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A Comparative Study of Optical Fluorescent Nanosensors ("PEBBLEs") and Fiber Optic Microsensors for Oxygen Sensing

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ABSTRACT

In this paper we report the use of phase sensitive fluorometry to obtain preliminary results from opto-chemical fluorescent oxygen nanosensors. PEBBLE (Probe Encapsulated By Biologically Localized Embedding) sensors were fabricated by immobilizing tris(4,7-diphenyl–1,10-phenanthroline)Ru(II) chloride and tris(1,10-phenanthroline)Ru(II) chloride within a polyacrylamide matrix. PEBBLEs have diameters of 20-200 nm and exhibit excellent performance for dissolved oxygen detection. Their performance is compared with micrometer-sized (10-20 μm) optical fiber sensors and free dye in solution. Oxygen sensing ability of PEBBLEs was tested in the presence of other quenchers and compared with free dyes in solution. While PEBBLEs have been developed for minimally invasive intracellular chemical analysis, they show additional advantages , such as increased dynamic range, compared to microsensors, and an absence of interference (quenching) by heavy ions, in contrast to free dye solutions.

Key words: Nanosensor; Microsensor; Optode; Oxygen sensor; Phase modulation.

1. INTRODUCTION

The detection and quantification of molecular oxygen levels are of prime interest in analytical, bio-medical and environmental applications. Extensive studies have been carried out, based on intensity methods of oxygen detection, in particular, where a dye has been immobilized in a thin film or coating[1,2,3,4]. However, intensity based measurements are susceptible to a number of errors resulting from dye bleaching, washout, changes in excitation intensity, and light losses in the optical fibers. These problems cause signal instabilities and, therefore, require frequent re-calibration of the sensor. Since the fluorescent lifetime of a species is an intrinsic property, it offers the possibility of inherent referencing. In the well-known phase modulation technique[5,6], the fluorescent dye is excited with a continuous, sinusoidal-modulated light source. Because of the finite lifetime of the excited state of the dye, the emission is delayed in time relative to the modulated excitation and described as the phase shift. Recent technological advances in the phase modulation technique have promoted intensive research using lifetime-based phase modulation methods to molecular oxygen sensing [7,8,9,10]. In recent years, considerable effort has been directed to create optodes incorporated into low-cost solid state opto-electronic systems based on phase modulation techniques[10,11,12,13].

Our group has specialized in producing micrometer and sub-micrometer fiber optic steady-state fluorescence sensors (optodes) for pH, O_2, K^+, Cl^-, NO_2^- and other analytes[14,15,16,17,18]. In addition, we have developed the smallest nano-optodes or PEBBLEs (Probe Encapsulated By Biologically Localized Embedding) capable of detecting pH, O_2, Ca^{+2}, Mg^{+2}, and K^+ [19,20]. For oxygen sensitive PEBBLEs this work was done using tris(4,7-diphenyl–1,10-phenanthroline)Ru(II) chloride (RuDPP) or tris(1,10-phenanthroline)Ru(II) chloride (RuPhen), immobilized in polyacrylamide matrix. The ruthenium complexes belong to a widely investigated family of oxygen sensitive dyes that undergo collisional photoluminescence intensity and lifetime quenching by molecular oxygen[10]. In the present work we report on the oxygen sensing properties of PEBBLEs as lifetime-based sensors, using the phase modulation technique, and compare their performance with this of fiber-optic microsensors and "naked" indicator dyes.

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2. MATERIALS AND METHODS

Tris(4,7-diphenyl–1,10-phenanthroline)Ru(II) chloride (RuDPP) and tris(1,10-phenanthroline)Ru(II) chloride (RuPhen) were purchased from GFS and Aldrich Chemicals. De-ionized water and phosphate buffer of ionic strength 100mM were used for all the experiments. Oxygen (>99%) and Nitrogen (>99%) were taken from a cylinder and the gas was bubbled through the PEBBLE solution in a sample vial for at least 20 min, sealed with a septum and the solution was extracted through a hypodermic needle.

