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Quenching of Cascade Reaction Between Triplet and Photochrome Probes with Nitroxide Radicals

A Novel Labeling Method in Study of Membranes and Surface Systems

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Abstract

We proposed a new method for the study of molecular dynamics and fluidity of the living and model biomembranes and surface systems. The method is based on the measurements of the sensitized photoisomerization kinetics of a photochrome probe. The cascade triplet *cis-trans* photoisomerization of the excited stilbene derivative sensitized with the excited triplet Erythrosin B has been studied in a model liposome membrane. The photoisomerization reaction is depressed with nitroxide radicals quenching the excited triplet state of the sensitizer. The enhanced fluorescence polarization of the stilbene probe incorporated into liposome membranes indicates that the stilbene molecules are squeezed in a relatively viscous media of the phospholipids. Calibration of the "triple" cascade system is based on a previously proposed method that allows the measurement of the product of the quenching rate constant and the sensitizer's triplet lifetime, as well as the quantitative detection of the nitroxide radicals in the vicinity of the membrane surface. The experiment was conducted using the constant-illumination fluorescence technique. Sensitivity of the method using a standard commercial spectrofluorimeter is about 10^{-12} mol of fluorescence molecules per sample and can be improved using an advanced fluorescence technique. The minimal local concentration of nitroxide radicals or any other quenchers being detected is about 10^{-5} *M*. This method enables the investigation of any chemical and biological surface processes of microscopic scale when the minimal volume is about 10^{-3} μL or less.

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Index Entries: Stilbene; Erythrosin B; nitroxide radical; liposome membrane; triplet-triplet energy transfer; sensitized *cis-trans* photoisomerization; quenching of triplet state; cascade reaction.

Introduction

The role of biological membranes and surface systems in the fundamental biochemical and biophysical processes producing the vital activities of all organisms cannot be overestimated. Biological membranes act as the barriers to separate cells and subcellular structures and contain the specific transportation channels for physiologically active compounds. They also form the enzymes' ensembles that catalyze the biochemical reactions. Practically all biologically essential phenomena, such as intercell interaction, cell proliferation, mobility, and pathological processes, are associated with the peculiarities or structure and dynamics of the biological membranes. The molecular dynamics of surface systems, such as liquid films, nanoscale particles, and quartz-contaminated optical fibers modified with a variety of dyes, is another important field of many basic and applied studies.

For the past four decades, the increasing number of publications on the role of free radicals in the biological and chemical processes indicates the growing interest concerning this problem *(1–5)*. The various radicals are capable of catalyzing or inhibiting the physical and biochemical processes and thus cause damage to living organisms. In particular, scientists speculate that the active radicals cause the cancer cell proliferation. Therefore, it is extremely important to detect the traces of radicals in living cells, because even a very low concentration can be harmful.

The biophysical labeling methods, mainly spin trapping and fluorescent, have been widely used to investigate the radical processes affecting the functionality and dynamics of the biological membranes *(6)*. Modifications of the specific parts of the biological and model membranes by a whole set of labels and probes allow the biological labeling methods to be extremely effective. Detailed conclusions on the dynamics of the various fragments of lipid layers in the liposomes have become possible by using these methods *(7–15)*. Along with the advantages, these methods have some limitations.

The sensitivity of the spin-trapping methods, based on the radicals' transformation to stable nitroxides, permits the detection of radicals' only up to 10^{-4} *M (9–11)*. This limitation restricts the use of the standard electron spin resonance (ESR) techniques. Another principle limitation of the ESR spectroscopy (based on the analysis of the ESR forms of spectral lines) and of the constant-illumination fluorescence technique is their characteristic times, which rank from 10^{-8} to 10^{-10} s. This means that these methods can be applied only for the investigation of the fast rotational and translational processes and only in low viscous solutions. The use of some special ESR methods and the phosphorescence polarization technique expands the limit of the luminescent measurements to the microsecond region *(12)*. Never-

theless, some essential limitations for the investigation of the mechanism and translational diffusion in the heterogeneous asymmetric biomembranes still exist. The ESR technique requires expensive and complicated ESR spectrophotometers. This technique, and consequently the method of spin traps, cannot be applied to the surface processes, individual cells, and other objects where high sensitivity is important.

To overcome the limitations of the spin-trapping and fluorescent methods, the fluorescence-photochrome and triplet-photochrome labeling techniques have been developed to study the dynamics of the biomembranes and surface systems *(7,8,16,17)*. These novel techniques are based on the constant-illumination measurements of the *trans-cis* photoisomerization kinetics of the stilbene fluorescence label incorporated into a membrane. Monitoring the fluorescence decay of the *trans*-stilbene derivative probe by the constant-illumination fluorescence technique makes it possible to follow the *trans-cis* photoisomerization, which is the rotation of the stilbene fragments in the excited state around the olefinic bond at approx 180°. It was found that the relaxation of the viscous medium is the limiting stage of the photoisomerization. The apparent rate constant of the photoisomerization in a viscous medium, such as the biological membranes, was found dependent on the relaxation rate of the medium. Those rate constants were related to the microviscosity of the medium in the vicinity of the stilbene chromophore *(16)*. Therefore, after an appropriate calibration, it is possible to estimate the rotational frequency of the stilbene fragments around the olefinic double bond in the photochemically excited state. The combined data on the fluorescence and photoisomerization properties of the stilbene-derivative probes make it possible to establish the location and detailed mechanism of the label motion in the investigated biomembrane and to estimate the motion parameters *(7,16,18)*.

The fluorescence-photochrome labeling method enables one to ascertain the location and mobility and to measure the quantitative parameters of the stilbene probe, *trans*-4'-dimethylamino-4-aminostilbene (*trans*-DMAAS), in *Escherichia coli* membrane. These studies have shown that the photochemically excited stilbene fragments were involved in a high amplitude rotation in the membrane at the angle of 180° with a rotation frequency of 4×10^7 s^{-1} at $T = 24°C$.

