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Quenching of the cascade reaction between triplet and photochrome probes by nitroxide radicals

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Abstract

A cascade of photochemical reactions between the triplet sensitizer Erythrosin B and the photochrome stilbene-derivative probe exhibiting the phenomenon of *cis–trans* photoisomerization has been investigated. The cascade triplet *cis–trans* photoisomerization of the excited stilbene chromophore is sensitized with the excited triplet Erythrosin B and depressed with nitroxide radicals quenching the excited triplet state of sensitizer. The rate of the cascade photoisomerization is sensitive to the molecular dynamics of media and radicals' concentration. The proposed method allows to measure the product of quenching rate constant and the sensitizer's triplet lifetime $k_q \times \tau_{ph}$. Calibration of the 'triple' system permits the quantitative detection of the nitroxide radicals in the vicinity of the stilbene-derivative photochrome and sensitizing triplet probes. The experiment is carried out with the constant-illumination fluorescence technique. Sensitivity of the method is close to 10^{-12} mole of fluorescence molecules per sample and the minimal local concentration of nitroxide radicals being detected is about 10^{-5} M. This method allows to investigate any chemical, biological objects and surface processes of microscopic scale when the minimal volume is about 10^{-3} μl.

Keywords: Stilbene; Erythrosin; Nitroxide radical; Triplet–triplet energy transfer; Sensitized *cis–trans* photoisomerization; Quenching of triplet state; Cascade reaction

1. Introduction

For the past two decades, the physical labeling methods have been extensively developed and applied to solve many structural and molecular dynamics problems. Recently, spin, fluorescent and phosphorescent labeling methods are widely used in biophysics, biochemistry and polymer science. The increasingly prominent role of the physical labels in biology, medicine and technology has been reflected in a number of monographs and reviews [1–6]. The annually growing number of publications indicates an incessant interest to the physical labeling methods, in particular, to triplet, photochrome and triplet–photochrome labels and probes, which allow to measure the rotational and translational diffusion of the probes and, consequently, the molecular dynamics of object under investigation, when the traditional spin and fluorescent labeling techniques are not available for some reasons [7–15]. Recently different radicals, which are the active intermediates in many chemical and biological reactions, are detected by spin-trapping method with the electron spin-resonance technique (ESR). It allows to detect about 10^{-10} mole of free radicals in aqueous solution per sample when the sample volume is 0.1 ml. It means the ESR sensitivity is limited to 10^{-6} M. Frequently, this sensitivity is not enough for investigation of the small heterogeneous systems, like a single lyposome and cell or radicals on a surface. Radicals can also be detected indirectly by their quenching effects on the excited triplet states of the chromophores, following the phosphorescence decay kinetics [3,16]. This method is more sensitive than the regular ESR technique. It permits to detect up to 10^{-12} mole of radicals per sample and makes it possible to follow the local concentration of radicals up to 10^{-5} M during their free diffusion through the sample. However, this method requires an expensive experimental set-up and cannot be readily adopted to the field measurements.

Molecular dynamics of biological objects, like biomembranes, may be studied by monitoring the encounters between paramagnetic particles, fluorescent or phosphorescent chromophores and quenchers, like stable nitroxide radicals [3,5,11,16]. However, the efficiency of those methods is limited to the sensitivity mentioned above of ESR

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spectroscopy and luminescent techniques. Due to a short life-time of the chromophores excited singlet state, which is about 10^{-9} s, only the low viscous solutions are available for investigation of their molecular dynamics with the fluorescence technique. It means the concentration of quencher should be at least 5–10 mM. To overcome this limitation, we propose a new labeling method which combines the three types of probes, such as a stable nitroxide-radical probe, photosensitizing chromophore in the exited triplet state (triplet probe) and a stilbene-derivative photochrome probe. Such a combination keeps some facilities of probes mentioned above and has an essential advantage in study of molecular dynamics and measurements of the local concentration of radicals.