The preparation of acrylamide PEBBLEs has been previously described[19]. Dissolving the crosslinker into the continuous phase (hexane) and the acrylamide into the non-continuous phase (water) forms a microemulsion. The polymerization solution consisted of 0.4 mM fluorescent ionophore, 27% by weight acrylamide, 3% N,N-methylenebisacrylamide, and 100mM phosphate buffer (pH=6.5). One ml of this solution was added to a solution containing 20 mL hexane, 1.8 mmol dioctyl sulfosuccinate, sodium salt and 4.24 mmol Brij 30. The solution was stirred under nitrogen for 20min while cooling in an ice bath. The polymerization was initiated with 24 µL of a 10% ammonium persulfate solution and 12 µL tetraethylene methylene diamine (TEMED). The polymerizing solution was allowed to stir at room temperature for 2 hours. Hexane was removed by rotary evaporation, then the probes were rinsed of surfactant with ethanol to give a product consisting of 20-200 nm diameter PEBBLEs.

The detailed fabrication of optical fiber microsensors has been described previously[21]. To produce micro-sized optical fiber sensors, the multimode silica optical fibers of 100 µm core diameter (Polymicro Inc.) were heated (25W CO_2 infrared laser Synrad Co.), pulled (micropipette puller P-87 Sutter Instrument Co.) and separated to form a pair of 5-10 µm pulled tips. Silanization is accomplished by immersion in a solution of gama-methacryloxy propyl trimethoxy silane. The sensor tip is attached via photopolymerization using the He 488 nm or the 532 nm line of the Nd:Yag. The monomer solution contains 30% acrylamide, 2.5% N,N methylene bisacrylamide, and 15 µl triethylamine is added to act as photoinitiator. The pulled tip of the optical fiber is placed into the beaker, while the distal end is coupled to the laser light source. The laser is shuttered on or off for 1-3 minutes producing the 15-20 µm oxygen sensor.

3. MEASURING SYSTEM

3.1 Phase sensitive method

For a single-exponential decay, the phase angle shift ϕ and modulation factor m, are related to the apparent phase lifetime τ_p and τ_m by[6]:

$$tan\phi_\omega = \omega\tau_p \tag{1}$$

$$m_\omega = (1 + \omega^2\tau_m^2)^{-1/2} \tag{2}$$

where ϕ - phase shift, m – demodulation factor, $\omega = 2\pi f$, and f is the linear modulation frequency.

The term "apparent" is used, because the apparent decay times are equal to the true decay times only for single exponential decays. In many cases, in particular when the dye is immobilized in a matrix, the ideal behavior displayed in solution is lost, and a multi-exponential type of lifetime decay is observed:

$$I(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \tag{3}$$

where the α_i are the amplitudes of components with decay times τ_i.

In the case of the excited-state population decaying exponentially, the intensity and lifetime can be described by the ideal Stern-Volmer equations:

$$I_0/I = 1 + k_{SV}[O_2] \tag{4}$$

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$$\tau_o/\tau = 1 + k_{SV}[O_2] \tag{5}$$

where I – luminescence intensity, τ -luminescence lifetime, $k_{SV} = k_2\tau_0$ – Stern-Volmer quenching constant, $[O_2]$ – oxygen concentration and the subscript 0 denotes values in the absence of oxygen.

A calibration curve of I_o/I vs. $[O_2]$ is linear, with the slope equal to k_{SV}. The Stern-Volmer quenching constant k_{SV} quantifies the quenching efficiency and therefore the sensitivity of the sensor. In solution, most dyes that undergo collisional quenching display ideal Stern-Volmer behavior. However, for sensors where the dye is immobilized in a support matrix, such as the polyacryliamide matrices in our work, the luminescence decay becomes multi-exponential or non-exponential and displays nonlinear Stern-Volmer plots[10,22,23]. An advantage of using the phase-sensitive technique is the ability to analyze multi-exponential decays, such that probes with complex intensity decays become suitable for sensing.

