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Polarization Oxygen Sensor: a Template for a Class of Fluorescence-Based Sensors

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We describe a novel oxygen polarization sensor based on a single molecule that changes the *observed* anisotropy of its emission with variation of the oxygen concentration. The approach is demonstrated both in solution and in solid films. A simple mathematical description of the sensor is included and experimentally verified. The technique demonstrated here creates opportunity for development of a new class of polarization-based dyes and sensors.

INTRODUCTION

Fluorescence-sensing methods have been widely investigated over the past decade, resulting in significant advances in both development of new fluorophores[1−4] and measurement techniques. Fluorescence methods and the associated instrumentation are valued for their high sensitivity and selectivity. In particular, the accuracy of the straightforward intensity measurements in real-world applications is not high, and the results are not sufficiently reliable. Significant efforts in calibration are required; otherwise, the measurements are prone to errors originating from different chemical and instrumental factors.

Self-referencing methods with increased reliability have been developed to circumvent the limitations of the intensity based techniques. One widely used approach is to measure the variation in the fluorescence lifetime.[5−7] This methodology has been used to develop sensors based on dynamic quenching,[8,9] resonance energy transfer,[10] and fluorescent indicators that exhibit two different lifetimes in free and associated forms.[11−13] An alternate approach is to implement wavelength-ratiometric detection. This technique relies on probes that exhibit large changes in emission or excitation spectra when associated with the analyte and has been used mainly for cation determination (pH,[14,15] Ca^{2+},[16] and Mg^{2+} [17]). Ratiometric indicators have also been engineered for dynamic quenchers, such as Cl^- [18] and O_2.[19] Recently, low-frequency modulation sensing[20] was developed. It uses a long-lifetime reference and measures the ratio of long- to short-lifetime emission intensities.

Anisotropy is another fundamental property of the fluorescent emission that is often used in analytical sensing. When the emission dipole of a molecule rotates around its axis at a rate comparable to the emissive rate, depolarization of the fluorescence occurs. In the case of rotational motion, the measured anisotropy r will obey the Perrin equation $r_0/r = 1 + \tau/\theta$, where r_0 is the initial anisotropy in the absence of rotation, r is the measured anisotropy, τ is the fluorescence lifetime, and θ is the rotational correlation time. Since rotational correlation time directly relates to the molecular size, polarization is usually used for the determination of antigen−antibody reactions. Polarization assays are simple, accurate, relatively free of artifacts, and do not require sophisticated instrumentation. A small labeled molecule (the antigen) with fluorescence lifetime of several nanoseconds has subnanosecond rotational correlation time, resulting in low anisotropy. Binding to a large antibody with a much longer correlation time of tens or hundreds of nanoseconds results in significant

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increase in anisotropy.[21] Recently, the approach was used for the detection of low concentrations of heavy metals.[22]

An alternate approach is to combine an external highly polarized reference fluorophore with a nonpolarized intensity-based fluorescent probe.[23] Stretched films incorporating dyes with linear structure are used as the reference. Because the total anisotropy is the weighted sum of the anisotropy contributions of the separate emissions, an increase in the intensity of the sensing fluorophore results in *decreased* anisotropy and vice versa. However, to create sensors, one has to use a suitable external reference.

In this paper, we present what we believe to be the first intrinsic polarization oxygen sensor. It is based on the changes in the emission polarization of a single dye that exhibits both a short and a long lifetime emission. The sensing principle is demonstrated both in solution and in solid films. The polarization sensor described here has the advantages of the ratiometric measurements and does not rely on changes in rotational correlation time for changes in anisotropy. No external reference is needed. The approach is generic and can be extended to the development of polarization probes and sensors for a variety of analytes.

THEORY

In an isotropic system, fluorescence anisotropy is calculated from the fluorescence intensities observed through polarizers oriented parallel ($I_{\|}$) and perpendicular ($I_{\perp}$) to the polarization of the excitation light.

$$r = \frac{I_{\|} - I_{\perp}}{I_{\|} + 2I_{\perp}} \tag{1}$$

If two emitting species with steady-state intensities, I_1 and I_2, are present, then the *observed* anisotropy is given by the equation

$$r = \alpha_1 r_1 + \alpha_2 r_2, \tag{2}$$

where r_1 and r_2 are the anisotropies, and α_1 and α_2 are the intensity fractions of the respective species. Here, $\alpha_1 = I_1/(I_1 + I_2)$, $\alpha_2 = I_2/(I_1 + I_2)$.