The proposed method is based on labeling the investigated object with the three types of physical probes:

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

This method employs the 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 widely accepted model for light-induced reversible isomerization of *trans*-stilbene [17–20] proceeds from the lowest exited singlet state through $^1t^*$ the twisted singlet intermediate $^1p^*$:

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

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

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

Here, $^3t^*$ and $^3p^*$ are the *trans* and twisted configurations (perpendicular with respect to the C=C double bond), respectively, of the lowest triplet, ^1p is the twisted ground state, $(1-\alpha)$ is the fraction of triplet decay into the *cis*-form and $(1-\beta)$ is the fraction of perpendicular singlet configuration decaying into the *cis*-form.

The triplet state can be populated not only due to the intersystem crossing of the excited singlet and triplet states of the stilbene molecule, but also by the triplet–triplet energy transfer from the excited triplet donor molecule, like erythrosin, which sensitizes the triplet excitation of the stilbene molecule. Following it the *cis–trans* photoisomerization occurs through the triplet excited potential surface. Previously, this phenomenon was investigated by Saltiel and his coworkers [21–23]. They determined a triplet lifetime of *trans*-stilbene in solution at room temperature with naphthalene-sensitized photoisomerization which have been performed in the presence of the ferrocene quencher. On increasing the quencher concentration, the position of the stationary state was shifted to the *trans*-side and the rate of photoisomerization was reduced owing to the absorption by ferrocene at $\lambda_{irr} = 254$ nm.

Görner and his coworkers investigated the sensitized *cis–trans* photoisomerization which have been performed for substituted stilbenes. The *cis/trans* ratio at sensitization with different sensitizers in the photostationary state was plotted as a function of their triplet energy E_T. From this plot the fraction $\alpha = 0.42$ of triplet which decays to the *trans*-ground state ^1t was derived. It was found that the plots of the *trans/cis* ratio are linearly dependent on the concentration of quencher. Obtaining the values for slope/intercept ratio and substituting the previously derived α-value, a triplet lifetime of the stilbene derivatives in different solvents at room temperature was calculated [24–26].

A similar approach has been used to study encounters in a model system containing the erythrosin sensitizer and the stilbene photochrome label [11]. Both types of molecules were covalently bound to the chymotrypsin macromolecule. The isomerization kinetics was monitored by fluorescence decay of the *trans*-stilbene photochrome molecule. At room temperature and pH 7, the rate constants of the triplet–triplet energy transfer between the sensitizer and the photochrome molecule were found to be $k_A = 2 \times 10^7\ M^{-1}\ s^{-1}$ and $k_B = 10^7\ M^{-1}\ s^{-1}$. It should be emphasized that the concentration of the triplet sensitizer did not exceed 10^{-7} M in those experiments, and the collision frequency was close to $1\ s^{-1}$ which is several orders of magnitude less than that measured with the regular luminescence or ESR techniques.

The free radicals are capable to quench very efficiently the triplet states of the excited molecules. It means the *cis–trans* isomerization of the stilbene label through the excited triplet state is affected by the presence of free nitroxide radicals. This effect can be used to estimate quantitatively the concentration of the nitroxide radicals in any investigated system.

The proposed method makes it possible to study the molecular dynamics of many heterogeneous systems like biomembranes, cells, Lambert–Blouget films, polymers and surfaces. The quantitative detection of the nitroxide radicals in the vicinity of the photochrome and triplet probes is carried out with the regular constant-illumination fluorescence technique which has been proved to be very sensitive, relatively simple and commonly available. All together, it permits the sensitivity of the method up to 10^{-12} mole per sample with the use of the commercial spectrofluorimeter. That means the local concentration of radicals which could be detected is up to 10^{-5} M when the investigated object has the volume at least 10^{-3} μl. Using the single-photon counting technique can improve the sensitivity of the fluorescence technique and reduce the minimal size of the investigated object.

