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Novel Fluorescent Methods for Biotechnological and Biomedical Sensoring: Assessing Antioxidants, Reactive Radicals, NO Dynamics, Immunoassay, and Biomembranes Fluidity

Gertz I. Likhtenshtein

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Abstract We proposed and developed a series of fluorescent methods for analysis and investigation of biological systems with a view of future biotechnological and biomedical applications. The methods we describe have been built upon several photochemical and photophysical phenomena including fluorescent quenching, photochrome photoisomerization, and energy transfer. Three new types of molecular probes have been developed and employed for such studies: (1) dual fluorophore–nitroxide compounds, (2) fluorescence–photochrome molecules, and (3) super molecules containing both fluorescence and fluorescent quenching segments. The fluorescent properties of the new probes were intensively exploited for several practical applications including a real-time analysis of antioxidants, nitric oxide, superoxide, reactive radicals, trinitrotoluene, and metal ions, investigation of molecular dynamics of biomembranes in a wide range characteristic times, detection of protein conformational transition, and characterization of surface system. Owning high sensitivity, simplicity, and availability of fluorescent techniques, these methods can be widely employed and are adaptable to fibrooptic sensoring. A general survey of the physical principles and application of the new fluorescent methods has been provided.

Keywords Fluorescence · Fluorescent quenching · Singlet–singlet energy transfer · Photochrome isomerization · Fluorephore-nitroxides · Antioxidants · Nitric oxides · Superoxide · Immunoassay · Stilbenes · Antibodies · Biomembrane fluidity

Introduction

A burst in development of modern biotechnology and biomedicine calls for new sensitive, rapid, and widely available methods of analysis of biologically important molecules and reactive radicals and measurement of biomembrane fluidity. Though a large body of literature exists on this subject, sensitivity, specificity, and reliability of commonly used methods do not satisfy the modern requirements.

During last decades, we proposed and developed a series of fluorescent methods for detecting biological molecules and radicals and for investigating molecular dynamics and microstructure of systems that are potentially important for biotechnology and biomedicine.

Fluorescence spectroscopy is exclusively suitable for such studies, as commercial fluorimeters are widely available and the method possesses record sensitivity. In this paper, we review the use of three new types of molecular probes: (1) dual fluorophore–nitroxide

G. I. Likhtenshtein (✉)
Department of Chemistry, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105, Israel
e-mail: gertz@bgu.ac.il

compounds, (2) fluorescent-photochrome molecules, and (3) super molecules containing fluorescence and fluorescent quenching segments. We have exploited the fluorescent properties of these probes as the basis of several methodologies, including analysis of antioxidants, nitric oxide (NO), superoxide, reactive radicals, trinitrotoluene, and metal ions, investigations of molecular dynamics of biomembranes in a wide range of characteristic times, and characterization of surface systems [1–10 and references therein]. Owning high sensitivity, simplicity, and easy availability of fluorescent techniques, these methods can be now widely employed in these and other fields. The techniques are also adaptable to fibrooptic sensoring.

This review is intended to provide the physical principles and outline potential applications of methods based on the use of the abovementioned fluorescent probes.

Dual FNO Compounds

The initial idea of combining a chromophore and a nitroxide in one molecule that could be used for studying molecular dynamics, intramolecular fluorescent quenching, and nitroxide fragment reduction has been suggested in the 1,980th [11, 12]. Keeping all properties of spin and fluorescent probes, the dual fluorophore–nitroxide compounds (FNO) possess an important new advantage. Specifically, as first demonstrated in [12], in such a super molecule, the nitroxide serves as a strong intramolecular quencher of the fluorescence from the chromophore fragment. Then, any chemical or photo-reduction of this fragment to a corresponding hydroxylamine derivative, oxidation of the nitroxide fragment, or addition of an active radical yield would result in a decrease of electron spin resonance (ESR) signal that would be accompanied by an increase in fluorescence. Another fruitful area of the FNO application appeared to be a metal ion analysis. A complex formation between FNO and a metal ion could yield a market change of the fluorescent quenching.

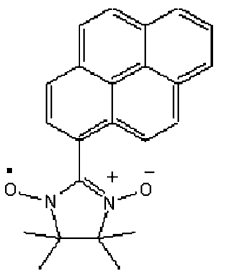
The organic synthetic chemistry allows for the easy modifications of the chemical structure of the dual molecules of different fluorescence as well as redox properties of the nitroxide segment with a variety of spacers [1].

The property of stable nitroxide radical moiety to quench the excited state of the chromophore segment could be exploited not only for developing new molecular probes, but also for modeling intramolecular photochemical and photophysical processes in the course of the light energy conversion and construction of new photoswitching magnetic materials [1, 4–7, 12–15].

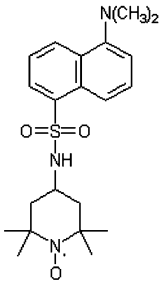
Analysis of Biological Important Molecules and Radicals

Antioxidant Status

A series of the dual FNO molecules including pyrene–nitronyl (PN) and DT



PN



DT

have been used in the analysis of antioxidants of different reducing capacity. On the basis of the abovementioned unique properties of the dual molecules, a method for the quantitative analysis of vitamin C in biological and chemical liquids has been proposed [16–18]. It was shown that, in the presence of ascorbic acid, an increase in the fluorescent intensity and a decay of the EPR signal of the dual probe DT occurred with the same rate constant (Figs. 1 and 2). By performing a series of pseudo-first-order reactions between the dual molecule and ascorbic acid and by consequently plotting the rate constants versus the ascorbic acid concentrations, the calibration curves for the vitamin C analysis were obtained (Fig. 2).

The use of the FNO probes for determination of the ascorbate concentration in complex biological systems that, for example, could contain proteins, is hindered because of the concentration-dependent interactions between the proteins and the ascorbate and the probe. The FNO reduction by ascorbic acid was accelerated with the increase of the bovine serum albumin concentration [17]. Thus, in complex systems, it is not the antioxidant concentration but the value of the rate constant of the nitroxide reduction that is the most important quantitative characteristic of the system's antioxidant status. The proposed method was used for the analysis of the antioxidant status of commercial juices and human blood [16, 18].

It is well established that six member pyridinyl nitroxides are reduced by such compounds as ascorbic acid and semiquinones with relative low negative redox potential but are not able to react with antioxidants of a higher redox potential. To overcome this limitation, the dual PN

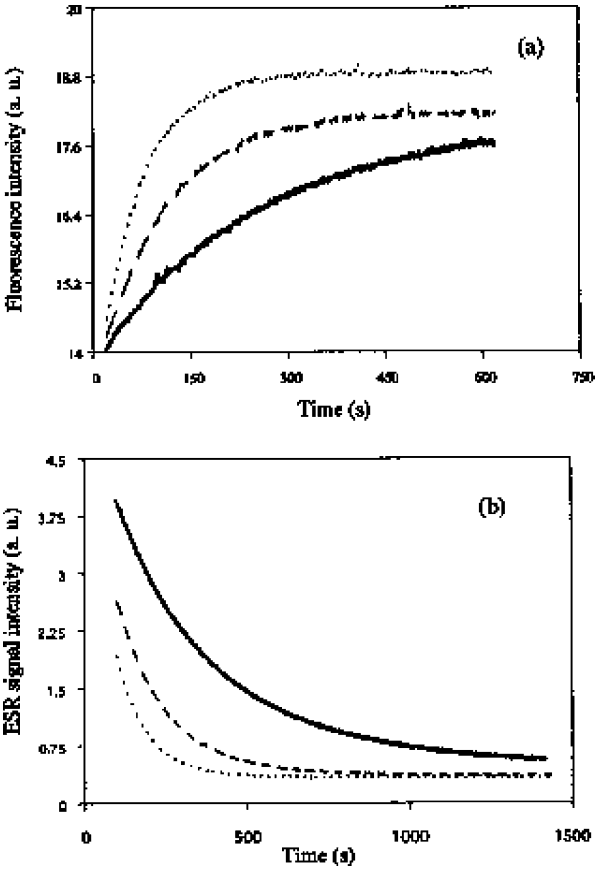


Fig. 1 Fluorescent enhancement (**a**) and ESR decay (**b**) of the probe caused by excess of ascorbic acid. *Solid line* 0.1 mM, *dashed line* 0.4 mM, *dotted line* 0.8 mM. Acquisition parameters of fluorescence: excitation slit 1 nm, emission slit 16 nm, voltage 480 V, T=25°C, phosphate buffer (pH 7; reprinted from [16], with permission from Elsevier)

