UC Irvine
UC Irvine Previously Published Works

Title

Effect of cholesterol on membrane microheterogeneity: a study using 1,6-diphenyl-1,3,5-hexatriene fluorescence lifetime distributions

Permalink

https://escholarship.org/uc/item/05n1k06g

Journal

Biochimica et Biophysica Acta, 1006(2)

ISSN

0006-3002

Authors

Fiorini, Rosamaria
Gratton, Enrico
Curatola, Giovanna

Publication Date

1989-11-01

DOI

10.1016/0005-2760(89)90196-3

Copyright Information

Peer reviewed

BBALIP 53264

Effect of cholesterol on membrane microheterogeneity: a study using 1,6-diphenyl-1,3,5-hexatriene fluorescence lifetime distributions

Rosamaria Fiorini [a], Enrico Gratton [b] and Giovanna Curatola [a]

[a] Institute of Biochemistry, Faculty of Medicine, University of Ancona, Ancona (Italy); [b] Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois, Urbana (U.S.A.)

The effect of cholesterol on microheterogeneity of liposomes obtained from saturated and unsaturated phospholipids was studied by measuring the fluorescence decay of 1,6-diphenyl-1,3,5-hexatriene (DPH). Data obtained by frequency domain fluorometry have been analyzed either by discrete exponential or continuous lifetime distribution approaches. In egg phosphatidylcholine liposomes, the addition of cholesterol increases the lifetime value or the centre of the lifetime distribution. At high cholesterol concentration, good fits are obtained using a monomodal distribution analysis or single exponential component. At low cholesterol concentration an additional short component of low fractional intensity must be included to obtain a good fit. In dipalmitoylphosphatidylcholine, the addition of cholesterol decreases the long lifetime component centre value both in the gel and in the liquid-crystalline state. The DPH lifetime value is sensitive to the dielectric constant of the probe microenvironment, and cholesterol has been shown to modify water penetration in the bilayer. Using this information our data indicate that cholesterol affects the polarity of the microenvironment in liposomes of unsaturated phosphatidylcholine and saturated phosphatidylcholine in different ways. Although the major conclusions of this paper are obtained using changes of the distribution centre upon cholesterol addition, there are also preliminary indications that the lifetime distribution width decreases as cholesterol is added. We have interpreted this observation as being due to the homogenizing effect of cholesterol.

Introduction

Cholesterol is an important component of biological membranes, constituting up to 50% of the total lipid content. The involvement of cholesterol in physiological events such as homeoviscous adaptation [1] and in pathological processes such as atherosclerosis has stimulated the study of its structural and functional role in natural membranes and model systems.

A number of methodological approaches have been used to study different aspects of cholesterol effect on membrane organization. Spectroscopic studies on liposomes have shown an increase of 'rigidity' of phospho-

lipid fatty acyl chains in the presence of cholesterol [2,3]. Moreover, using differential scanning calorimetry a decrease of order has been detected in the gel phase [4]. From the observations of the different effect of cholesterol in gel and liquid crystalline phases a possible role of this molecule has been proposed in which cholesterol controls an intermediate-state fluidity in natural membranes [5]. In LM cells this hypothesis is supported by the disappearance of phase separation when cholesterol is added to previously cholesterol-depleted cells [6]. A similar effect has been shown in cholesterol-depleted erythrocytes, in which there is a segregation of protein rich domains [7]. In previous studies [8] using DPH fluorescence decay we have shown that, in the erythrocyte membrane, cholesterol depletion induces an increase of membrane microheterogeneity, as evaluated from the lifetime distribution width. We proposed that the origin of the lifetime distribution is due to different dielectric microenvironments in which the DPH molecules are located [9]. Therefore the homogenizing effects of cholesterol observed in erythrocyte

membrane have been related to changes in membrane water permeability [10]. A possible role has been ascribed to the hydroxyl group, since different 3-OH-blocked cholesterol derivatives are unable to affect some physicochemical properties of lipid bilayer [11]. In this respect, Demel et al. [12] have pointed out that the presence and the orientation of the oxygen function are the principal factors to observe a sterol-like effect in membranes.

Theoretical considerations and experimental evidence suggest that biological membrane are organized into domains of specific composition and properties [8,13]; however, this structural aspect of membrane organization is at present difficult to define on a molecular basis due to methodological and technical limitations.

To investigate the role of cholesterol in controlling the degree of membrane heterogeneity we studied the modification induced by this molecule on liposomes of unsaturated and saturated phospholipids using the changes produced on the fluorescence decay of DPH. The decay was measured using multifrequency phase fluorometry, which we have shown to be a suitable technique to describe membrane heterogeneity by means of the distribution analysis of complex excited state decay [8,9].