The experimentally measured anisotropy value r of the stilbene fluorescence polarization in the membrane showed the fast wobbling of the stilbene chromophore with the frequency much higher than the radiative decay rate constant k_f (approx 10^9 s^{-1}) within a small angle of $\varphi_w = 22.7°$ *(16)*. The obtained data allowed researchers to speculate that the polar $(CH_3)_2N$-group of DMAAS would be unable to penetrate the hydrophobic membrane and would be located in the interface region of the membrane *(16)*. The current research proved this hypothesis.

The previously developed cascade method is based on the cascade scheme involving the stilbene photoisomerization that is sensitized by the triplet-triplet energy transfer between a chromophore in the excited triplet

state, such as Erythrosin B, and the stilbene photochrome probe *(17)*. The encounters were studied in a model system containing the Erythrosin B sensitizer and the stilbene photochrome label. Both types of molecules were covalently bound to α-chymotrypsin. The *trans-cis* isomerization kinetics was monitored by the fluorescence decay of the *trans*-stilbene photochrome molecule. The rate constants of the triplet-triplet energy transfer between the sensitizer and the photochrome molecule were measured at room temperature and pH 7.0. Note that the concentration of the triplet sensitizer did not exceed 10^{-7} *M* in those experiments, and that the collision frequency was close to 1 s^{-1}, which is several orders of magnitude less than that measured with the standard luminescence or ESR techniques.

The next step done to expand the applicability of the fluorescence-photochrome technique for investigation of the molecular dynamics and for measurements of the concentration of stable radicals in solution was the development of the spin-triplet-photochrome labeling method *(19)*. This technique combines these three types of biophysical probes:

1. Stable nitroxide-radical spin probe (*R*).
2. Triplet probe, which has the high quantum yield of the triplet excited state and can be used as a photosensitizer (*E*).
3. Stilbene-derivative photochrome probe, which is fluorescent only in its *trans*-form (*A*).

The combination of these three probes keeps their facilities and thus has an essential advantage in the study of molecular dynamics and measurements of the local concentration of radicals' using the standard very sensitive fluorescence technique *(19)*.

The cascade technique employs these three well-known physical processes:

1. *Trans-cis* and *cis-trans* photoisomerization of the excited molecule.
2. Triplet-triplet energy transfer.
3. Quenching of the excited triplet state by a nitroxide radical.

The light-induced reversible *trans-cis* photoisomerization of the *trans*-stilbene molecules is the main quenching funnel of their fluorescence, and it proceeds from the lowest excited singlet state $^1t^*$ through the twisted singlet intermediate $^1p^*$ *(18,20–22)*:

$$^1t^* \rightarrow {}^1p^* \rightarrow {}^1p \rightarrow (1 - \beta)^1c + \beta^1t$$

or, alternatively, by the intersystem crossing pathway via the biradical twisted triplet state $^3p^*$:

$$^1t^* \rightarrow {}^3t^* \rightarrow {}^3p^* \rightarrow {}^1p \rightarrow (1 - \alpha)^1c + \alpha^1t$$

in which $^3t^*$ and $^3p^*$ are the *trans* and twisted configurations (perpendicular with respect to the olefinic double bond), respectively, of the lowest triplet; ^1p is the twisted ground state; $(1 - \alpha)$ is the fraction of triplet decay into the

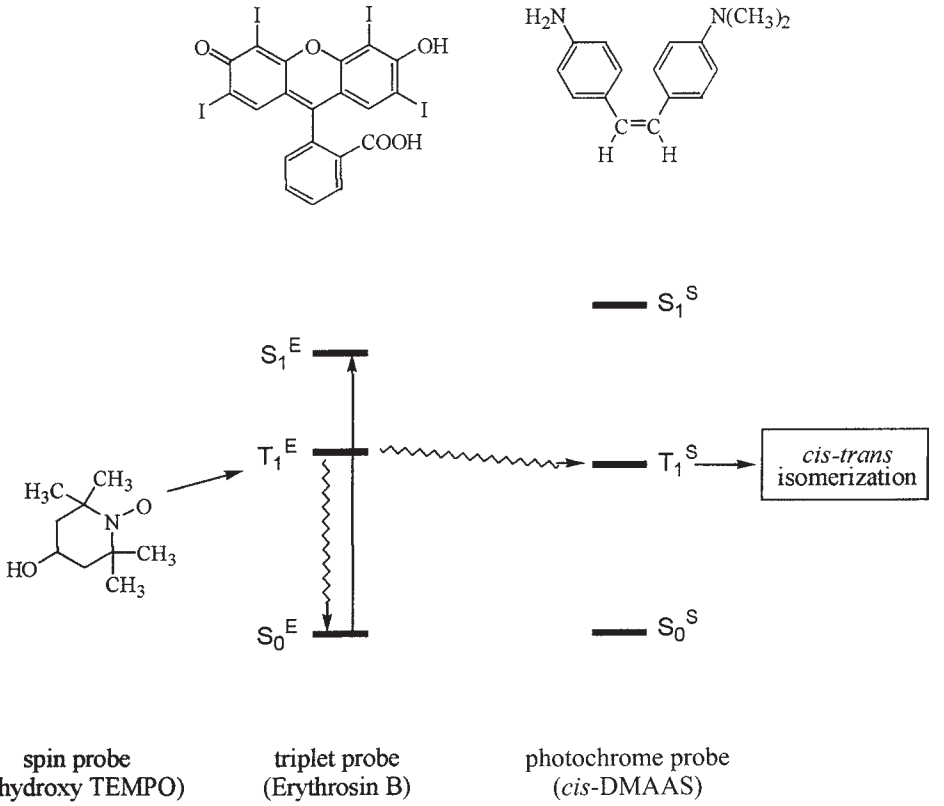

Fig. 1. Representation of the energy levels of the cascade reactants and a competition between the $T_1^E \longrightarrow T_1^S$ and $T_1^E \longrightarrow S_0^S$ processes.

cis-form; and (1 – β) is the fraction of the perpendicular singlet configuration decaying into the *cis*-form.