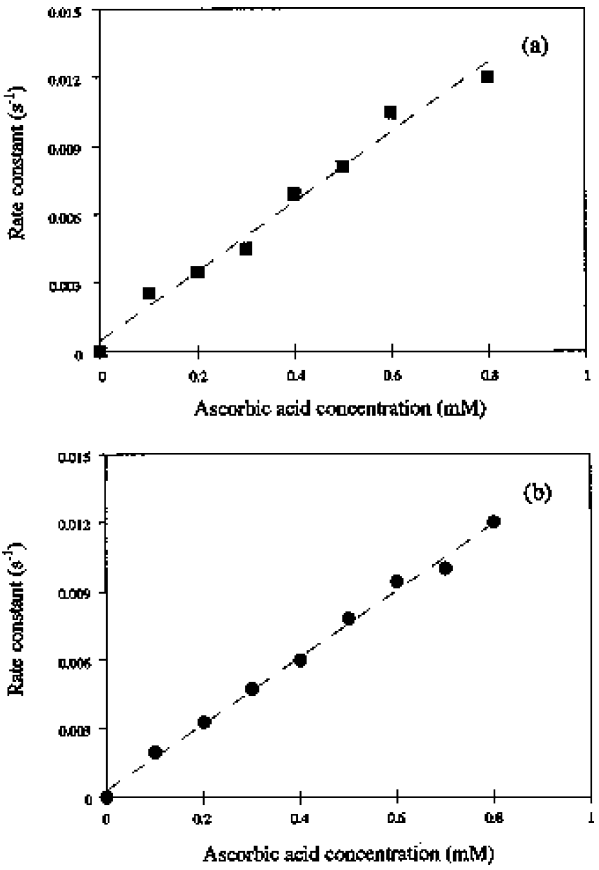


Fig. 2 Dependence of rate constant of reduction of dansyl–2,2,6,6-tetramethyl-4-hydroxylpiperidine-*N*-oxide on ascorbic acid concentration. Fluorescence (**a**) and ESR (**b**) measurements; T=25°C, phosphate buffer (pH 7; reprinted from [16], with permission from Elsevier)

probe possessing a high redox potential was employed [10]. It was shown that the fluorescent PN assay is suitable for analyzing such antioxidants as quercetin and galangin down to a submicro-concentration scale. The linear dependencies of the experimental pseudo-first-order rate constant on the concentration of these antioxidants in the range 5×10^{-8}–10^{-6} M and in the presence of catalase gave the rate constant values equal to 7.6×10^{-1} M^{-1} s^{-1} and 1.8×10^{-2} M^{-1} s^{-1} for quercetin and galangin, respectively. These values are close to those obtained independently by following the decay of the PN ESR signal.

NO Analysis

NO is a tiny molecule with an enormous physiological impact [19, 20]. Real-time monitoring of NO dynamics under physiological conditions of nanomolar concentrations is a difficult analytical problem. This has been attributed to the labile nature of NO molecule, which both rapidly diffuses through the medium and readily reacts with many scavenger targets. The latter include oxygen, superoxide, amino and mercapto compounds, hemeproteins, and so forth. Although a variety of methods for NO detection have been proposed, they are not generally amenable to *real-time in situ measurement of endogenous NO*.

A new method for NO analysis in nano-concentration scale and real-time monitoring NO outflax from tissues with the use a dual FNO has been developed [21]. It was shown that the PN

reacts with NO to yield a pyrene–imino nitroxide radical (PI) and NO_2. Conversion of PN to PI is accompanied by changes in the EPR spectrum from a five-line pattern (two equivalent N nuclei) into a seven-line pattern (two nonequivalent N nuclei). The transformation of the EPR signal is accompanied by a drastic increase in the fluorescence intensity, as the imino nitroxide radical is a weaker quencher than the nitronyl. The EPR methods were employed for the calibration of NO in the micromole concentration scale (Fig. 3), while the much more sensitive fluorescent technique allowed for the calibration of NO in the nanomolar concentration scale.

This method was applied to follow up on concentrations of NO and *S*-nitroso compounds in tissue from pig *trachea epithelia*. The measured basal flux of *S*-nitroso compounds obtained from the tissues was about 1.2 nmol/g×min and NO-synthase stimulated by extracellular adenosine 5′-triphosphate produced NO· flux of 0.9 nmol/g×min [21].

Superoxide Analysis

A new rapid and highly sensitive method for the superoxide analysis has been developed based on a reaction of fluorophore–nitronyl with $O_2^{-\cdot}$ [10]. Specifically, the reaction of PN with superoxide radicals generated by the xanthine/xanthine oxidase system offered a drastic increase (about 2,000 times) in the fluorescent intensity and a decrease of the EPR signal [10]. When the rate of superoxide production is slow ($\omega_i < 2\times10^{-7}$ M/min), the reaction followed the zero-order law. Under these conditions, the rate of fluorescent increase (dI/dt) is proportional to the rate of superoxide production (ω_i). The dependence dI/dt on ω_i was used as a calibration curve in subsequent experiments (Fig. 4). At higher concentrations, the kinetics of fluorescent change follows the first order. The kinetic analysis allowed for calculating the rate constant of the PN reduction by superoxide as $k_1 = 1.54\times10^{6}$ M^{-1} s^{-1} (pH 7.4, T=300 K).

Because of its very high sensitivity and simplicity, the proposed method will, under certain conditions, have advantages over conventional light absorption, EPR and chemiluminescent techniques and will be able to monitor biological processes *in a real time.* The contribution of other reactive oxygen species could be estimated quantitatively by previous treatment with appropriative inhibitors. Thus, the contribution of superoxide radical can be estimated in the presence of superoxide dismutase and catalase.

The ability of dual FNO molecules to detect alkyl radicals, superoxide, and hydroxyl radicals was demonstrated in several literature reports [22–26].

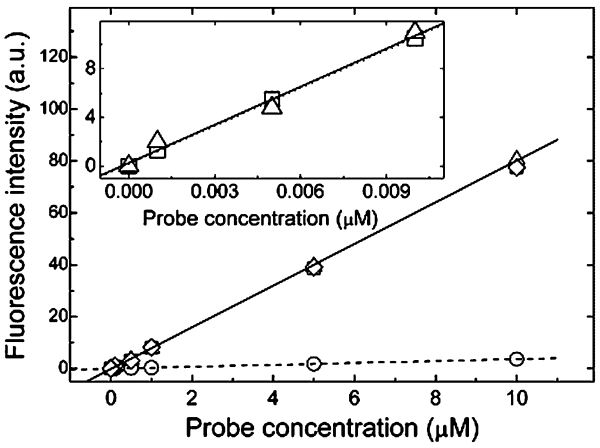


Fig. 3 Integrated fluorescence measured before (*circles*) and after a 5-h incubation of PN with excess *N*-acetyl-*S*-nitrosopenicillamine (10 μM; *squares*) and a 10-min incubation with NO (10 μM; *diamonds*). The fluorescence of the pyrene–imino (*triangles*) is measured for comparison (reprinted from [21], with permission from Elsevier)

