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Estimation of Membrane Activity of Water-Soluble Polysubstituted Fullerene Derivatives by Luminescence Methods

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Abstract—Patterns of the interaction between water-soluble polysubstituted fullerene derivatives (PFDs) and the lipid bilayer of phosphatidylcholine liposomes were investigated by applying triplet and fluorescent probes. Objective quantitative criteria have been proposed for the evaluation of membranotropic action of chemical substances, notably, fullerene derivatives that quench fluorescent probes with different localizations within the membrane. Thus, the defined criteria are the rate constants for the quenching of the fluorescence of triplet probes and the equilibrium constants for PFD-probe complexes, which characterize their stability. The localization of PFDs in the membrane was determined by comparing rate constants for the quenching of eosin phosphorescence and equilibrium constants for PFD–chromophore complexes. In addition, the efficiency of the interaction of PFDs with various sites of the phospholipid membrane has been seen to depend on the charge of addends that are attached to the polysubstituted derivatives.

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INTRODUCTION

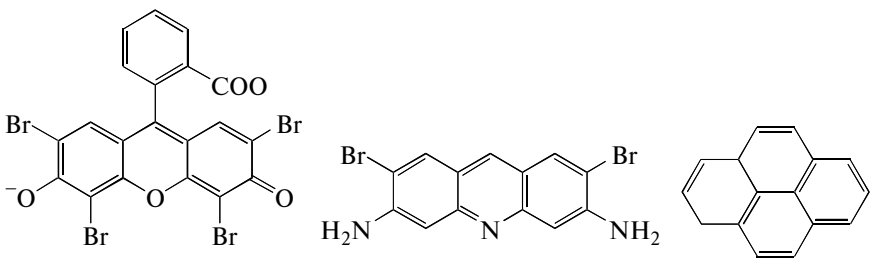
According to numerous scientific reports that have examined fullerene C_{60} derivatives, the prospects for their application in biological and pharmacological practices deserve special consideration. The interest in these materials is aroused due to the unique structure of their carbon spheroid, the presence of lipophilic and membranotropic properties, the ability to transform oxygen into the singlet state, antioxidant and antiviral activities, and cytotoxicity against cancer cells. Thus, it has become apparent that the study of physicochemical mechanisms of the action of fullerene derivatives on biological structures is very important. To address this issue, fluorescent and triplet probes are successfully applied.

The transport mechanisms of substances through living cells and, thus, the interaction of these substances with cell membranes are one of the most important issues for investigating the biological action of chemical substances.

This study is dedicated to evaluating the effect of polysubstituted derivatives of fullerenes synthesized by P.A. Troshin at the Institute of Problems of Chemical Physics (IPCP) on phospholipid membranes by fluorescence and phosphorescence techniques.