The triplet state can be populated by the intersystem crossing of the excited singlet and triplet states of the stilbene molecule as well as by the triplet-triplet energy transfer from the excited triplet donor molecule, such as Erythrosin B, which sensitizes the triplet excitation of the stilbene molecule. Following the sensitization, the *cis-trans* photoisomerization occurs through the triplet excited potential surface *(23–25)*.

Figure 1 schematically shows the relative position of the energetic levels of Erythrosin B and the stilbene photochrome probe and a competition between the $T_1^E \rightarrow T_1^S$ and $T_1^E \rightarrow S_0^S$ processes.

The free nitroxide radicals are capable of quenching quite efficiently the triplet state of the excited molecule *(5)*. Consequently, the *cis-trans* photoisomerization of the stilbene label through the excited triplet state is affected by the presence of radicals. This effect has been used, after a previous calibration, to estimate quantitatively the concentration of the nitroxide radicals in the investigated system. The product of the quenching rate constant of the synthesizer quenched by nitroxide and the sensitizer's

triplet lifetime $k_q \times \tau_{ph}$ can be measured, if the τ_{ph} is known *(19)*. Those values are considered molecular dynamics parameters in the investigated system.

The proposed spin-triplet-photochrome labeling method enables the experimental measurement of the following important parameters:

1. Local concentration of the stable radicals (spin labels, products of active radicals, spin probes, reactions, spin-redox probes, and so on).
2. Mobility (rotation, wobbling) of the fluorescence photochrome probes and their fragments in the excited singlet state using the fluorescence and fluorescence-polarization techniques.
3. Rotational mobility of the triplet probes using the phosphorescence polarization.
4. Rate constant of encounters between the photochrome and triplet probes.
5. Rate constant of encounters between the triplet probes and nitroxide radicals.
6. Rotation of the nitroxide radicals measured by ESR spectroscopy.

The combination of these data could elucidate the sophisticated molecular dynamics of the object under investigation.

The spin-triplet-photochrome method can be used for the study of biological and model membranes, surface systems, and objects of microscopic size in which the minimal volume of a sample available for the standard fluorescence measurements is about 10^{-3} μL. The method can be applied to fiberoptics spectroscopy and fluorescence microscopy.

The present study demonstrates the application of the cascade method for the study of molecular dynamics and the quantitative detection of the nitroxide radicals in the model liposome membranes using the standard commercial fluorescence technique.

Materials and Methods

The synthesis of 4-dimethylamino-4'-aminostilbene (DMAAS) has been described previously *(16)*. Erythrosin B, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (4-hydroxy-TEMPO), and L-α-phosphatidylcholine (PPDC) were purchased from Sigma Israel.

Multilamellar liposomes were prepared according to the conventional procedure *(26)*. The 1.2×10^{-3} *M* PPDC stock solution was prepared in chloroform and mixed. The mixture was dried under a stream of nitrogen for 45 min and then further dried for several hours under reduced pressure. The dry lipid was dispersed in nitrogen-saturated water by vortexing in a nitrogen atmosphere at 45°C.

All the measurements were performed in 0.1 *M* phosphate buffer solution (pH 7.1) at 25°C. Concentrations of Erythrosin B and DMAAS in their phosphate buffer solutions were 7.5×10^{-7} and 3×10^{-6} *M*, respectively. The *trans*-DMAAS was preliminarily transformed into its *cis*-form by irradiation with a Hg lamp at the absorption maximum of the *trans*-isomer with

λ_{ex} = 370 nm. Erythrosin B was excited near its absorption maximum at λ_{irr} = 546 nm. The fluorescence emission and fluorescence polarization of the *trans*-DMAAS were recorded at λ_{em} = 430 nm with an SLM-4800 Aminco-Bowman spectrofluorimeter after excitation near its absorption maximum at λ_{ex} = 370 nm using typically a 16-nm slit width for excitation and emission. All the sample solutions were degassed with nitrogen before measurements were made.

ESR spectra were recorded on a Bruker EMX-220 digital X-band spectrometer equipped with a Bruker EP 4241VT temperature-controlled system at 297 K. The ESR measurements were conducted with the following parameters: 9.40-GHz microwave frequency, 20.12-mW nonsaturated microwave power, 100-kHz field modulation of 1-G amplitude. Absolute concentrations of the nitroxide radical were obtained by the numerical double integration of ESR signal vs 10^{-3} *M* water Tempol solution used as an external standard.

Results and Discussion

Liposomes are commonly used as the model membranes in experimental studies *(8)*. Large multilamellar vesicles (of the order of 10^4 Å, or 1 μm, in diameter) were obtained by slowly evaporating chloroform from a suspension of phospholipid in a mixed solvent system *(27,28)*.

DMAAS was used as a photochrome probe because of its high fluorescence yield and hydrophobicity, which is extremely important for biomembrane studies *(16)*.

The motion and orientation of DMAAS in a liposome bilayer can be deduced from the fluorescence emission spectrum and fluorescence decay kinetics. The maximum fluorescence wavelength of the *trans*-isomer in a buffer solution is λ_{em} = 444 nm while it is blue-shifted to λ_{em} = 422 nm when the stilbene emission is measured in the liposomes (Fig. 2). This shift to the blue is obvious and indicates that the stilbene label is located in a relatively nonpolar medium of the liposomes. The apparent local micropolarity of E_T^{30} = 45 kcal/mol in the vicinity of the stilbene label was estimated using a calibration of the empirical solvent parameter E_T^{30} *(29)* vs the fluorescence emission energy of DMAAS measured in different solvents *(30)*.