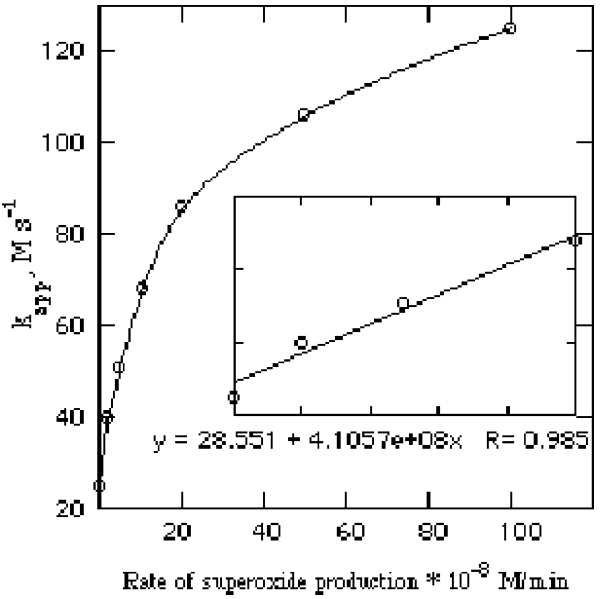
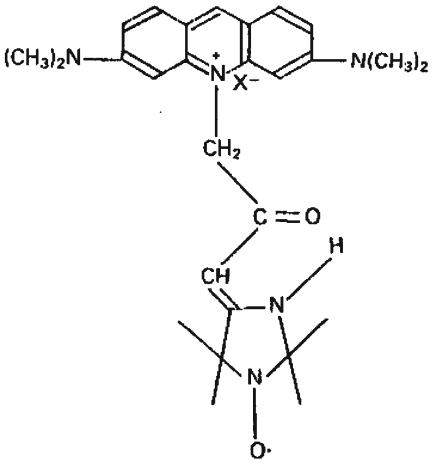


Fig. 4 Dependence of the apparent rate constant of FNNRO reduction on the rate of superoxide production; PBS (pH 7.4, T=300 K). The *inset* shows the linear segment of the curve for low rates of superoxide production corresponding to the zero order process (ω_i<2×10^{-7} M/min) [19] (reprinted from [10], with permission from Elsevier)

Analysis of Metal Ions

A method of determination of metals in a solution by chelate formation with intramolecular fluorescence quenched by nitroxide segment was reported [3]. Chelating reagents whose molecules contain both a fluorescent fragment and a spin label near a chelating group are suggested for use in the spectrofluorometric detection of metal ions. In solutions of such reagents,



I, X = Cl
II, X = I

a considerable intramolecular quenching of the fluorescence of the luminescent fragment by the adjacent paramagnetic spin label was observed. The proposed method is based on the change in the degree of fluorescent quenching resulting from an alteration of the conformation of the

reagent molecules during the chelation and a consequent change in the distance between the luminescent group and the spin label. Measurement of the change in the fluorescent intensity of the reagent solution by addition of the metal ions (Zn^{II}, Al^{III}, Mo^{VI}, Ga^{III}) makes it possible to achieve detection limits of 10^{-9} M.

Fluorescence–Photochrome Probes

A necessary stage in the olefinic photoisomerization process in stilbene fluorescence–fluorophore molecules, in the singlet or triplet excited state, involves twisting (about the former double bond) of stilbene fragments relative to one another (Fig. 5) [2, 8, 9, 27–30].

As *trans*-stilbene is fluorescent, and *cis*-stilbene is fluorescent silent; the process can be readily monitored by a single steady-state fluorescent technique.

Theoretical considerations and existing experimental data indicate that, under certain conditions, the rate of photoisomerization depends strongly on the microviscosity around the isomerized molecule and upon the effect of steric hindrance. In a viscous medium, the apparent rate constant of *trans–cis* photoisomerization k_{iso} is controlled by the reorganization rate of the process in the medium:

$$k_{iso} = \sigma I_{in} \phi k_r \tau_d \text{ , here } k_r = 1/\tau_r. \quad (1)$$

where τ_r is the characteristic time of the medium reorganization, I_{in} is the intensity of incident light, τ_d is the characteristic time of the exited-state deactivation, ϕ is the quantum yield of the exited state, σ is the absorption cross-section. Thus, by measuring the kinetic photo-isomerization of the stilbene derivative molecules and proper calibration, one can acquire information about the microviscosity and steric hindrance of their surrounding environment.

This method was used for the measurement fluidity of biological membranes [2, 31–34]. Information about the microviscosity of the binding site region is obtained when the label is bound to a specific site of a protein (as for an antigen, in a binding site of an antibody, or for a substrate, in the binding site of an enzyme, etc.) [8, 38–41]. The results of the study of dynamic contacts of macromolecules in a solution and a biomembrane according to kinetics of isomerization of fluorescent label sensitized by the dye in a triplet state were reported [30, 35]. In some systems, it is expected that steric hindrance at the binding site would prevent the twisting of stilbene fragments of the label in the exited state. On this theoretical basis, fluorescence–photochrome immunoassay were developed [8].

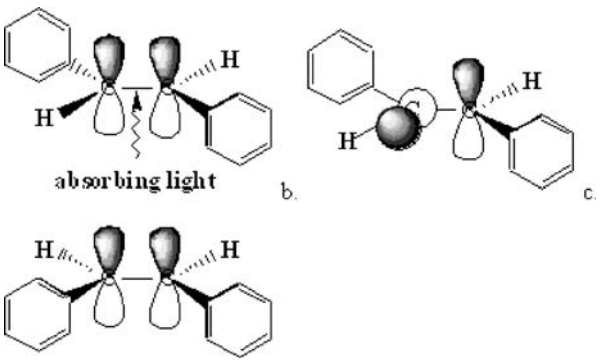


Fig. 5 Scheme of *p*-orbitals in *trans*-stilbene (**a**), intermediate excited state (**b**), and *cis*-stilbene (**c**)

Molecular Dynamics (Fluidity) of Biomembranes

The proposed fluorescence–photochrome method (FPM) is based on monitoring fluorescent parameters and kinetics of photochrome photoisomerization of para-substituted stilbenes (PSS). The method was employed for studying molecular dynamics of biological membranes [2, 31–35]. It was shown that PSS exhibits fluorescent characteristics that are similar to those of typical membrane fluorescence probes such as diphenylhexatriene and, therefore, is suitable for studying molecular dynamics and micropolarity of media using the steady-state and time-domain fluorescent polarization and the spectral relaxation shift techniques. In addition, a study of the kinetics of PSS *trans–cis* and *cis–trans* photoisomerization makes it possible to estimate, under certain conditions, the twisting correlation time of the stilbene fragments in the excited state of PSS for the fixed angle of 180°. In viscous media, this process is the rate-limiting stage. Taken together, both techniques, fluorescence and photochrome, make it possible to establish a detailed mechanism and, under certain calibration, to measure quantitative parameters of stilbene probe mobility in a membrane.

FPM was applied to the study of *Escherichia coli* membrane dynamics using 4-dimethyl amino-4′-aminostilbene (DMAAS) [31]. Considering the dependence of the glycerol viscosity on temperature and then the effect of viscosity on the rotational correlation time of a nitroxide radical (τ_c), ln k_{iso} was plotted against τ_c. It was found that, in the low temperature region:

$$\ln k_{iso} = 28.5 - 1.77 \ln \tau_c \quad (2)$$

Because the volume of the stilbene fragments, twisted in the excited state, and the nitroxide radicals are close to each other, this dependence was used to estimate the twisting correlation time of the stilbene fragments $\tau_{tw} \approx \tau_c$. Important note here is that, owing ability to integrate results of the stilbene photoisomerization, this method allows one to measure k_{iso} over an exceptionally wide range and, therefore, to expand values of τ_{tw} for the twisting around of single bond in the excited states for fixed angle of 180°.

Figure 6 shows the location and parameter mobility of DMAAS in the *E. coli* membrane.

To conclude, the fluorescence–photocrome method might, under certain conditions, gain an advantage over conventional fluorescence and spin-labeling because of being simpler, more sensitive, and informative.