2. Materials and methods

4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), Erythrosin B and 4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (4-hydroxy-TEMPO) were purchased from Sigma Israel Chemicals. All the measurements were performed in 0.1 M phosphate buffer solution (pH 7.1) at 25°C. Concentrations of Erythrosin B and SITS in their phosphate buffer solutions were 7.5×10^{-7} and 3×10^{-6} M, respectively. The *trans*-SITS was preliminary transformed into its *cis*-form being irradiated with the Hg-lamp at the absorption maximum of the *trans*-isomer with $\lambda_{ex} = 366$ nm. Erythrosin B was excited near its absorption maximum at $\lambda_{irr} = 546$ nm. Fluorescence emission of the *trans*-SITS was recorded at $\lambda_{em} = 428$ nm with an SLM-4800 Aminco-Bowman spectrofluorimeter after excitation near its absorption maximum at $\lambda_{ex} = 366$ nm using typically a 16 nm slit-width for excitation and emission. All the sample solutions were degassed with nitrogen before measurements.

3. Results and discussion

The triplet state of the erythrosin sensitizer populated with the long-wave irradiation is energetically much more lower than the singlet state excitation of the photochrome stilbene-derivative molecule. The encounters between the stilbene photochrome and the excited triplet erythrosin lead to a triplet–triplet energy transfer from the erythrosin excited molecule to the stilbene probe. Thus, the sensitizer and the photochrome involve the cascade which results in a *cis–trans* photo-isomerization of the stilbene molecule:

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

$$E^T + A \xrightarrow{k_A^T} E + A^T$$

$$E^T + B \xrightarrow{k_B^T} E + B^T$$

$$A^T \xrightarrow{k'_{iso}} B$$

$$B^T \xrightarrow{k''_{iso}} A$$

where A and B are the *cis*- and *trans*-stilbene isomers, respectively.

Since the photoisomerization rate in a low-viscous aqueous media is much more higher than the triplet state deactivation rate, $k_{iso} \gg (\tau_T)^{-1}$, the processes of $A^T \rightarrow A$ and $B^T \rightarrow B$ can be ignored and not considered here. The values of k_A^T and k_B^T can be obtained from the measurement of the experimental rate constant with the method of laser flash photolysis [27,28].

The quenching process can be described by an additional step in the reaction scheme discussed above:

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

The quenching of the excited triplet stilbene will be not considered here, since its triplet lifetime is essentially less than the lifetime of the excited triplet erythrosin molecule [11,29].

The triplet lifetime of Erythrosin B was measured to be 75 μ s [29]. The reciprocal value $(\tau_{ph})^{-1}$, which is the phosphorescent decay rate constant, is much higher than the collision frequencies k_A^T [A] and k_B^T [B] which are about $10\ s^{-1}$ under conditions of our experiment.

Solving the kinetics equation based on the total cascade reaction given above with the consequent quenching by radicals at the steady-state conditions and taking into account all the approximations, we obtain:

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

$$[A^T] = \frac{k_A^T}{k_{iso}}[A][E^T] \quad (2)$$

Trans-isomer appears in the solution with the rate:

$$\frac{d[B]}{dt} = -k_B^T[B][E^T] + k_{iso}[A^T] = (k_A^T[A] - k_B^T[B])[E^T] \quad (3)$$

when

$$[A] = [C_0] - [B]$$

$$\frac{d[B]}{dt} = k_A^T[C_0][E^T] - (k_A^T + k_B^T)[B][E^T] = -k_{exp}[B] + \text{const} \quad (4)$$

where the experimental rate constant $k_{exp} = (k_A^T + k_B^T)[E^T]$.

In the cascade scheme above, the concentration of the *trans*-stilbene [B] excited at $\lambda_{ex} = 366$ nm approaches the photostationary level exponentially with an increase of fluorescence at $\lambda_{em} = 428$ nm. The sensitized *cis–trans* photoisomerization of the *cis*-stilbene derivative is observed since the resulting fluorescence of the *trans*-stilbene appearing in solution increases with irradiation time at 546 nm. The experimental rate constant k_{exp} is a slope of the following first-order kinetic equation:

$$\frac{dI_t}{dt} = -k_{exp}I_t + \text{const} \quad (5)$$

Thus, k_{exp} can be calculated by plotting dI_t/dt against I_t, where I_t is the momentary intensity of the *trans*-isomer fluorescence and t is the time of light exposure at the absorption maximum of the erythrosin sensitizer ($\lambda_{irr} = 546$ nm). The constant value of $I_0[E^T]$ can be calculated as an intercept of the linear plot dI_t/dt against I_t, where I_0 is the initial intensity of fluorescence due to presence of some unconverted *trans*-stilbene in the sample at the start of reaction.