It was mentioned before *(19)* that the whole stilbene molecules could not be involved in a high-amplitude rotation with the correlation time close to the fluorescence lifetime (approx 10^{-9} s) in a viscous environment of *E. coli* membrane *(16)*. The similar conclusion can be made in the case of the liposome membranes. The fluorescence anisotropy *r* of *trans*-DMAAS measured in the liposomes at *T* = 24°C was found to be (0.225 ± 0.018) whereas the same parameter measured in a phosphate buffer solution was (0.130 ± 0.011). This means that the stilbene molecules were incorporated almost totally in the lipid bilayers, which caused the significant increase in the fluorescence anisotropy. Now, the anisotropy in the model liposome membrane (*r* = 0.225) can be compared with the anisotropy of the same probe imbedded in the *E. coli* membrane (*r* = 0.281) *(16)*.

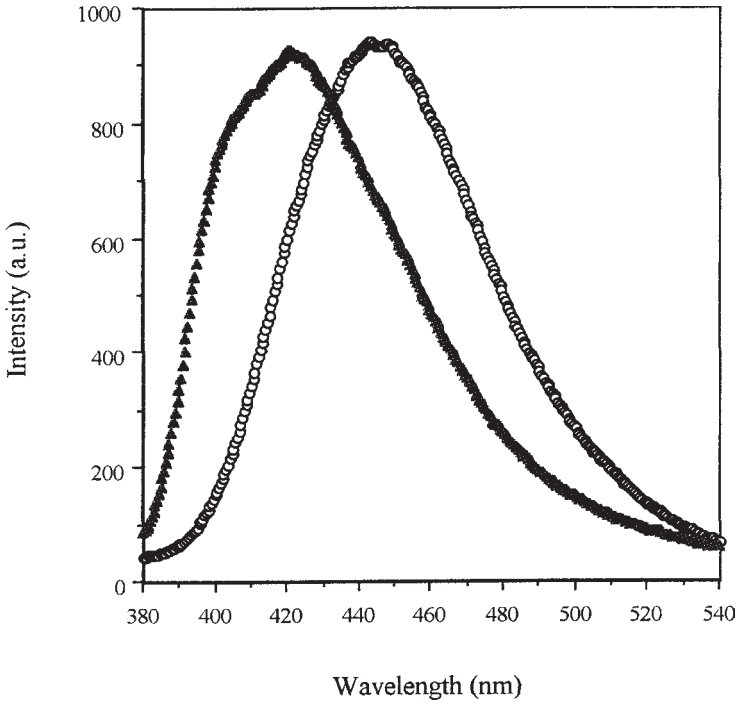

Fig. 2. Fluorescence emission spectra of *trans*-DMAAS in aqueous buffer solution (3×10^{-6} *M*) in the absence (○) and presence (▲) of liposomes (a.u., arbitrary units).

The experimentally measured anisotropy value makes it possible to estimate an angle of the stilbene probe wobbling in a liposome membrane $\varphi_w = 30.2°$ with the frequency much higher than the radiative decay rate constant k_f (approx 10^9 s^{-1}) using the following equation:

$$r = r_o[(3\cos^2\varphi_w - 1)/2] \quad (1)$$

in which $r_o = 0.362$ is the previously measured anisotropy when the stilbene molecule is "frozen" *(16)*.

This angle value is higher then $\varphi_w = 22.7°$ previously calculated for *E. coli* membrane by the same equation *(16)*. Therefore, the model liposome membrane is less fluid than the cell membrane.

Previously, it was found that Erythrosin B and the stilbene probe involve the cascade reaction that results in a *cis-trans* isomerization of the stilbene photochrome molecule *(17,19)*. The triplet state of the Erythrosin B sensitizer populated with the long-wave irradiation is energetically lower than the excited singlet state of the photochrome stilbene-probe molecule. The encounters between the stilbene photochrome and the excited triplet Erythrosin B lead to a triplet-triplet energy transfer from the Erythrosin B excited molecule to the stilbene derivative. The sensitized isomerization of the *cis*-stilbene derivative is observed because the resulting fluorescence of the *trans*-stilbene appearing in solution gradually grows with an increase

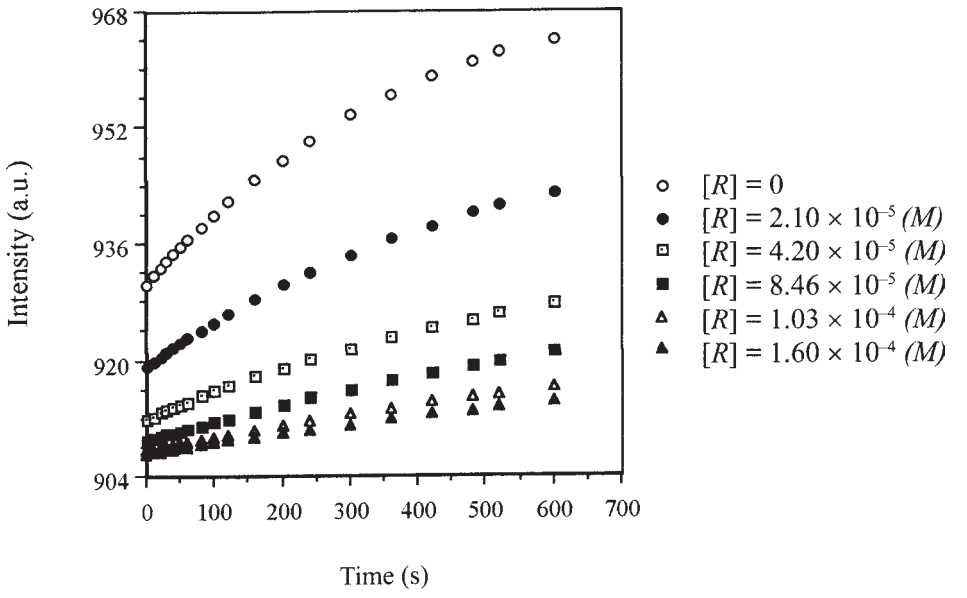

Fig. 3. Experimental fluorescence intensity of the excited *trans*-DMAAS vs the sample irradiation time at 546 nm for various 4-hydroxy-TEMPO concentrations. *trans*-DMAAS was excited at λ_{ex} = 370 nm, and its fluorescence intensity was measured at λ_{ex} = 430 nm (a.u., arbitrary units).

of the irradiation time at the excitation maximum of the Erythrosin B sensitizer at 546 nm.

