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Lifetime-Based Sensing: Influence of the Microenvironment

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The influence of the microenvironment on the fluorescence behavior of indicator molecules is investigated. A model is developed to describe the fluorescence decay of indicator molecules in a nonuniform medium. Its consequences for fluorescence lifetime-based chemical sensors are discussed and verified in two examples, namely, a pH sensor using a pyrene compound in a hydrogel and a ruthenium complex for oxygen sensing embedded in a polystyrene membrane.

Over the last decade there has been considerable interest in the development of optical chemical sensors based on fluorescence measurements (see, for example, ref 1 and references therein). The concept of using the fluorescence decay time has proved especially to be very attractive for optical sensing, and for certain applications, it has important advantages compared to intensity-based schemes. The feasibility of this method has been demonstrated for measuring various analytes,^{2–5} from oxygen over ions and glucose to pH.

Luminescence studies are usually performed on molecules that are in some way dispersed in a medium. It is well appreciated that the properties of the medium affect the luminescence properties of the molecules. As long as the temporal fluctuations of the environmental influences are much faster than the luminescence decay, each molecule can be regarded as subject to the same interactions and the kinetics of the ensemble is uniform. When the medium is a high-viscosity fluid or a solid, this presumption need not to be valid. Different molecules have different influence from their respective microenvironment, and the kinetics becomes more complex.

Usually for sensor purposes indicator molecules are immobilized in polymer matrices. In this case, the medium may consist of domains with more or less crystalline or amorphous character, and in the amorphous parts the local density may vary considerably. As a consequence, the observed decay is, even in the simplest case, no longer a single exponential. In recent years it has been shown that fluorophores in nonuniform environments exhibit luminescence decays that are best understood by a model of continuous distributions of decay times (see, for example ref 6 and references therein). However, the form of the distribution

cannot unambiguously be deduced from the measured decay data. It was shown^{7–9} that fitting procedures cannot distinguish sufficiently between, for example, a single Gaussian distribution of decay times and a sum of two exponentials or between a bimodal Gaussian distribution and a sum of at least three exponentials. Thus, a description based on discrete decay time components should only be regarded as truly representing discrete molecular states if it is supported by supplementary data.⁹

It is the aim of this work to give a simple theoretical model for the fluorescence decay in a nonuniform environment, including also the action of additional quenchers. This model has a better physical basis than the usual fits with multiple exponentials or lifetime distributions of arbitrary shape. It was first developed to describe oxygen sensors based on fluorescence decay time.¹⁰ In those devices the excited-state interaction of a luminophore with oxygen acts as a deexcitation process, competing with the luminescence emission (dynamic quenching). The lifetime of the excited state and, accordingly, the quantum efficiency of the luminescence become dependent on the oxygen concentration. Hence both quantities can be used for measuring purposes. In most cases of proposed oxygen sensors, the respective indicators had been incorporated in a polymer matrix. However, quenching did not follow, as was originally expected, a linear Stern–Volmer relation, and the luminescence decay turned out not to be single exponential. Approaches using multiple exponentials or even distributions of lifetimes gave acceptable fits but yielded no reasonable physical basis for understanding the kinetics. The model given in this paper aims at accounting for that insufficiency. The versatility of this model is demonstrated on two examples, namely, sensing of pH and oxygen. Its consequences for the calibration curve of a sensor will be discussed.

THEORETICAL CONSIDERATIONS

When indicator molecules are immobilized in polymer matrices, the fluorescence decay is influenced by interactions between the fluorophores and the polymer. Most polymers are nonuniform media in a sense that there is no long-range structural order. The fluorescence decay profiles of molecules in a nonuniform medium can be assumed as being an average over a distribution of relaxation rates. As a consequence, the decay function of the molecules becomes nonexponential.

The time-dependent probability $p(t)$ for an excited luminophore to be in the excited state at a time t after excitation is found

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from the solution of the following differential equation:

$$-dp(t)/dt = p(t)(k_r + k_{nr}) \quad (1)$$

In this equation k_r is the radiative deexcitation rate and k_{nr} is the rate for nonradiative relaxation of the luminophore. Both rates are determined by internal properties of the luminophore as well as by the properties of the surrounding medium. However, due to spatial nonuniformities in the medium, the rates may be different for luminophores situated at different sites inside the medium. To deal with this fact, a discrete or quasi-continuous distribution of rates may be applied, leading to distributions of lifetimes.⁶ However, the form of the distribution cannot without further assumptions be unambiguously recovered from static or time-resolved fluorescence measurements.

