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Analysis of cell membrane micro-heterogeneity using the fluorescence lifetime of DPH-type fluorophores

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Heterogeneity in the lipid organization in lipid bilayers and cell membranes was probed by using the fluorescence decay of 1,6-diphenyl-1,3,5-hexatriene (DPH) and DPH attached to the *sn*-2 position of phosphatidylcholine (DPH-PC). In the presence of protein, it is proposed that the bulk lipids and boundary lipids can potentially provide distinct enough fluorophore environments for two different lifetime centers to be recovered from the analysis of the fluorescence decay. To test this model experiments were performed with cytochrome b_5 in 1-palmitoyl-2-oleoylphosphatidylcholine bilayers. The number of boundary lipids of cytochrome b_5 is known from the literature or can be calculated from known dimensions, so that for a known protein:lipid ratio the fraction of lipids in the bulk and boundary lipid regions is known. These values were found to closely correspond to the fractions associated with the lifetime centers recovered from an analysis of the fluorescence decay assuming two major fluorophore populations. This indicated that the DPH distributed in a similar manner to the lipids and that its boundary lipid residency time was greater than the excited state lifetime, showing the validity of the approach. An important requirement was that the protein should influence the fluorophore decay sufficiently enough to enable separate lifetime centers for the bulk and boundary lipid fluorophores to be recovered by the analysis. Attempts were made to analyze the fluorescence decay of DPH in liver plasma membranes and microsomes as arising from two distinct fluorophore populations, however, the basic condition was not satisfied. By contrast, using DPH-PC it was possible to extract two separate lifetime centers. The limitations and potential of this approach are critically assessed and it is concluded that in certain circumstances information pertaining to the protein-lipid interfacial region of membranes can be extracted from fluorescence decay heterogeneity properties.

Introduction

The physical properties of membrane lipids are the focus of much attention due to their important role in the support and modulation of membrane protein function. To investigate these properties fluorescent molecules are often introduced into the membrane and a number of physical parameters obtained. The fluorescence lifetime is a parameter of primary importance since it is highly sensitive to the membrane lipid environment surrounding the fluorophore. Usually a discrete single fluorescence lifetime is assumed in the analysis of the decay of the excited state. This assumes a homogeneous fluorophore environment and an implied homogeneity in the lipid organization, a situation inherently unsatisfactory for cell membranes which have a complex composition.

A potentially important region of cell membranes is the first shell of lipids immediately adjacent to the protein, defined as the boundary lipid region, which is of interest since perturbation of protein function by hydrophobic agents is mediated through this region. For some years there was debate as to whether the lipids in this region were compositionally distinct from the bulk lipids (reviewed in Ref. 1). While it is clear

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-PC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-PC; EPR, electron paramagnetic resonance; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; ns, nanoseconds (10^{-9} s); POPC, 1-palmitoyl-2-oleoyl-PC; PC, phosphatidylcholine.

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that bulk and boundary lipids exchange on the order of 10^{-7} seconds [2] and are therefore not distinct species, lipid orientations and dynamics in the two regions may be quite different. This could lead to differences in the degree and nature of a specific perturbation of the boundary lipid region compared to that in the bulk lipid region.

A wide range of physical techniques have been used to probe the boundary lipid region of membrane proteins. Apart from fluorescence techniques, differential scanning calorimetry (e.g. see Ref. 3), ^{13}C-NMR (e.g. see Ref. 4), Raman spectroscopy (e.g. see Ref. 5) and EPR (reviewed in Ref. 1) have been used. EPR has been widely used for the study of boundary lipids although the focus has been more on determining the number of boundary lipids rather than the properties of the region as such. Although DPH lifetime heterogeneity has been used to assess membrane micro-heterogeneity in natural membranes [6] and lipid bilayers [7] these studies were not aimed at the question of the protein-lipid interfacial region. Studies of lipid selectivity of the protein/lipid interface have been carried out using a fluorescence quenching approach (see also Refs. 8 and 9) as have studies of the lipid order in this region [10,11]. Also in one study the number of boundary lipids in the sarcoplasmic reticulum Ca^{2+}-ATPase was estimated from DPH fluorescence anisotropy data [12]. Apart from these studies the vast majority of fluorescence spectroscopic studies of the hydrophobic region of cell membranes treat the protein-lipid interfacial region and the bulk lipid region as a single homogeneous environment.