It has previously been shown that the excited triplet state of Erythrosin B is quenched by molecules located in both aqueous and lipid phases of the liposome membrane *(31)*. Therefore, the triplet probe is located in a superficial portion of the membrane.

After Erythrosin B and *cis*-DMAAS solutions were mixed, the sample was irradiated at λ_{irr} = 546 nm, at which only Erythrosin B could be excited. Neither *cis*- nor *trans*-DMAAS are excited with this wavelength of light. Nevertheless, the increase in fluorescence was observed in the sample because the irradiation time at 546 nm increases owing to the photoisomerization of the *cis*-stilbene into its *trans*-form sensitized by Erythrosin B itself. Figure 3 shows the experimental fluorescence intensities of *trans*-DMAAS vs irradiation time at λ_{irr} = 546 nm for different radical concentrations. The experimentally measured fluorescence intensity increases not from zero, owing to the *trans-cis* equilibrium attained by preliminary irradiation at 370 nm. Actually, the first measurement in each case has been done not at $t_o = 0$, but a few seconds later, owing to some technical reasons in performing the experiment. That is why the initial intensity I_o varies from one radical concentration to another. However, all the fluorescence curves can be extrapolated to a single point at t_o, which is the apparent initial intensity I_0^* owing to the presence of some unconverted *trans*-stilbene in a sample at the start of the reaction. The concentration of the *trans*-DMAAS

excited at λ_{ex} = 370 nm approaches the photostationary level exponentially with an increase in fluorescence at λ_{em} = 430 nm. Following the gradual growth of the fluorescence intensity with an increase of the irradiation time at the excitation maximum of Erythrosin B sensitizer, the apparent rate constant k_{exp} was calculated as a slope of the plot dI_t/dt vs I_t. Here, I_t is the momentary intensity of the *trans*-isomer fluorescence and t is the time of the light exposure at the excitation maximum of the Erythrosin B sensitizer (λ_{irr} = 546 nm):

$$dI_t/dt = -k_{exp}I_t + \text{const} \tag{2}$$

The constant value of $I_o[E^T]$ was calculated as an intercept of the linear plot dI_t/dt vs I_t. Here, $[E^T]$ is the steady-state concentration of the excited triplet Erythrosin B.

Figure 4 shows the ESR spectra of the polar nitroxide-radical. There is no difference between the spectrum in the presence of the liposomes (Fig. 4A) and the spectrum in their absence (Fig. 4B). Therefore, the nitroxide probe is located in the aqueous phase of the investigated system. Figure 5 shows the location of the spin, triplet, and stilbene photochrome probes in the model liposome membrane.

By introducing the nitroxide radicals, which quench the excited state of Erythrosin B, the rise in fluorescence was depressed and the rate constant value k_{exp} was reduced (Fig. 3). We have shown previously that this phenomenon differs from the standard fluorescence quenching processes that occur through the exchange mechanism when the concentration of radicals $<10^{-3}$ *M* could not be monitored *(19,32)*. The concentration of radicals in our experiment was much lower than that necessary for the direct quenching of the *trans*-DMAAS fluorescence and varied from 1.6×10^{-4} to 2.0×10^{-5} *M*.

The experimental rate constant of the entire investigated process follows as *(19)*:

$$k_{exp} = (k_A^T + k_B^T)[E^T] \tag{3}$$

in which k_A^T and k_B^T are the rate constants of the triplet-triplet energy transfer between the sensitizer and the photochrome molecule for the *cis*-stilbene isomer (A) and *trans*-stilbene isomer (B), respectively.

The values of k_A^T and k_B^T and can be obtained from the measurement of the experimental rate constant with the method of laser flash photolysis *(33,34)*. The following equations perform the formation of the triplet Erythrosin B and its quenching with radicals in the cascade reaction:

$$E \underset{1/\tau_{ph}}{\overset{\sigma I_{ex}}{\rightleftharpoons}} E^T$$

$$R + E^T \xrightarrow{k_q} R + E$$

in which σ is the absorption cross-section; I_{ex} is the excitation intensity; k_q is the quenching rate constant; and τ_{ph} is the triplet lifetime of Erythrosin B.