Cascade Spin-Triplet-Photochrome Methods

A cascade of photochemical and photophysical reactions between a triplet sensitizer and a fluorescence–photochrome probe exhibiting the phenomena of *cis–trans* photoisomerization, triplet–triplet energy transfer, and the triplet exited-state quenching by a stable radical has been proposed and developed [2, 32–36].

The sensitized cascade of triplet *cis–trans* photoisomerization of the excited stilbene includes a triplet sensitizer (erythrosin B), a photochrome stilbene-derivative probe (DMAAS), and a nitroxide radical (5-doxyl stearic acid) quenching the excited triplet state of the sensitizer (Fig. 7).

The spin-triplet-photochrome labeling technique was applied to investigate the dynamic processes in L-α-phosphatidylcholine (PPDC) multilamellar liposomes [34]. The experimental results give the evidence of an efficient nitroxide-radical inhibitor effect upon the sensitized *cis–trans* photoisomerization of DMAAS by quenching the sensitizer triplet state. The phosphorescence triplet lifetime of erythrosin B in PPDC membranes was measured to be 3×

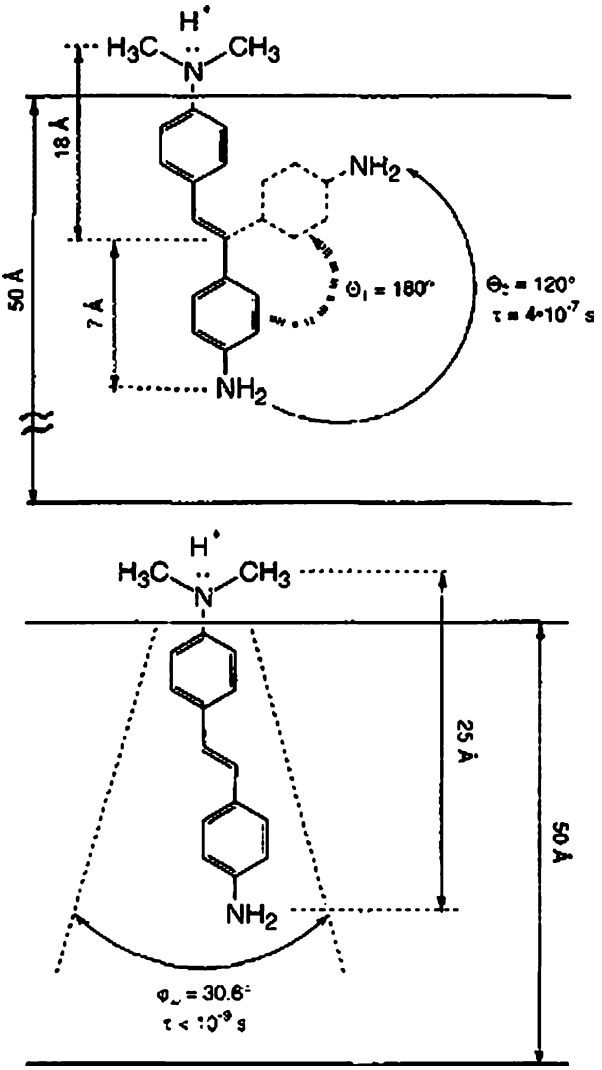


Fig. 6 Proposed location and parameters mobility of DMAAS in *E. coli* membranes. *Top panel* high amplitude low frequency twisting of the stilbene fragment in the exited state. θ_1 and θ_2 are the angles of the fragment twisting. *Bottom panel* low-amplitude high frequency wobbling of the probe as a whole [31]

10^{-4} s. The experimental quenching rate constant of the cascade reaction k_q, and the rate constant of the triplet–triplet energy transfer k^T evaluated in two-dimensional terms were obtained as $k_q=(1.05\pm0.08)\times10^{15}$ cm^2 mol^{-1} s^{-1} and $k^T=(1.26\pm0.21)\times10^{12}$ cm^2 mol^{-1} s^{-1}. Taking into consideration the efficiency of the triplet–triplet energy transfer $\gamma=3.5\%$, the rate constant of encounters between the sensitizer and the photochrome was found as $k_{en}=3.4\times10^{13}$ cm^2 mol^{-1} s^{-1}.

The obtained values of k_q and k^T in multilamellar liposomes were examined in frames of a two-dimensional model describing steady-state diffusion-controlled reactions that also accounted for the dependence of the diffusion-controlled rate constant on the lifetime of the exited species [39]. The value of k_{en} obtained in [34] together with the data on diffusion-controlled rate constants obtained from other methods were found to be in good agreement with the abovementioned advanced theory of diffusion-controlled reactions [Fig. 8]. To summarize, the theory provided by Razi Naqvi et al. [39] can be recommended for the

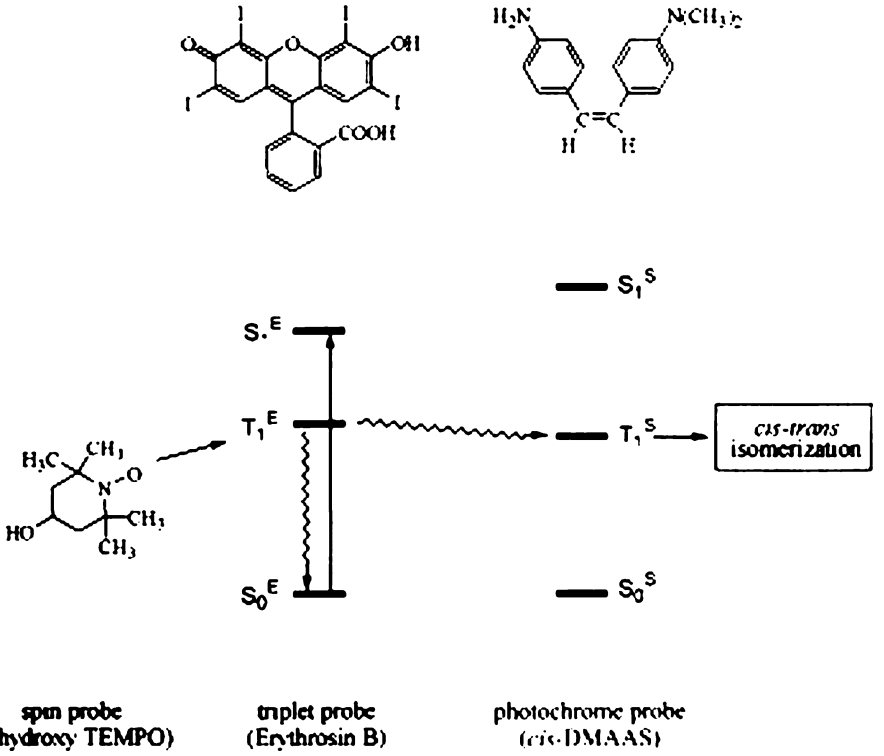


Fig. 7 Representation of energy levels of cascade reactants and competition between the $T_1^E \rightarrow T_1^S$ and $T_1^E \rightarrow S_o^S$ processes [34]

analysis of the experimental quenching data under long-irradiation steady-state conditions in a two-dimensional geometry.

In the frame of CSTPM, the following dynamic parameters of the cascade system components can be experimentally measured: the spin label rotation correlation time and spin relaxation parameters, the fluorescence and phosphorescence polarization correlation times, the

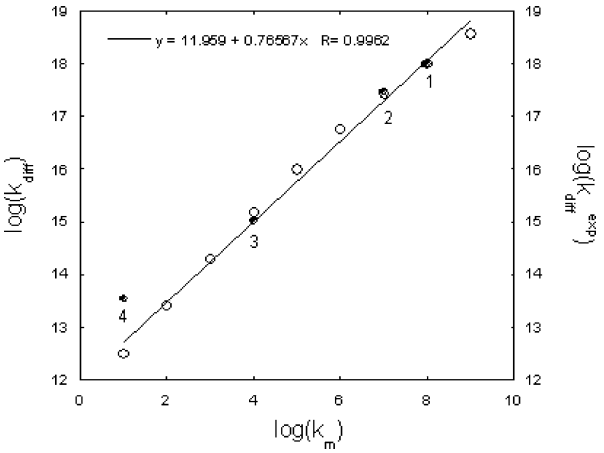


Fig. 8 Theoretical (*open circle*) and experimental (*filled circle*) dependences of logarithms of the diffusion-controlled rate constants (k_{diff}) on logarithms of the unimolecular decay of the excited species ($k_M=1/\tau_M$), which characterizes the time scale of different methods. Experimental diffusion-controlled rate constants are marked as (*filled circle*) *1* for [23], *2* for [1], *3* and *4* for k_q and k^T obtained in the present work [34]

singlet- and triplet-state quenching rate constants, the rate constant of photoisomerization, and the rate constant of the triplet–triplet energy transfer. It appears that this set of parameters constitutes cumulative characteristics of the dynamic state of biomembranes in the wide range of the probes' amplitudes and characteristic times.