In previous studies from this laboratory [13] the influence of the protein on the fluorescence decay was noted and subsequently evidence presented that the protein/lipid interface presents a heterogeneous environment leading to a range of decay rates for fluorophores in this region of membranes [14]. In the present study the possibility that fluorophore environments in the bulk and boundary lipid regions are different enough for the recovery of two distinct fluorescence lifetime centers from analysis of the fluorescence decay of DPH and DPH-PC was critically assessed. Although it is recognized that there may be a more complex structural compartmentalization in membranes, this appeared to be a reasonable beginning for this type of analysis. First, a model system was examined consisting of cytochrome b_5 in bilayers of a single molecular species PC from which the basic requirements for analysis of fluorescence decay in membranes arising from two fluorophore populations were established. Attempts were then made to apply the approach to liver cell membrane fractions using DPH and DPH-PC. It was concluded that within certain limitations, the technique is valid for discerning two distinct environments in model lipid bilayers and cell membranes.

Experimental Procedures

Materials

DPH, and DPH-PC were obtained from Molecular Probes (Eugene, OR). Phospholipids were from Avanti Polar Lipids (Birmingham, AL). Cytochrome b_5 was prepared as previously described [15], except for an additional gel filtration step in deoxycholate and was kindly donated by Dr. P.W. Holloway (Department of Biochemistry, University of Virginia Medical School).

Methods

Preparation of membrane fractions

Rat liver microsome preparation, determination of enzyme markers, cholesterol/phospholipid molar ratio and phospholipid class analyses were as previously published [16]. The low levels of 5'-nucleotidase and succinate dehydrogenase indicated only small amounts of plasma membrane and mitochondrial contamination.

A plasma membrane-enriched fraction was prepared largely according to established procedures [17]. Briefly, a liver homogenate (250 mM sucrose, 5 mM Hepes-KOH, 1 mM EGTA pH 7.4) was filtered and then centrifuged at $1500 \times g$ for 10 min. The resuspended pellet (8 vol) was mixed with 1 vol of Percoll (Pharmacia) and centrifuged at $35\,000 \times g$ for 30 min. The band near the top of the tube was washed twice in 50 mM Tris-HCl (pH 7.5) ($15\,000 \times g$, 20 min). The plasma membrane marker 5'-nucleotidase, showed a 25-fold enrichment over the homogenate. Cytochrome-*c* oxidase (mitochondrial marker) was found only in trace amounts. Glucose-6-phosphatase (endoplasmic reticulum marker) was depleted by a factor of 0.26 over the homogenate.

Lipid extraction and separation

Lipids were extracted from the isolated membranes [18] and the phospholipids were separated from the neutral lipids using silicic acid columns. The neutral lipids were first eluted by chloroform, followed by elution with methanol to obtain the phospholipids. The phospholipids were then quantified by the determination of lipid phosphorus [19].

Preparation of vesicles

Aliquots of lipids (in chloroform) and fluorophores (in tetrahydrofuran) were placed together in a test tube, the solvent removed by a stream of nitrogen, and the lipids dispersed to form MLV by the addition of 10 mM Tris-HCl, 150 mM NaCl (pH 7.4), followed by vortexing. LUV (diameter $\approx$ 100nm) were then prepared by extrusion of freeze-thawed suspensions of

phospholipids using a Lipex Extruder (Vancouver, BC) as previously described [20]. DPH was introduced into intact membranes by injecting a solution (≈ 1 μl) of the probe in tetrahydrofuran (DPH) to achieve a final probe/phospholipid ratio of 1:400 (DPH). DPH-PC was introduced into intact membranes by first preparing a MLV suspension of the fluorophore as described above then sonicating for 3 min at 4°C using a Fisher sonicator with a micro-tip at 50% full power. DPH-PC sonicated vesicles were then incubated at room temperature in the dark for 2 h with the membranes. The membranes were washed by centrifugation (100 000 × g, 30 min) and resuspension in buffer followed by re-centrifugation, to remove DPH-PC which had not incorporated into the membrane. The extent of incorporation of DPH-PC into microsomes was determined in a control experiment, by following the change in fluorescence anisotropy with time, after addition of DPH-PC vesicles to microsomes. The incorporation was slow, but was complete after ≈ 17 h since by this time the fluorescence anisotropy had reached a plateau value. The fluorescence anisotropy of the microsomes after 2 h incubation and washing (as used in the lifetime measurements) was close to the 17 h value, this indicating that the DPH-PC vesicles which remained must have been mostly removed.

