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Fluorescence lifetime distributions of 1,6-diphenyl-1,3,5-hexatriene reveal the effect of cholesterol on the microheterogeneity of erythrocyte membrane

R.M. Fiorini ^a, M. Valentino ^a, M. Glaser ^b, E. Gratton ^c and G. Curatola ^a

^a *Istituto di Biochimica e Istituto di Medicina del Lavoro, Università di Ancona (Italy) and Departments of* ^b *Biochemistry and* ^c *Physics, University of Illinois, Urbana-Champaign, IL (U.S.A.)*

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The fluorescence decay of 1,6 diphenyl-1,3,5-hexatriene (DPH) has been used to characterize aspects of the erythrocyte membrane structure related to the microheterogeneity of the lipid bilayer. The DPH decay has been studied using frequency domain fluorometry and the data analyzed either by a model of discrete exponential components or a model that assumes a continuous distribution of lifetime values. The main intensity fraction was associated with a lifetime value centered at about 11 ns in the erythrocyte membrane, but a short component of very low fractional intensity had to be considered to obtain a good fit to the data. The lifetime value of the long component was insensitive to temperature, while the width of the distribution decreased with increasing temperature. In multilamellar liposomes prepared from phospholipids extracted from the erythrocytes, the long lifetime component showed a temperature dependence. The depletion of 27% of the cholesterol in the erythrocyte membrane induced a broadening of the distribution, suggesting a homogenizing effect of cholesterol. This effect has also been detected in egg phosphatidylcholine at a very low cholesterol/phospholipid molar ratio. The role of cholesterol on membrane heterogeneity is discussed in relation to the effect of cholesterol on water penetration.

Introduction

A basic assumption of the fluid mosaic model of the membrane structure is that there is free diffusion of membrane components. However, an asymmetric distribution of membrane components between the two monolayers [1], as well as the

linkage of membrane lipids and proteins with elements of cytoskeleton and cytomusculature [2], can induce a macroheterogeneity in the membrane. Moreover, theoretical models and experimental evidence suggest the possibility of microheterogeneity in the structural organization of model and natural membranes [3–8]. In a bilayer containing a single phospholipid, structural defects have been demonstrated by a variety of techniques, and these defects have been ascribed to conformational disorder of acyl chains related to *trans-gauche* isomerization [3]. Although these defects are transient, they could be important for transport processes in the membranes [9,10]. Structural defects increase with increasing temperature and defects exist at the boundary between

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; FWHM, full width at half maximum; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene.

Correspondence: G. Curatola, Istituto di Biochimica, Facoltà di Medicina, Via Ranieri, 60131 Ancona, Italy.

fluid and gel phases [11]. In multicomponent systems, the coexistence of different phases has been shown by means of phase diagrams generated from calorimetric [12] and spectroscopic [13] data. Interestingly, Wunderlich et al. [14] were able to observe the presence of 'lipid clusters' in total lipid extracts from *Tetrahymena pyriformis*. In lipid mixtures below the phase transition, cholesterol can induce a phase separation, giving rise to cholesterol-rich and cholesterol-poor phases [15,16]. Moreover, the enthalpy and the cooperativity of the gel-to-liquid-crystalline phase transition is reduced by increasing cholesterol concentrations [17]. This effect is also observed for mixed chain phospholipids [18]. Cholesterol affects phospholipid phase behaviour in intact cell membranes also. In mouse LM cells, cholesterol depletion induces a phase transition which disappears after partial sterol repletion [19,20]. Similarly, Hui et al. [21], in cholesterol-depleted erythrocyte membrane, have shown the appearance of lipid-phase separation, which probably induces a segregation of integral membrane proteins into domains. These observations suggest that cholesterol could regulate the degree of membrane heterogeneity by means of its tendency to reduce the cooperativity of the interactions between acyl chains; on the other hand, cholesterol increases membrane ordering [19,22], preventing the segregation of membrane components. Besides cholesterol, membrane proteins could also be very important in giving rise to membrane domains. However, the influence of proteins on acyl chain order and mobility as well as on the segregation of specific lipids is still controversial.

