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INTERACTIONS OF NOVEL PHOSPHONIUM DYE WITH LIPID BILAYERS: A FLUORESCENCE STUDY†

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The phosphonium-based optical probes attract ever growing interest due to their excellent chemical and photophysical stability, high aqueous solubility, long wavelength absorption and emission, large extinction coefficient, high fluorescence quantum yield, low cytotoxicity, etc. The present study was focused on assessing the ability of the novel phosphonium dye TDV to monitor the changes in physicochemical properties of the model lipid membranes. To this end, the fluorescence spectral properties of TDV have been explored in lipid bilayers composed of zwitterionic lipid phosphatidylcholine (PC) and its mixtures with cholesterol (Chol) or/and anionic phospholipid cardiolipin (CL). It was observed that in the buffer solution TDV possesses one well-defined fluorescence peak with the emission maximum at 533 nm. The dye transfer from the aqueous to lipid phase was followed by the enhancement of the fluorescence intensity coupled with a red shift of the emission maximum up to 67 nm, depending on the liposome composition. The quantitative information about the dye partitioning into lipid phase of the model membranes was obtained through approximating the experimental dependencies of the fluorescence intensity increase *vs* lipid concentration by the partition model. Analysis of the partition coefficients showed that TDV has a rather high lipid-associating ability and displays sensitivity to the changes in physicochemical properties of the model lipid membranes. The addition of CL, Chol or both lipids to the PC bilayer gives rise to the increase of the TDV partition coefficients compared to the neat PC membranes. The enhancement of the phosphonium dye partitioning in the CL and Chol-containing lipid bilayers has been attributed to the cardiolopin- and cholesterol-induced changes in the structure and physicochemical characteristics of the polar membrane region.
KEYWORDS: phosphonium probe, lipid membranes, fluorescence, partitioning
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During the last decades the phosphonium-based optical probes attract ever growing interest due to their favorable characteristics, namely, the excellent chemical and photophysical stability, high aqueous solubility, long wavelength absorption and emission, large extinction coefficient, high fluorescence quantum yield, low cytotoxicity, etc [1-14]. The above properties render phosphonium dyes highly suitable for the selective staining of mitochondria [3-5], antioxidants detection [6, 7], probing the structural differences of DNA/RNA grooves [1, 2], exploring the tumor multidrug resistance [8], to name only a few. More specifically, the cyanine-based phosphonium dyes can be used for the kinetic differentiation between homo-and alternating AT-DNA forming dimers within DNA minor grooves [1, 2]. Moreover, the cyanine-based phosphonium dyes demonstrated the strongly selective antiproliferative activity toward HeLa cancer cell lines [1]. The phosphonium-tagged coumarin derivatives can be used for the identification of the peptide fragment [9]. In recent years it was shown that phosphonium-based fluorophores are highly efficient for mitochondria imaging with phosphonium groups serving as specific targeting sites toward mithochondria [3-5]. Specifically, Li and colleagues developed a perylene-based phosphonium fluorophore possessing the dual-emissive luminescence in living cells [3]. The possibility of mitochondria imaging in living cells was described also for a new conjugated phosphonium salt TPP characterized by the high intracellular selectivity toward mitochondria and the aggregation-induced emission [4]. A highly efficient cellular uptake and specific accumulation in mitochondria was observed for cyanine-based phosphonium probes possessing the potential dependent mitochondria-related fluorescent signal without exhibiting the cell toxicity [5]. Moreover, the phosphonium conjugates proved to be very effective in monitoring the oxygen variations within mitochondria [10] and measuring the hydrogen peroxide levels [11]. Furthermore, numerous studies have demonstrated that phosphonium fluorescent dyes can be effectively used for optical detection of disease-related protein aggregates, amyloid fibrils [12,13]. In particular, a phosphonium dye TDV was used as a mediator in the amyloid-scaffolded multichromophoric systems for monitoring the amyloid transformation of the N-terminal fragment of apolipoprotein A-I [12] and insulin [13]. Moreover, previous studies suggested that phosphonium dyes may prove of value in elucidating the mechanism of DNA interactions with pathogenic protein aggregates [14].