Proficiency of the method can be further expanded by a choice of the cascade participants with a higher efficiency of the triplet–triplet energy transfer, higher sensitizer lifetime, and by increasing the time of integration of experimental data on a photochrome photoisomerization. It should be noted that the diffusion-controlled rate constants depend on k_M and on concentration and, therefore, more adequately depict the actual molecular dynamics in complex systems such as biological membranes. Eventually, for applications in biological system, it is also important to use photochrome probes with the excitation and emission wavelengths in the near infrared area.

Fluorochrome Immunoassay

A rapid, sensitive, and quantitative novel immunoassay technique was developed to auspiciously combine both the high sensitivity of fluorescent measurements with the high specificity of an antibody [8]. Fluorochrome immunoassay (FCIA) is performed without separation of antibody-bound haptens from those that are free and utilizes fluorescence measurements from widely available standard commercial fluorometers. FCIA is based on the hypothesis that an appropriately designed stilbene-antigen analogue probe will suffer considerable steric hindrance to *trans–cis* photoisomerization when bound within the combined constraints of both an antibody binding site and a second globular protein.

Specifically, an appropriately designed 2,4-dinitrophenyl (DNP)–hapten derivative of fluorescent *trans*-4,4′-diaminostilbene (DAS) was squeezed between two large globular proteins: a lysozyme (Lys) from one side, and anti-2,4,6-trinitrophenyl antibody (anti-TNP) from the other side, to provide the desired constricted environment to restrict *trans*/*cis*-stilbene isomerization within the anti-TNP–DNP–DAS–Lys adduct {Fig. 9}

As was theoretically predicted and then experimentally verified, the *trans–cis* photoisomerization rate for the bound probe was found to be markedly inhibited, compared to that expected for the free probe in solution. The fluorescence/photochrome-labeled probe was competitively displaced from the anti-TNP binding site in the presence of the picric acid hapten, and photoisomerization then commenced to produce the fluorescent-silent *cis*-stilbene diastereomer (Fig. 10). The process of association and dissociation of a hapten-antibody complex was readily monitored by the fluorescent technique in the presence of both the antibody-bound and free haptens.

The FCIA method can be potentially used either independently or as an additional complementary method to enzyme-linked immunosorbent assay and fluorescence polarization immunoassays technique. FCIA does not need polarization equipment and is not markedly influenced by light scattering effects. The FCIA technique can be expanded for analyses of enzymes and receptors including adaptation to fibro-optic techniques.

Super Molecules Containing Fluorescence and Fluorescent Quenching Groups

Super molecules containing a fluorescent group and fluorescent quenching segments, chelated metals, can be utilized for analysis of molecules and reactive radicals. In such super molecules, the quenching of the fluorophore fluorescence can occur under certain conditions, by a singlet–singlet energy transfer mechanism. The quenching efficiency is strongly dependent on the

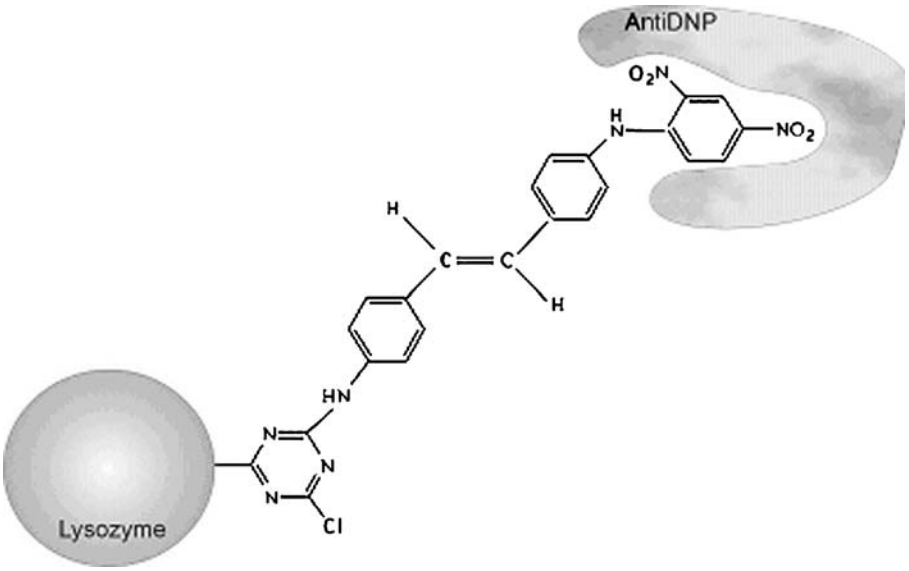


Fig. 9 Scheme of the anti-TNP–DNP–DAS–Lys complex. The DNP–DAS label bound between two large protein molecules by means of non-covalent binding to an anti-TNP antibody at one side and on the other side via covalent linkage to Lys using cyanuric chloride as the cross linker [8]

overlap integral between the donor fluorescence and acceptor absorption spectra and the distance between the donor and acceptor segments. Therefore, any change of chemical structure of the quenching segment can be monitored by the sensitive fluorescent technique. This approach is illustrated by the examples of analysis of NO and superoxide [40, 41].

NO Analysis

A novel assay was developed for the measurement of NO [40]. The proposed method is based on fluorescence, using a fluorophore–heme dual functionality probe (FHP). The heme group can serve as an effective NO trap, because of its very fast reaction with NO and the high stability of the resulting complex. As the heme is connected with a fluorophore as a part of the FHP dual-functionality probe, the heme quenches the fluorophore fluorescence, under certain conditions, by a singlet–singlet energy transfer mechanism.

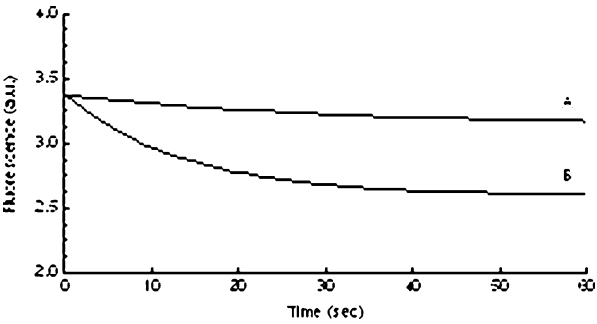


Fig. 10 Time-trace of anti-TNP–DNP–DAS–Lys solution before (*A*) and after (*B*) the addition of picric acid. Experimental conditions: excitation 360 nm, emission 410 nm, [DNP–DAS–Lys]= 2 μM, [anti-TNP]=1.5 μM, [picric acid]=20 μM, solution composition: *N*,*N*′-dimethylformamide (20%), glycerol (30%), PBS buffer B (50%); *T*=25°C [8]

This method was tested using myoglobin covalently modified by stilbene label 4-acetamido-4′-isothiocyanatostilbene- 2,2′-disulfonic acid (SITS). The change in emission intensity of the stilbene fragment, versus an increasing concentration of NO precursors, clearly demonstrated the spectral sensitivity required to monitor the formation of a heme–NO complex in a concentration range of 10 nM–2 μM (Fig. 11) [40].

