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DPH Lifetime Distributions in Vesicles Containing
Phospholipid HydroperoxidesMary Lou Wratten⁺, Enrico Gratton^{*}, Martin van de Ven^{*} and Alex Sevanian⁺⁺ Department of Pathology and Toxicology, University of Southern
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Multifrequency phase fluorometry was used to determine the lifetime distributions of 1,6 diphenyl-1,3,5-hexatriene in 1-palmitoyl-2 linoleoyl phosphatidylcholine small unilamellar vesicles containing 2% incorporation of phospholipid hydroperoxides. A biexponential decay was observed in both vesicle preparations over a temperature range of 5 to 35°C. Vesicles containing phospholipid hydroperoxides showed an overall longer lifetime as well as a greater distribution in width. These findings suggest that phospholipid hydroperoxides create structural heterogeneity in membrane structure.

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Lipid peroxidation occurs in membranes during periods of oxidant stress (1-5). Oxidative modifications to component phospholipids may in turn compromise the normal structure and functions of the cell membrane (6,7). There is also growing evidence that even trace amounts of oxidized phospholipids can have a profound effect on the biophysical characteristics of phospholipid bilayers (8,9).

Relatively little is known about the specific mechanisms by which these modifications affect the structural characteristics of the membrane. Oxidative modifications may affect membrane hydration, the packing order of the phospholipids or they may create microdomains within the phospholipid bilayer. DPH serves as a sensitive probe to changes in the membrane environment. There is a direct correlation between the lifetime decay of the probe and its surrounding dielectric constant. If the probe is in a

Abbreviation: DPH, 1,6 diphenyl - 1,3,5-hexatriene.

relatively polar environment, its lifetime is shortened compared to the lifetimes observed in hydrophobic environments.

In this investigation we measured the fluorescence decay of DPH in control and oxidatively modified small unilamellar vesicles. Our observations suggest that the presence of even small amounts of phospholipid hydroperoxides in membrane bilayers dramatically alters the environment of the probe via changes in the dielectric constant. Moreover, small amounts of phospholipid hydroperoxides specifically appear to create disturbance in membrane phospholipids elucidating further the question of how lipid peroxidation affects biomembrane structure. Our findings may also have an important bearing on spectroscopic studies utilizing unsaturated phospholipids which often contain trace amounts of lipid peroxides.

Materials and Methods

1-palmitoyl 2-linoleoyl phosphatidylcholine (PLPC) was purchased from Sigma and was purified by HPLC (10). 1-palmitoyl (~2-(9,13-hydroperoxy) linoleoyl-phosphatidylcholine (PLPC-OOH) was prepared from PLPC by using soybean lipoxygenase (Sigma) as described previously (11). PLPC-OOH was purified by reverse phase HPLC (12) and stored in dichloromethane. Background levels of peroxides of both control and modified phospholipids were checked before and immediately after the experiments by HPLC (12) and gas chromatography (13).

Small unilamellar vesicles (100 nm) were prepared under N_2 , in 10 mM Tris/150 mM KCl as described previously (8). Control vesicles were made entirely of PLPC; modified vesicles contained PLPC with 2 mole percent incorporation of PLPC-OOH. Size distributions were analyzed by electron microscopy (8) and dynamic light scattering. DPH in tetrahydrofuran was added to the vesicles 30 minutes prior to measurements to give a final probe concentration of 10^{-6} M and a 1:1000 ratio of probe to lipid.

Measurements were taken on a multifrequency cross correlation phase fluorometer (14) equipped with a 325 nm HeCd laser (Liconix) and an ISS ADC interface for data collection and global analysis package. A long wavelength cut-on filter RG 370 (Janos) was used to eliminate contributions from scattered light. The modulation frequencies used were: 2,4,7,11,16,20,25, 40,60, and 90 MHz. p-bis[1-5-phenyloxazoyl]benzene (POPOP) in ethanol was used as a reference compound (1.35 ns lifetime). The sample cuvette was kept under N_2 for all measurements. Temperature were regulated by a circulating water bath and monitored by means of a thermistor probe inserted into the reference cuvette. The data was analyzed using Global Analysis Software (University of Illinois, LFD) as described elsewhere (15).