The results are discussed in relation to the changes of membrane order as detected by steady-state anisotropy.

Materials and Methods

Materials

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

Methods

Multilamellar liposomes were prepared by drying both pure lipids and mixtures under N_2, resuspending them in 10 mM Hepes/100 mM KCl (pH 7.4) and shaking the lipid suspension vigorously.

DPH previously dissolved in tetrahydrofuran was added to the sample to produce a probe to phospholipid molar ratio of 1 : 1000. The suspensions were incubated for 1 h in the dark at room temperature. The effect of very small addition of THF was separately studied. At the concentration used in this work no effect on the spectroscopic properties of DPH was detected.

The background fluorescence was checked prior to each measurement, and was less than 0.5% of the fluorescence when DPH was added.

Steady-state fluorescence anisotropy measurements were performed with a Perkin Elmer Fluorescence Spectrophotometer MPF-66 equipped with Series 7000 Pro-

fessional Computer. The sample was excited by polarized light at 360 nm and the fluorescence anisotropy was detected at 430 nm by using a cut-off filter at 390 nm.

DPH lifetime measurements were performed with the multifrequency phase fluorometer described by Gratton and Limkeman [14], 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 Liconix Model 4240NB laser) and a large range of modulation frequencies (from 2 to 70 MHz) were used. Data were acumulated at each modulation frequency until the standard deviations of the phase and modulation values were below 0.1° and 0.002, respectively.

All lifetime measurements were obtained using POPOP in the reference cell. The POPOP lifetime was 1.35 ns [15]. The fluorescence was measured through a long-pass filter type RG 370 from Janos Technology (Townshed, VT) which showed negligible luminescence. Data were analyzed assuming either a sum of exponential or a continuous distribution of lifetime values [9]. For both the exponential and distribution analysis, the programs minimize the reduced chi-square defined by an equation reported elsewhere [16].

Results

EPC liposomes

Table I shows the analysis using two exponentials of DPH fluorescence decay in EPC multilamellar liposomes at different cholesterol concentrations. In EPC liposomes (without cholesterol) a component of 7.44 ns with a fractional intensity of 0.96 and a short component of 1.84 ns were obtained. Both lifetime values increased with increasing cholesterol concentration.

The same set of data was also analyzed by using a sum of one of two continuous distributions of lifetime values characterized by a Lorentzian shape centered at a

TABLE I

Double-exponential analysis of the fluorescence emission decay of DPH in egg PC and egg PC/cholesterol mixtures

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

mol% cholesterol	τ_1	f_1	τ_2	f_2	χ^2
–	7.44	0.96	1.84	0.04	1.39
1.2	7.34	0.97	1.58	0.03	1.55
2.4	7.34	0.97	1.15	0.03	1.72
4.7	7.60	0.95	2.25	0.05	0.87
9.0	7.65	0.94	2.62	0.06	0.47
13.0	8.00	0.94	2.83	0.06	0.90
16.6	8.09	0.92	3.69	0.08	0.59
23.0	8.37	0.94	3.68	0.06	0.96
29.0	8.61	0.94	4.24	0.06	0.76
33.3	9.43	0.69	6.91	0.31	2.43

TABLE II

One and two component distributions analysis of fluorescence emission decay in egg PC and egg PC/cholest. rol mixtures

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

One-component distribution analysis

mol% cholesterol	C	W	f	x^2
–	7.20	0.93	1.00	2.10
1.2	7.13	0.96	1.00	2.51
2.4	7.20	1.03	1.00	3.70
4.7	7.28	0.93	1.00	1.55
9.0	7.26	0.89	1.00	1.31
13.0	7.61	0.89	1.00	0.99
16.6	7.68	0.56	1.00	0.95
23.0	8.05	0.49	1.00	1.12
29.0	8.28	0.41	1.00	0.62
33.3	8.47	0.08	1.00	1.95