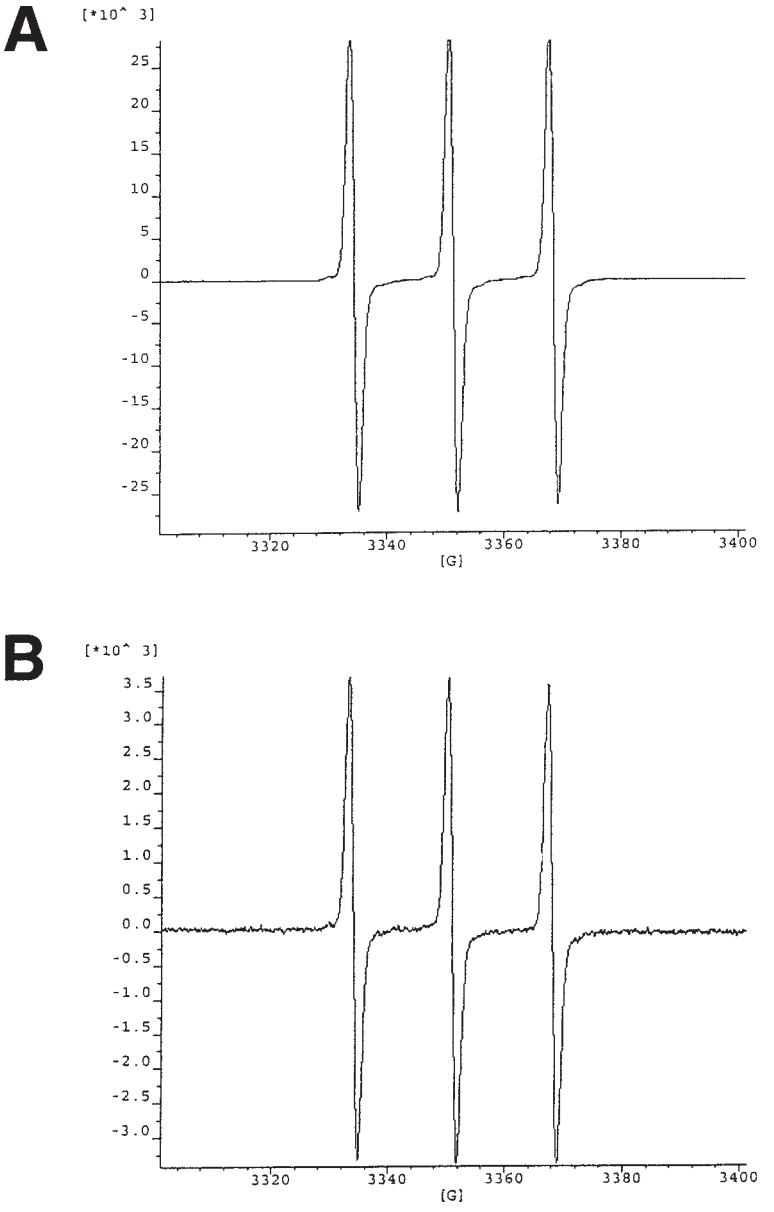

Fig. 4. ESR spectra of 4-hydroxy-TEMPO **(A)** in the presence and **(B)** in the absence of liposomes.

The quenching of the excited triplet stilbene will be not considered here because its triplet lifetime is essentially shorter than the lifetime of the excited triplet Erythrosin B molecule *(25,35)*.

Taking into account the consequent quenching by radicals at the steady-state conditions, the experimental rate constant of the cascade photoisomerization follows as *(19)*:

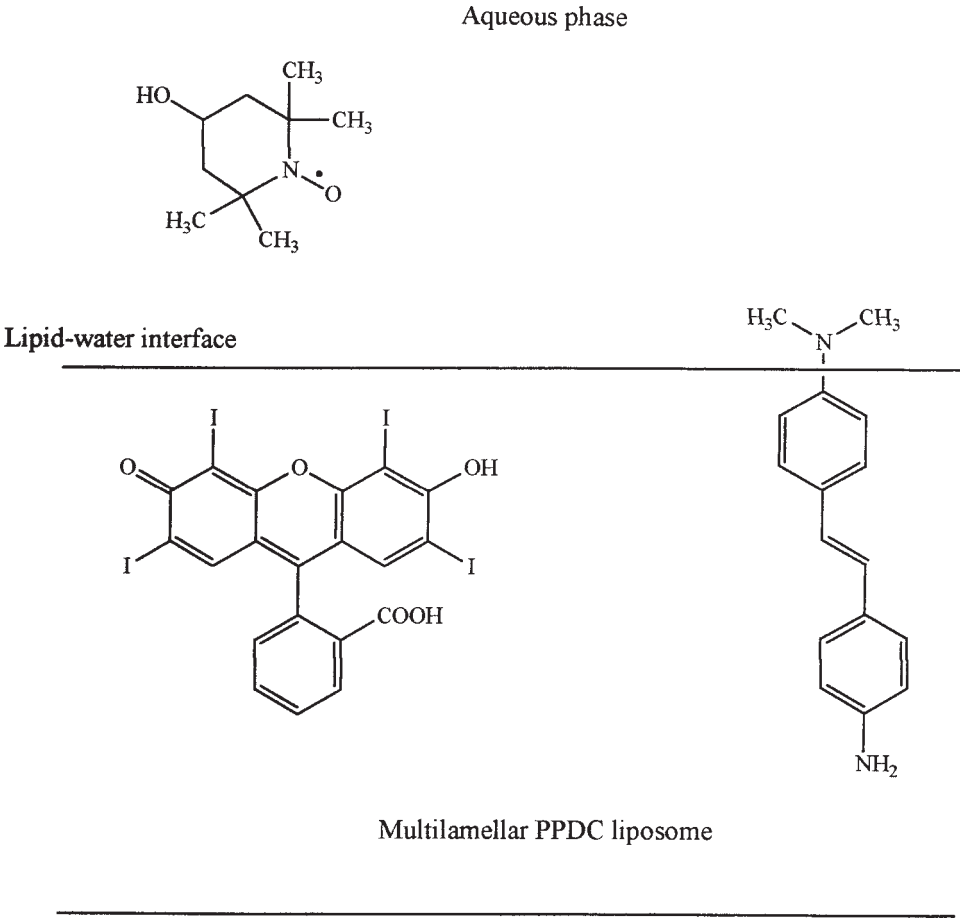

Fig. 5. A location of the spin, triplet, and photochrome probes in the model liposome membranes.

$$k_{exp} = \frac{\sigma I_{ex}(k_A^T + k_B^T)[E]}{k_q[R] + (\tau_{ph})^{-1}} \quad (4)$$

Figure 6 shows the fluorescence kinetic plots in their derivative forms dI_t/dt against fluorescence intensity, which are linear according to Eq. 2.