Furthermore, the new methodology for NO measurement was also found to be effective for assaying tissues from rabbit and porcine trachea epithelium in the presence adenosine triphosphate (ATP), and a specific inhibitor of NO syntase (NOS) L-NG-nitroarginine methyl ester hydrochloride (L-NAME) (Fig. 12). The measured NO flux in the initial time interval for tissue sample from rabbit trachea epithelia and porcine trachea epithelia was found to be as ~7.9×10^{-12} mol/s×g and ~3.0×10^{-12} mol/s×g, respectively.

Because of a fast oxidation of ferrous hemes in aerobic media, the abovementioned method has a limitation of being used only under anaerobic condition. To overcome such a limitation, a new method for the direct real-time monitoring of secreted NO at nanomolar concentrations in physiological aerobic conditions has been developed [41]. The latter method is based on preventing fast oxidation of ferrous heme in aerobic media by its incorporation in human serum albumin and labeling the albumin cystein-34 with 5-((((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulfonic acid (IAEDANS). This method is also based upon the change of fluorescent resonance energy transfer extent as NO binds to a hemalbumin trap. The sensitivity limit of this method is about 20 nM of NO (Fig. 13), which is much lower than of other existing methods except the chemiluminescent detection (the later is not highly selective).

According to the preliminary results [41], the use of reagents such as arginine, ATP, and a specific inhibitor of NOS, namely L-NG-nitroarginine methyl ester hydrochloride (L-NAME), which affects the NO production, showed the high specificity of the method for real-time monitoring NO in tissues (Fig. 14). The method was employed for quantitative estimation of secreted NO from several kinds of tissue: (a) tracheal porcine epithelium, (b) rabbit trachea, and (c) human polyps.

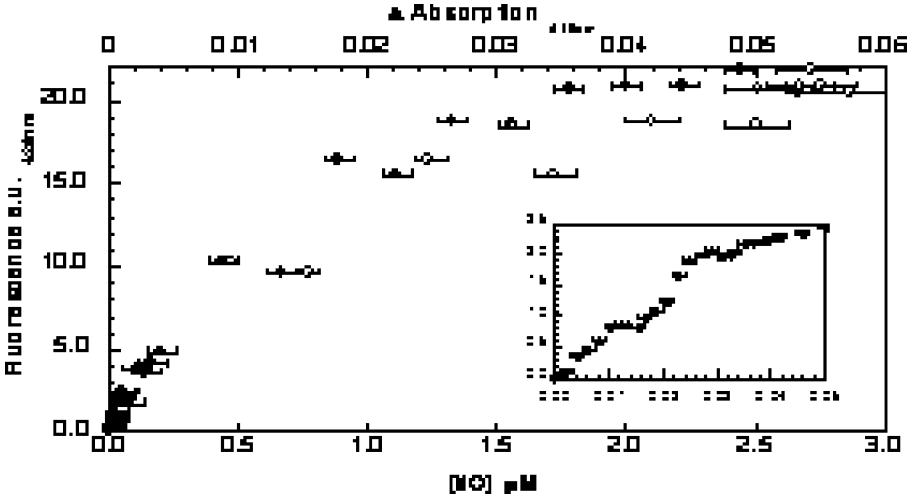


Fig. 11 Calibration curve drawn as fluorescent intensity versus absorption (*upper x-axis*), which is proportional to NO concentration (*lower x-axis*). The *graph inset* is an extended version of the low concentration part of the calibration curve [40]

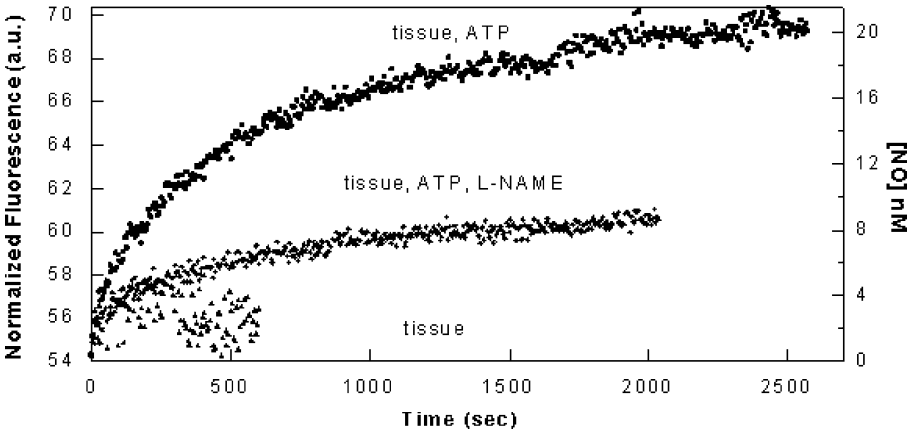


Fig. 12 Time trace of fluorescence (*left scale*) and NO concentration (*right scale*) of Mb(Fe^{2+})–SITS solution (0.05 μM) with: pieces of tissue (*from rabbit trachea epithelium*, 0.0192 g) and ATP (*upper circle points*), piece of tissue (0.0203 g) with ATP including L-NAME (*lower triangle points*), and with piece of tissue without ATP. ATP was added at t=0 s; L-NAME was added 10 min before time trace [40]

Superoxide Dynamics

A method for monitoring the superoxide dynamics used the same principle as in labeled myogblobin and hemalbumin probe [41]. For this purpose, cytochrome C from horse heart was labeled by fluorescein isothiocyanate (FITC). Ferrocytochrome C, when reduced by superoxide, changes its absorption spectrum and, therefore, the label fluorescence. Figure 15 shows kinetics of the fluorescent changes after a ferro hemeprotein was reduced with different rates by xanthine/xanthine oxidase system.

The proposed fluorescent method for analysis of superoxide in submicromolar concentrations scale, even in its current state, is remarkably sensitive as compared with traditional methods based on measurement of absorption and/or EPR techniques (the sensitivity gain is by at least one order of magnitude). The method sensitivity can be further increased by more than

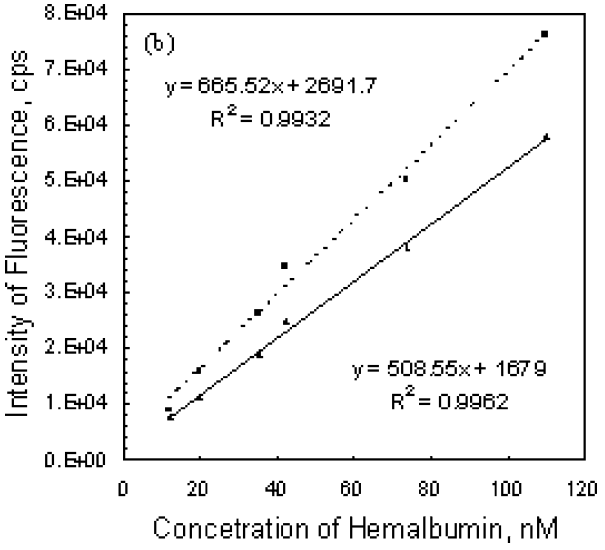


Fig. 13 Calibration curve for fluorescent emission of free (*triangles*, *solid line*) and nitrosilated (*squares*, *dashed line*) hemalbumin complex according to IAEDANS fluorescence (λex= 340 nm, λem=475 nm). PBS (pH=7.4), 25°C [41]

