIALS AND METHODS

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 (bPrPp) were obtained from Neosystem Laboratories (Strasbourg, France).

Large unilamellar vesicle preparation

LUVs were prepared as described previously (24-26). Briefly, lipids were dissolved in a mixture of chloroform: methanol (65:25 v/v). To produce a film of lipids, the solution was dried to under a stream of nitrogen for 30 minutes. The lipid solutions were then lyophilized for 30 minutes. 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 1h. 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). 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:lipid ratio before drying the samples. Di-4-ANEPPDHQ from water stock was added to lipid mixture in 1:1000 dye:lipid ratio after dissolving the lipids in buffer.

Fluorescence spectroscopy

All fluorescence spectroscopy measurements were carried out using a Fluorolog 3 spectrometer (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 phase 1mM LUVs before and after the addition of polypeptides. ~~Laurdan~~ 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 nm to 700 nm with excitation at 488 nm. Laurdan excitation spectra were measured between 280 nm and 420 nm (with the emission measured

at 430 nm and 450 nm for lo and ld phase, respectively) and di-4-ANEPPDHQ excitation spectra were measured between 400 nm and 500 nm (with the emission measured at 580 nm and 600 nm for l_o and l_d phase, respectively). The fluctuations in lamp intensity over the selected wavelength were corrected for in the excitation spectra. Both emission and excitation spectra were acquired ~~at~~ with a 2 nm bandwidth and a background subtraction ~~for~~ of a PBS spectrum was performed in all cases ~~for all spectra~~. Where indicated, the spectra were subjected to either rolling average smoothing over four consecutive data points or automated Fourier transform smoothing, but most spectra displayed are from raw data. Two ~~trans~~membrane interacting ~~poly~~peptides, mastoparan (a 14-residue peptide toxin isolated from wasp venom) and the 30 N-terminal residues from bovine prion protein ~~(N-terminal residues 1–30~~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.

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 various secondary structure elements was evaluated using the DICHROWEB software (27-29).

Dynamic Light Scattering

The integrity and size distribution properties 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 a ~~–~~ALV/CGS3 goniometer system (ALV, Langen, Germany) using 5 mm glass tubes. ~~Zetasizer instrument (Nano ZS, Malvern Instruments, Worcestershire, UK) with a glass cuvette with a 1 cm path length~~. 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 ~~(DTS)~~ to obtain the unweighted radius.

RESULTS

For decades laurdan has been used to characterise phase co-existence in both model membranes and cells, due to its different spectra in ld relative to lo/gel phase (19, 30). Di-4-ANEPPDHQ was recently introduced as an attractive alternative to laurdan, primarily for live cells, ~~work~~ mainly due to its solubility in water rather than organic solvent and its excitability within the visible rather than the uv range (10, 12). Both dyes could be excellent tools for examining order in biological membranes if it can be demonstrated that the change in their emission responds solely to membrane order and not to other parameters. The very crucial characterisation of the response of laurdan and di-4-ANEPPDHQ to membrane inserted peptides has now been performed.

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 (31). Laurdan was mixed with the lipids prior to LUV formation whereas di-4-ANEPPDHQ that is water-soluble was added 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 probably the result of different batches of DOPC. For the purpose of this study, phosphate determination was considered unnecessary, vad är det?.

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 of the same lipid compositions were identical to those of the LUVs (not shown). Mastoparan or bovine prion protein-derived peptide, bPrPp, was added to the LUVs to sequentially achieve a 1:1000, 1:100 and 1:10 ratio. This did not cause any alteration in either in the appearance of the spectra or in the positions of the excitation or 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 ld LUVs had a peak maximum around 585 nm and in lo 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. 2 C and D). The addition of either mastoparan or bPrPp did not affect the di-4-ANEPPDHQ excitation spectra in either ld or lo phase.