The extent of incorporation was also followed by measuring the fluorescence lifetime and the distributional width of the major lifetime center (for a bimodal Lorentzian analysis, as described below) at 30-min intervals. The lifetime was initially low due to fluorophore self-quenching and the distributional width was relatively high. By 150 min the lifetime had increased to within 0.25 ns of the value for washed membranes and the distributional width was within 0.9 ns (due to the relief of self-quenching as the DPH-PC dispersed into the membrane), in addition the values were clearly approaching plateau values close to those for the washed membranes.

After the fluorescence lifetime measurements were made on the microsomes with incorporated DPH-PC the lipids were extracted using chloroform/methanol and separated on high performance TLC plates [21]. Illumination of the plate with UV light showed that all the fluorescence had remained associated with the PC and none was observed elsewhere on the plate. It is estimated that the DPH-PC/phospholipid ratio achieved was $\approx (1:400)$.

Incorporation of cytochrome b_5 into POPC vesicles was achieved by addition of the protein to 2 ml of 200 μM POPC LUV in a cuvette followed by 30–45 min incubation in the dark at 37°C. For removal of the chromophoric heme, L-1-tosylamide-2-phenylethyl chloromethyl ketone treated bovine pancreatic trypsin (Sigma) was added as powder (1 mg) to 2 ml of vesicles incubated with protein and the total suspension allowed to further incubate for 4 h at room temperature in the dark [22,23].

Fluorescence measurements

Fluorescence lifetime data were obtained using an SLM 48000 multifrequency phase-modulation fluorimeter [24,25]. The source of excitation was from a Liconix Model 4210NB HeCd laser at 325 nm, modulated by a Pockels cell to obtain a range of frequencies from 5 to 150 MHz. Emission was observed through a 420 nm red-pass filter and a Glan-Thompson polarizer, set at the magic angle. For a reference either POPOP (*p*-bis[2-(5-phenyloxazolyl)]benzene) in ethanol (with a lifetime of 1.35 ns, [26]) or an aqueous solution of rabbit liver glycogen as a scatterer was used.

The estimated experimental error used in the analyses was generally taken as 0.2° in the phase and 0.002 in the modulation, based on the standard deviation of averaged values for phase and modulation at each frequency. In some instances individual experiments may have had errors outside this range, this procedure was used to facilitate comparison between experiments and to remain consistent with common practise. For most of the results, analyses from single data sets are given, except for some data in Table III where standard deviations from three experiments are given to give some idea of reproducibility.

Data analysis

Phase and modulation data were subject to analyses using the GLOBAL analysis software [27,28] and SLM software (which uses an optimized version of a non-linear least-squares algorithm [29] to fit the data to minimal values of the reduced χ^2 parameter for the particular analysis and errors chosen. χ^2-surface plots, with confidence levels were calculated using GLOBAL. Various analyses were explored as alternatives to exponential fits, such as a unimodal Lorentzian distribution, but on the basis of the χ^2, a minor component typical of DPH type fluorophores was still required in the analysis. A bimodal Gaussian analysis were also performed in initial studies but in keeping with studies from other laboratories [30–41] a Lorentzian distributional analysis was used. However, the alternative of using Gaussian distributions has to be given serious consideration [42]. The parameters recovered include the lifetime, fractional intensities (relating to the fraction of molecules associated with the lifetime or distribution), distributional width at half peak maximum (for distributions) and the χ^2. For biexponential analyses both programs gave very similar results, although most of the data was analyzed using GLOBAL. For the Lorentzian distributions the analyses were taken out to ten half widths. Previously [13,14] two-half widths were used so that the values obtained for the distributional widths tended to be broader than those obtained in the

present work. For comparative purposes it is important to use the same value for truncation of the tails of the Lorentzians. It should be noted, however, that the it may not be fully appropriate to talk of real values for the distributional width since Lorentzian distribution is only being used as an approximation.

Results and Discussion

The aim of this study was to evaluate the use of membrane fluorophore decay heterogeneity as a tool for probing structural micro-heterogeneity in membranes. To accomplish this initial studies on the fluorescence decay of DPH in cytochrome b_5 in a single species lipid bilayer (POPC) were performed. The results in Table I compare two types of analysis. The first (shown on the left side of the table) is for a single fluorophore population, which is described by a double exponential or bimodal Lorentzian continuous distribution.