Assume a fluorophore with two emissions of significantly different lifetime: τ_S that is significantly shorter than the rotational correlation lifetime θ and τ_L that is significantly longer. According to the Perrin equation, when $\tau_S \ll \theta$, the anisotropy r_S of the short lifetime emission equals $r_S \approx r_{0S}$; because $\tau_L \gg \theta$, the long-lifetime emission will be completely depolarized, that is, $r_L \approx 0$.

Rearranging (2) with these values yields

$$r = \frac{1}{n+1} r_S, \tag{3}$$

where n is the ratio of the intensity contributions of the long and short-lifetime emissions, $n = I_L/I_S$. Consequently, any change of the intensity of the long lifetime will result in change of the observed anisotropy r. This effect can be exploited to develop a new class of sensors based on changes in the fluorescence anisotropy.

In our particular case, the intensity of the long-lifetime emission depends only on the oxygen concentration in the sample according to the Stern−Volmer equation.

$$\frac{I_{L0}}{I_L} = 1 + k_{SV}[O_2] \tag{4}$$

Substitution of eq 3 in eq 4 gives the oxygen polarization sensor transfer function,

$$r = \frac{r_S}{\dfrac{n_0}{1 + k_{SV}[O_2]} + 1} \tag{5}$$

where n_0 is the ratio of the long and short-lifetime intensities in absence of O_2.

EXPERIMENTAL SECTION

Materials. As a polarization-sensing dye, [1,2-bis(diphenylphosphino) ethane−Pt{$S_2C_2(CH_2CH_2$-N-2-pyridinium)}] [BPh_4] (dppe−Pt2P) was used.[19] Triethyl citrate and cellulose acetate were from Sigma (St. Louis, MO). All other materials were of analytical grade. Nitrogen and oxygen (>99%) were from Air Products (Allentown, PA) and used without further purification. Nitrogen−oxygen gas mixtures were prepared with a gas blender equipped with two Series 150 flowmeters from Advanced Specialty Gas Equipment (Middlesex, NJ).

Preparation of the Sensors. For each sensor, 1 g of cellulose acetate (CA) was dissolved in 15 mL of acetone and combined with the desired amount of triethyl citrate (TEC): 0, 25, 50, 75, or 100% (w/w CA). The dye dppe−Pt2P was dissolved in acetone at a concentration of 10% (w/w) and added to the acetone/CA/TEC cocktails to give a final concentration of 0.5% dppe−Pt2P (based on the combined weight of CA and TEC). The resulting mixture was spread-cast on a polyester laser printer transparency (3M, St. Paul, MN) with a thickness of 1 mm, covered with a glass bell to decrease the evaporation rate of the solvent, and allowed to dry for 24 h. The resulting films were ~0.2 mm thick.

Measurements. Corrected steady-state and polarization spectra were obtained on a SLM (Rochester, NY) AB-2 fluorimeter. Solution measurements were performed in L-format, and the film measurements were performed in front-face format. Time-resolved luminescence decays were recorded on a frequency domain lifetime fluorimeter ISS-Koala (ISS, Shampaign, IL). Blue LED LNG992CFBW (Panasonic, Secaucus, NJ) driven by a current source was used as the excitation source. The modulation voltage was applied through bias T.[24] The standard radio frequency amplifier for the photomultiplier tubes was replaced with a ZHL-6A (Mini-Circuits, Brooklyn, NY) to enhance the low-frequency performance. The excitation light was filtered by 500-, 550-, and

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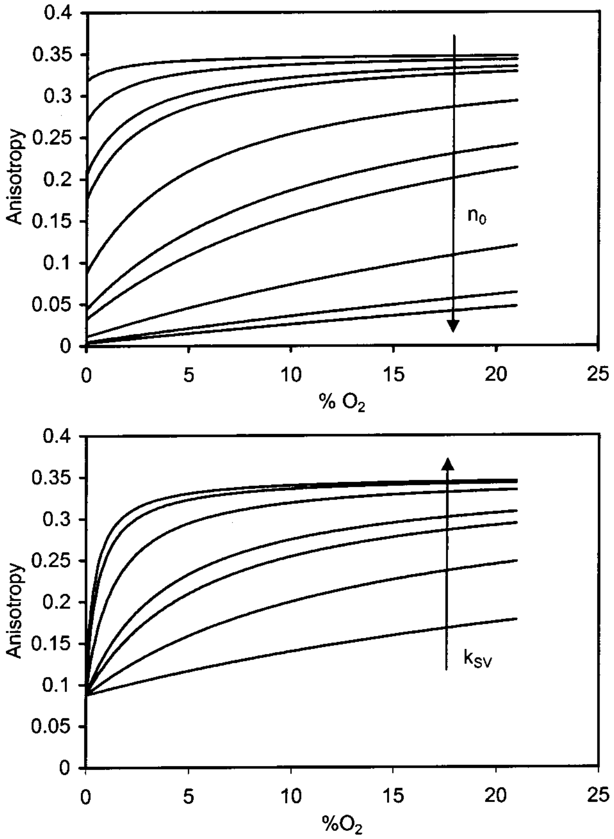