As a next logical step in evaluating the biomedical potential of phosphonium-based fluorescent probes, the present study was directed at assessing an ability of the novel phosphonium dye TDV to monitor the properties of lipid bilayers. More specifically, the aim of the present study was two fold: i) to obtain the quantitative information about the dye partitioning into lipid phase of the model membranes and ii) to assess the TDV sensitivity to the changes in the physicochemical properties of the lipid bilayer.

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EXPERIMENTAL SECTION

Materials

Egg yolk phosphatidylcholine, beef heart cardiolipin and cholesterol were purchased from Sigma (St. Louis. MO, USA). The phosphonium dye TDV (Fig.1) was provided by Professor Todor Deligeorgiev, University of Sofia, Bulgaria. All other materials were commercial products of analytical grade and were used without further purification.

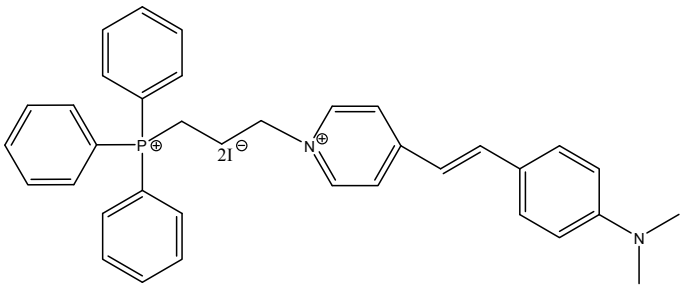

Figure 1. The structural formula of TDV

Preparation of lipid vesicles

Unilamellar lipid vesicles composed of zwitterionic lipid phospatidylcholine (PC) or PC mixtures with anionic lipid cardiolipin (CL) and sterol cholesterol (Chol) were prepared by the extrusion method [15]. The thin lipid films were obtained by evaporation of lipids' ethanol solutions. The dry lipid residues were subsequently hydrated with 5 mM sodium phosphate buffer, pH 7.4 at room temperature to yield lipid concentration of 1 mM. Thereafter, lipid suspension was extruded through a 100 nm pore size polycarbonate filter (Millipore, Bedford, USA). In this way, 6 types of lipid vesicles containing PC and 5, 10 or 20 mol% CL, 30 mol% of Chol or the combination of 10 mol% CL and 30 mol% of Chol with the content of phosphate being identical for all liposome preparations. Hereafter, the liposomes containing 5, 10 or 20 mol% CL are referred to as CL5, CL10 or CL20, respectively, while the liposomes bearing 30 mol% Chol are denoted as Chol30, respectively. Accordingly, the liposomes with cardiolipin content 10 mol% and cholesterol content 30 mol% were marked as CL10/Chol30.

Spectroscopic measurements

The stock solution of TDV was prepared by dissolving the dye in 10 mM Tris buffer, pH 7.4. The concentration of TDV was determined spectrophotometrically, using the extinction coefficient $\varepsilon_{480} = 2.1\times10^5 M^{-1}cm^{-1}$ The dye-liposome mixtures were prepared by adding the proper amounts of the probe stock solutions in buffer to the liposome suspension of different composition varying the lipid concentration from 0 to 4.76 μM. The dye-liposome mixtures were incubated for an hour. The steady-state fluorescence spectra were recorded with FL-6500 spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, UK) at 20°C using 10 mm path-length quartz cuvettes. The excitation wavelength was 480 nm. The excitation and emission slit widths were set at 10 nm.