The fluorescence lifetime analyses include a minor lifetime component typical of DPH type fluorophores. The photophysical properties of DPH are best described according to a model of DPH having two closely spaced excited states ($^1Ag^*$ and $^1Bu^*$) as recently discussed in a review [43]. It has been suggested that the short lifetime component of DPH is due to a photoproduct of DPH formed after exposure to light [7,44] or that it may explained in terms of emission from the $^1Bu^*$ state possibly stabilized relative to the $^1Ag^*$ state. Further reports have appeared on this subject [45,46] also concerning the two-state model. At present the uncertainty concerning the origin of the short lifetime component and its lack of correlation with a bilayer property do not suggest that it is a parameter which can presently be used to yield useful information concerning lipid bilayers.

Analysis of the fluorescence decay as a continuous distribution recognizes that the fluorophore may experience micro-heterogeneity during the excited state. A second type of analysis (shown on the right side of Table I) is a 'two-fluorophore population analysis' which allows for two separate major lifetime components and is described by a triple exponential or trimodal Lorentzian distribution. In this case there are two distinct fluorophore environments each yielding separate lifetime centers, again with a third minor component.

For DPH in POPC bilayers, a double exponential decay gave a major lifetime of 8.21 ns, with an additional minor lifetime component. Attempts to fit to a bimodal Lorentzian resulted in a very small distributional width, as previously described [13], so that for POPC, DPH experienced an essentially homogeneous

TABLE I

Fluorescence decay analysis of DPH in cytochrome b_5-POPC bilayers

2-exp: biexponential analysis; 3-exp: triple exponential analysis; 2-Lor: bimodal Lorentzian analysis; 3-Lor: trimodal Lorentzian analysis; τ: lifetime centers (ns); W: widths at half-height of distribution (ns); f: fractional intensities; χ^2: chi-squared. The average errors in the phase and modulation were 0.2° and 0.002, respectively. Data were collected for 10–12 frequencies for 5–120 MHz.

		τ	W	f	χ^2		τ	W	f	χ^2
POPC	2-exp	8.21		0.93	0.35					
		2.50		0.07						
cytb_5/POPC (1:50)	2-exp:	6.79		0.80	0.96	3-exp:	7.20		0.70	0.51
		2.21		0.20			3.34		0.25	
							1.37		0.05	
	2-Lor:	6.44	1.31	0.86	0.40	3-Lor:	6.74	1.39	0.70	0.52
		2.38	0.28	0.14			3.98	1.50	0.25	
							2.12	0.10	0.05	
cytb_5/POPC (1:50) [a]	2-exp:	7.74		0.90	0.46	3-exp:	8.37		0.70	0.31
		2.08		0.10			5.16		0.25	
							1.50		0.05	
	2-Lor:	7.72	0.72	0.87	0.29	3-Lor:	8.06	0.00	0.63	0.35
		2.78	2.08	0.13			6.49	1.55	0.28	
							2.28	1.03	0.09	
cytb_5/POPC (1:100)	2-exp:	7.09		0.85	0.25	3-exp:	7.27		0.80	0.15
		2.15		0.15			3.06		0.15	
							1.50		0.05	
	2-Lor:	6.96	0.78	0.88	0.15	3-Lor:	7.13	0.49	0.80	0.22
		2.21	0.17	0.12			4.13	1.41	0.10	
							2.01	0.23	0.10	

[a] Heme removed by trypsination.

single fluorophore environment. Upon addition of cytochrome b_5 (cytochrome b_5/POPC, 1:50) the lifetime center was reduced to 6.79 ns (double-exponential) or 6.44 for a bimodal Lorentzian distributional analysis, which gave an improved χ^2. The latter fit also gave a distributional width for the major lifetime center. Previous studies [14] have shown that the heme group in cytochrome b_5 will quench the fluorescence of DPH by resonance-energy transfer, so the reduced lifetime and distributional width are expected. The distribution is therefore at least partly attributable to the range of DPH-heme distances, leading to a range of decay rates.

When the heme was removed by trypsin treatment, as verified by absorption measurements, the major lifetime center for the double-exponential or bimodal Lorentzian, while higher (7.74 and 7.72 ns, respectively), reflecting an alleviation of quenching, was still reduced compared to the lifetime of DPH in POPC alone. This effect can be ascribed to the influence of the protein.

The fluorophores affected by the protein are essentially only those in the boundary lipid region, defined as the first lipid shell around the protein. This is because the lateral diffusion rate of the lipids is of the order of 10^{-7} cm/s [2], which means that fluorophores in this region, when excited, will have decayed to the ground state before migrating into the bulk lipid region. This leaves a second population of fluorophores resident in the bulk lipid region during the excited state and out of the sphere of protein influence (provided the protein-lipid ratio is low enough). Since in this model, the lifetime of these bulk lipid fluorophores should be the same as in bilayers of POPC alone this suggests that there are two distinct fluorophore populations.