To properly describe the decay of the excited state, one has to take into account the influence of the indicator's environment on both rates in eq 1. The radiative rate can be calculated from the well-known Strickler–Berg relation,¹¹ which shows that the medium may influence the rate by shifting the absorption maximum or distorting the shape of the absorption band. This can easily be verified by accurately measuring the absorption. Further influence by the medium is described by its refractive index. In a broad fluorescence band, some dispersion may be encountered. In addition, local variations in the refractive index have to be considered. Since the influence on the emission comes from the whole near field of the emitting dipole, local variations on a scale smaller than $\lambda/2$ are averaged out. If large-scale variations are present, they yield a corresponding variation in the decay rate. In a well-thermostated, chemically homogeneous medium, variations in refractive index can be brought about only by variations in local density. In most cases, long-range density variations may be small enough that they have no measurable effect on the decay rate. The same usually is true for the dispersion effect.

The main influence of the medium surrounding the luminophore on its luminescence decay should enter via the nonradiative relaxation rate k_{nr} , which is the sum of the rates of internal conversion k_{in} , intersystem crossing k_{ic} , and quenching k_q . The intersystem crossing probability depends on the spin–orbit coupling in the excited luminophore which could be influenced by an external heavy atom effect, if such atoms were present in the matrix. For most polymers this is not the case, so this effect can be neglected. The internal conversion rate usually decreases on inclusion of the luminophore in a rigid environment because of the limitations imposed on the molecular flexibility, leading to a modified rate k'_{in} . The magnitude of this influence cannot be expressed in general terms but depends on the specificities of the luminophore and the matrix, respectively. If the stabilizing effect of the matrix shows spatial variations, this may lead to a distribution of rates and hence to a nonexponential decay. The form of the distribution function, and hence that of the decay curve, cannot be predicted without extensive knowledge of the structure of the matrix in the surroundings of the luminophore.

The quenching rate is important not only in the presence of external quenchers. Even the matrix itself can, and obviously in many cases does, act as a quencher. It has to be assumed that quenching of the fluorophore is brought about by interactions with neighboring regions of the polymer. The influence of these

interactions depends on the distance between the luminophore and the nearest interacting polymer site. So the quenching rate $k_q^{(i)}$ for the i th excited molecule is the sum over the distance-dependent interactions with j quenching sites:

$$k_q^{(i)} = \sum_j k_{ij}(r_{ij}) \quad (2)$$

(k_{ij} quenching rate for the i th molecule caused by the j th interaction site, r_{ij} distance between the interacting partners). This rate has to be included in eq 1. The resulting differential equation can be solved, if the distribution of distances is assumed to be homogeneous and isotropic.

The distance dependence of k_{ij} can have different forms, depending on the quenching mechanism. The two physically most important mechanisms are electron transfer and electromagnetic near-field interactions. In the case of electron transfer, the distance dependence is exponential. The prerequisite for this mechanism is the presence of strong electron acceptors in the matrix, which is usually not the case in polymers used for chemical sensing. So the most important mechanism remains an electromagnetic one.

Electromagnetic interactions between close-lying molecules were first treated by Förster.¹² He considered the resonant case only and came to the result of strong energy transfer. Resonant energy transfer can only occur when the emission band of the luminophore and the absorption band of the energy acceptor match. In transparent polymers, this is certainly not the case. This may be the reason that energy transfer has not been taken into account as a relevant interaction between luminophores and a polymer matrix.

For luminophores with a rather long excited-state lifetime, however, even nonresonant interactions may contribute significantly. After excitation of the luminophore, the equilibrium in the microenvironment of the indicator is disturbed due to a change in the dipole moment; therefore, a reorientation of the local environment due to minimization of the local field energy leads to an inhomogeneous spectral broadening of the luminescence.¹³ However, in contrast to solvents where only a solvation shell around the indicator has to be taken into account, in a membrane, the whole volume surrounding the indicator has to be considered.

Assuming the distances between indicator and polymer interaction sites being homogeneously distributed and the quenching rate to depend on r with a power law, $k_{ij} \propto r_{ij}^{-n}$, eq 1 becomes

$$-dp_i(t)/dt = p_i(t) \left[\frac{1}{\tau} + \frac{1}{\tau} \sum_j (r_o/r_{ij})^n \right] \quad (3)$$

with $\tau = (k_r + k_{in} + k_{ic})^{-1}$. The critical radius r_o ("Förster radius") is the distance where the rate caused by the energy transfer is equal to the sum of the other rates. The total decay function can be calculated by integrating this differential equation and summing up over all sites.