MATERIALS AND METHODS

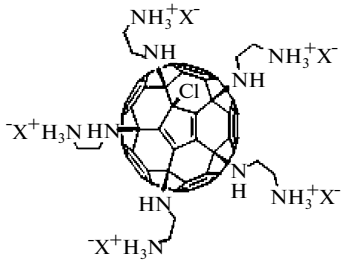
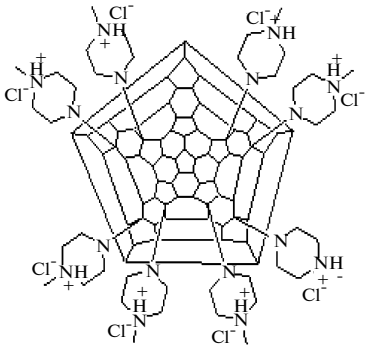
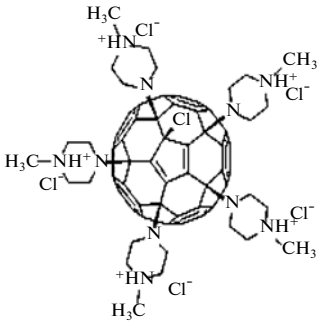
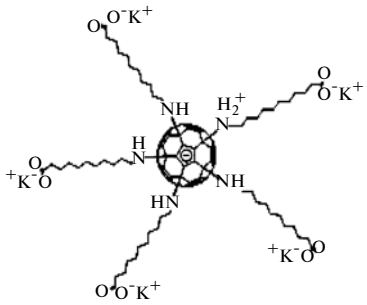
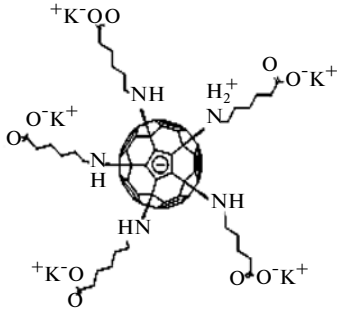
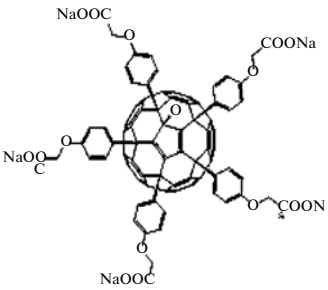
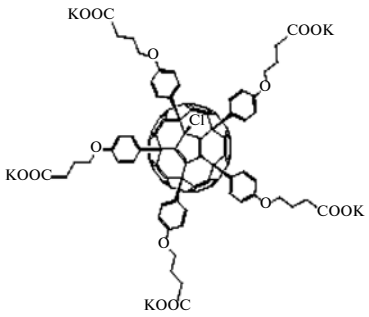
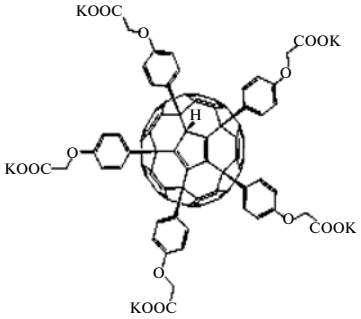
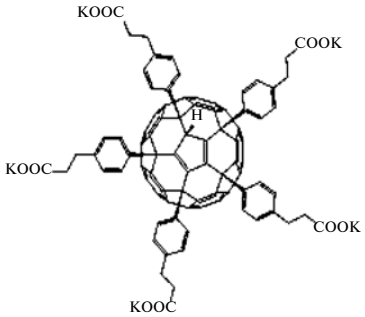
In this study, the water-soluble polysubstituted fullerene derivatives (PFDs) presented in the Table 1 were used. The following dyes were used as probes: eosin Y (Sigma), 2,7-Br-proflavine, synthesized by V.S. Romanova (Institute of Organoelement Compounds, Russian Academy of Sciences (INEOS RAS)), and pyrene (Sigma) (Fig. 1).



Eosin Y 2,7-Br-proflavine Pyrene

Fig. 1. Luminescent probes used in the study.

Table 1. Water-soluble polysubstituted fullerene derivatives

 KB 318	 KB 392	 TP 1105
 NK 205	 NK 212	 KB 445
 KB 446	 KB 449	 KB 450

Liposomes were formed according to the protocol [1]. Phosphatidylcholine (Fluka) was dissolved in ethanol (40 mg/ml), and the ethanol was removed by a rotary evaporator (150 mbar, 270 rpm). A white phospholipid film that was formed on the walls of a flask was then transferred into a water buffer solution (Tris-HCl, pH = 7.2, 0.02 M) by shaking for 10–20 min at room temperature under argon flow. The resulting suspension was subjected to ultrasound at 4°C for 10 min under argon flow on a UZDN-1 U4.2 ultrasonic disperser [2].

The kinetics of quenching of the phosphorescence of eosin and the fluorescence spectra of 2,7-Br-proflavine and pyrene were registered by a Cary-Eclipse spectrofluorometer (Varian). Prior to the measurements, oxygen was removed from the samples enzymatically by adding 10 mg of glucose (Sigma) and 100 μg of catalase (Sigma) [2, 3].