Partitioning model

The TDV binding to the model lipid membranes has been analyzed in terms of the partition model [16]. The total concentration of the dye distributing between aqueous and lipid phases (Z_{tot}) can be represented as:

$$Z_{tot} = Z_F + Z_L\,, \tag{1}$$

where subscripts *F* and *L* denote free and lipid-bound dye, respectively. The coefficient of dye partitioning between the two phases (K_P) is defined as [16]:

$$K_P = \frac{Z_L V_W}{Z_F V_L}\,, \tag{2}$$

where V_W , V_L are the volumes of the aqueous and lipid phases, respectively. Given that under the employed experimental conditions the volume of lipid phase is much less than the total volume of the system V_t , we assume that $V_W \approx V_t = 1$ sm^3. Therefore

$$Z_F = \frac{Z_{tot} V_W}{V_W + K_P V_L} = \frac{Z_{tot}}{1 + K_P V_L}\,. \tag{3}$$

The dye fluorescence intensity measured at a certain lipid concentration can be calculated as:

$$I = a_f Z_F + a_L Z_L = Z_F \left(a_f + a_L \frac{K_P V_L}{V_W} \right) = Z_F \left(a_f + a_L K_P V_L \right), \tag{4}$$

where a_f, a_L represent molar fluorescence of the dye free in solution and in a lipid environment, respectively. From the Eqs. (3) and (4) one obtains:

$$I = \frac{Z_{tot}(a_f + a_L K_P V_L)}{1 + K_P V_L}. \quad (5)$$

The volume of lipid phase can be determined from:

$$V_L = N_A C_L \sum v_i f_i, \quad (6)$$

where C_L is the molar lipid concentration, f_i is mole fraction of the i-th bilayer constituent, v_i is its molecular volume taken as 1.58 nm^3, 3 nm^3 and 0.74 nm^3 for PC, CL and Chol respectively.

The relationship between K_p and fluorescence intensity increase (ΔI) upon the dye transfer from water to lipid phase can be written as [16]:

$$\Delta I = I_L - I_W = \frac{K_p V_L (I_{\max} - I_W)}{1 + K_p V_L}, \quad (7)$$

where I_L is the fluorescence intensity observed in the liposome suspension at a certain lipid concentration C_L, I_W is the dye fluorescence intensity in a buffer, $I_{\max}$ is the limit fluorescence in a lipid environment.

Lipophilicity calculation

The resources of the virtual computational laboratories (http://biosig.unimelb.edu.au/pkcsm/prediction#, http://www.swissadme.ch/ and https://mcule.com/apps/property-calculator/) were used for the calculation of the lipophilicity of the examined dye.

RESULTS AND DISCUSSION

To examine how the TDV lipid associating ability depends on the membrane physical properties, at the first step of our study the emission spectra of this phosphonium dye were recorded in the buffer solution and liposomal suspensions of different composition. TDV was found to be weakly-emissive in the buffer with the fluorescence maximum at 533 nm. Typical fluorescence spectra measured at increasing lipid concentration are presented in Fig. 1.