Two component distribution analysis

mol% cholesterol	C_1	W_1	f_1	C_2	W_2	f_2	x^2
–	7.34	0.29	0.97	1.84	2.22	0.03	1.25
1.2	7.25	0.25	0.97	1.58	2.00	0.03	1.37
2.4	7.33	0.18	0.98	1.15	0.24	0.02	1.57
4.7	7.57	0.06	0.96	2.25	0.14	0.04	0.83
9.0	7.62	0.05	0.94	2.62	0.05	0.06	0.47
13.0	8.00	0.06	0.97	2.00	0.46	0.03	0.48
16.6	8.05	0.05	0.93	3.69	0.05	0.07	0.65
23.3	8.35	0.05	0.95	3.68	0.05	0.05	1.02
29.0	8.60	0.05	0.92	4.81	0.27	0.08	0.76
33.3	0.84	0.05	0.83	6.91	0.05	0.17	2.08

decay time C and having a width W (Table II). Both the exponential and the two component distribution analysis gave similar reduced chi-square values. The one
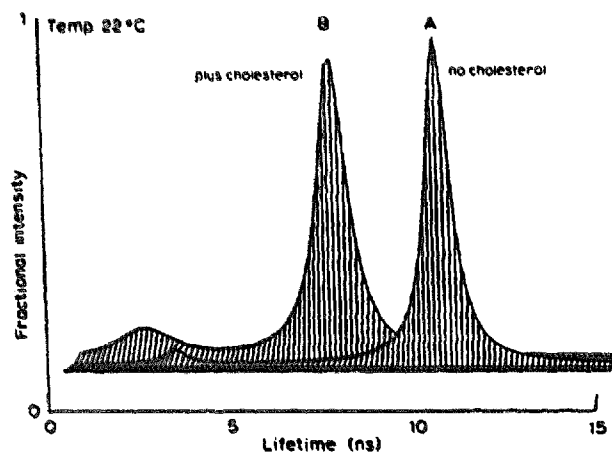

Fig. 1. (A) Distribution analysis of DPH at 22°C in DPPC multilamellar liposomes. The recovered distribution has a centre at 10.43 ns and a FWHM of 0.770 ns. (B) Distribution analysis of DPH at 22°C in a DPPC/cholesterol mixture (16.6 mol% cholesterol). The recovered distribution has a centre at 7.444 ns and a FWHM of 0.997 ns.
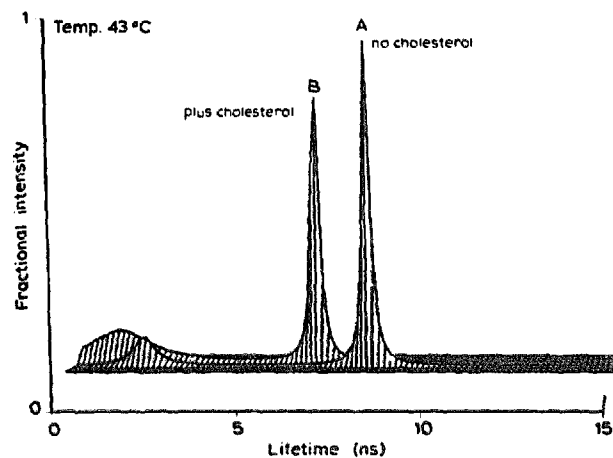

Fig. 2. (A) Distribution analysis of DPH at 43°C in DPPC multilamellar liposomes. The recovered distribution has a centre at 8.222 ns and a FWHM of 0.05 ns. (B) Distribution analysis of DPH at 43°C in a DPPC/cholesterol mixture (16.6 mol% cholesterol). The recovered distribution has a centre of 6.713 ns and a FWHM of 0.05 ns.

component distribution analysis shows a slight improvement of the fit only for the last two concentrations of cholesterol used. Both centres of the two-component distribution have values similar to those of the long and short components obtained by the exponential analysis. The width of the distribution shows a dependence on the cholesterol concentration. The width is gradually modified until 4.7 mol% cholesterol. At the last two higher cholesterol concentrations used (29.0 and 33.3 mol% cholesterol) the one-component distribution analysis gives a slightly better fit relative to the two-component analysis.

DPPC

Since EPC is an highly unsaturated phospholipid species and under our experimental conditions it is in the liquid-crystalline state, we have studied the effect of cholesterol on saturated phosphatidylcholine (DPPC) in gel and liquid-crystalline phase.

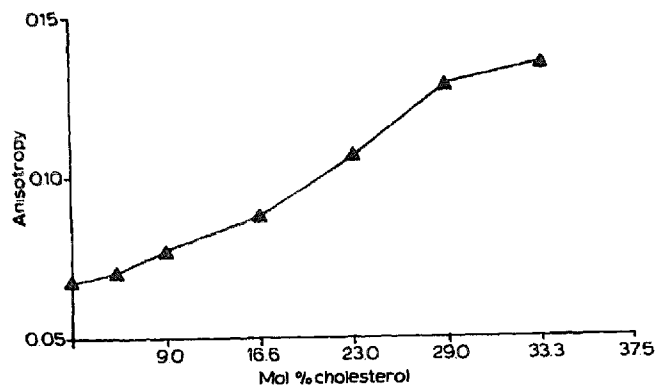In Figs. 1 and 2 are reported the lifetime distributions obtained in DPPC multilamellar liposomes with

Fig. 3. Fluorescence anisotropy of DPH in EPC as a function of cholesterol concentrations in multilamellar liposomes.