The location of PFDs in the membrane was assessed by the method of fluorescence probes. The following dyes were used as probes: eosin Y, having two negative charges at neutral pH; 2,7-Br-proflavine, having one positive charge; and hydrophobic pyrene, having no charges and located in hydrophobic sites of the membrane, which corresponds to the region of fatty acid residues of phospholipids. Two milliliters of phosphatidylcholine liposomes in Tris-HCl buffer, pH = 7.2, 0.02 M, were placed into a 1-cm quartz cell,

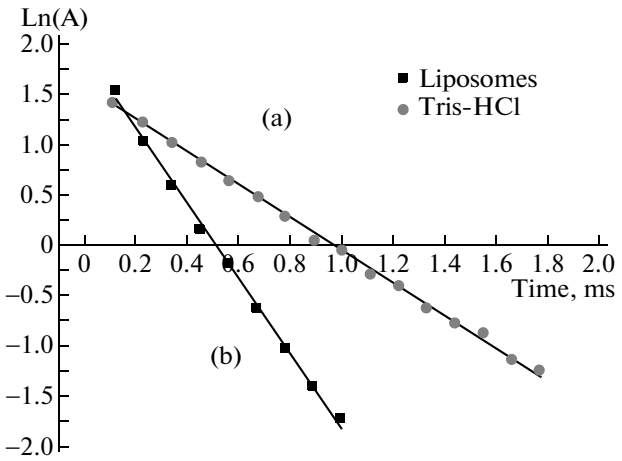


Fig. 2. Phosphorescence decay rate of eosin Y: (a) in water solution; (b) within the phosphatidylcholine liposomes.

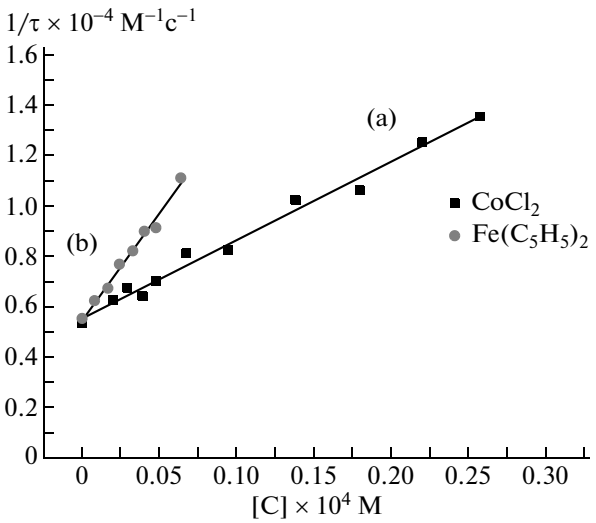


Fig. 3. Phosphorescence decay rate of eosin within phosphatidylcholine liposomes with the addition of the following substances: (a) $CoCl_2$; (b) $Fe(C_5H_5)_2$.

followed by the addition of chromophores in a concentration of 2×10^{-6} M. The samples were titrated by aliquots of PFDs. The decrease in the fluorescence amplitude of the probes was registered at 541 nm for eosin Y, 510 nm for 2,7-Br-proflavine, and 370 and 475 nm for pyrene. The equilibrium constant (Keq), characterizing the stability of the PFD-probe complex in the water solution and within phosphatidylcholine liposomes, was determined from the inclination of the experimental curve by the Stern–Volmer relationship.