3.2 Instrumentation and experimental set-up

The photoluminescence spectra and frequency responses for the PEBBLEs and free dyes were measured with an ISS K2 lifetime phase sensitive fluorimeter. The excitation source was an air-cooled continuous-wave Argon ion laser (MKW Industries) tuned to 488 nm. The light source was coupled through the instrument's Pockels cell and amplitude modulated with a Marconi Instruments 2022D synthesizers and low-frequency modulator (K2.LF, ISS). Data was collected at 12 to 20 distinct frequencies between 1-500 kHz. Sensor emission was collected through a 515 nm high pass filter (Newport) using laser light scattered from a diluted glycogen solution as a reference signal during the data acquisition. A schematic drawing of the fluorescence detection system used for steady state and lifetime measurements is shown in Figure 1.
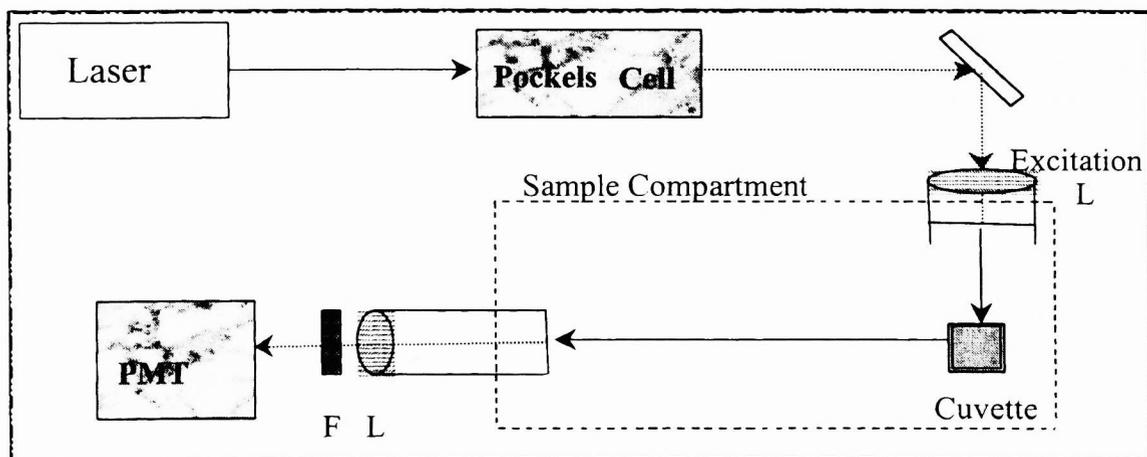

Figure 1: Schematic drawing of optical set-up for photoluminescence and time-resolved photoluminescence experiments. The emission is collected with ISS K2 Fluorimeter. L– lenses, F – filters.

The photoluminescence spectra of the microsensors were back collected using an Ocean Optics (Dunedin, FL) 2000 spectrometer, bifurcated fiber, and Argon ion laser tuned to 488 nm. As with the PEBBLES, the microsensors are immersed in gas saturated solutions to measure the sensor response to the analyte.

4. RESULTS AND DISCUSSION

4.1 Photoluminescence

The RuDPP and RuPhen are water-soluble molecular oxygen sensitive dyes with excitation and emission wavelength in the visible region (see Figure 2). Representative PL emission responses to molecular oxygen in gas-equilibrated buffer solutions of polyacrylimide PEBBLEs, containing RuDPP and RuPhen, are shown in Figure 2(a) and (b), respectively. The O_2 dependent PL spectra of both types of PEBBLEs have one broad emission, centered at 630nm (Figure 2(a)) and 610nm (Figure 2(b)). The emissions are centered at 630nm and 610nm in PL emission spectra, as is the free dye. The PL measurements for the microsensors show a blue shift of 15 to 20 nm, related to the matrix immobilization of the dye molecules (spectra not shown).