The possibility of studying membrane heterogeneity using some physical or physicochemical methods is important and will improve our knowledge of membrane structure and its physiological implications. However, there are a number of potential methodological and technical difficulties: domains might be very small and changes of physical properties in the domains can be difficult to analyze. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has been widely used to study the structure and dynamic properties of model and natural membranes. The fluorescence decay of DPH is single exponential in isotropic solvent at room temperature, while it presents a

multiple exponential decay in mixed phospholipid vesicles [13]. In phospholipid vesicles, we are able to describe the decay using a continuous distribution of lifetime values. The width of the lifetime distribution can be related to the different physical properties of the environment in which the DPH molecules are located, i.e., the membrane microheterogeneity [23].

In this article, we apply this approach to study the microheterogeneity of the erythrocyte membrane. We chose the erythrocyte membrane because the erythrocyte has only a single membrane, and consequently it can be prepared without contamination by other membrane fractions. Moreover, its cholesterol content can easily be modified without any other compositional change. In the present article, we have analyzed the DPH fluorescence decay in cholesterol-depleted and intact erythrocyte membranes. We have also studied liposomes prepared from erythrocyte phospholipid extracts and from egg phosphatidylcholine. The decay data have been analyzed using either the exponential approach or the continuous distribution analysis.

Materials and Methods

Materials

Cholesterol was obtained from Sigma, St. Louis, (MO), egg phosphatidylcholine was obtained from Avanti Polar Lipids, Birmingham (AL). DPH was obtained from Molecular Probes, Eugene (OR). Both lipids and probe were used without further purification.

Membrane preparations

Hemoglobin-free erythrocyte membranes were prepared from fresh human blood by hypotonic hemolysis according to Bramley et al. [24]. The extraction of lipids from erythrocyte membranes was performed according to Zwaal et al. [25]. Neutral lipids and phospholipids were separated by chromatography on short Unisil columns according to Ferguson et al. [26]. Multilamellar liposomes were formed from erythrocyte phospholipids by resuspending the extracts in 10 mM Hepes/100 mM KCl (pH 7.4) and shaking the suspension vigorously. Modification of the cholesterol to phospholipid ratio in erythrocyte

membranes using DPPC liposomes was carried out according to Cooper et al. [27]. 25 mg DPPC was added to 10 ml of 0.155 M NaCl and sonicated for 60 min with a Branson bath sonifier. After sonication, 4.0 ml of human serum albumin (2.0%, w/v) were added and the albumin/phospholipid mixture was centrifuged at $21\,800 \times g$ for 30 min to sediment any undispersed lipid. Fresh normal human blood was washed three times with Hanks' balanced salt solution and resuspended at a hematocrit of 10% in Hanks' solution containing penicillin (100 U/ml). The red-cell suspension was mixed with equal volumes of the albumin/phospholipid mixture and incubated in a shaking water-bath for 16 h. The liposome-free control consisted of equal volumes of the erythrocyte suspension and 0.155 M NaCl with albumin. After incubation, the samples were washed three times and used to prepare hemoglobin-free erythrocyte membranes. The total lipid extracted from the erythrocytes incubated with liposomes has been shown by thin-layer chromatography [28] to have a phospholipid composition similar to that observed in control erythrocyte membranes. Egg phosphatidylcholine and cholesterol-egg phosphatidylcholine (5 to 100, molar ratio) multilamellar liposomes were prepared at room temperature by drying the lipids under N_2 and resuspending them in 0.15 M NaCl/5 mM phosphate buffer (pH 8) with vigorous shaking.

Assay procedures

Cholesterol was determined by the cholesterol oxidase assay [29]. This assay showed that the depletion procedure used with the intact erythrocytes had extracted about 27% of the membrane cholesterol. Phosphate was determined according to Kates [30] and protein concentration was determined by the method of Lowry et al. [31].