The reciprocal value of the experimental rate constant is dependent linearly on the radical concentration $[R]$:

$$1/k_{exp} = a + b[R] \quad (5)$$

in which

$$a = 1/\sigma I_{ex}(k_A^T + k_B^T)\tau_{ph}[E] \quad (6)$$

and

$$b = k_q/\sigma I_{ex}(k_A^T + k_B^T)[E] \quad (7)$$

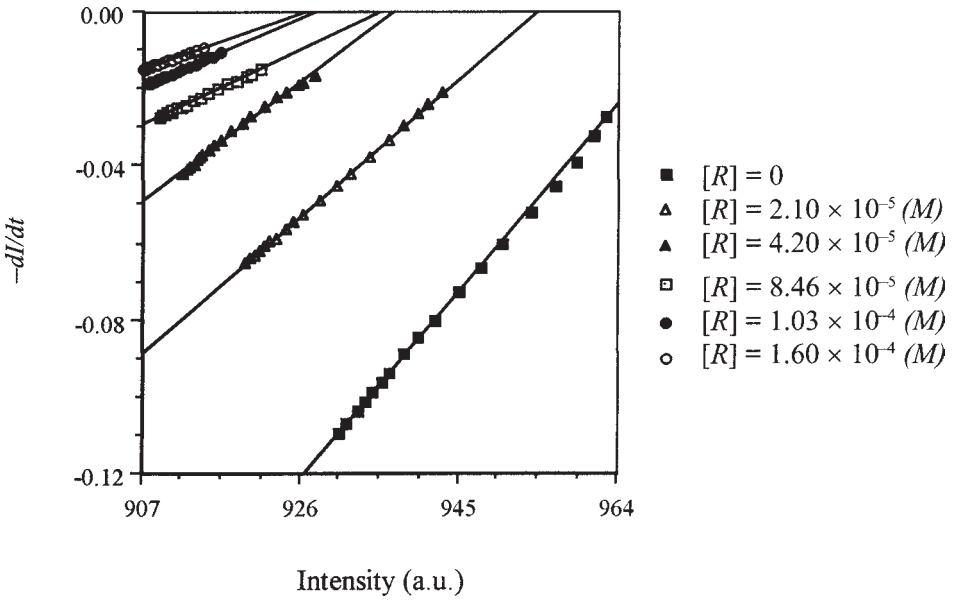

Fig. 6. *Trans*-DMAAS fluorescence increase rate vs fluorescence intensity for various 4-hydroxy-TEMPO quencher concentrations.

The quenching rate constant k_q is as follows:

$$k_q = (b/a)(\tau_{ph})^{-1} \tag{8}$$

Figure 7 presents the experimental data that give evidence of the nitroxide-radical inhibitor effect on the sensitized *cis-trans* isomerization of the stilbene derivative by quenching the sensitizer's triplet state in the interface of the liposome membrane. It shows the linear dependence of the reciprocal experimental rate constant k_{exp}^{-1} on the radical concentration. According to Eq. 8, the slope-to-intercept ratio b/a of this linear curve is equal to the product $k_q \times \tau_{ph}$. Thus, by measuring the fluorescence intensity of the excited stilbene molecule as a time function of the Erythrosin B excitation in the investigated "triple" system (Erythrosin B-DMAAS-nitroxide radical), we can calculate the quenching rate constant k_q if the τ_{ph} is known. Using the appropriate calibration, we can determine the radical concentration in a vicinity of the probes.

The triplet lifetime of Erythrosin B in the model liposome membrane was measured to be $\tau_{ph} = 0.96 \times 10^{-3}$ s *(31)*. Taking into account this value, the quenching rate constant $k_q = (1.75 \pm 0.15) \times 10^7\ M^{-1}s^{-1}$ was obtained from the product $k_q \times \tau_{ph}$. The quenching rate constant of the cascade reaction previously measured in aqueous solution has a value of $1.3 \times 10^9\ M^{-1}s^{-1}$ *(19)*. The conclusion is obvious. While the cascade reaction between Erythrosin B and the stilbene probes occurs within the liposome membrane, the radicals quench this reaction on the membrane lipid-water interface. However, the quenching process on the membrane interface is not as effective as for the free triplet probe in solution.

$$(k_{exp})^{-1} = (380 \pm 42) + (6.39 \pm 0.48) \times 10^6 [R]$$

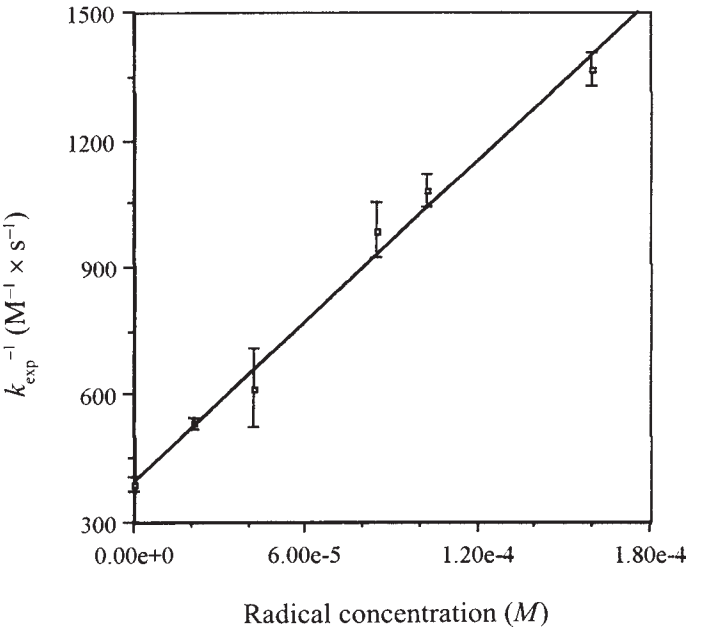

Fig. 7. Linear dependence of the reciprocal experimental rate constants on the 4-hydroxy-TEMPO radical concentration.

Figure 8 presents the proposed location and mobility diagram of the *trans*-DMAAS photochrome probe in the liposome membrane. Based on the theoretical considerations and experimental data, two types of problems in the study of the biomembranes and surface systems can be solved using the proposed spin-triplet-photochrome technique. First, varying the concentration of the spin probe introduced into an object of interest and measuring the sensitized stilbene photoisomerization makes it possible to calculate the product $k_q \times \tau_{ph}$. This product may be used as an apparent parameter of the local microviscosity. The quenching rate constant k_q is derived from the product $k_q \times \tau_{ph}$ after a measurement of the τ_{ph} in an independent experiment. Second, the local concentration of the nitroxide radicals in a sample can be estimated according to a calibration curve. It is possible to calibrate the experimental rate constant value k_{exp}^{-1} vs the radical concentration for the systems with a known macro- and microviscosity. For example, the kinetics of a reaction between the spin-trap and active radicals or the dual fluorophore-nitroxide probe reduction can be followed by such a technique *(36)*. If the reaction product locates in an aqueous phase, the calibration should be performed in a water solution. If the reaction occurs in a membrane phase, the system must be calibrated using the nitroxide probe incorporated into the same investigated object.