The dissolved oxygen Stern-Volmer plot based on PL spectra in the wide region of oxygen concentration is shown in Figure 3, for both types of PEBBLEs. For the RuDPP dye the curve exhibits a linear behavior with a sharp rise at lower oxygen concentrations and a slight down-curved quenching behavior at very high (oxygen saturated solution) concentrations. For the RuPhen dye the curve exhibits a linear behavior in all range. All the intensity quenching quotients (Q_{DO}) for free dyes, PEBBLEs and microsensors are summarized in Table 1. The calculation was done according to the following equation: $Q_{DO}=(I_0-I)/I$, where I_0 is the luminescence intensity and the subscript 0 denotes values in the absence of oxygen. In other
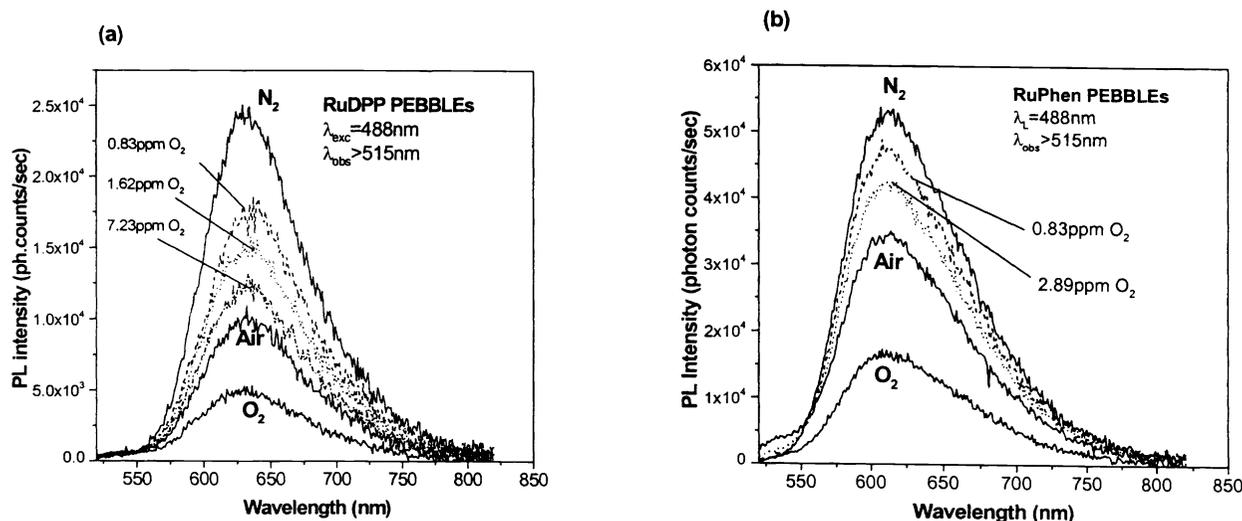

Figure 2: Photoluminescence spectra of RuDPP (a) and RuPhen (b) polyacrylamide PEBBLEs. Dye concentration in PEBBLE solution is 4.3µM (this concentration calculated according to the initial parameters of the PEBBLEs' preparation procedure). PEBBLEs are dispersed in gas-saturated phosphate buffer of ionic strength 100mM, pH=7.6. The emission has been excited with 488 nm Argon ion laser and collected through 515 nm high pass filter.

words, the quenching quotient quantifies the dynamic range of the opto-chemical system.

A linear behavior of the PEBBLEs in oxygen concentrations up to 15ppm (it should be noted that air oxygen content is 8.8ppm) is expected, since the intensity quenching quotients (Q_{DO}) for PEBBLEs and free dyes are similar (see Table 1). The quenching quotients for the microsensors for both dyes are considerably smaller (see Table 1), 5-6 times less for RuDPP and 2 times less for RuPhen[15,21], in comparison to the corresponding PEBBLEs. Therefore, the dynamic range of the PL

intensity-based measurements of Ru complexes in PEBBLEs remains almost as extended as for the free dye. This result indicates that the PEBBLEs do not show a large matrix effect, in contrast to the microsensors immobilized onto optical fibers.