The experimental rate constant of the entire investigated process follows as:

$$k_{\text{exp}} = \frac{(k_{\text{A}}^{\text{T}} + k_{\text{B}}^{\text{T}})\sigma I_{\text{ex}}[\text{E}]}{k_{\text{q}}[\text{R}] + (\tau_{\text{ph}})^{-1}} \quad (6)$$

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

$$\frac{1}{k_{\text{exp}}} = a + b[\text{R}] \quad (7)$$

where

$$a = \frac{1}{\sigma I_{\text{ex}}(k_{\text{A}}^{\text{T}} + k_{\text{B}}^{\text{T}})[\text{E}]\tau_{\text{ph}}} \quad \text{and} \quad b = \frac{k_{\text{q}}}{\sigma I_{\text{ex}}(k_{\text{A}}^{\text{T}} + k_{\text{B}}^{\text{T}})}[\text{E}]$$

The quenching rate constant k_{q} can be calculated from the slope to intercept ratio, $b/a = k_{\text{q}} \times \tau_{\text{ph}}$,

$$k_{\text{q}} = \frac{b}{a}(\tau_{\text{ph}})^{-1} \quad (8)$$

Thus, measuring the fluorescence intensity of the excited stilbene molecule as a time-function of erythrosin excitation in the investigated 'triple' system (erythrosin–stilbene–nitroxide radical), we can calculate the quenching rate constant k_{q}, if τ_{ph} is known, and determine the radical concentration in vicinity of the probes with the use of an appropriate calibration.

After the erythrosin and *cis*-SITS solutions were mixed, the sample was irradiated at 546 nm where only the erythrosin could be excited. Neither *cis*- nor *trans*-SITS are excited with this wavelength light. Nevertheless, the increase of fluorescence was observed in the sample due to the isomerization of the *cis*-SITS into its *trans*-form which was sensitized by erythrosin itself. Following the fluorescence intensity rise gradually with increasing irradiation time, the experimental rate constant k_{exp} was calculated as a slope of the plot $\text{d}I_t/\text{d}t$ versus I_t. Introducing the nitroxide radicals, which quench the excited state of erythrosin molecule, the fluorescence increase was depressed and the rate constant value k_{exp} was reduced.

Fig. 1 shows the experimental fluorescence intensity of the *trans*-stilbene molecule versus irradiation time at 546 nm for different radical concentrations. The fluorescence intensity increases not from zero due to the *trans–cis* equilibrium attained by preliminary irradiation at 366 nm. Fig. 2 shows the plots in the derivative forms $\text{d}I_t/\text{d}t$ against fluorescence intensity, which are linear according to Eq. (5). Increase of the radical concentration diminishes the rate of the *trans*-SITS formation in the sensitized *cis–trans* isomerization process, and as a result, the fluorescence increase slows down. This phenomenon differs from the regular fluorescence quenching processes which occur through the exchange mechanism when radicals in concentration less than 10^{-3} M could not be detected [5]. This statement is supported with the Stern–Volmer plot for quenching of the *trans*-SITS fluorescence with nitroxide radicals shown in Fig. 3. The following Stern–Volmer equation describes the collisional quenching of fluorescence:

$$\frac{I_0}{I} = 1 + k_0\tau_{\text{f}}[\text{Q}] = 1 + K_{\text{D}}[\text{Q}] \quad (9)$$

where [Q] is the concentration of quencher, I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively, k_0 is the bimolecular quenching constant, τ_{f} is the lifetime of the fluorophore in the absence of quencher, and K_{D} is the Stern–Volmer quenching constant which is calculated from a plot I_0/I versus [Q].