Fluorescence measurements

DPH dissolved previously in tetrahydrofuran was added to the samples to give a final concentration of 10^{-6} M, and a DPH/phospholipid molar ratio of 1:1000. The suspension was incubated for 1 h in the dark. Under these experimental conditions, the background phospholipid fluorescence was less than 0.5% of the total fluo-

rescence of the DPH-labeled samples. Lifetime measurements were performed with the multi-frequency phase fluorometer described by Gratton and Limkeman [32]. In order to eliminate polarization effects in the fluorescence measurements, the experiments were carried out using an excitation polarizer at 55° with respect to the horizontal direction and no polarizer in the emission side [33]. The instrument was equipped with an ISS-ADC interface for data collection and analysis. The wavelength of excitation was set at 325 nm (ultraviolet line of an HeCd laser, Liconix Model 4240 NB). The modulation frequencies used were 2, 5, 10, 15, 20, 25, 30, 50 and 70 MHz. Data were accumulated at each modulation frequency until the standard deviations of the phase and modulation values were below 0.1° and 0.002, respectively. The sample temperature was controlled using an external bath circulator and the temperature was measured in the sample cuvette prior to and after each measurement using a digital thermometer. All lifetime measurements were obtained using POPOP in the reference cell. The POPOP lifetime was 1.35 ns [34]. The fluorescence was measured through a long-pass filter type RG 370 from Janos Technology (Townshend, VT) which showed negligible luminescence. The experimental data were analyzed assuming either a sum of exponential or a continuous distribution of lifetime values [23]. A program provided by ISS (La Spezia, Italy) was used for the distribution analysis. For both the exponential and distribution analysis, the programs minimize the reduced chi-square defined by an equation reported elsewhere [23]. The applicability of distribution analysis in comparison with exponential approach to study lifetime heterogeneity has been already discussed in detail by Alcalá et al. [35].

Results

The DPH emission decay in all samples was measured in the temperature range of 4 – 50°C . In erythrocyte membranes (Table I), the fluorescence decay analyzed using two exponentials showed a long component of about 11.5 ns with a fractional intensity of 0.9, that was insensitive to temperature. A short component of about 2–4 ns was also observed. The same data were also analyzed using

TABLE IA

EXPONENTIAL ANALYSIS OF THE FLUORESCENCE EMISSION DECAY OF DPH IN ERYTHROCYTE MEMBRANES

Abbreviations: τ_1 , τ_2 , lifetime in ns; f_1 , f_2 , fractional intensity; χ^2 , reduced chi-square.

T (°C)	τ_1	f_1	τ_2	f_2	χ^2
3.6	11.76 ± 0.17	0.94 ± 0.01	2.18 ± 0.36	0.06	3.42
10.0	11.64 ± 0.20	0.95 ± 0.01	2.20 ± 0.51	0.05	4.60
20.5	11.80 ± 0.16	0.94 ± 0.01	3.19 ± 0.45	0.06	1.80
30.9	11.95 ± 0.19	0.91 ± 0.02	4.60 ± 0.50	0.09	0.91
40.3	11.58 ± 0.12	0.93 ± 0.01	3.88 ± 0.35	0.07	0.65
50.0	11.02 ± 0.06	0.95 ± 0.00	2.81 ± 0.19	0.05	0.31

TABLE IB

DISTRIBUTION ANALYSIS OF THE DECAY

Abbreviations: C , center of the distribution in nanoseconds; W , full width at half maximum (FWHM) in nanoseconds; f , fractional intensity; χ^2 , reduced chi-square.

T (°C)	C	W	f	χ^2
3.6	11.30	1.47	0.98	1.63
10.0	11.21	1.47	0.99	2.17
20.5	11.33	1.15	0.98	1.01
30.9	11.51	0.80	0.95	0.67
40.3	11.53	0.10	0.93	0.76
50.0	11.00	0.10	0.95	0.37

TABLE IIA

EXPONENTIAL ANALYSIS OF THE FLUORESCENCE EMISSION DECAY OF DPH IN LIPOSOMES MADE FROM PHOSPHOLIPIDS EXTRACTED FROM THE ERYTHROCYTES

Abbreviations: τ_1 , τ_2 , lifetime in nanoseconds; f_1 , f_2 , fractional intensity; χ^2 , reduced chi-square.