TABLE III

Fluorescence anisotropy of DPH in DPPC and DPPC/cholesterol mixture below (21°C) and above (43°C) the main transition temperature

T (°C)	Anisotropy	
	DPPC	DPPC/Chol. (16.6 mol% cholesterol)
21	0.329	0.309
43	0.094	0.185

the addition of cholesterol (16.6 mol% cholesterol) above and below the DPPC transition temperature (T_m). Below the transition temperature (22°C) the presence of the cholesterol changes the DPH fluorescence lifetime value from 10.9 ns to 7.4 ns. The effect on the distribution width is minor with an increase from 0.830 to 0.997 corresponding to a 12% change. Above T_m the presence of cholesterol induces a very slight decrease of the lifetime value without any significant changes on the distribution width.

Steady-state anisotropy

The steady-state anisotropy of DPH in EPC and DPPC in the presence and absence of cholesterol are reported in Fig. 3 and Table III, respectively. Fig. 3 shows that the presence of increasing cholesterol concentrations induces an increase of anisotropy. Below the DPPC transition temperature the presence of cholesterol induces a significant decrease of the anisotropy, while above the transition the anisotropy increases (Table III).

Discussion

Using a double-exponential analysis of the DPH fluorescence decay we have shown that cholesterol affects both long- and short-component lifetime values. The small fractional intensity associated with this short component makes its interpretation difficult. However, its sensitivity to increasing cholesterol concentration, as well as the abrupt change of its fractional intensity observed at 33.3 mol% cholesterol, is compatible with the proposal of Karnovsky et al. [17] that the short component represents DPH molecules located in polar membrane regions.

The two-component distributional analysis is very similar to that obtained using two exponentials. Moreover, the distributional approach shows a narrowing distribution width of the long component as the concentration of cholesterol is increased. The increase in EPC of the average lifetime value with increasing cholesterol concentration could be related to the effect of this molecule in reducing the penetration of water [10]. Through this action, cholesterol could also induce a more homogeneous environment in the membrane

hydrophobic core. Since the DPH lifetime is single exponential in isotropic solvents at room temperature and is directly related to the dielectric constant of its surroundings [18], the homogenizing effect of cholesterol can also induce a decrease of microenvironments heterogeneity. The results using EPC liposomes are in agreement with our previous observations [8] of higher values for the main lifetime component in erythrocyte membranes with respect to the phospholipid extracts. Moreover, in erythrocyte membranes depleted of cholesterol we also observed an increase of the distribution width.

The effect of cholesterol in inducing a higher degree of membrane homogeneity seems to be important from a structural point of view, since it is already apparent after an addition of one cholesterol molecule per 22 EPC molecules (4.7 mol% cholesterol). This concentration is also the minimum cholesterol content which induces changes of cooperative behaviour observed by differential scanning calorimetry [19].

Under our experimental conditions the increase of membrane order as measured by DPH steady-state anisotropy is also evident.

It has been shown that cholesterol is incorporated into the structure of aqueous lamellar phospholipids even at the higher concentrations used in this study [20]. The polar hydroxyl group of cholesterol enables it to orient in the membrane bilayer parallel to the phospholipid molecules [21]. It has been suggested that cholesterol exerts its influence by the hydration of the -OH group, ordering the water near the membrane surface [22]. Moreover, cholesterol decreases lipid chain mobility, thus reducing structural defects and therefore the space in which water molecules can be accommodated [23]. Kao et al. [24] have shown that the presence of cholesterol reduces the apparent polarity of the hydrocarbon region of the phospholipid bilayer. In unsaturated phosphatidylcholine, Davenport et al. [25] have shown that DPH is located in the central part of the bilayer. Therefore, in our experimental conditions it cannot be excluded that cholesterol favours this tendency so that the probe is located in a more hydrophobic and homogeneous environment. The aforementioned aspects of cholesterol interactions with membrane phospholipids could explain both the decrease of DPH lifetime value and distributional width, further supporting its effect in reducing the degree of membrane heterogeneity. As mentioned before, the homogenizing effect could be directly related to the decrease of water penetration; however, the reduction of the number of structural defects could be of importance in this respect. The effects exerted by cholesterol are dependent upon the phase in which the molecules are introduced. The presence of cholesterol induces a decrease of order as detected by steady-state anisotropy in the gel phase of DPPC and an increase above the transition in agree-

ment with calorimetric data [4]. However, the disordering effect in the gel phase induces also a decrease in long lifetime component value. In our interpretation, the decrease of lifetime value depends on the increase of the dielectric constant of the medium. This hypothetical increase of water permeability below the transition temperature is in agreement with data obtained on phosphatidylcholines by De Gier et al. [26].