4.0 — RuDPP and RuPhen PEBBLEs
3.5 — Stern-Volmer plots
3.0 — of intensity-based data
2.5
$(I_0/I)-1$ 2.0
1.5
1.0
0.5
0.0

0   10   20   30   40

Oxygen concentration (ppm)
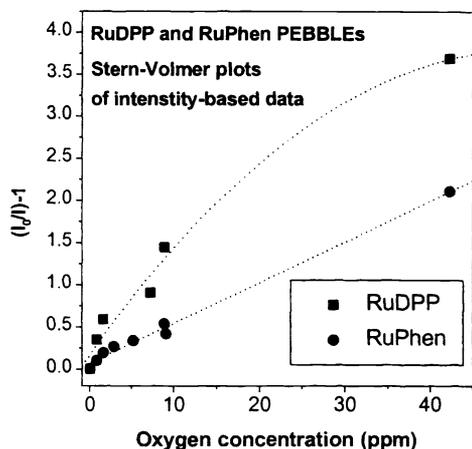
■ RuDPP
● RuPhen

Figure 4: Oxygen Stern-Volmer plot for RuDPP and RuPhen dyes immobilized in polyacrylamide PEBBLE matrix, dispersed in gas-saturated buffer at 22°C. The dotted line is a guide to the eye.

Table 1: Intensity Quenching parameters of RuDPP and RuPhen as free dyes, immobilized i the polyacrylamide matrix in PEBBLEs and in the microsensors.

	Free Dyes		PEBBLEs		Microsensors	
	RuDPP	RuPhen	RuDPP	RuPhen	RuDPP	RuPhen
Q_{DO} (%)	81	95	79	69	14	31
$N_{2\ sat.}$ $I_{PL}\ x10^5$ (ph.counts/sec)	2.3	5.2	0.24	0.52	0.68	0.19
$Air_{sat.}$ $I_{PL}\ x10^5$ (ph.counts/sec)	1.7	2.6	0.09	0.33		
$O_{2\ sat.}$ $I_{PL}\ x10^5$ (ph.counts/sec)	0.4	0.28	0.05	0.16	0.59	0.13

The dynamic range of PEBBLE nano-optodes may be associated with the relative size of the PEBBLE and the microsensor. The PEBBLE size is no more than 200 nm, whereas the microsensor size is on the order of 20 μm. However, the sensor sizes are related to the different methods that are used to make the sensors, and that may make the difference.

The different structures of the crosslinker and monomer suggest different water solubilities[24,25]. Acrylamide is water-soluble while the crosslinker is significantly less water soluble. The different solubilities indicate that the monomers will assume configurations that form aggregates having the more hydrophilic portions (i.e. the amide) of the molecules jutting out from the aggregate and the hydrophobic parts (i.e. the double bonds) of the molecules coming together to minimize contact with the aqueous environment. This affects reactivity by preventing the photo-initiator from having easy molecular level access to the different monomers. What may actually happen is the formation of a short chain of crosslinker molecules followed by addition of the acrylamide molecules. While there will be crosslinks, they may not be effective at altering the diffusivity of the forming hydrogel or the crosslinks may create localized regions of highly crosslinked gel and regions with very low crosslink density.

The synthesis of the PEBBLE sensors is different from the fiber sensors because they are made in a microemulsion with hexane as the continuous phase. The surfactants emulsify the water, monomer mixture, and hexane creating the microemulsion[23]. The polymerization occurs inside the micellar structures containing water and monomer. Here the initiator is the thermal initiator, ammonium persulfate.

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The microemulsion creates an environment that dissolves the crosslinker into the continuous phase (hexane) and the acrylamide into the non-continuous phase (water). The surfactant is responsible for emulsifying the two monomers together during the polymerization reaction[26]. This environment will provide a more evenly dispersed level of monomer and crosslinker. The resultant polymer or hydrogel will have a more consistent degree of crosslink density, without regions of very high and very low crosslink densities. The effect on the resultant sensors will be to have a more controlled and, probably, open structure, which allows for freer diffusion of analyte into and out of the PEEBLE sensor.