T (°C)	τ_1	f_1	τ_2	f_2	χ^2
2.4	10.20 ± 0.19	0.87 ± 0.02	2.65 ± 0.24	0.13	2.82
10.1	10.00 ± 0.21	0.86 ± 0.02	2.70 ± 0.25	0.14	3.11
20.1	9.43 ± 0.09	0.89 ± 0.01	2.48 ± 0.15	0.10	0.74
30.3	9.17 ± 0.13	0.89 ± 0.01	2.37 ± 0.21	0.11	1.52
40.2	8.50 ± 0.09	0.90 ± 0.01	2.07 ± 0.18	0.10	0.96
50.3	7.88 ± 0.11	0.89 ± 0.01	1.84 ± 0.20	0.11	1.48

TABLE IIB

DISTRIBUTION ANALYSIS OF THE DECAY

Abbreviations: C , center of the distribution in nanoseconds; W , full width at half maximum (FWHM) in nanoseconds; f , fractional intensity; χ^2 , reduced chi-square.

T (°C)	C	W	f	χ^2
2.4	9.56	1.47	0.91	0.91
10.1	9.58	0.93	0.89	2.43
20.1	9.44	0.62	0.88	0.78
30.3	9.00	0.45	0.90	1.46
40.2	8.39	0.28	0.91	0.95
50.3	7.67	0.10	0.85	1.13

a continuous distribution of lifetime values characterized by a sum of two Lorentzians centered at a decay time C , and having a full width at half maximum (FWHM). Only the major lifetime component is reported in Table I. Although the center of the distribution had a value similar to that of the long component obtained from the exponential analysis, the width of the distribution showed a definite dependence on temperature. The lifetime distribution was relatively broad up to 30°C (W , 1 ns), becoming very narrow at 40 and 50°C (W , 0.1 ns). In multilamellar liposomes made from phospholipids extracted from the erythrocytes, a double-exponential analysis gave a long component and a short component, both sensitive to the temperature (Table II). The longer lifetime value gradually decreased from 10.20 ns to 7.88 ns as the temperature increased. The average lifetime

using a distributional analysis gave similar results, except that the center of the lifetime distribution at 2.4 and 10.1°C was slightly lower than the lifetime value obtained using the exponential analysis. The distribution width was broad at low temperature (W , 1.4 ns), and it decreased gradually as the temperature was increased up to 50°C, although at 40°C it was broader than that of the erythrocyte membrane.

Since the width of the distribution in phospholipids is larger than that previously observed in total lipid extracted from erythrocyte [36], we thought it would be of interest to study a possible role of cholesterol in controlling the degree of membrane heterogeneity. Therefore, we investigated the DPH lifetime in membrane from cholesterol-depleted erythrocytes and in egg phosphatidylcholine/cholesterol liposomes. The measurements were performed at 40°C, since both the

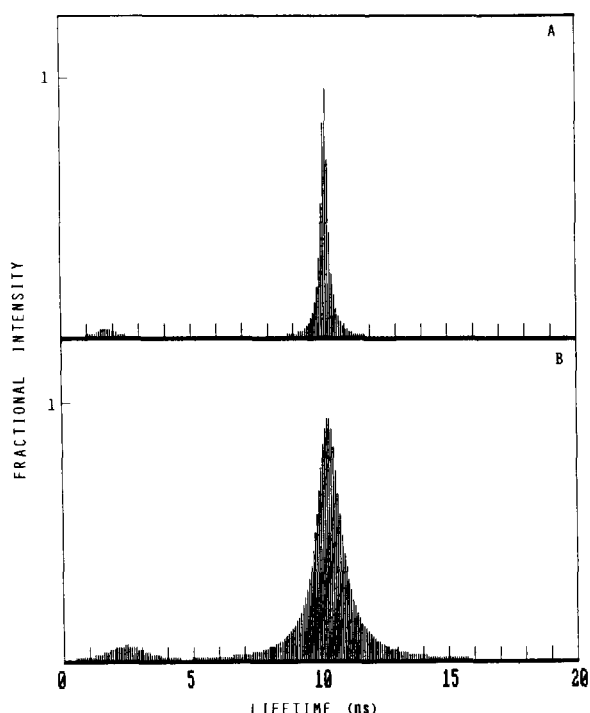


Fig. 1. (A) Distribution analysis of DPH at 40°C in erythrocyte membranes obtained from erythrocytes incubated overnight at 37°C. The recovered distribution has a center at 10.27 ns and a FWHM of 0.05 ns. Chi-square, 2.70. (B) Distribution analysis of DPH at 40°C in erythrocyte membranes obtained from cholesterol-depleted erythrocytes incubated overnight at 37°C. The recovered distribution has a center at 10.32 ns and a FWHM of 0.63 ns. Chi-square, 0.39.