The Stern–Volmer constant is found as a slope of the plot to be $K_{\text{D}} = (44.7 \pm 4.6)\ \text{M}^{-1}$. The nitroxide-radical quenching effect on the *trans*-SITS fluorescence in the absence of

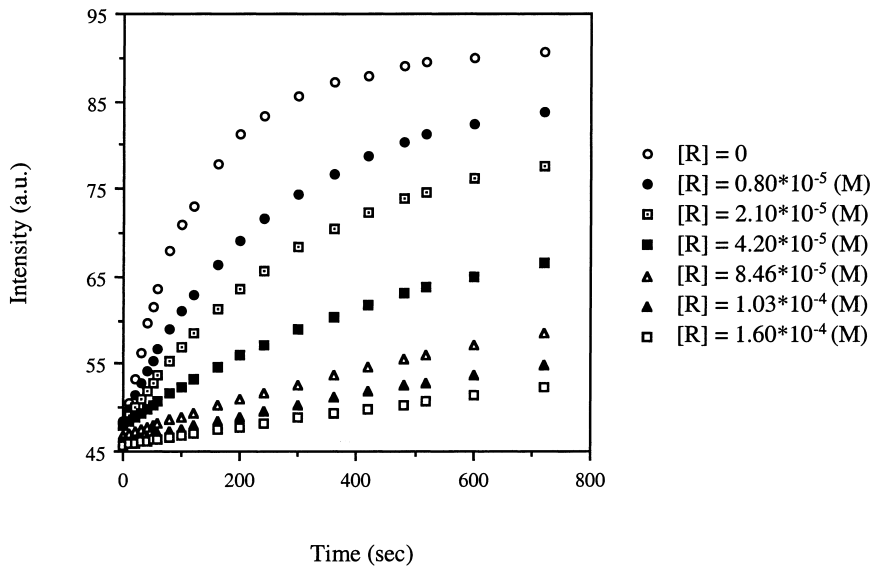

Fig. 1. Experimental fluorescence intensity of the excited *trans*-SITS against the sample irradiation time at 546 nm for different 4-hydroxy-TEMPO concentrations. *Trans*-SITS was excited at $\lambda_{\text{ex}} = 366$ nm, and the fluorescence intensity was measured at $\lambda_{\text{em}} = 428$ nm.

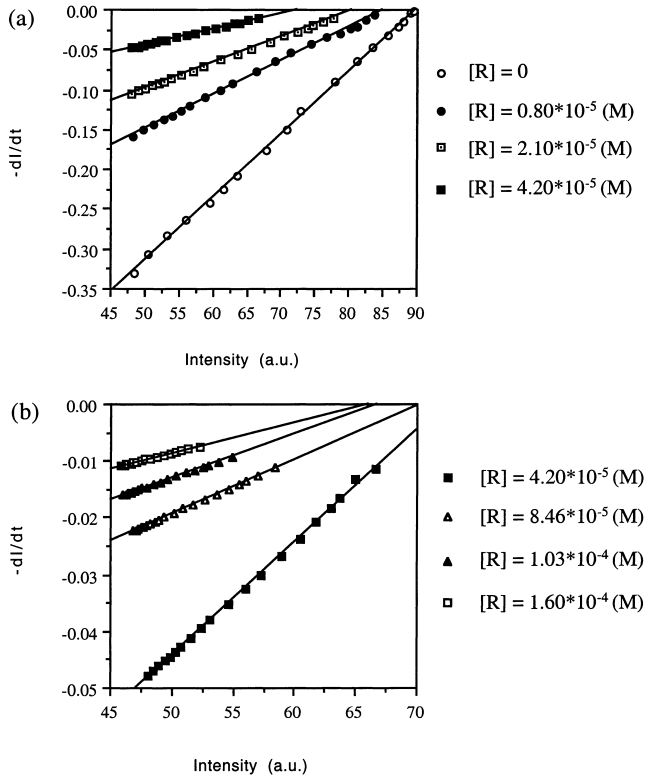

Fig. 2. *Trans*-SITS fluorescence increase rate d*I*/d*t* against fluorescence intensity for different quencher concentrations: (a) [R] = 0; 0.8×10^{-5}; 2.1×10^{-5}; 4.2×10^{-5} M, (b) [R] = 4.2×10^{-5}, 8.46×10^{-5}, 1.03×10^{-4}; 1.60×10^{-4} M.