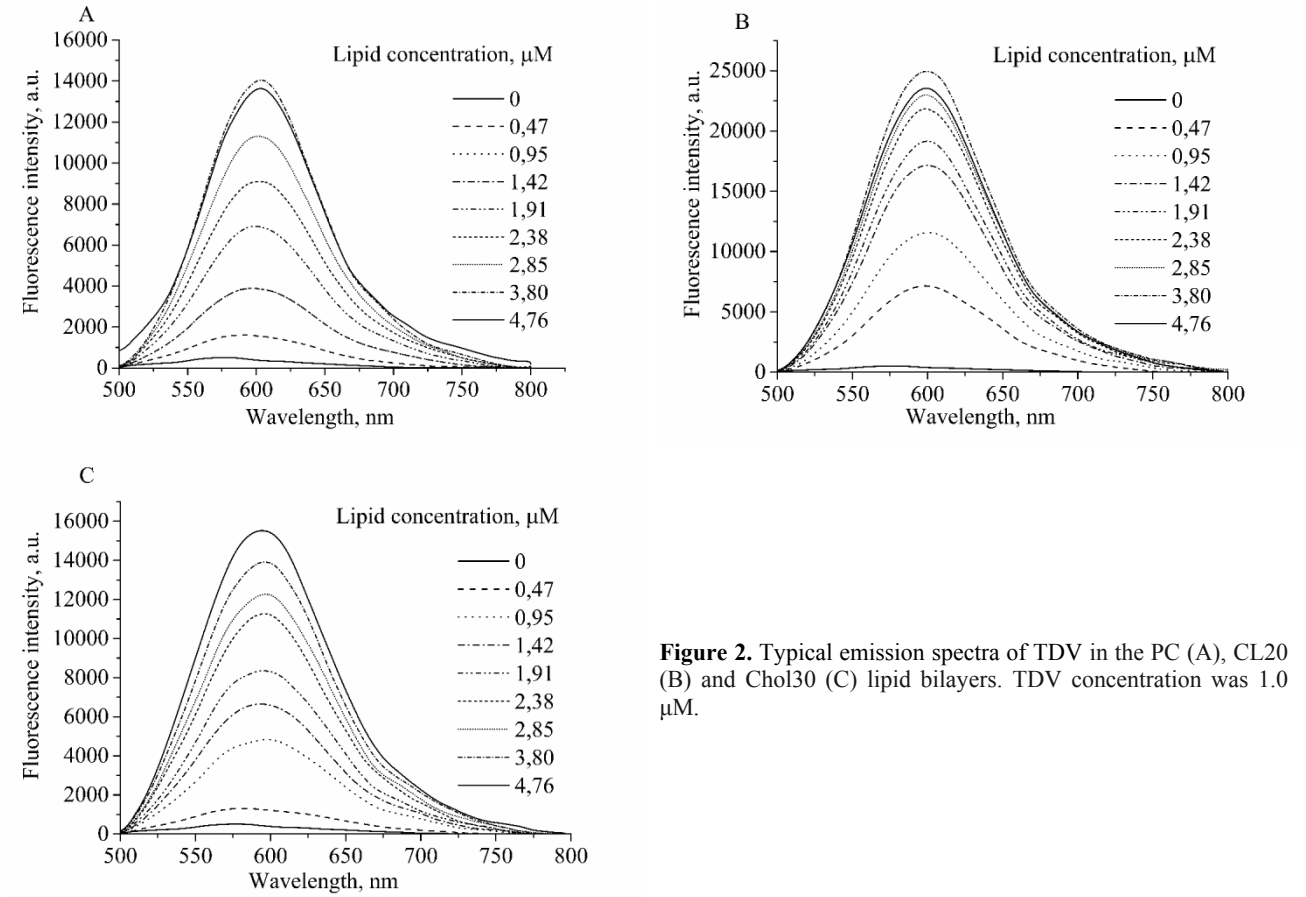

Figure 2. Typical emission spectra of TDV in the PC (A), CL20 (B) and Chol30 (C) lipid bilayers. TDV concentration was 1.0 μM.

As seen in Fig. 1, the TDV transfer from the aqueous to lipid phase resulted in a significant increase of the fluorescence intensity coupled with a red shift of the emission maximum λ_F up to 67 nm, depending on liposome composition (Table 1). The observed fluorescence enhancement is most likely to arise from the decreased mobility of the fluorophore and the reduced polarity of its surroundings. To quantitate the dye partitioning in the lipid phase of the model membranes, the experimental dependencies $\Delta I(C_L)$ were obtained (Fig. 2). The resulting binding isotherms were hyperbolic in shape for all dye-lipid systems under study. To obtain the quantitative parameters of the TDV partitioning into lipid bilayers of varying composition the binding curves were approximated by the equation (7).