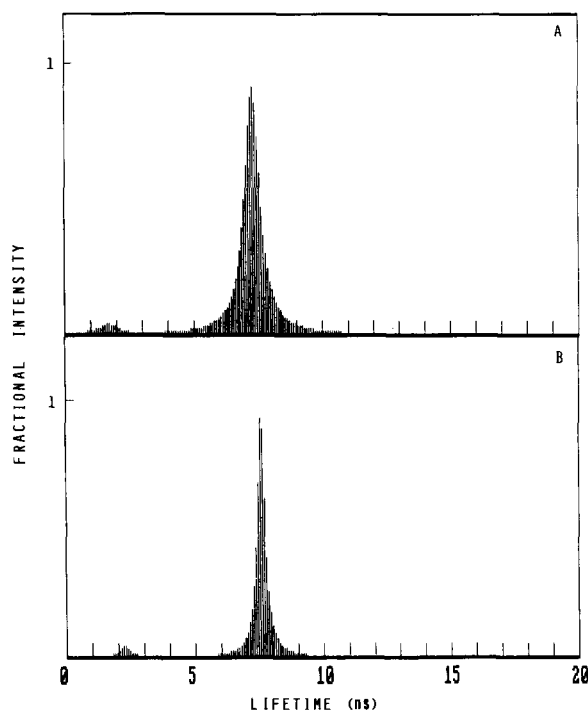


Fig. 2. (A) Distribution analysis of DPH in egg phosphatidylcholine multilamellar liposomes at 40°C. The recovered distribution has a center at 7.28 ns and a FWHM of 0.25 ns. Chi-square, 0.29. (B) Distribution analysis of DPH in cholesterol-egg phosphatidylcholine multilamellar liposomes (5:100, molar ratio) at 40°C. The recovered distribution has a center at 7.57 ns and a FWHM of 0.06 ns. Chi-square, 0.83.

erythrocyte membranes and the liposomes made from extracted phospholipids showed a narrower distribution at this temperature. In cholesterol-depleted erythrocyte membranes and in the control, the distribution analysis gave almost the same average lifetime value around 10.30 ns (Fig. 1A, B). The value was slightly lower than that observed in freshly prepared erythrocyte membranes. However, in cholesterol-depleted erythrocyte membranes, the width of the distribution was larger than the control (Fig. 1A, B).

In egg phosphatidylcholine liposomes, the DPH lifetime was 7.28 ns (Fig. 2A). This value is similar to those for DPPC and DMPC above the main transition temperature [23]. Moreover, the width of the distribution had the same value as that observed in phospholipid extracts. The addition of cholesterol (5:100, mole ratio) induced a very slight increase of the lifetime value to 7.57 ns and a remarkable decrease of the distribution width to a value similar to that of the erythrocyte membranes obtained from erythrocytes incubated overnight at 37°C (Figs. 2B and 1A). This trend was maintained at higher cholesterol concentrations (data not shown). The results of the study of the effect of cholesterol concentration on the DPH decay in egg phosphatidylcholine is in preparation.

Discussion

Using a discrete exponential analysis, we have shown that DPH decay in erythrocyte membranes can be characterized by two exponential components. The long component of 11 ns represents about 90% of the fractional intensity, in agreement with previous observations [37]. In analogy with previous results in model and natural membranes [23,27], a short component of 2–3 ns associated with about 5–10% of the fractional intensity was also observed. The origin of the short component is debated at present, although it is known to be sensitive to the time of sample irradiation and may be due in part to a photolysis artefact [13]. In membranes, it cannot be excluded that the short component can represent a small DPH fraction localized in a very polar environment [37]. The lifetime of the long component was largely insensitive to temperature. In membranes, the DPH

steady-state polarization changes with temperature over a wide temperature range (data not shown), which implies that the value of DPH lifetime is not strongly affected by membrane fluidity, in agreement with the observation made in isotropic solvents of different viscosity [38].