Our results indicate that the effect of cholesterol on membrane heterogeneity is related to membrane composition. The effect of cholesterol on unsaturated phosphatidylcholine is different from that exerted on saturated species, indicating that the double bonds in the fatty acid chains are very important in determining structural defects which can be related to the degree of water penetration.

At high cholesterol concentration the lifetime analysis shows that there is only one narrow distribution component, which indicates a homogeneous environment for the DPH molecules.

The study of the effect of cholesterol on saturated and unsaturated phosphatidylcholine by using the changes of the fluorescence properties of DPH has enabled us to define an homogenizing effect of cholesterol due to a decrease in water permeability on unsaturated phosphatidylcholine in analogy with the observation made on natural membrane [8]. This effect is shown by the changes of the average lifetime values both using double-exponential or distributional analysis.

Acknowledgements

References

1 Sinensky, M. (1974) Proc. Natl. Acad. Sci. USA 71, 522–527.
2 Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132.
3 Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265–279.
4 Chapman, D. (1973) in Biological Membranes, Vol. 2 (Chapman, D., Wallach, D.F., eds.), pp. 91–112, Academic Press, New York.
5 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–136.
6 Welti, R., Rintoul, D.A., Goodsaid-Zalduondo, F., Felder, S. and Silbert, D.F. (1981) J. Biol. Chem. 256, 7528–7535.
7 Hui, S.W., Stewart, C.M., Carpenter, M.P. and Stewart, T.P. (1980) J.Cell Biol. 85, 283–291.
8 Fiorini, R.M., Valentino, M., Glaser, M., Gratton, E. and Curatola, G. (1988) Biochim. Biophys. Acta 939, 485–492.
9 Fiorini, R.M., Valentino, M., Wang, S., Glaser, M. and Gratton, E. (1987) Biochemistry 26, 3864–70.
10 Finkelstein, A. and Cass, A. (1967) Nature 216, 717–718.
11 Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132.
12 Demel, R.A., Lala, A.K., Nanda Kumari, S. and Van Deenen, L.L.M. (1984) Biochim. Biophys. Acta 771, 142–150.
13 Klausner, R.D. and Kleinfeld, A.M. (1984) in Cell Surface Dynamics (Perelson, A.S., Delisi, C. and Wiegel, F.W., eds.), pp. 23–58, Marcel Dekker, New York.
14 Gratton, E. and Limkeman, M. (1983) Biophys. J. 44, 315–325.
15 Lakowicz, J.R., Cherek, H. and Balter, A.J. (1981) Biochem. Biophys. Methods 5, 131–146.
16 Alcalà, J.R., Gratton, E. and Prendergast, F.G. (1987) Biophys. J. 51, 587–596.
17 Karnovsky, M.J., Kleinfeld, A.M., Hoover, R.L. and Klausner, R.D. (1982) J. Cell. Biol. 94, 1–6.
18 Zannoni, C., Arcioni, A. and Cavatorta, P. (1983) Chem. Phys. Lipids 32, 179–250.
19 Mabrey, S., Mateo, P.L. and Sturtevant, J.M. (1978) Biochemistry 17, 2464–68.
20 Reiber, H. (1978) Biochim. Biophys. Acta 512, 72–83.
21 Presti, F.T. (1985) in Membrane Fluidity in Biology, Vol. 4 (Aloia, R.C. and Boggs, J.M., eds.), pp. 97–145, Academic Press, New York.
22 De Kruijff, B., Demel, R.A. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 331–347.
23 Blume, A. (1983) Biochemistry 22, 5436–42.
24 Kao, Y.J., Soutar, A.K., Yi Hong, K., Pownall, H.J. and Smith, L.C. (1978) Biochemistry 17, 2689–96.
25 Davenport, L., Dale, R.E., Bisby, R.H. and Cundall, R.B. (1985) Biochemistry 24, 4097–4108.
26 De Gier, J., Mandersloot, J.G. and Van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 150, 666–675.