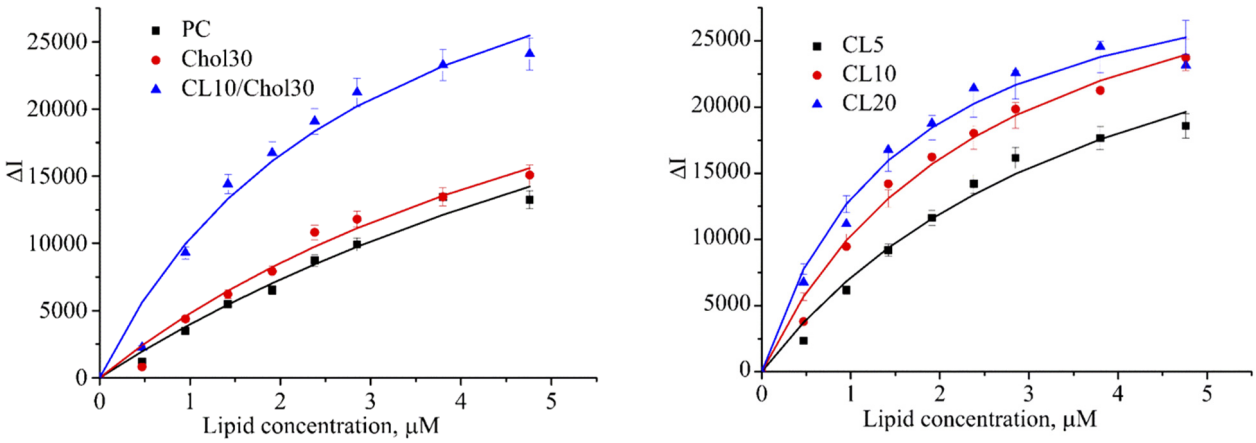

Figure 2. Isotherms of TDV binding to the model lipid membranes. TDV concentration was 1.0 μM. Solid lines represent an approximation of the experimental profiles $\Delta I(C_L)$ by equation (7).

The parameters of TDV partitioning into lipid bilayers are presented in Table 1. The analysis of partition coefficients showed that TDV possesses a rather high lipid-associating ability. Importantly, the estimated K_p values for the model membranes of different lipid composition are in good agreement with the lipophilicity of TDV evaluated using the resources of different virtual computing laboratories (Table 2). A molecular parameter, such as lipophilicity, is commonly used to characterize the tendency of a molecule to distribute between water and water-immiscible solvent [17] and can be expressed by a term accounting for hydrophobic and dispersion forces, and polarity term [18,19].

Table 1. Parameters of TDV partitioning into lipid systems

	λ_F, nm	$K_p \times 10^5$	$\Delta I_{max} \times 10^4$	Fluorescence anisotropy
PC	602	0.96±0.2	4.8±0.9	0.165
CL5	600	2.4±0.4	3.7±0.6	0.161
CL10	600	3.9±0.6	3.5±0.6	0.161
CL20	600	6.4±1.1	3.7±0.7	0.163
Chol30	594	1.4±0.4	5.7±0.8	0.169
CL10/Chol30	596	3.3±0.5	6.1±0.6	0.160

It turned out that addition of CL and Chol to PC bilayer gives rise to the increase of partition coefficients compared to the neat PC membrane. In terms of the modern theories of membrane electrostatics partition coefficient can be represented as consisting of electrostatic and nonelectrostatic terms [20,21]:

$$K_p = exp\left(\left\{w_{el} + w_{Born} + w_h + w_n + w_d\right\} / kT\right) \quad (8)$$

where w_{el} characterizes the Coulombic ion-membrane interactions; w_{Born} corresponds to the free energy of charge transfer between the media with different dielectric constants; w_n is the term determined by hydrophobic, van der Waals and steric factors; w_h related to the membrane hydration; w_d depends on the membrane dipole potential [21-23]. Considering the cationic nature of TDV, the observed increase in K_p values with an increase of the CL content can be explained by electrostatic dye-lipid interaction.