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

Mastoparan and bPrPp insertion does not affect the integrity of LUVs

The insertion of peptides into LUVs may 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 (Fig. 3). 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 any of the peptide to lipid ratios tested in either ld or lo phase, but at the highest concentration there was an asymmetric shift in the correlation curve in both phases, indicating that there were ~~more large~~on average larger vesicles as well as a wider distribution of vesicle sizes (Fig. 3A-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 the peak was shifted to a higher value in most experiments (Fig. 3E-H). In all experiments at 1:10 bPrPp, ~~was~~ an asymmetric shift in the correlation curve was observed, indicative of ~~more large~~on average larger vesicles together with a wider distribution of vesicle sizes. The peak shift was accompanied by a similar asymmetric shift in the correlation curve, consistent with a wider distribution of vesicle sizes and somewhat larger vesicles after peptide addition.

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 were interacting with the vesicles, but ~~it remained possible that they were associated with rather than inserted into the membranes~~we wished to examine the membrane-binding further, by examining 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~~ing~~ 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 larger content 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 (23), which was one of our reasons for the inclusion of the negatively charged DPPG in the LUVs.

The secondary structure content was evaluated using DICHROWEB (27-29). A fairly large uncertainty in the predicted amount of structure can however be anticipated, due to among other things uncertainties in peptide concentration. 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 from mastoparan's interaction with model membranes (21, 32). 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 (23). The CD analysis clearly demonstrates that the peptides insert into the bilayers of the different vesicles, and furthermore, in a fashion that is independent of the added dyes.

DISCUSSION

There is a heated debate about whether lipid domains exist in biological membranes. It mainly reflects a lack of methods for studying membrane regions that may be smaller than 20 nm in diameter (33, 34). Laurdan and di-4-ANEPPDHQ are able 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 frequently objections are raised that the dyes may not report exclusively on lipid packing but that the spectra may be affected by interactions with proteins being the most common objection. To address to these caveats, we have performed a careful study on the effect membrane inserted peptides of both α-helical and β-strand structure on the spectra of the two dyes in different membrane phases and found none.

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. ~~The~~ This ~~result is~~implies that higher laurdan excitation GP values ~~the~~ indicate ~~higher the~~a higher degree of curvature (35). However, GP values report on the mobility of the water molecules and low GP values do not necessarily mean that more water molecules ~~do~~ enter the bilayer. In a study of laurdan's relative Prodan, it was concluded that although the membrane fluidity and solvent relaxation rates increase with membrane curvature the Stokes shift was not affected (36). This is as compatible with an increase in the mobility rather than an increase in the number of water molecules in the bilayer or an increase in both.

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 *T. marmorata* with that in liposomes of extracted lipids from the same tissue no differences were detected (37). 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 (14). However, no measures were taken to ensure that the peptide was inserted in the LUVs. In the present study a peptide to lipid ratio of 1 to 10 was used as the highest concentration and, based on the structure induction in the peptides, the level of insertion was at least 60%. This is compatible with estimations of the plasma membrane being crowded with proteins (38).

It has been reported that high concentrations of laurdan in cells enables FRET between the donor tryptophan (Trp) residues in membrane proteins and the acceptor laurdan (39). bPrPp contains two Trp residues at positions 9 and 18 that are in its membrane inserted region

(23). FRET between these Trps and laurdan could effectively cause a new peak to appear in the laurdan excitation spectra below 310 nm, due to Trp excitation, ~~where Trp is excited,~~ 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 that laurdan is not found in the vicinity of Trps. 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 (40, 41). It is also possible that the membrane inserted peptides aggregate and that laurdan prefers a pure lipid environment and therefore stays out of the aggregated areas. However, most setups for laurdan excitation would not cause any excitation of Trp so FRET should generally not have to be considered. In a study where the laurdan excitation GP values were reported to decrease as the laurdan to cell ratio increased, FRET between Trp and laurdan could not have been the cause of the change (42). Since laurdan partitions equally to ordered and disordered phases (43), self-quenching seems an unlikely cause of the decrease in GP values but the distribution of phases may cause preferential self-quenching in the ordered phase.