RESULTS AND DISCUSSIONS

The negatively charged eosin Y xanthene dye is known to adsorb onto the surface of lecithin membranes near the region of polar head groups of the lipids [2]. The deactivation of the phosphorescence of eosin Y can be achieved by both a polar quencher in the water solution and as a result of lateral diffusion through the membrane bilayer by a hydrophobic quencher. A comparison of the quenching rate of the triplet probes by different compounds such as fullerene derivatives in water solution, on the surface of liposomes (in the region of polar heads), and within the hydrophobic sites of the lipid bilayer of liposomes can reflect the ability of chemical compounds to be incorporated into lipid membranes. Thus, such a comparison reveals the membrane-acting properties of the substances. PFDs were shown to be quenchers of the phosphorescence of eosin Y in both water solutions and within phosphatidylcholine liposomes. It was found that, when the lipid : dye ratio is 50 : 1 in a liposome membrane, almost all eosin Y molecules are incorporated into the lipid bilayer in the region of

Table 2. The constants of luminescence quenching of probes by water-soluble polysubstituted derivatives of fullerenes

PFDs	Phosphorescence of eosin Y		Fluorescence of 2,7-Br-proflavine		Fluorescence of pyrene	
	K_{Tris}, M^{-1} s^{-1}	K_{Lip}, M^{-1} s^{-1}	K_{Tris}, M^{-1}	K_{Lip}, M^{-1}	$K_{394\ nm}$, M^{-1}	$K_{475\ nm}$, M^{-1}
KB 197	0.31×10^8	0.05×10^8	5.83×10^5	3.6×10^5	1.37×10^5	1.34×10^5
KB 450	0.09×10^8	0.06×10^8	6.64×10^5	3.20×10^5	0.91×10^5	0.95×10^5
KB 445	0.45×10^8	0.42×10^8	20.0×10^5	10.0×10^5	1.00×10^5	0.87×10^5
KB 446	0.61×10^8	0.59×10^8	20.0×10^5	8.30×10^5	1.08×10^5	1.28×10^5
KB 449	0.97×10^8	0.84×10^8	10.0×10^5	7.00×10^5	0.69×10^5	0.83×10^5
NK 205	$<10^6$	$<10^6$	0.95×10^5	0.60×10^5	0.36×10^5	0.41×10^5
NK 212	$<10^6$	$<10^6$	0.80×10^5	0.45×10^5	0.86×10^5	0.88×10^5
KB 318			$*3.35 \times 10^5$	$*1.03 \times 10^5$	3.06×10^5	3.46×10^5
KB 392	2.00×10^9	1.60×10^9	$*3.49 \times 10^5$	$*1.55 \times 10^5$	1.25×10^5	1.83×10^5
TP 1105	0.90×10^9	0.74×10^9	$*3.28 \times 10^5$	$*0.57 \times 10^5$	1.53×10^5	2.20×10^5

* Eosin Y is used as a dye.

polar heads. Moreover, the molecules from the water part do not affect the eosin phosphorescence signal, because the phosphorescence decay curve of eosin Y is exponential in the water solution and within the liposomes, with the absence of quenchers and with the presence of PFDs (Fig. 2).

This conclusion is confirmed by the profile of quenching of phosphorescence of the dye achieved by the hydrophilic ion Co^{2+} from the water phase and by the neutral hydrophobic quencher ferrocene, which can attack dyes only from a hydrophobic region of the membrane and which is not capable of quenching the phosphorescence of dyes in the water phase (Fig. 3).

The efficiency of quenching of the probe was observed to depend on the character of a charge localized on addends of PFDs in both the water solution and within phospholipid membranes.

As was shown in Table 2, when the phosphorescence of negatively charged eosin Y is quenched by fullerene derivatives that exhibit negative charges on their addends (KB 445, KB 446, KB 449, KB 450) and by fullerene derivatives NK 205 and NK212 with a negative charge on the fullerene spheroid, the rate constants of quenching in water solutions and within phosphatidylcholine liposomes have orders of 10^6–10^7 M^{-1} s^{-1}, which is approximately 2–3 order lower than the diffusion constant. This phenomenon is a result of the repulsive interaction between electrostatic charges of chromophore and PFDs.