Figure 1. Dependence of the sensor response on changes in n_0 and k_{SV}: (a) $k_{SV} = 1$; $n_0 = 0.1, 0.3, 0.7, 1, 3, 7, 10, 30, 70, 100$; (b) $n_0 = 3$; $k_{SV} = 0.1, 0.3, 0.7, 1, 3, 7, 10$. The arrow shows the direction of increase in the variable parameter.

650FL07 short-wave-pass filters (Andover Corp., Salem, NH). The emission light was filtered by a 500FH90 long-wave-pass filter (Andover Corp.). Viscosity measurements were performed on a Rheometric Fluid Spectrometer RFS III (Rheometrics Scientific, Inc., Piscataway, NJ).

RESULTS AND DISCUSSION

As follows from eq 5, the behavior of the sensor is influenced by the initial ratio of the intensities n_0 and the Stern−Volmer coefficient k_{SV}. These two parameters affect both the linearity and the sensitivity, but in the opposite manner. As shown in Figure 1a and b, an increase in n_0 or a decrease in k_{SV}, improves the linearity of the sensor. The range is extended with an increase in k_{SV}. This suggests the use of high-oxygen-permeability materials is likely to expand the practical range, since the apparent k_{SV} is related to the solubility of oxygen in the polymer. At a given value of k_{SV}, the range is maximized, and further increases in k_{SV} simply increase the sensitivity of the sensor. When varying n_0, the range has a well-defined maximum; however, the corresponding value of n_0 strongly depends on the k_{SV}.

For theory validation, dual-emission dye with two dramatically different lifetimes is required in order to demonstrate the predicted anisotropy changes. One of the few existing dyes with these properties is dppe−Pt2P. It has been shown to emit from both singlet and triplet states at room temperature.[19] Elsewhere, this dye has proven to be useful for ratiometric oxygen determination.[25,26] In this study, the dye was used to demonstrate the feasibility of a polarization sensor. As a first step, dppe−Pt2P in solution was investigated. The solvent for the dye was carefully selected to meet certain criteria. Primarily, sufficient viscosity is necessary to ensure that the rotational diffusion of the dye is low enough that the singlet is strongly polarized, but high enough that the triplet is depolarized. Furthermore, it was desirable to choose a solvent that could be used as a plasticizer for solid films. TEC was selected as the solvent because of its relatively high viscosity (9.7 cP) and its ability to dissolve the dye. The spectrum of dppe−Pt2P dissolved in TEC at different O_2 concentrations is presented in Figure 2. The shape and position of the peaks ($\lambda^1_{max} = 588$ nm, $\lambda^3_{max} = 712$ nm) are similar to those recorded in DMSO.[19] In air, the emission is mainly from the singlet, since the triplet intensity is significantly quenched ,even at atmospheric pressure. The difference between the spectra in air and oxygen is minimal. The phase-modulation data used in lifetime determination are shown in Figure 3. Because of its high intensity, the singlet emission was extensively filtered in this measurement. The frequency behavior clearly indicates the presence of two largely

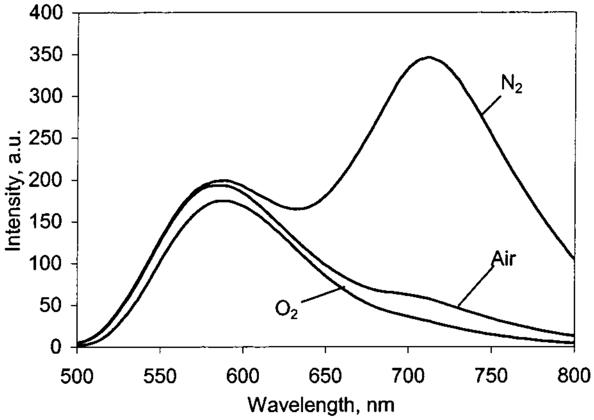

Figure 2. Emission spectra of dppe−Pt2P solutions in TEC saturated with N_2, air, and O_2, respectively. Excitation wavelength $\lambda_{ex} = 470$ nm, SD = 4.6.