As was shown by various authors,^{12,14–16} integration of eq 3 gives an expression of the following form:

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$$N(t) = N(0) \exp[-t/\tau - a(t/\tau)^{\Delta/n}] \quad (4)$$

Here $N(t)$ is the number of excited fluorophore molecules at time t , Δ is the dimensionality of the system, and a is a parameter proportional to the density of quenching sites. In the three-dimensional case, $a \equiv 4/3\pi^{3/2}\rho r_0^3$, where ρ stands for the number of quenching sites per volume.

Assuming an electromagnetic interaction between indicator and polymer and no point charges to be present in the system, only dipole interactions have to be considered (higher multipoles will give negligible contributions). It is well-known from electromagnetic theories that the distance of dipole–dipole energy transfer depends on the sixth power of r ; hence $n = 6$. Since the interaction sites in the polymer are assumed to be homogeneously distributed in space, the dimension of the system is 3 ($\Delta = 3$). Hence we got $\Delta/n = 1/2$ corresponding to a dipole–dipole interaction in three dimensions.

Thus, by taking into account the interactions of the fluorophore with the environment, the fluorescence decay profile even in the absence of any external quencher is found to deviate from the usual exponential form where the exponent is a linear function of time. In addition, eq 6 has a physical meaning in relating the form of the decay curve to a certain quenching mechanism, namely, energy transfer by off-resonance electromagnetic coupling, and two structural quantities, namely, the density of quenching sites and the critical radius. The number of quenching sites within the critical radius is given by $N = a\pi^{-1/2}$.

For the case of optical sensors, it is interesting to examine the influence of this luminophore–matrix interaction on a dynamical quenching process. The following considerations neglect, however, any interaction between quencher and matrix, which is in fact only a rough approximation. The presence of a dynamical quencher introduces a new deexcitation path into eq 1:

$$-dp_i(t)/dt = p_i(t) \left[\frac{1}{\tau} + \frac{1}{\tau} \sum_j (r_0/r_{ij})^6 + \frac{c}{\tau} \right] \quad (5)$$

where $c = \tau k_Q$ with k_Q the bimolecular dynamic quenching rate, which is proportional to the molar concentration of the quencher. The dynamical quenching parameter c is independent of r_{ij} and, in the usual and most simple case, constant in time. Integration gives the following decay function:

$$N(t) = N(0) \exp[-(1 + c)(t/\tau) - a\sqrt{(t/\tau)}] \quad (6)$$

It is interesting to note that the dynamical quenching parameter c enters only into the term linear in t , while the square-root term remains unaffected. If no matrix interactions were present ($a = 0$), the decay would remain single exponential but would be shortened and the decay time τ would obey the well-known Stern–Volmer relation,¹⁷ with the dynamical quenching parameter related to the Stern–Volmer constant by $c = K_{SV}[Q]$, where $[Q]$ is the molar concentration of the dynamical quencher. With matrix interactions, the form of the decay curve is concentration dependent. The relative contribution of the square-root term decreases with increasing quencher concentration.