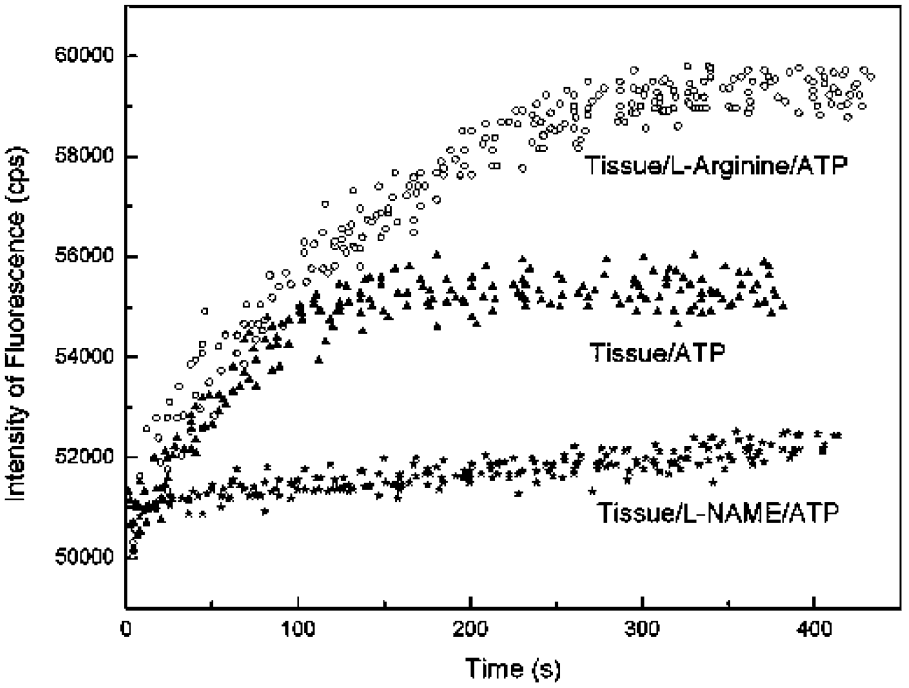


Fig. 14 Fluorescent responses to addition of ATP 0.5 mM after previous treatment with L-NAME (*stars*), L-arginine (*circles*), and without additional treatment (*triangles*). Porcine trachea, 37°C, Ringer buffer, aerobic conditions [41]

two order of magnitudes by optimizing the choice of a fluorescent label and its position within the protein globule.

Systems Immobilized on Quartz Slides

With an aim to establish the bases for a fibrooptic biosensoring, a number fluorescent molecular systems were immobilized on quartz surface and investigated.

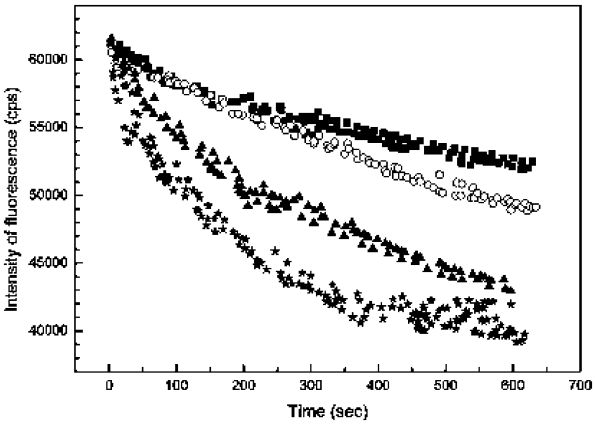


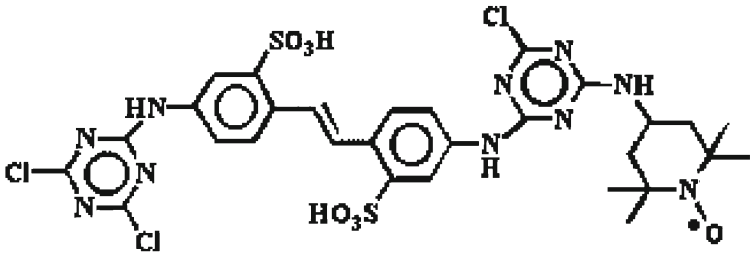
Fig. 15 Reduction of FITC-labeled cytochrome C (500 nM in PBS pH 7.4) by superoxide at different rates of O_2-production, followed fluorimetrically (λex= 480 nm, λem=520 nm). The rates of superoxide synthesis were 12 nM/min (*filled squares*), 16 nM/min (*circles*), 25 nM/min (*filled triangles*), and 920 nM/min (*filled stars*) [41]

Local Medium Effects in Photochemical Behavior of Immobilized Stilbenes

Several substituted *trans*-stilbene derivatives have been prepared and immobilized on a quartz surface [42]. Several immobilization methods have been tried including the silanization technique, cross-linking with cyanuric chloride, surface activation with cyanogen bromide, and surface smoothing with coating proteins. Studies of solvent polar effects on the fluorescent spectrum of the immobilized stilbenes indicated that the maximum wavelength of the fluorescent emission is not very sensitive to solvent polarity. The apparent local polarity of the medium in the vicinity of the stilbene label was estimated. The *trans–cis* photoisomerization kinetics of the stilbene derivatives in the immobilized and free state in a medium of different viscosity was monitored with the fluorescent technique at constant illumination conditions. The apparent photoisomerization rate constant of the process was found to be three to four times less for the immobilized label than in the free state. Investigation of the microviscosity effect on the photoisomerization of the immobilized and free stilbene label was carried out changing the relative concentration of glycerin in a glycerin–water mixtures used as a solvent. Upon appropriate calibration, the microviscosity in the vicinity of the stilbene label could be estimated.

Sensor for Ascorbic Acid and Surface Microviscosity

A new photochrome/fluorescence-spin method for the simultaneous quantitative analysis of the redox status and viscosity of a medium has been developed [43]. The method of the viscosity measurement is based on the use of double FNO molecules. In such hybrid compounds, the nitroxide moiety quenches the fluorescence of the fluorophore (stilbene moiety). The reduction of nitroxide segment by an antioxidant (ascorbic acid for example) causes a rise of fluorescence of the fluorophore. The rate constant of the stilbene fragment photoisomerization in such systems is dependent upon the viscosity of the media. The synthesized dual stilbene–nitroxide probe



BFL1

was covalently immobilized onto the surface of a quartz plate as an eventual sensor. The immobilization procedure included a cyanogen bromide surface activation followed by smoothing with a protein tether. The rate of fluorescent change was monitored in aqueous glycerol solution of different viscosities and content of ascorbic acid. A good correlation was found (a) between the concentration of ascorbic acid in the sample and the rate of fluorescence increase because of the reduction of the nitroxide moiety and (b) between the rate constant of photoisomerization and the viscosity of the media. An appropriate calibration would make possible the determination of the viscosity of a media (in a range 1–500 cP) as well as the ascorbate content in a range $(1–9)\times10^{-4}$ M (Fig. 16).

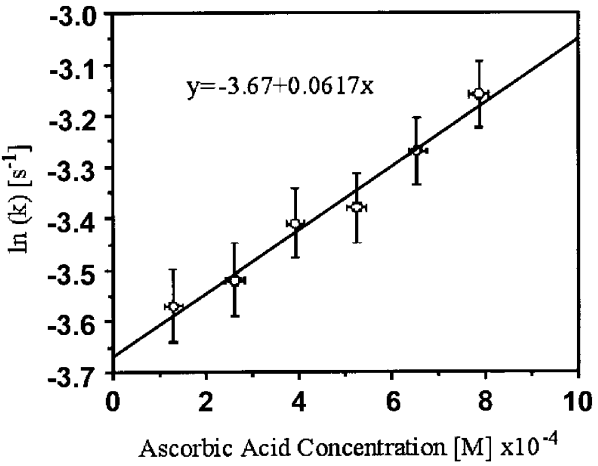


Fig. 16 Logarithmic dependence of the nitroxide moiety reduction rate for the immobilized BFL1 molecule versus the ascorbic acid concentration in aqueous solution. *T*=298°K, PBS pH 7.4 [43]

All the abovementioned experiments were performed with the use of a very sensitive fluorescent technique by means of commercial fluorometer and modified quartz plates in a single fast measurement. The covalent immobilization of the label enables multiple use of the same plate without the need to prepare and calibrate additional solutions—an important advantage for possible industrial use. Replacing the quartz plates by quartz optical fibers