Laurdan interaction with proteins can occur but there is to our knowledge no evidence suggesting that laurdan interacts with proteins in membranes. When offered the choice between water-based media and proteins, laurdan has a preference for the proteins, presumably caused by an affinity for the hydrophobic protein cores (3). More relevantly, by measuring FRET between tryptophans in acetylcholine receptors and laurdan it was concluded that the dye was randomly distributed in the membranes with no affinity for the protein (37). Nor did laurdan avoid the acetylcholine receptors. Laurdan has frequently been used to study what effect proteins like apolipoprotein A-1, that interact with membranes, exert on membrane order but in these studies it is not an interaction between laurdan and the protein that is assessed (30).

Laur~~ru~~dan in DOPC spiked with 5% DPPG emits more in the blue part of the spectra than laurdan in pure DOPC (17), with the peaks at 452 and 483 nm sometimes at almost identical intensities. This is in contrast to a study claiming that the head groups of phospholipids are not responsible for the spectral properties of laurdan (44). However, considerable variations in GP values were found between different head groups but there was no trend that could be ascribed to charge (44). We have chosen the peak at 483 nm for comparisons in this study since it gives the shift of 50 nm between lo and ld phase 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. For di-4-ANEPPDHQ the effect of the negatively charged DPPG on the emission in ld phase is more pronounced than for laurdan, with a blue shift from 630 nm (10) to 590 nm suggesting that the emission of di-4-ANEPPDHQ with its double positive charge is very sensitive to head group charge.

It has previously been reported that bPrPp has a significant effect on membrane dynamics and order (45), and that it can cause leakage in both cells and model membrane systems (23). The secondary structure of this fragment has previously been found to depend on factors such as relative peptide concentration, salt concentration and lipid head-group properties, and the structure can vary between α-helix and β-sheet (23, 45). Here we see 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 containing some negatively charged phospholipids (23). 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 in agreement with previous work (21, 46, 47). Mastoparan has the ability to form transient pores (48), and can alter between a transmembrane and in-plane orientation, which is believed to contribute to membrane leakage effects (49). The different properties of the peptides make them good representatives of membrane-inserted proteins.

Our results demonstrate that, despite variations in secondary structure and the known membrane-perturbing effects of the peptides, neither peptide has any significant effect on either of the dyes. Hence, we demonstrate that laurdan and di-4-ANEPPDHQ report on changes in the membrane phase properties and are not influenced by the presence of peptides or proteins.

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

FIGURE 2 The effect of membrane inserted peptides on the excitation spectra of laurdan and di-4-ANEPPDHQ. l_o phase LUVs were prepared mixing sphingomyelin, DOPC spiked with 5% DPPG and cholesterol in 1:1:2 ratio. l_d phase LUVs were made of DOPC spiked with 5% DPPG. Two different transmembrane polypeptides, mastoparan or bPrPp, were added to l_o or l_d phase LUVs stained with laurdan or di-4-ANEPPDHQ in 1:1000, 1:100 and 1:10 protein/total lipid ratio and their excitation spectra were measured from 300 nm to 420 nm for laurdan and from 400 nm to 520 nm for di-4-ANEPPDHQ. Fluorescence intensity values were normalized to the sample with no added peptide. (A) l_o and l_d excitation spectra of laurdan after addition of mastoparan. (B) l_o and l_d excitation spectra of laurdan after addition of bovine prion protein. (C) l_o and l_d excitation spectra of di-4-ANEPPDHQ after addition of mastoparan. (D) l_o and l_d excitation spectra of di-4-ANEPPDHQ after addition of bPrPp. Spectra shown are representative of three experiments.

FIGURE 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 or bovine prion protein in 1:1000, 1:100 and 1:10 protein/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) Laurdan, lo phase and mastoparan. (C) Di-4-ANEPPDHQ, ld phase and mastoparan. (D). Di-4-ANEPPDHQ, lo phase and mastoparan. (E) Laurdan, ld phase and bPrPp. (F) Laurdan, lo phase and bPrPp. (G) Di-4-ANEPPDHQ, ld phase and bPrPp. (H). Di-4-ANEPPDHQ, lo phase and bPrPp. The experiments were repeated between two and four times.

FIGURE 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.