The basic criteria for such analysis to be attempted was that the fluorophores at the protein/lipid interface should experience a lifetime center distinct from that in the bulk lipids a condition which was met by the cytochrome b_5/POPC system. Analysis of the fluorescence decay according to a two fluorophore populations was tested by using a three-lifetime center analysis to account for the two fluorophore populations and the third minor component (as shown in Table I, triple-exponential). This yielded (for cytochrome b_5/POPC, 1:50, without heme) two lifetime centers, one of 8.37 ns, which is close to that for POPC alone, and which may arise from the bulk lipid fluorophore population. According to the two fluorophore population model the other major lifetime center of 5.16 ns could therefore have been due to the protein influenced fluorophore population. One difficulty is that the χ^2 for the triple exponential was not significantly improved over that for the double-exponential or bimodal Lorentzian distribution. At present there is no satisfactory method for choosing between the different

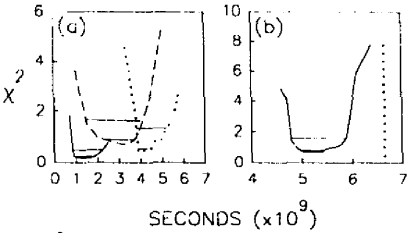

Fig. 1. The χ^2-surface and confidence analyses (at the 67 and 98% levels) for: (a) the distributional width for DPH in cytochrome b_5/POPC (1:100) (solid line) and for DPH-PC in liver microsomes (dashed line) and plasma membranes (dotted line); (b): the lifetime center of the boundary lipid DPH-PC fluorophore population in microsomes (solid line), determined using a fixed bulk lifetime value as recovered from the analysis (shown as the dotted line), illustrating the resolution of the two lifetime centers.

fits unless one describes a more feasible physical model. A further aid to assessing the appropriateness of the chosen model is to examine the χ^2-surface and confidence levels. This is shown, as an example, for the distributional width of the protein influenced fluorophore population (for cytochrome b_5/POPC, 1:100) in Fig 1a.

In the presence of heme, the bulk fluorophore lifetime was 7.2 ns (using a triple exponential analysis), due to quenching by the heme. For a protein/lipid ratio considerably lower than used here, there would also be a third population of bulk lipid fluorophores outside the energy transfer distance of ≈ 7 nm not experiencing quenching.

If instead of a triple exponential, a trimodal Lorentzian distributional analysis is used, a distributional width for cytochrome b_5/POPC (in the presence of heme) of 1.39 ns is recovered for the bulk lipid fluorophore population (center 6.74 ns), reflecting the range of fluorophore distances from the heme. This distributional width narrows to essentially a very small value when the heme is removed, along with the recovery of the lifetime center to 8.06 ns, which was close to the lifetime of DPH in POPC alone, as expected. By contrast, the distributional width associated with the protein influenced fluorophore population (1.50 ns) remained. This would appear to be due to the DPH experiencing environmental heterogeneity in the boundary lipid region. Balanced against the extra information obtained from fitting to the trimodal Lorentzian distribution, as apart from the triple-exponential, is the increased number of fitting parameters which are required. Thus although the Lorentzian distributional has considerable attraction, since the χ^2 for the two fits are comparable (contrasting with DPH-PC in membranes - see below), the case for this particular solution is weakened.

Although removal of the heme allowed the lifetime centers of the bulk and boundary lipid fluorophores to recover to higher unquenched values, the fractions associated with the lifetimes were not affected (using a two-fluorophore population model). These values were 0.25 for the boundary lipid fluorophore lifetime center and 0.7 for the bulk lipid fluorophore lifetime center. Taking, for example, the fraction of boundary lipid fluorophores (without heme) from the trimodal Lorentzian (0.28) and correcting to allow for an assumed non-contributing minor component, gives a number for the percentage of fluorophores in the boundary lipid region of 25%. This value can also be calculated from the known cytochrome b_5 dimensions.