At the same time, when the negatively charged eosin Y is quenched by fullerene derivatives KB 318, KB 392, and TP 1105, with five and eight positive charges on the addends, the process of quenching differs significantly from one that uses the study of molecules having negative charges as quenchers. The rate constants of the quenching of the phosphorescence of eosin Y by KB 392 and TP 1105 derivatives have orders of 10^9, which is two to three orders higher than those of negatively charged derivatives and which is comparable with the rate constants of quenching of phosphorescence of the probe in case of diffusion interactions (Table 2). The addition of even low concentrations of the derivative KB 318 into the sample containing the dye lead to a sharp decrease of the phosphorescence intensity of eosin and did not have any considerable effect on its lifetime. This effect can be related to the formation of a strong fullerene-dye complex by electrostatic interactions between the negatively charged chromophore and the positively charged fullerene derivative. Consequently, the generation of the stable PFD-probe complex causes a complete quenching of the excited state of the dye within the complex structure and makes it impossible to measure the rate constants of quenching of the phosphorescence of eosin Y for the positively charged KB 318.

Thus, it was found that the efficiency of quenching of the phosphorescence of the triplet probes in water solutions and within phospholipid membranes depends on the character of the electrostatic charge of the triplet probe and the fullerene derivatives. This result provides a basis for selecting the cations and anions from a set of fullerene derivatives by comparing the rate constants of the phosphorescence quenching of oppositely charged probes by the derivatives. This issue is very important for investigations into the biological activity of this class of compounds, because, on the one hand, electrostatic interactions play an important role in biochemical processes in the organism and, on the other hand, polysubstituted derivatives of fullerenes possess a quite complicated structure; thus, it is often not very easy to assess their electrostatic conditions.

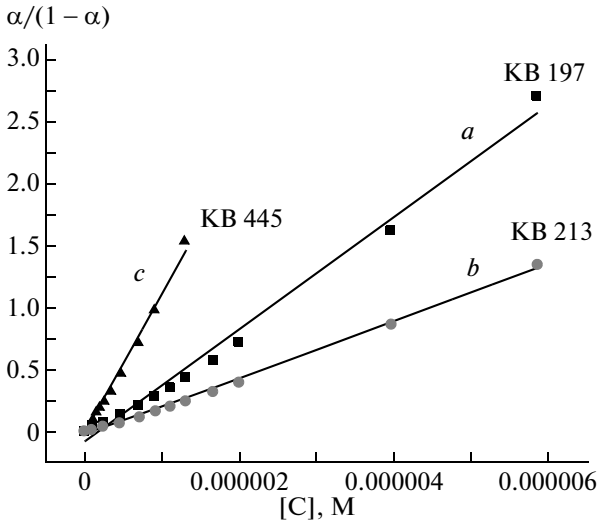


Fig. 4. Quenching of the fluorescence of 2,7-Br-proflavine in liposomes by the following substances: (a) KB197, (b) KB213, and (c) KB445 in the coordinates of Stern–Volmer

The localization of PFDs within the membranes of phosphatidylcholine liposomes was determined by a fluorescence technique with the following fluorescence probes: the negatively charged eosin Y, the positively charged 2,7-Br-proflavine localized in the membrane in the region of the polar heads of phospholipids, and the uncharged hydrophobic probe pyrene localized in hydrophobic sites of the membrane in the region of fatty acid residues of phospholipids.

The quenching of the fluorescence of chromophores in water solutions and membranes in the presence of quenchers is known to be achieved by two mechanisms, notably, by the diffusion interactions between chromophore and the quencher and by a complex formation between them [5]. Thus, in the case of dynamic collisions of excited molecules of chromophores and quenchers, the registration of the quenching processes of fluorescence can be performed at molar concentrations of quenchers [5].