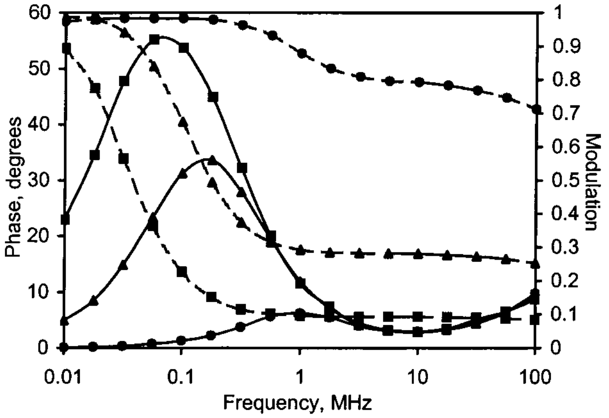

Figure 3. Frequency domain response of dppe−Pt2P solution in TEC: solid line, phase; dashed line, modulation; ■; N_2 saturated solution; ▲, air-saturated solution; ●, O_2-saturated solution. $SD_{phase} = 0.7°$, $SD_{modulation} = 0.021$.

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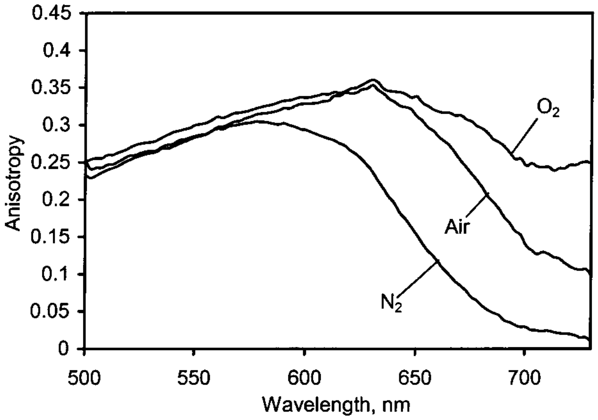

Figure 4. Anisotropy spectra of dppe−Pt2P solution in TEC saturated with N_2, air, and O_2. SD = 0.018.

Table 1. Peak Ratios, Triplet Lifetime, and Anisotropy Range of Cellulose Acetate Films with Varying Plasticizer Contents

	% TEC in CA				
	0%	25%	50%	75%	100%
	Peak Ratio				
N_2	4.57	2.69	2.79	2.23	2.54
air	2.72	1.65	1.28	0.62	0.47
O_2	1.26	0.74	0.89	0.52	0.46
	Triplet Lifetime, μs				
N_2	27.8	27.3	26.3	28.0	28.8
air	17.5	16.6	12.9	7.1	2.7
O_2	7.8	7.7	5.1	3.2	1.2
range ($r_{N2} - r_{air}$)	0.03	0.05	0.06	0.07	0.09

different lifetimes. The least-squares fit suggests that the singlet lifetime increased to 0.3 ns (as opposed to 0.2 ns in DMSO). The triplet lifetime was determined to be 1900 ns in air-saturated solution, 7200 ns in N_2-saturated solution, and 180 ns in O_2-saturated solution. These results are expected, given that the dye emission is quite sensitive to the polarity of the environment.[19] The lifetime values indicate that at room temperature, the emission anisotropy should vary with the changes in the oxygen content, since the calculated rotational correlation lifetime of dppe−Pt2P in TEC is 3.16 ns. Indeed, the results in Figure 4 show significant and oxygen-independent anisotropy of the singlet emission (~0.3−0.35), but the anisotropy around the triplet (710 nm) varies from ~0.025 in N_2 to ~0.224 in oxygen.

Furthermore, although there is little change in the intensity when the solution is saturated with air or oxygen, there is a significant (~0.12 above 630 nm) change in fluorescence polarization. Another remarkable (though predictable) feature is the potential to vary the sensitivity to the analyte through proper selection of the wavelength of the emission detection.

To develop a solid film sensor that preserves the oxygen-dependent anisotropy, TEC with dppe dissolved in it was used as a plasticizer of CA. Films with different amounts of plasticizer (25, 50, 75, and 100% TEC/CA w/w) were prepared and evaluated. Additionally, a sample containing dye but without plasticizer was also investigated. The results are summarized in Table 1. The range of the sensor was defined as the difference between the measured anisotropies in nitrogen and in air.