From the approximate radius of cytochrome b_5 of 1.6 nm [47], and of the POPC head group of 0.47 nm [48] a value for the number of boundary lipids of 14 can be calculated. The number has also been experimentally determined [3]. From this number the fraction of lipids in the boundary lipid region can be calculated for bilayers with a known protein/lipid ratio and for the cytochrome b_5/POPC (1:50) the value is 14:50 or 28%. While it should be noted that the cytochrome b_5 is not a true transmembrane protein and that an asymmetric disposition could partially contribute to fluorophore heterogeneity, the fraction recovered is in agreement with the fraction of fluorophores in the boundary lipid region recovered from the two-fluorophore population analysis. This suggests that the distribution of the DPH between the boundary and bulk lipid regions follows that of the lipids and provides support for the possibility that a two population model was at least as appropriate as the other possible solution of a single fluorophore population.

DPH in microsomes and plasma membranes

The fluorescence decay analyses for DPH in microsomes and plasma membranes and in bilayers made from extracted total lipids and phospholipids are shown in Table II. In terms of χ^2, for bilayers of microsomal total lipids, a bimodal Lorentzian fit to the data was superior to the double-exponential at least implying fluorophore environmental heterogeneity. One basis of fluorophore environmental heterogeneity in lipid bilayers has been ascribed to the dielectric constant gradient [33]. Water penetrates into the lipid bilayer in a depth dependent manner so that the dielectric constant varies from ≈ 2–5 at the bilayer center to 70 for water at the membrane surface. The rate of decay of the excited state of DPH is highly sensitive to the dielectric constant [49] and is significantly reduced by the presence of water. Therefore for an ensemble of fluorophores positioned across the bilayer, a range of decay rates will be expected. The sampling of the dielectric constant gradient by DPH in phospholipid bilayers has been previously observed for bilayers of microsomal phospholipids alone [13] and is shown again here for comparison. In comparison, the distributional width was less for bilayers of total microsomal lipids. This would be expected since due to the presence of cholesterol which has been previously shown to decrease the distributional width due to its decreasing the water penetration into the bilayer [35,36,39–41] although since the cholesterol level is small in microsomes the effect is barely significant in this case.

The bimodal Lorentzian, while allowing for a range of fluorophore environments in the membrane, does not allow for the possibility that fluorophores influenced by the bulk lipids and by protein, while in the excited state, may be represented by distinctly separate lifetime centers. However, the basic requirement for the two-fluorophore population analysis to be able to recover two lifetime centers, that the fluorescence lifetime center for the fluorophore in the total lipids alone (here 8.55 ns, for a bimodal Lorentzian) should be different from that in the presence of protein (i.e. for intact microsomes, 8.42 ns) is not met for DPH in microsomes. This means that a two-fluorophore population analysis is not appropriate. The same applied to DPH in liver plasma membranes (Table II). This does not imply that the DPH fluorescence lifetime is not influenced by protein. Also this does not necessarily

TABLE II

Fluorescence decay analysis of DPH in microsomes and plasma membranes

		τ	f	χ^2		τ	W	f	χ^2
Microsomes	2-exp:	8.78	0.89	2.67	2-Lor:	8.42	1.90	0.93	0.25
		2.29	0.11			2.80	1.20	0.07	
Total lipids	2-exp:	8.51	0.94	1.18	2-Lor:	8.55	0.66	0.93	0.72
		2.30	0.06			2.77	1.22	0.07	
Phospholipids	2-exp:	8.22	0.94	1.96	2-Lor:	8.14	0.88	0.95	0.58
		1.21	0.06			0.74	0.69	0.05	
Plasma membranes	2-exp:	9.70	0.88	7.32	2-Lor:	9.38	2.53	0.90	1.14
		1.91	0.12			1.98	1.29	0.10	
Total lipids	2-exp:	9.61	0.89	0.92	2-Lor:	9.17	0.35	0.87	0.71
		2.49	0.11			3.10	0.74	0.13	

mean that there are not two distinct environments but that using DPH the respective lifetime centers are too close for recovery by the analysis.

DPH-PC in microsomes and plasma membranes

DPH-PC has been used in a number of studies [41,50–53] and offers the advantage that it is a phospholipid and that the DPH position in the membrane is known since it is attached to the *sn*-2 fatty acyl chain. The fluorescence decay analyses for DPH-PC in microsomes and plasma membranes and in bilayers of derived total lipids are shown in Table III. A striking observation is that the major lifetime center for a bimodal analysis of DPH-PC, in the total lipids or phospholipids, has a very small distributional width. While for DPH the distributional width may be explained as being due to the DPH sampling across the bilayer, tethering the DPH to the PC *sn*-2 chain appears to prevent this by forcing it to locate at an approximately fixed distance from the bilayer center.