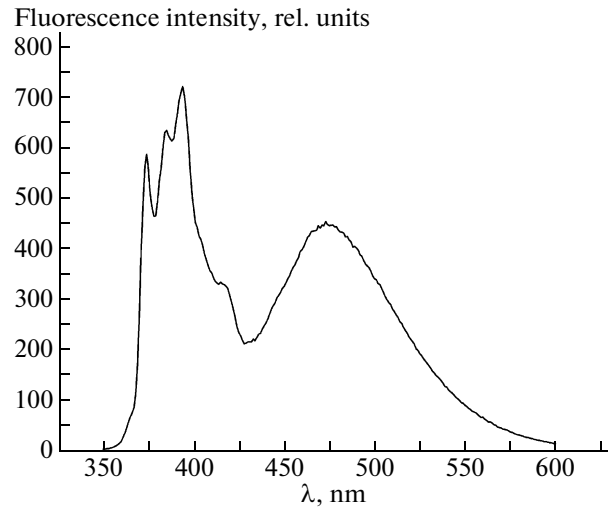


Fig. 5. Fluorescence spectrum of pyrene incorporated into phosphatidylcholine liposomes.

Since the quenching of the fluorescence of the probes occurs at low concentrations of the fullerene derivatives ~10^{-6} M, it is possible to conclude that quenching of the studied dyes in the excited singlet state (with a lifetime of ~1 ns) is not a result of diffusion interactions but is achieved due to the formation of a long-living complex PFD dye held together by the mutual electrostatic attraction of opposite charges on the dye and PFDs.

The interaction between a luminescent molecule and a quencher and the subsequent formation of the complex is defined by the Stern–Volmer equation as follows [6]:

$$\frac{\alpha}{1-\alpha} = K_p[q], \quad \text{where} \quad \alpha = \frac{I_0 - I}{I_0 - I_k},$$

where I_0 is the luminescence intensity of a probe in the absence of a quencher, I_k is the luminescence intensity of chromophore molecules in a complex with a quencher, and I is the luminescence intensity of a probe upon the concentration of a quencher [q].

The equilibrium constant K_{eq} (*Fig. 4*) can be calculated from the angulation of linear anamorphoses in the Stern–Volmer coordinates by considering $I_k = 0$ in all experiments. For a set of oppositely charged dyes and PFDs, the K_p values lie in diapason of 10^5–10^6 M^{-1} (Table 2).

As was shown in *Table 2*, the values of K_p depend on the nature of the dye and fullerene derivatives, as well as on the matrix that solvates a current complex, where the higher the salvation of charges of a dye and PFDs is, the lower the complex stability is. For example, the values of K_p in the 0.02 M Tris-HCl buffer solution are higher than in liposomes for the eosin Y – KB318 and 2,7-Br-proflavine – KB-445 pairs.

Thus, it was demonstrated that, by applying the method of triplet probes, the selected lipophilic charged dyes adsorb efficiently on the membrane when the particular lipid : dye ratio is used. The following part of the study proposes a quantitative method for estimating the efficiency of the interaction of fullerene derivatives with dyes in the region of polar heads of the membrane based on the equilibrium constants K_{eq} that were obtained by analyzing the dependence of the fluorescence intensity of a dye from the concentration of PFDs.

Similar to eosin Y, the 2,7-Br-proflavine dye adsorbs onto the membrane in the region of polar heads of phospholipids, because proflavine is a lipophilic positively charged molecule.

A study of the interaction between PFDs and nonpolar sites of the membrane was performed by analyzing the effect of PFDs on the fluorescence of the hydrophobic probe pyrene interacting with the lipids of a membrane in the region of fatty acid residues.

Due to the fact that the lifetime of pyrene in the excited singlet state is ~100 ns, which is about 100 times higher than that of the fluorescent hydrophilic probes used in the study, it is possible to examine very slow diffusion processes in solutions and within a membrane by measuring the changes in pyrene fluorescence [5].