Results

The fluorescence decay of DPH was measured in PLPC and PLPC-OOH containing vesicles over the temperature range of 5 to 35°C (Table 1). Data

Table 1. Discrete biexponential fluorescence decay for DPH in pure PLPC and 2% PLPC-OOH containing small unilamellar vesicles

TEMP (°C)	5	10	15	20	25	30	35
<u>PLPC VESICLES</u>							
LIFETIME 1 (ns)	3.74	3.62	2.80	2.64	2.36	2.11	2.00
LIFETIME 2 (ns)	9.67	9.62	9.19	8.94	8.73	8.48	8.23
FRACTION 2	0.877	0.874	0.915	0.915	0.920	0.920	0.910
X ²	0.636	0.126	1.01	0.445	0.233	0.315	0.351
<u>PLPC Vesicles with 2% PLPC-OOH</u>							
LIFETIME 1 (ns)	4.61	4.00	3.29	3.24	2.91	2.30	2.19
LIFETIME 2 (ns)	10.08	9.92	9.53	9.48	9.18	8.79	8.61
FRACTION 2	0.846	0.877	0.911	0.903	0.912	0.930	0.923
X ²	0.753	0.801	0.512	0.66	0.791	0.557	1.488

were analyzed both for a discrete multi-exponential decay and a continuous distribution of lifetimes (16). For a discrete biexponential decay, data were best characterized by a short (approximately 3 ns) and a long (approximately 9 ns) component (Table 1).

Both PLPC and PLPC-OOH vesicles showed a decrease in DPH lifetimes as the temperature was increased. In addition, when analyzed by a Lorentzian distribution, the full width at half maximum decreased with increasing

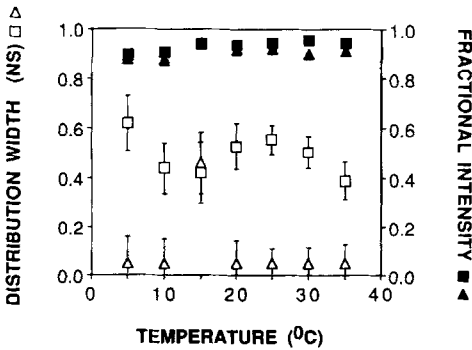


FIGURE 1. Width (FWHM) in nsec. and fractional intensity for the longer component of a continuous bimodal lifetime distribution (Lorentzian) for pure PLPC and 2% PLPC-OOH containing small unilamellar vesicles. Error bars indicate 67% confidence interval.