However, in an attempt to interpret the observed increase of the partition coefficients for TDV in the presence of the anionic lipid CL, one should bear in mind the ability of CL to modify the physicochemical properties of the lipid bilayers [24, 25]. Specifically, Shibata et al, based on the FTIR data, demonstrated that CL negative charge tends to move the N^+ end of P-N dipole parallel to the membrane surface, causing the rearrangement of water bridges at the

bilayer surface and stabilizing the intermolecular hydrogen-bonded network including hydrational water [24]. They hypothesized that the above perturbations in the bilayer surface are coupled with the increased amount of the membrane-bound water in the presence of a cone-shaped CL molecules, leading to the enhancement of the hydration of ester C=O groups in the presence of cardiolipin [24]. Moreover, the ability of CL to increase bilayer hydration was also evidenced from the molecular dynamic simulations of lipid membranes with cardiolipin [25].

Table 2. Lipophilicity of TDV calculated using the resources of the virtual computational laboratories

Resource	Method	LogP
http://www.swissadme.ch/	XLOGP3	8.01
	WLOGP	6.21
	MLOGP	6.25
	SILICOS-IT	7.61
	Consensus	6.00
https://mcule.com/apps/property-calculator/		6.2095
http://biosig.unimelb.edu.au/pkcsm/prediction#)		6.2095

To determine whether i) the novel phosphonium dye is sensitive to the changes in lipid packing density and reflects the CL-induced changes in the hydrophobic membrane part; or ii) TDV preferentially resides in the polar part of the lipid bilayer and the increase in K_p values in the CL-containing membranes is caused by alterations in the bilayer physicochemical properties produced by cardiolipin, the fluorescence anisotropy of TDV was measured (Table 1). It is known that the fluorescence anisotropy of the membrane-bound dye is determined by the rate of its rotational diffusion and reflects the changes in lipid packing density. As illustrated in Table 1, the anisotropy values of TDV appeared to be insensitive to the changes in membrane composition, indicating that the dye is most probably located in the polar membrane region. Most likely, the phosphonium part of TDV resides in the polar water/membrane interface, while the rest part of its molecule penetrates more deeply into the membrane interior.

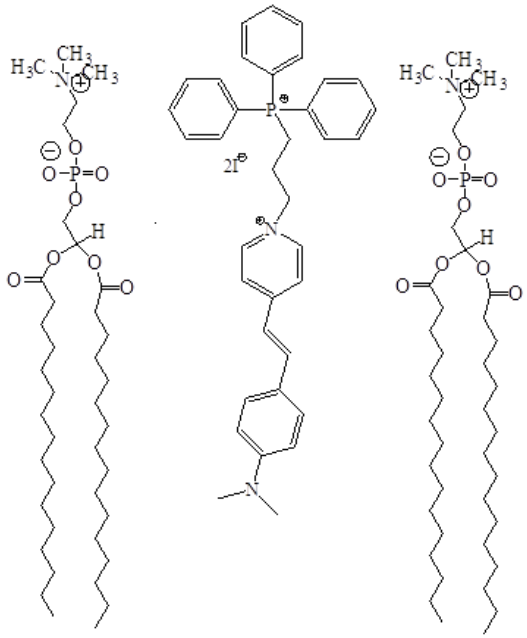

Figure 3. Possible bilayer location of TDV

Additional argument in favour of the TDV ability to monitor the physicochemical properties of the polar membrane part comes from the fluorescence response of this phosphonium dye to the presence of Chol. As seen in Table 1, the measured anisotropy values in the Chol-containing membranes are comparable with those obtained in pure PC and PC/CL membranes, therefore the TDV location in the non-polar membrane part seems less probable due to a well-known ability of Chol to produce tighter lateral packing of lipid molecules (condensing effect) [26]. An assumption was made that the observed increase in K_p value in the presence of cholesterol is associated with the ability of Chol to alter hydration and packing density of lipid membranes [27-30]. It was previously shown that cholesterol is