The second essential property of pyrene is the formation of so-called excimers, which are complexes of excited molecules of the probe with unexcited molecules [5]. The fluorescence spectrum of the excimer is shifted into a long-wave region compared to a monomer (Fig. 5). When pyrene is used as a fluorescent probe, the monomers of pyrene, during the period of the excited state (80–120 ns in liposomes), dislocate by 20–40 Å in the membrane. This feature allows one to analyze the membrane structure at these distances. Various events of static and dynamic quenching of pyrene in the excimeric and monomeric states by other molecules in the membrane can provide valuable information about the structure of the membrane and the membrane activity of molecules. As was shown in Fig. 5, the fluorescence spectrum of pyrene in the liposomal membrane consist of several peaks in the range of 350–400 nm corresponding to the monomeric state of pyrene and a wide peak in the range of 430–500 nm corresponding to the excimeric state of pyrene.

The titration of pyrene by different PFDs resulted in the efficient quenching of the fluorescence of pyrene, while the ratio between "monomeric" and "excimeric" peaks was changed negligibly. This indicates that the interaction between PFDs and excited molecules of the probe is efficient in both monomeric and excimeric states and leads to the complete quenching of excited molecules of pyrene. By taking into account the fact that the quenching of fluorescence of the probe is observed at concentrations of

PFDs of around 10^{-6}–10^{-5} M (Table 2), it is possible to conclude that quenching is achieved not due to the presence of dynamic complexes, but through the formation of long-living complexes of PFDs with pyrene in both monomeric and excimeric states. Since the pyrene molecule is uncharged and has an aromatic saturated structure, most likely the interaction of pyrene with PFDs is achieved through the direct formation of the complex between the aromatic pyrene structure and the spheroid of fullerene or its addends in the hydrophobic region of a membrane. The observed differences in K_{eq} for the interaction of pyrene with different PFDs (table) can be the result of a partial involvement of the addends in the process of the formation of the complex, or they can be caused by a different extent of immersion of the fullerene core into the hydrophobic region of a membrane. In particular, higher values of K_{eq} were observed for positively charged PFDs.

Thus, this study suggests objective and quantitative criteria to estimate the membrane activity of chemical substances, in particular, fullerene derivatives, which are quenchers of fluorescence for probes with different localizations in the membrane. These criteria are the rate constants for the quenching of the phosphorescence of triplet probes by fullerene derivatives and the equilibrium constants for the PFD-probe complexes, which display the stability of the complexes. This in turn allows one to determine the location of PFDs in the membrane when fluorescence probes interacting with different membrane sites are involved.

A comparison of the rate constants of the quenching of the phosphorescence of eosin and the equilibrium constants for the PFD–chromophore complexes has revealed that the efficiency of the interaction of PFDs with different sites of the phospholipid membrane depends significantly on the charge in addends attached to the spheroid of polysubstituted derivatives.

ACKNOWLEDGMENTS

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REFERENCES

1. N. van Rooijen and A. Sanders, "Liposome Mediated Depletion of Macrophages: Mechanism of Action, Preparation of Liposomes and Applications," J. Immunol. Methods **174**, 83–93 (1994).
2. V. M. Mekler, A. I. Kotel'nikov, G. I. Likhtenshtein, and M. A. Berkovich, "Application of Phosphorescent Probes for Study of Model and Biological Membranes," Biofizika **27**, 641–645 (1982).
3. R. A. Kotelnikova, A. I. Kotelnikov, G. N. Bogdanov, V. S. Romanova, E. F. Kuleshova, Z. N. Parnes, and M. E. Vol'pin, "Membranotropic Properties of the Water Soluble Amino Acid and Peptide Derivatives of Fullerene C_{60}," J. FEBS Lett. **389**, 111–114 (1996).
4. Yu. A. Vladimirov and E. V. Proskurnina, "Free Radicals and Cell Chemoluminescence," Usp. Biol. Khim. **49**, 341 (2009); Biochemistry (Moscow) **74**, 1545 (2009).
5. Yu. A. Vladimirov and G. E. Dobretsov, *Fluorescent Probes in Biological Membrane Studies* (Nauka, Moscow, 1980) [in Russian].
6. C. A. Parker, *Photoluminescence of Solutions* (Elsevier, Amsterdam, 1968).