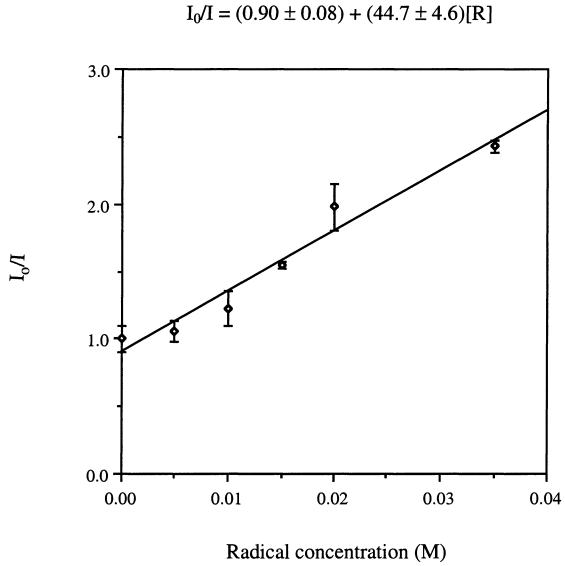

Fig. 3. Stern–Volmer plot for quenching of the *trans*-SITS fluorescence with nitroxide radicals.

sensitizer is observed even only with the direct excitation at $\lambda_{ex} = 366$ nm when the radical concentration is higher than 10^{-3} M. The radical concentration in our experiment is substantially lower than that necessary for the direct quenching of the *trans*-SITS fluorescence and varies from 1.6×10^{-4} to 8.0×10^{-6} M. The increase of the *trans*–

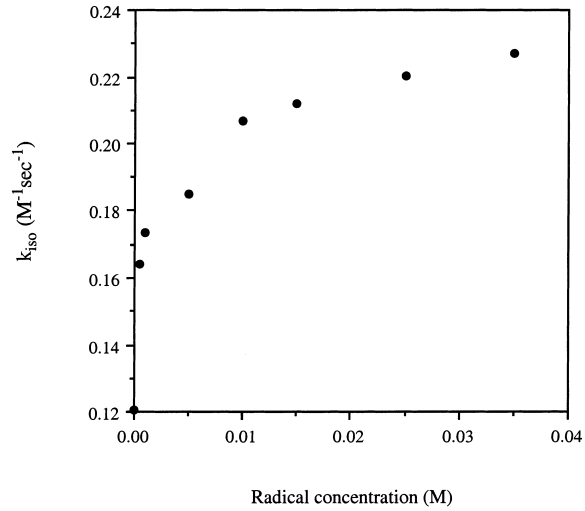

Fig. 4. The SITS *trans–cis* photoisomerization rate dependence on radical concentration.

cis photoisomerization rate as the radical concentration increases starting from 10^{-3} M shown in Fig. 4 supports the absence of quenching effect on the stilbene isomerization at low radical concentrations.

The experimental data gives the evidence of the nitroxide-radical inhibitor effect upon the sensitized *cis–trans* isomerization of the stilbene derivative by quenching the sensitizer's triplet state. This conclusion is supported with data presented in Fig. 5. It shows the linear dependence of the reciprocal experimental rate constant k_{exp}^{-1} on radical concentration. According to Eq. (8), the slope to intercept ratio *b*/*a* of this linear curve is equal to the product $k_q \times \tau_{ph}$. The quenching rate constant value k_q of $(1.3 \pm 0.1) \times 10^9$ $M^{-1}\,s^{-1}$ was obtained from this product taking into account the triplet lifetime of Erythrosin B which is 75 μs in aqueous