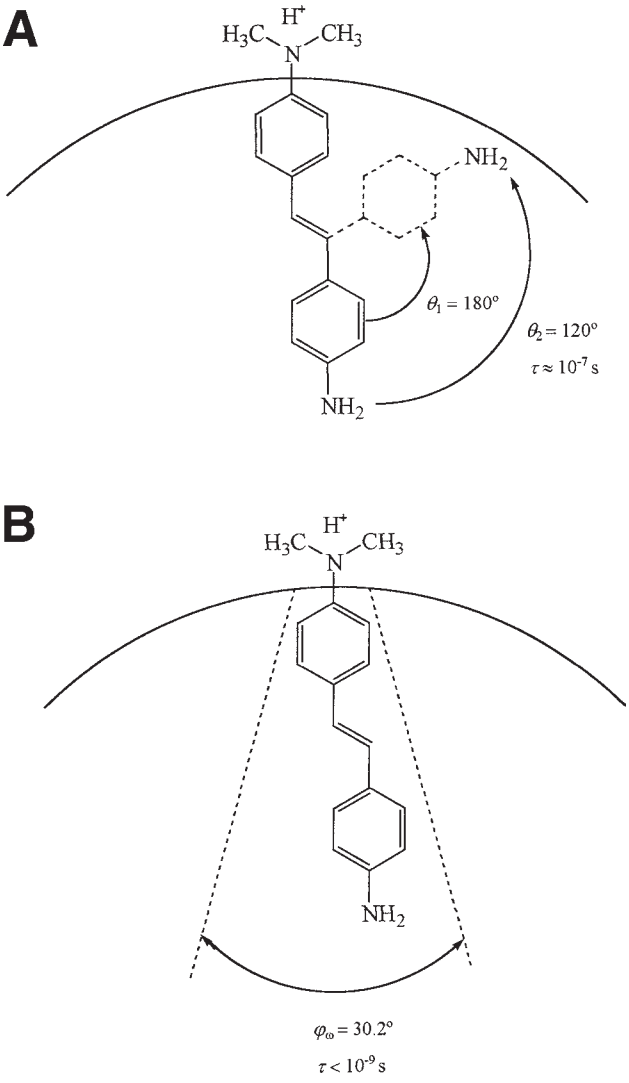

Fig. 8. The allocation and mobility of *trans*-DMAAS inside *E. coli* membrane. **(A)** High-amplitude low-frequency rotation of the stilbene aromatic rings around the olefinic bond in the excited state (θ_1 and θ_2 are the rotation angles between the aromatic fragments in the stilbene molecular plane and perpendicular to it, respectively, and τ is the correlation time of this rotation); **(B)** low-amplitude high-frequency wobbling of the stilbene probe inside the membrane with the wobbling angle φ_w and the wobbling correlation time τ.

The investigated lipid-water interface system may be considered, as well, as a model of a surface system in general, in which the nitroxide probe diffuses freely in the aqueous phase and the triplet and stilbene probes are immobilized onto the surface. Note that the proposed method can be applied to studies of any heterogeneous or homogeneous system contain-

ing any molecules that are capable of quenching the excited triplet state of a sensitizer.

Conclusion

The previously proposed spin-triplet-photochrome labeling method performed in aqueous solution has proved to be versatile technique in studies of the biological objects, such as biomembranes. The fluorescence spectra and polarization of *trans*-DMAAS measured in the PPDC liposomes indicated that the stilbene molecule had been incorporated almost totally into the lipid bilayers. The triplet probe Erythrosin B was found located in a lipid-water interface of the liposome membrane.

The ESR spectrum of the polar nitroxide-radical did not change in the presence or the absence of the liposomes. This means that the radicals were unable to enter the hydrophobic liposome membrane. While the cascade reaction between Erythrosin B and the stilbene probes occurs inside the liposome membrane, the radicals quench this reaction on the membrane's surface (at the lipid-water interface). The quenching rate constant value k_q of $(1.75 \pm 0.15) \times 10^7 M^{-1}s^{-1}$ was obtained. The same quenching rate constant of the cascade reaction previously measured in aqueous solution has a value of $1.3 \times 10^9 M^{-1}s^{-1}$ *(19)*.

This method helps solve experimentally several molecular dynamics problems using the standard constant-illumination fluorescence technique and allows the measurement of the product of the quenching rate constant and the sensitizer's triplet lifetime, $k_q \times \tau_{ph}$. This product can be used as a molecular dynamics parameter of the biological and surface systems. If the sensitizer's triplet lifetime τ_{ph} is measured independently, then the k_q value can be calculated. The local concentration of radicals or any other quenchers of the sensitizer's triplet state can be estimated using the calibration curve of the experimental rate constant k_{exp}^{-1} vs the quencher concentration. This labeling method enables one to perform the measurements in the microscopic scale when the minimal volume of a sample available for the fluorescence measurements is about 10^{-3} μL using a standard spectrofluorimeter.

Measuring the stilbene probe fluorescence and polarization, the triplet probe phosphorescence, and the rotational mobility of a spin probe, and then monitoring the stilbene photoisomerization kinetics after calibration of the whole system make it possible to estimate the local microviscosity of a biological object.

The proposed method, keeping all advantages of the spin, fluorescence, and photochrome labeling techniques, is simpler, more sensitive, and informative than these techniques. The novel cascade method can be applied to studies of a variety of biological and surface systems, including individual cells and surface lipid layers.

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