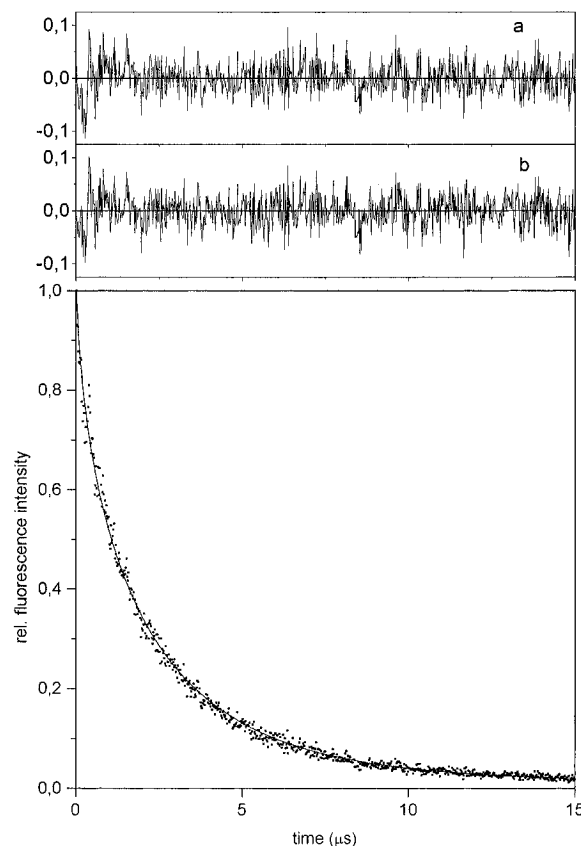


Figure 1. Fluorescence decay of ruthenium–diphenylphenanthroline in polystyrene, at an oxygen partial pressure of 369 Torr. Experimental decay, fitted curve according to eq 6, and comparison of the weighted residuals from this model (a) and a conventional multiexponential fit (b).

Using eq 6 to fit the decay curves of luminescent molecules in the polymer matrices is not only physically reasonable but it also has practical advantages over the usual sum-of-exponentials approach. A double-exponential fit has four independent parameters, while this model requires only two (or three, if a quencher is present). Nevertheless, the quality of the fit is as least as good using this model as is with the sum-of-exponentials approach. This is shown in Figure 1, where the luminescence decay of an oxygen-sensing material (ruthenium–diphenylphenanthroline) is plotted. Both fits have the same quality, as can be seen from the nearly identical weighted residuals, and is corroborated by equal coefficients of determination $r^2 = 0.998$.

The total luminescence emission is given by the area under the decay curve. This is obtained by integrating eq 6 from time zero to infinity.¹⁸ The result is equal to the luminescence intensity measured in conventional, non-time-resolved luminescence spectroscopy. The dependence of this intensity on the quencher concentration is now nonlinear, even though the Stern–Volmer relation is linear. The nonlinear contribution results from the square-root term. A nonlinear quenching behavior has been reported for many luminescence-based optical sensors.^{2,19–20}

EXPERIMENTAL SECTION

The applicability of the model was demonstrated in two different types of sensors. In the first one (diethylamino)-

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methylpyrene (DaPy, Lambda Fluoreszenztechnologie Ltd., Graz) was used as a pH indicator. The dye was dissolved (10^{-3} M) in a mixture (9:1 v/v) of ethanol (Merck p.a.) and distilled water. A 10 mL aliquot of this solution was added to 0.8 g of a polyurethane-based hydrogen (D6, Tyndale-Plains-Hunter, Ringo, NJ) and stirred vigorously. Films of approximately 0.1 mm thickness were prepared on various polymeric supports by drawing the support from the hydrogel, removing excess material by spinning, and curing the film at 60 °C. pH measurements were performed using standard buffer solutions (Merck).

The second example was on oxygen sensors. The transition metal complex ruthenium(II)–tris(4,7-diphenyl-1,10-phenanthroline) [Ru(dpp)] was prepared as described in ref 21 with perchlorate as the counterion. This dye was incorporated into a polystyrene membrane: 1.0 g of polystyrene (molecular weight 250 000; Aldrich) and 13 mg of Ru(dpp) (ClO_4)₂ were dissolved in 10 mL of chloroform, spread on a polyester foil, and dried at 90 °C for 2 h. The thickness of the layer was 10 μm . Gas mixtures were prepared using a precision gas mixer PGM-3 (Medicor Inc.).

Time-resolved measurements were performed using a nitrogen laser (pulse width 300 ps) as the excitation source ($\lambda_{\text{ex}} = 337$ nm). The fluorescence was monitored by a fast photomultiplier (rise time 1.5 ns) and processed by a 1 GHz digital signal analyzer (Tektronix DSA 601A). The decay data were fitted using various commercially available mathematical software packages. The temperature for all measurements was 22 °C.

RESULTS AND DISCUSSION

(Diethylamino)methylpyrene has a lone-pair electron on the nitrogen which at basic pH leads to an intramolecular excited-state electron transfer to the pyrene.²² This is a path for efficient radiationless deexcitation and hence yields a weak fluorescence and a short decay time. At acidic pH, the nitrogen is protonated. The positive charge impedes the electron transfer, and the result is a strong fluorescence and a rather long decay time. This acid–base reaction can be treated as static quenching; that means that there are two species present in the ground state. The first one is the neutral indicator, identical with the base form of the dye; the other one is the protonated or acid form of the indicator. The relative contributions of the two forms are pH dependent. Since no excited-state acid–base reaction occurs with this compound, only the preexponential factors are affected by pH.