Comparison of the lifetime centers for DPH-PC in microsomes of 6.08 ns, and 7.48 ns for bilayers of microsomal total lipids (from bimodal Lorentzian major lifetime centers) shows these two values were dif-

TABLE III

Fluorescence decay analysis of DPH-PC in microsomes and plasma membranes

		τ	W	f	χ^2		τ	W	f	χ^2
Microsomes										
Intact membranes	2-exp:	6.89		0.83	6.92	3-exp.	11.61		0.22	0.58
		(0.11) [a]		(0.02)			(0.46)		(0.03)	
		1.81		0.17			5.23		0.67	
		(0.30)		(0.02)			(0.14)		(0.01)	
							0.85	0.13		
							(0.11)	(0.02)		
	2-Lor:	6.08	2.17	0.92	1.70	3-Lor:	6.66	0.02	0.21	0.31
		(0.17)	(0.25)	(0.03)			(0.11)	(0.03)	(0.01)	
		1.33	0.43	0.08			5.13	3.02	0.72	
		(0.32)	(0.33)	(0.03)			(0.15)	(0.56)	(0.03)	
							1.06	0.82	0.07	
							(0.50)	(0.46)	(0.03)	
Total lipids	2-exp:	7.53		0.93	0.59					
		2.58		0.07						
	2-Lor:	7.48	0.01	0.94	0.71					
		2.51	0.10	0.06						
Phospholipids	2-exp:	7.31		0.89	0.74					
		(0.05)		(0.00)						
		3.07		0.11						
		(0.09)		(0.00)						
	2-Lor:	7.30	0.08	0.89	0.65					
		(0.05)	(0.11)	(0.00)						
		3.05	0.00	0.11						
		(0.11)	(0.00)	(0.00)						
Plasma membranes										
Intact membranes	2-exp:	7.70		0.76	5.14	3-exp:	15.52		0.16	1.64
		(0.13)		(0.03)			(1.57)		(0.01)	
		1.36		0.24			6.15		0.65	
		(0.07)		(0.03)			(0.06)		(0.02)	
							1.14	0.19		
							(0.08)	(0.03)		
	2-Lor:	6.64	3.53	0.87	1.16	3-Lor:	8.58	0.06	0.19	1.06
		(0.08)	(0.57)	(0.02)			(0.69)	(0.04)	(0.01)	
		1.11	0.36	0.13			5.92	4.04	0.66	
		(0.06)	(0.02)	(0.02)			(0.37)	(0.68)	(0.02)	
							1.16	0.62	0.14	
							(0.08)	(0.23)	(0.02)	
Total lipids	2-exp:	7.78		0.86	2.76					
		1.84		0.14						
	2-Lor:	7.81	0.00	0.86	2.73					
		1.95	0.25	0.14						

[a] S.D. in parentheses (where given) are for three independent experiments for which the χ^2 from the different fits are all from a single representative data set.

ferent enough to justify attempting a two fluorophore population analysis. In contrast to the results for cytochrome b_5, the χ^2 for a trimodal Lorentzian distributional analysis was superior to the triple exponential and the distributional width of 3.02 ns for the protein influenced fluorophores was considerable (see χ^2-surface plot, Fig. 1a). The distributional width for the DPH-PC population in the bulk region was very small, and in keeping with this, the value in the total lipid bilayers was also very narrow (due to the inability of the DPH of DPH-PC to sample the dielectric constant gradient). We note that for cytochrome b_5 a very small distributional width for the trimodal Lorentzian analysis was also found for the bulk lipid region (with DPH) but the reason was due to the POPC, which being a single species, packs in such a way as to prevent the same degree of water penetration found for the mixed species microsomal phospholipid bilayers, as noted previously [13]. The very narrow distributional width for the bulk lipids further facilitated the recovery of separate lifetime centers. This is illustrated in the χ^2-surface plot for the protein influenced fluorophore population lifetime center, generated using a fixed bulk lifetime value as shown in Fig 1b.

For DPH-PC in plasma membranes, again a two population analysis was considered appropriate since comparing the values for the bimodal Lorentzian lifetime center for intact plasma membranes (6.64 ns) and total lipid bilayers (7.81 ns) showed considerable protein influence (see Table III and Fig. 1a).

Plasma membranes contain considerable cholesterol which, as mentioned above, tends to increase the lifetime center and narrow the distributional width. A likely consequence would be that any fluorophore in the bulk lipid should have a lifetime center, higher and narrower, than any potential protein directed center. Thus cholesterol presence would be expected to facilitate recovery of the fluorophore populations in the bulk and protein-influenced regions. The results confirmed this, showing two clearly distinguishable lifetime centers which can again be tentatively ascribed to bulk lipid and protein-influenced fluorophore populations (see Table III).