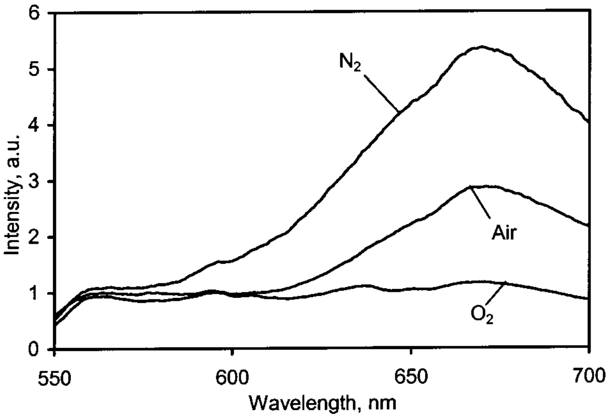

Figure 5. Spectra of dppe in CA film with 0% plasticizer in N_2, air, and O_2. SD = 0.036.

Immobilization of the dye in plasticized glasslike film significantly changed the its emission characteristics. The position of the singlet peak is slightly blue-shifted in comparison with the TEC solution. The peak ratio and the dye lifetimes were also different. As shown in Table 1, the singlet−triplet ratio varies with the percentage of the plasticizer used. The Stern−Volmer coefficient also varies significantly (~8 times), on the basis of the lifetime data. The singlet peak is largely quenched at low or absent plasticizer concentrations (Figure 5). One possible reason for the observed quenching is the polarity of the CA matrix. As the plasticizer concentration is increased, the singlet peak reappears (data not shown). In the presence of O_2, the triplet lifetime increases with a decrease in the plasticizer content (Table 1). As mentioned earlier, this may be the result of changes in the O_2 solubility of the film.[27]

As follows from Table 1, the variations in the peak ratio did not influence the range as predicted by the model. This may be attributed to the decreased amount of the plasticizer, which results in a significant decrease in the rotational diffusion of the dye. To some extent, this is confirmed by the increased anisotropy in the absence of O_2, as compared to that in solution: in air, the anisotropy reaches 0.24 at 665 nm in nonplasticized films. On the other hand, as the concentration of TEC (and consequently, k_{SV}) is increased, the range of the sensor is expanded. However, at TEC concentrations above 100%, the films could not be completely cured. These samples remained tacky and could not be used without additional support. Even the CA/100% TEC film was quite soft, but it maintained its integrity. For this reason, this formulation was used for sensor calibration and model verification.

The calibration curve of the sensor using the anisotropy at 665 nm is shown in Figure 6. The solid line represents the theoretical prediction based on eq 5. As can be seen, there is good agreement between the model and the experimental results (correlation coefficient, 0.9797). However, the values for n_0 and r_S are specific to this wavelength. If a different wavelength is selected, the model parameters should be recalculated.

The use of the polarization sensor at a single wavelength is not practical, because the intensity of the light is significantly decreased after passing the monochromators and polarizers, Thus, the simplicity of polarization determination is lost because of the

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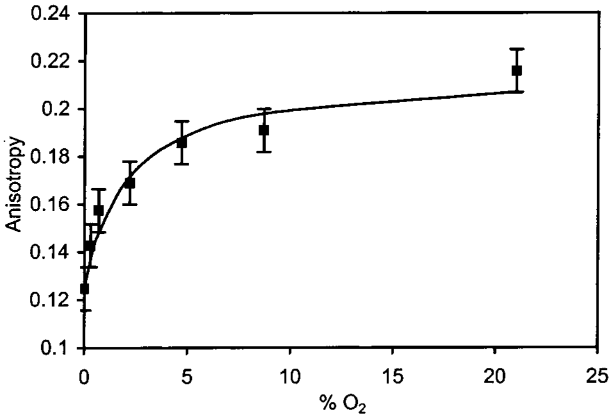

Figure 6. Calibration curve of the sensor. ■, experimental points; solid curve, model fit using the following parameters: $n_0 = 0.215$, $k_{SV} = 0.85$.

requirement for an extremely sensitive photodetector to compensate for the decrease in optical power. However, since the polarization spectra of the film run almost parallel between 625 and 700 nm (data not shown), it is possible to use this entire band for polarization determinations. In this case, the absolute value of the anisotropy will decrease, but the range will remain the same. This would not only enhance the output signal level, enabling the use of low-cost semiconductor photodetectors, but would also replace expensive monochromators with low-cost band-pass or long-pass filters.

In conclusion, we have shown that using a dual emission dye with largely different lifetimes enables simple anisotropy measurements, provided one of the peaks is affected by the analyte concentrations. In this particular case, the sensor was sensitive to oxygen. Using this approach, a dye with such properties could be used as a probe in viscous media (i.e., cell protoplasm) or for the development of polarization-based sensors.

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