When a sum of exponentials was used to fit the fluorescence decay data, two exponentials turned out to be necessary to give a suitable fit at any pH. Figure 2 shows the normalized preexponential factors, taken from a fit with two exponentials, plotted against pH. There is some resemblance to a sigmoidal, as expected for a pH indicator. However, the numerical evaluation shows that the measured values are widely at variance with those expected from the Henderson–Hasselbalch equation. At pH 3 and pH 14 only a single species should be present, namely, the acid or the base form of the indicator, respectively. In those cases a single exponential should be sufficient to fit the decay curve. However, to fit our experimental data, also with low and high pH, a sum of two exponentials was necessary to give a suitable fit. If, on the other hand, both species had a double-exponential decay, than around the $\text{p}K_{\text{a}}$ value, where both species are present in

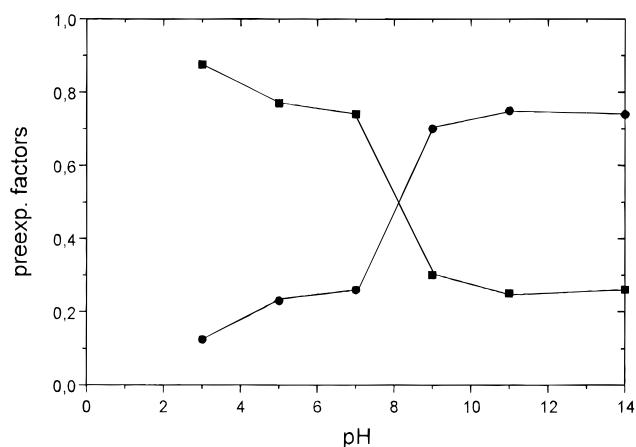


Figure 2. Conventional model. Normalized preexponential factors a (squares) and b (circles) of DaPy in hydrogel vs pH in a two-exponential fit, $I = a \exp(-t/\tau_1) + b \exp(-t/\tau_2)$. Lines connecting the symbols are only to guide the eye and have no physical meaning.

considerable amount, one would expect four exponential components to fit the decay adequately. Nevertheless, also in this pH region only two distinguishable components emerged in the fit. Thus it turns out that the fit with two exponentials is only a formal description, with no physical meaning for the fitting parameters. As can be seen from Figure 2, it is not possible to use the pH dependence of the preexponential factors to calibrate a sensor device.

On the other hand, when applying the new model to describe the fluorescence decay, a better understanding of the pH reaction is achieved. Using the new model, we get time constants of 160 ns for the acid form and 22 ns for the base form of the indicator. The parameter a , which represents the deviation from exponentiality induced by the environment, is 0.4 for the acid and 0.8 for the base form. The difference in a between acid and base forms of the indicator can be attributed partly to a change in the magnitude of the absorption of the indicator vs pH, which influences the strength of the energy transfer, and partly to the higher dipole moment of the protonated indicator. This leads to a reorientation between indicator and polymer environment, which influences the dipole interaction. Due to this, the effective density of quenching sites can be different between acid and base forms of the indicator. Last but not least, the hydrogel itself, especially the amount of swelling, is also affected by the change in pH, and therefore, the density of quenching sites is also influenced. All these effects contribute to the value of the interaction parameter a , which therefore is different between acid and base forms of the indicator.

Figure 3 shows the characteristic curve of the indicator, calculated from the new model and plotted as the relative contribution of the base form vs pH. From the measured values, a $\text{p}K_{\text{a}}$ of 7.7 is obtained. In a range of about pH 6–10 the determination of pH was possible with a resolution of better than 0.05. Changing of pH influences only the preexponential factors of the acid and base forms of the indicator; therefore, when the sum of amplitudes is normalized, only one independent parameter is necessary to describe the pH dependence of the indicator.