The second analysis approach used continuous distribution of lifetime values. As has been discussed for model membranes [23], the distribution analysis represents only a phenomenological approach, which can be used to characterize the heterogeneity of the DPH surroundings. In the erythrocyte membrane and in liposomes obtained from the extracted phospholipids, the width of the distribution was large and sensitive to temperature. Since the DPH lifetime is extremely sensitive to the value of the dielectric constant [38], the width of the distribution can be attributed to the heterogeneity of the membrane organization which influences the local value of the dielectric constant. In model membranes, we have hypothesized that the DPH lifetime heterogeneity might arise from the different position of DPH along the membrane normal [23]. Positional heterogeneity of DPH has also been suggested in oriented bilayers by Vos et al. [39] using angle-resolved steady-state fluorescence. The changes in the distribution width as a function of temperature in this latter case can be related to the different mobility of DPH molecules, which rapidly average over the different environments along the membrane normal during the excited state [23]. However, in a biological membrane, microenvironments of different dielectric constants could also be formed along the membrane plane due to the presence of cholesterol and protein molecules and to microdomains of specific phospholipid composition. In this respect, DPH is a useful probe, since it partitions equally between fluid and solid environments, insuring a homogeneous distribution in the membrane plane [40]. The DPH lifetime distribution width as a function of temperature in liposomes of extracted phospholipids showed a pattern similar to that of the erythrocyte membrane. Starting from a similar value at lower temperatures, the decrease of the distribution width in liposomes was more gradual as the temperature was increased. This observation suggests that the heterogeneity of the DPH lifetime values in the erythrocyte membrane is due

largely to the phospholipid component; however, the different temperature behavior indicates that other components can also be involved.

Cholesterol could be very important in this respect because its influence on the degree of water penetration [41] can reduce the heterogeneity along the membrane normal. In cholesterol-depleted erythrocyte membranes at 40°C, the width of the distribution was larger than that of both the erythrocyte membrane and the liposomes from extracted phospholipids. This result suggests that, in a partially depleted cholesterol membranes, the surroundings of the DPH molecules remain heterogeneous at higher temperature. These results are in agreement with the observations of Hui et al. [21], who have shown changes in membrane organization in cholesterol-depleted erythrocyte membranes with a cholesterol/phospholipid molar ratio < 0.5. Interestingly, the cholesterol concentration used in this article is also the minimum cholesterol content which induces the broadening of the calorimetric curve [42] and changes in membrane fluidity [43]. The observation that cholesterol decreases the DPH lifetime width in egg phosphatidylcholine liposomes agrees further with the interpretation that cholesterol affects membrane microheterogeneity. The center of the distribution in egg phosphatidylcholine liposomes is not strongly affected by small changes in cholesterol concentration. In liposomes, the effect of very low cholesterol concentrations on distribution width is quite large, suggesting that cholesterol could exert a bulk role in controlling membrane heterogeneity, due to the high cholesterol/phospholipid ratio, about 0.95 [44], in erythrocyte membranes. The decrease of water penetration induced by cholesterol could also explain the higher values of the main lifetime component in the erythrocyte with respect to the phospholipid extract. In erythrocyte membranes, the high cholesterol/phospholipid ratio could be responsible for the overall membrane dielectric properties, as suggested by the observation that 27% cholesterol depletion increased the width of the distribution by a factor of more than 10.

The distribution analysis of DPH fluorescence decay in erythrocyte membranes, although based on a phenomenological ground, offers a better description of membrane heterogeneity with re-

spect to the exponential approach. Moreover, the distribution analysis appears to be a sensitive tool to study the aforementioned aspects of membrane heterogeneity, since it has shown that the addition of one cholesterol molecule to 22 egg phosphatidylcholine molecules decreases the distribution width by a factor of about 4, in the absence of significant modifications of the average lifetime value.

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