Environmental heterogeneity of boundary lipid fluorophores

The recovery of a distributional width in the analyses indicates that the ensemble of fluorophores in the sample experience a range of different environments while in the excited state. There have been a number of studies showing that for lipid bilayers an analysis as a distribution of decay rates is more appropriate than a discrete single-exponential decay [13,14,30–42,54]. For lipid bilayers alone environmental heterogeneity has been ascribed to the dielectric constant gradient (see above). At present it is not known what causes the environmental heterogeneity at the protein/lipid interface, although it again could be due to the presence of water. There is also a possibility, as yet uncertain, that some contribution could arise from a range of orientations of the fluorophores [55–57]. It should be noted that for a fluorophore with a longer lifetime than DPH (e.g. pyrene) a distributional width could still be recovered. This would occur because the boundary lipid residency time for the fluorophore would then be shorter than the lifetime of the excited state so that the region encompassed by protein influenced fluorophores would then extend beyond the first lipid shell. In this case, each fluorophore would then reside at the protein surface, and be under its influence, for a different proportion of its time in the excited state which would lead to the recovery of a range of decay rates.

Fluorophore features important for heterogeneity analysis

It is important to recognize that to a considerable extent the properties of the fluorophore, as well as the form of analysis used, can 'impose' features on the extent of the heterogeneity which is revealed. Nevertheless, this presents interesting potential for varying the fluorophore, attaching to different lipids or using fluorophores which have a non-random distribution in the membrane. Also there is the possibility of extending the protein-influenced fluorophore population beyond the first shell of lipids as discussed above. The opposite of adding a collisional quencher to shorten the lifetime could also be envisaged.

In labeling intact membranes with fluorophores there is always the possibility that the fluorophore may not be evenly distributed in the membrane. This probably does not occur with DPH since its hydrophobic character without charge or tethering to another molecule would tend to allow it to reach most available areas. In addition, DPH is thought to distribute evenly, even amongst different lipid phases [12,58] although this latter point remains to be demonstrated for DPH-PC. With DPH-PC it is also difficult to be certain that it reaches all regions of the membrane, also for plasma membranes, the PC tends to locate more in the outer half of the bilayer. For microsomes a more random distribution may occur due to the presence of a non-specific flipase [59]. These features need to be considered when interpreting this type of experiment, although again it may be possible to take advantage of fluorophores locating in specific regions of membranes.

It is possible that other fluorophores with a conjugated diene structure which are tethered to PC would behave in a similar manner to DPH-PC, for example parinaric acid. The conjugated diene structure leads to a relatively inflexible rod shaped molecule, which because of neighboring phospholipid fatty acyl chains, cannot deviate far from the perpendicular to the bilayer normal. By contrast, one would expect anthroyl-

stearates attached to PC would still be able to sample to some extent across the bilayer [60].

Conclusions

In this work the use of fluorophore decay heterogeneity as a probe for membrane lipid organization has been assessed. The results of the studies with cytochrome b_5 suggest that a two-fluorophore population analysis of the fluorescence decay have the potential of providing useful information pertaining to the boundary lipid region. Fluorescence spectroscopic methods are widely employed in the study of natural membranes and the fluorescence lifetime is a common parameter which is determined. An important conclusion, with implications in the field of fluorescence spectroscopy of natural membranes in general, is that the common assumption of either a single homogeneous fluorophore population or a heterogeneous population centered around a single lifetime center (i.e. using the single fluorophore population model) has to be applied with caution in the decription of the physical situation in membranes and lipid bilayers containing proteins. The present study indicates that one possible solution is to move to a multiple fluorophore population analysis of the type examined here. At the same time multiple fluorophore population analysis show some dependence on the membrane composition and the fluorophore type and although these features may be potentially beneficial the method must be applied with caution. In this study we have at least shown that useful information can be obtained in spite of the present imperfection in methodology and theoretical understanding.

In summary, it has been shown that information on the boundary lipid region can be directly obtained from the fluorescence decay of membrane fluorophores. The present approach is novel in that from the fluorescence lifetime data from a single measurement it yields information on the number of boundary lipids, as well as on the degree of fluorophore environmental heterogeneity at the protein/lipid interface. This technique should be of some potential use in the study of this important region in cell membranes.

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