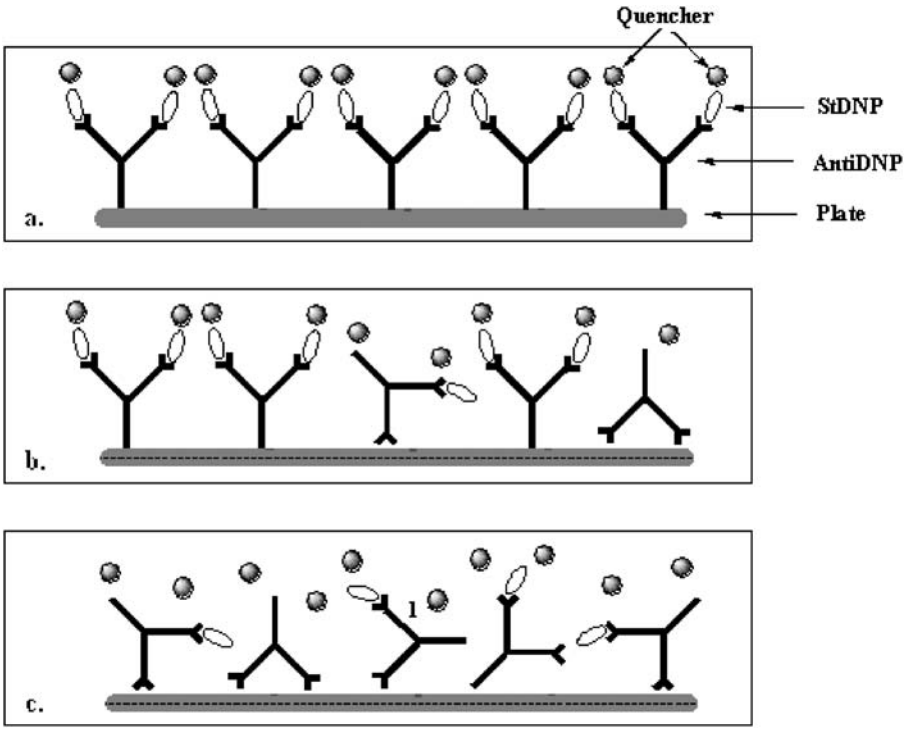


Fig. 17 Possible arrangements of solid-phase antibodies bound to a fluorescent antigen. **a** A totally ordered system, **b** a partially ordered system, **c** a completely disordered system [38]

will facilitate the use of the given methods for the continuous monitoring of chemical and biological processes.

A FPM for the Quantitative Characterization of Solid-Phase Antibody Orientation

A FPM for quantifying the orientation of solid-phase antibodies immobilized on a silica plate was proposed [38]. The method is based on measurements of fluorescent quenching by a quencher in solution and rates of *trans–cis* photoisomerization and photodestruction of a stilbene-labeled hapten in an antibody binding site. These experimental parameters enable a quantitative description of the order of binding sites of antibodies immobilized on a surface and can be used to characterize the microviscosity and steric hindrance in the vicinity of the binding site. Furthermore, a theoretical method for the determination of the depth of immersion of the fluorescent label in a two-phase system was developed [38, 44]. The model exploits the concept of dynamic interactions and is based on the empirical dependence of parameters of static exchange interactions on distances between the exchangeable centers.

In [38], anti-DNP antibodies and stilbene-labeled DNP as a hapten were used. Possible arrangements of solid-phase antibodies bound to a fluorescent antigen are presented in Fig. 17.

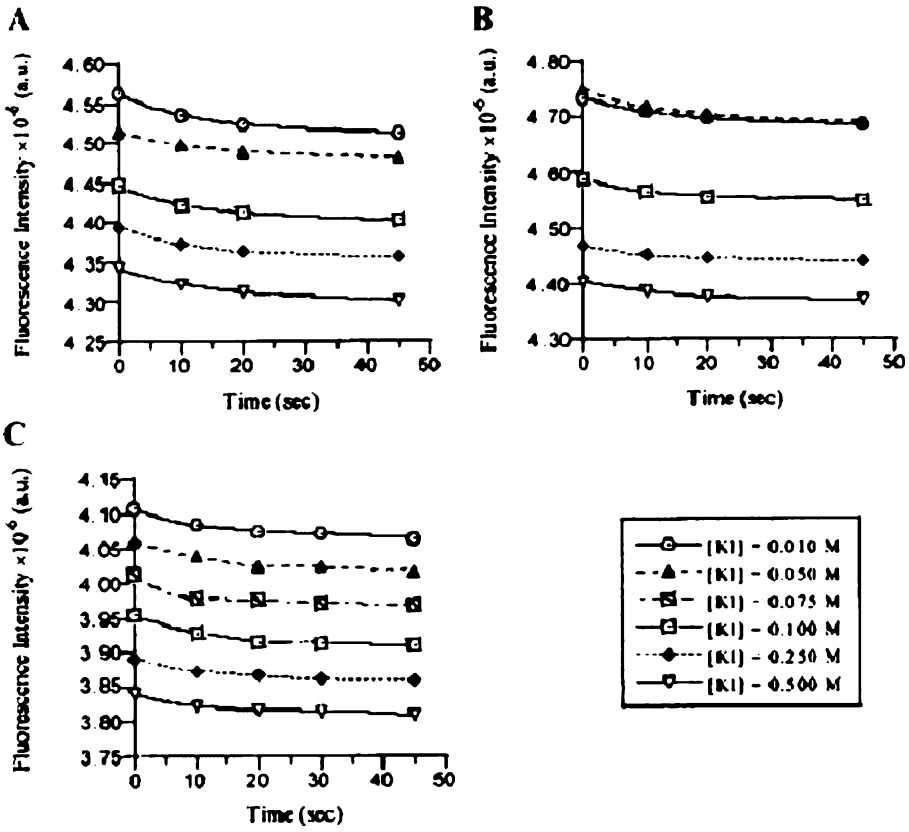


Fig. 18 Fluorescent decay kinetics of STDNP bound to immobilized anti-DNP measured in the presence of different concentration of potassium iodide [38]

Four different antibody immobilization techniques were examined: physical adsorption, covalent binding to a silane-activated surface, Langmuir–Blodgett films, and the oriented method [38]. Figure 18 showed fluorescent decay kinetics of a stilbene–DNP derivative (STDNP) bound to anti-DNP immobilized on quartz slides.

The kinetics depends on local microviscosity in the vicinity of bound STDNP, while initial intensity decreases in the presence of different concentrations of potassium iodide-indicated accessibility of the antibody binding. It was found that there is a well-ordered surface of monolayer antibodies when Langmuir–Blodgett films are used for immobilization.

The FPM is also suitable for the characterization of the physical parameters of wide range immobilized systems, such as proteins, enzymes, lipids, and polymer films.

Conclusion

The basis for a series of novel methods for the real-time monitoring of antioxidants, NO, reactive radicals, metal ions, and immunoassays in a solution and the investigation of molecular dynamics and the microstructure of biomembranes and surface systems has been described. A series of such compounds includes dual FNO probes, fluorescence–photochrome molecules and super molecules containing fluorescence and fluorescent quenching segments. The special advantages of the developed methods are in the use of very sensitive, rapid, simple and commonly available fluorescent techniques that provide possibility to measure the nanomole concentration of molecules under analysis in a dynamical regime.

The measurement of kinetics of photoisomerization of fluorescence–photochrome molecules, (stilbene derivatives for example) makes it possible to determine the dynamic parameters of biological membranes on the time scale from seconds to nanoseconds, to perform immunoassays in solution, and to investigate the microstructure and dynamics of surface systems (antibodies on silica plates, for example).

Proficiency of the method can be expanded by an optimum choice of the fluorescent probes with the excitation and emission wavelengths in the near infrared area, in particular, and the use of modern techniques as the fluorescent time-domain nano- and picosecond spectroscopy. The abovementioned methods may, after certain developments, be adapted for fibrooptic biosensoring and be employed in biotechnology and biomedicine for routine determinations (analysis of biologically important molecules and diagnostics) and in basic research as well.

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