capable of producing the increase in separation of phospholipid headgroups [27]. Moreover, numerous studies provide evidence for the decreased polarity at the level of glycerol backbone of phospholipids in the presence of cholesterol [28-30]. The TDV sensitivity to the membrane hydration is confirmed by the observed blue shift of the position of emission maxima in the presence of Chol in comparison with CL-containing and pure PC membranes, indicating that TDV accommodates in the less polar environment in the presence of cholesterol. The blue shift of the emission maxima in the presence of cholesterol was observed also for Prodan and Laurdan [30,31]. Given that addition of Chol to PC bilayer gives rise to the increase of partition coefficients compared to the neat PC membrane, the possibility of the TDV preferential interactions with cholesterol cannot be excluded. Most probably, similarly to Prodan, TDV tends to reside in the cholesterol-rich regions of the lipid bilayer [31]. Moreover, one cannot rule out the possibility that TDV is sensitive to the Chol-induced changes in the dipole potential of lipid bilayer [3,4,32]. To uncover the factors contributing to the membrane association of TDV, further studies are needed.

CONCLUSIONS

To summarize, the present study has been undertaken to evaluate the potential of the novel phosphonium dye to trace the changes in the physicochemical properties of the model lipid membranes. TDV was found to display a marked lipid-associating ability and high sensitivity to the physicochemical properties of lipid bilayers. Based on the comprehensive analysis of the binding parameters and spectral characteristics of TDV in the different lipid systems it was assumed that membrane electrostatics and hydration can contribute to the membrane association of TDV. High environmental sensitivity of the examined posphonium dye allowed us to recommend this probe for membrane studies.

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ВЗАЄМОДІЯ НОВОГО ФОСФОНІЄВОГО ЗОНДУ З ЛІПІДНИМИ МЕМБРАНАМИ: ФЛУОРЕСЦЕНТНЕ ДОСЛІДЖЕННЯ

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Останнім часом оптичні зонди на основі фосфонію привертають все більший інтерес завдяки їх чудовій хімічній та фотофізичній стабільності, високій розчинності у воді, поглинанню та випромінюванню в довгохвильовій області, великих коефіцієнтах екстинкції, високому квантовому виходу флуоресценції, низькій цитотоксичності, тощо. Дана робота була спрямована на оцінку чутливості нового фосфонієвого барвника TDV до змін фізико-хімічних властивостей модельних ліпідних мембран. З цією метою, було досліджено флуоресцентні спектральні властивості TDV в ліпідних бішарах, що складались із цвіттеріонного ліпіду фосфатидилхоліну (ФХ) та його сумішей з холестерином (Хол) та/або аніонним фосфоліпідом кардіоліпіном (КЛ). Виявилось, що в буферному розчині TDV має один добре виражений пік емісії з на довжині хвилі 533 нм. Перехід барвника з водної в ліпідну фазу супроводжувався зростанням інтенсивності флуоресценції зонду, поряд із червоним зсувом максимуму випромінювання, величина якого досягала 67 нм, залежно від складу ліпосом. Була отримана кількісна інформація щодо розподілу барвника в ліпідну фазу модельних мембран шляхом апроксимації експериментальних залежностей зміни інтенсивності флуоресценції зонду від концентрації ліпіду моделлю розподілу. Аналіз отриманих коефіцієнтів розподілу демонструє високу ліпід-асоціюючу здатність TDV та його чутливість до змін фізико-хімічних властивостей модельних ліпідних мембран. Включення КЛ, Хол або обох ліпідів до ФХ бішару спричиняло збільшення коефіцієнтів розподілу TDV, порівняно з чистими ФХ мембранами. Зростання коефіцієнтів розподілу фосфонієвого барвника в ліпідних мембранах, що містили КЛ та Хол, було інтерпретовано в рамках уявлень про зміни структури та фізико-хімічних характеристик полярної області мембрани під впливом кардіоліпіну та холестерину.

Ключові слова: фосфонієвий зонд, ліпідні мембрани, флуоресценція, розподіл.