The second example is Ru(dpp) in a polystyrene membrane. This compound can be used for sensing oxygen. Oxygen quenches the luminescence and hence yields a shorter decay time. In homogeneous media and under circumstances of purely dynamic quenching, the variation in luminescence intensity or in

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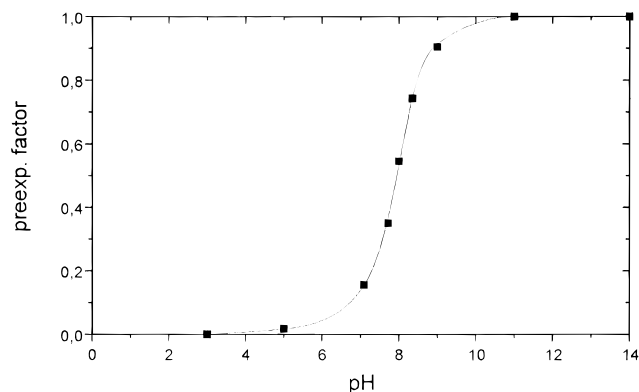


Figure 3. New model. Normalized preexponential factor for the base form of DaPy vs pH. Squares are values calculated from the experimental decay curves using the described theoretical model. The full line is calculated from the Henderson–Hasselbalch equation.

the luminescence decay time with externally applied oxygen partial pressure is described by the Stern–Volmer relation. However, when the indicator is embedded in a polymer matrix, deviations from the linear Stern–Volmer plot are observed.^{8,10}

When fitting the fluorescence decay curves with a sum of exponentials, three lifetimes are necessary to give a suitable fit.¹⁰ Two of the three exponentials do not depend on the oxygen pressure significantly; only the longest decay time decreases with increasing oxygen concentration. While the preexponential weighted mean lifetime²³ agrees with the intensity plot, the normalized preexponential factors vs oxygen pressure show a behavior that cannot be explained by the assumption of dynamic or static quenching.

Applying our new model to the experimental data set, a fit with $a = 1.92$ and $\tau = 11.42 \mu\text{s}$ is obtained. The only oxygen-dependent variable was the quenching parameter c . As shown in Figure 4, there is a linear relationship between the quenching parameter c and the oxygen concentration with $c = c' \times p\text{O}_2$ and $c' = 3.45 \times 10^{-3} \text{ Torr}^{-1}$. While with this model only one parameter is sufficient to describe the oxygen dependence of the sensor, in the case of using a sum of exponentials, six parameters had been necessary to get a fit of the same quality.

CONCLUSION

The interactions of a luminophore with a polymer surrounding influences its luminescence decay properties. In many cases, deviations from a single-exponential decay can be observed, which may be due to spatial nonuniformity in the refractive index of the matrix, variations in the steric stabilization of the chromophore by the polymer environment, or nonresonant energy transfer from

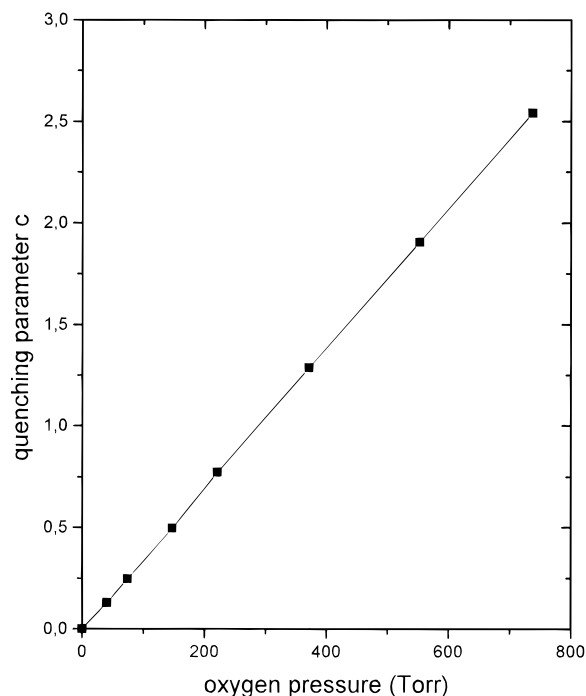


Figure 4. New model. Quenching parameter c for ruthenium–diphenylphenanthroline in a polystyrene membrane as a function of external oxygen partial pressure.

the luminophore to acceptor sites in the matrix. It has been shown in this work that this nonresonant energy transfer leads, at least in the case of a homogeneous distance distribution between luminophores and quenching sites, to a simple mathematical description of the luminescence decay which needs only one additional parameter. Experimental assessment with two different luminophores in two different polymers proves the suitability of this approach. At least for the examples provided, the present model gives a physically more reasonable and practically more tractable procedure in evaluating luminescence decays than do multiple-exponential or rate-distribution models. This may be of advantage in the quantitative treatment of luminescent chemical sensors, to which problem numerous publications have been devoted.

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