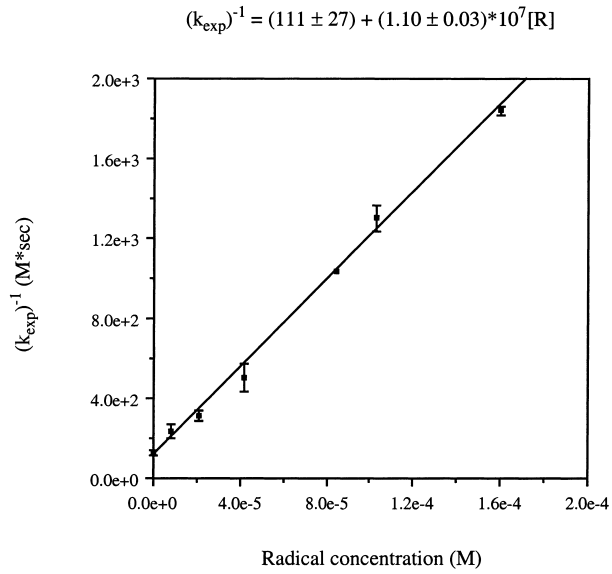

Fig. 5. Linear dependence of the reciprocal experimental rate constant k_{exp}^{-1} on the radical concentration [R].

solution [29]. The value of the quenching rate constant k_q was found to be within the rank of the diffusion rate constant and rate constants which are typical for quenching of excited triplet molecules in low viscous solutions [30]. For example, quenching of the Mg-phthalocyanine phosphorescence with benzophenone in aqueous solution occurs with a rate constant value of $1.4 \times 10^9\ M^{-1}\ s^{-1}$ [3].

It is possible to calibrate the experimental rate constant value k_{exp}^{-1} versus radical concentration for systems with known macro- and micro-viscosity if the triplet lifetime of sensitizer can be obtained from an independent experiment. From the calibration plot described by Eq. (7), the product $k_q \times \tau_{ph}$, is obtained and can be used as an apparent parameter of microviscosity in the vicinity of sensitizer. The quenching rate constant k_q is derived from the product $k_q \times \tau_{ph}$ after measuring the τ_{ph} in an independent experiment, as it was mentioned above. Finally, according to the calibration curve, the local concentration of radicals in the sample can be estimated.

The following protocol based on the experimental results can be proposed:

1. Introduction of a stilbene probe into the object of interest followed by monitoring the photoisomerization kinetics with the regular constant-illumination fluorescence technique and calculating the isomerization rate constant. The calibration plot of this constant versus microviscosity or a spin-probe rotational frequency in a model system will allow to estimate the micro-viscosity parameters, like wobbling angle of the probe and rotational viscosity of the medium [14].
2. Introduction of a stilbene probe and a triplet sensitizer followed by monitoring the sensitized isomerization of the stilbene molecule with the same steady-state fluorescence set-up and calculating the apparent rate constant of the sensitized isomerization [11].
3. Calibration of a triple system (spin–photochrome–triplet), followed by measurement of a quenching rate constant k_q and calculation of a local molecular dynamics parameter $k_q \times \tau_{ph}$.
4. Calculation of a local radical concentration in the vicinity of the triplet-photochrome probes using the above mentioned calibration plot of k_{exp}^{-1} versus radical concentration.

The sensitivity of the constant-illumination fluorescence technique is close to 10^{-9} M. Hence, the approximate volume of a sample available for the fluorescence measurement in the method proposed above can be 10^{-3} μl when the local concentration of fluorophore is close to 10^{-3} M and the local radical concentration is about 10^{-5} M.

4. Conclusions

The proposed spin-triplet-photochrome labeling method helps to solve experimentally a number of molecular dynamics problems using the regular constant-illumination fluorescence technique. It allows to measure the product of 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 investigated system including objects of microscopic size where the minimal volume of a sample available for the fluorescence measurements with this method is about 10^{-3} μl. If the sensitizer's triplet lifetime τ_{ph} is measured independently, then the k_q value could be calculated. The local concentration of radicals can be estimated from the calibration curve of the experimental rate constant k_{exp}^{-1} versus radical concentration. The following *b*-parameter, which is the ratio k_q/k_{exp}, is derived from this calibration plot for a model system and can be extended for any system because both the rate constants characterize the encounters in the same medium. It means the *b*-value is expected to be approximately the same in the model and investigated systems. Local microviscosity in the vicinity of the stilbene label can be estimated by monitoring the stilbene photoisomerization kinetics after calibration of the system.

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