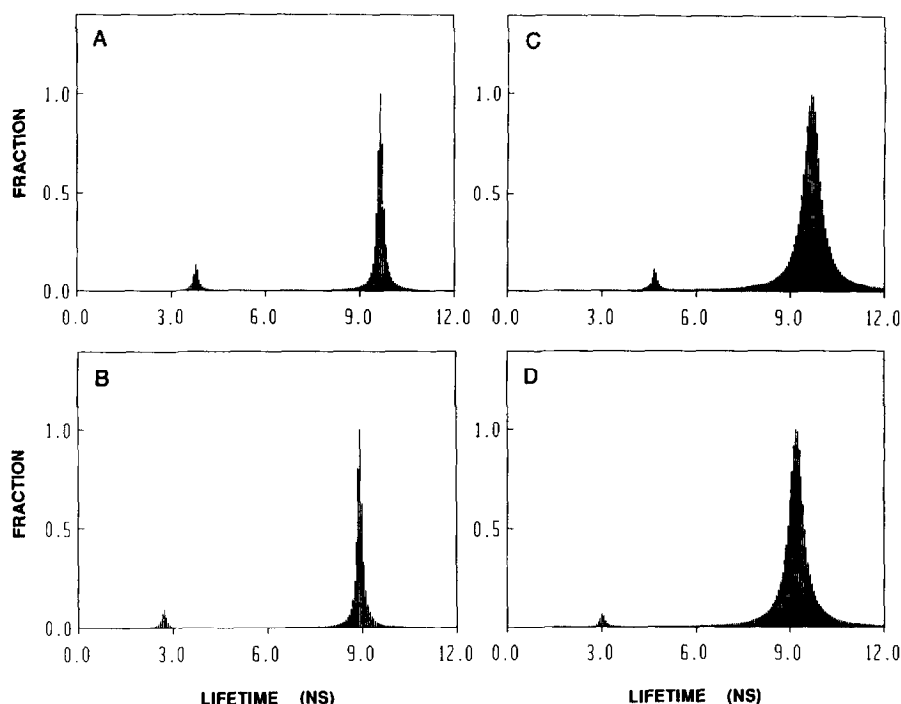


FIGURE 2. A typical bimodal Lorentzian lifetime distribution for a) PLPC vesicles at 5°C ($X^2 = 0.745$), b) PLPC vesicles at 20°C ($X^2 = 0.501$), c) PLPC-OOH vesicles at 5°C ($X^2 = 0.622$), and d) PLPC-OOH vesicles at 20°C ($X^2 = 0.756$).

temperature for both PLPC and PLPC-OOH vesicles. It is also interesting to note that while the distribution width decreased, fractional contribution (which indicates the relative amount of lifetime species for each component), did not change significantly (Fig 1).

The lifetime of DPH for both the short and long components was longer in PLPC-OOH vesicles than in PLPC vesicles. PLPC-OOH vesicles also had a much larger width of the long component as compared to control PLPC vesicles (Fig 2).

Discussion

Because DPH is assumed to partition equally between the various phases, it should reflect differences in local environments. Previous studies have confirmed that DPH undergoes a biexponential decay in both saturated (17,18) and unsaturated phospholipid bilayers (19); and that the lifetime decay of the

probe is temperature dependent (17). The purpose of our present study is to determine how the introduction of an oxidatively modified phospholipid affects the fluorescence decay and lifetime distributions of DPH.

Although there is still considerable debate as to the nature of the short lifetime component, it has been proposed that the short lifetime component derives from different transbilayer locations of the probe (20).

There are several possibilities as to the difference in lifetimes and lifetime distribution between control and oxidatively modified vesicles. First, the incorporation of hydroperoxyphospholipids may cause a physical reorientation or shift in location of the probe within the bilayer. A physical reorientation may cause the probe to be positioned in an environment with a lower dielectric constant. The modified phospholipids may also cause differences in packing or create microdomains in the bilayer. These types of changes may increase the lifetime values.

The increase in the distribution width of the PLPC-OOH containing vesicle may be due in part to a more heterogeneous probe environment induced by different structural constraints imparting a variety of orientations placed on the probe. Previous studies with peroxide free vesicles have shown that as the temperature is increased, the full width at half maximum decreases (17,18). This may be due to a higher diffusion rate of the probe moving in and out of the membrane as well as a more "average environment" of the probe within the bilayer. The PLPC-OOH may hinder the probe motion or diffusion rate within the membrane causing both a shift in the overall lifetimes as well as a gradient of probe environments, and hence a broader distribution of lifetimes.

Another explanation of these differences could be due to interactions of the probe with the peroxide moiety. The peroxide groups are weakly ionized and could play a role in having a repulsive effect on DPH affecting its orientation in the membrane. This may be clarified further through direct

measurements of probe orientation in bilayers containing oxidatively modified phospholipids.

Hydroperoxyphospholipids represent the primary product of lipid peroxidation in vivo (3). It must be stressed that studies of membrane structure in systems where oxidative modifications have been imposed by induced peroxidation of the phospholipid components are likely complicated by the variety of primary and secondary decomposition products that are generated. In addition, the levels of these products can be considerably higher than those formed during oxidant stress in vivo. These factors are likely to complicate the interpretation of biophysical data derived from membranes by virtue of the chemical complexity imposed by lipid peroxidation. Our study has shown that even small amounts of a specific phospholipid peroxide significantly change the biophysical characteristics of the membrane.

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