4.2 Time-resolved photoluminescence

Representative frequency responses of RuDPP and RuPhen PEBBLEs to molecular oxygen in gas-equilibrated solutions are shown in Figure 4(a) and (b), respectively. Both RuDPP and RuPhen PEBBLEs show similar behavior with regard to lifetime quenching. Double-exponential analysis reveals a major component (71%-83%) and a minor component with very short lifetime, presumably due to the excess light scattering from the dispersed PEBBLEs. The apparent lifetime of the dominant component decreases with increasing oxygen concentration and its values are summarized in Table 2. The minor component's lifetime seems to be unaffected by the changes and is not shown. The lifetime quenching quotient is calculated according to the following equation: $Q_{DO} = (\tau_0 - \tau)/\tau_0$, where τ is the luminescence lifetime and the subscript 0 denotes values in the absence of oxygen. For RuDPP the phase shift and modulation factor maxima, $\Delta\phi$ and Δm at 97kHz, are significantly large, and are 50% and 46%, respectively. For RuPhen these values were obtained at 190kHz, and are large as well: 45% and 40%. Frequency responses of "naked' Ru complexes dissolved in phosphate buffer (pH = 7.6) show single exponential decays, with dynamic ranges only 8 to 12 percent better than those of the corresponding PEBBLEs (see Table 2). These results confirm our earlier observation, based on PL intensity-based measurements, that the dye exhibits no significant matrix effect in the PEBBLE matrix, with regard to the dynamic range.
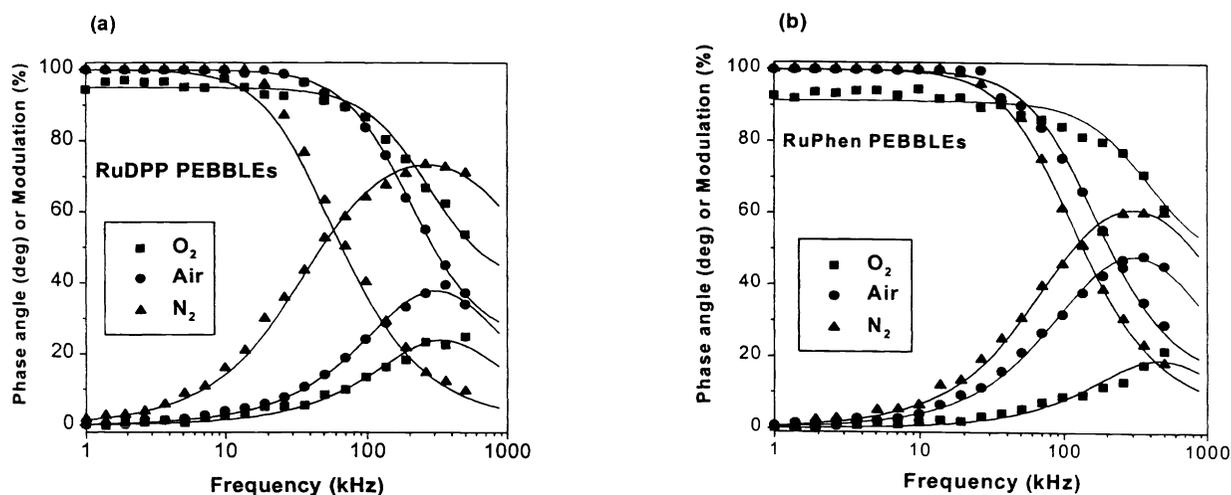

Figure 4: Frequency response of RuDPP (a) and RuPhen (b) polyacrylamide PEBBLEs despersed in gas-saturated phosphate buffer of ionic strength 100mM, pH=7.6, with dye concentration in PEBBLE solution of 4.3μM (this concentration calculated according to the initial parameters of the PEBBLEs' preparation procedure). The PL has been excited with 488 nm Argon ion laser and collected through 515 nm high pass filter.

Table 2: Lifetime Quenching parameters of RuDPP and RuPhen as free dyes, immobilized in polyacrylamide matrix in PEBBLEs. τ denotes the apparent lifetime obtained from single- or double-exponential analysis (major component) of the specie in gas-saturated buffer solutions.

	Free Dyes		PEBBLEs	
	RuDPP	RuPhen	RuDPP	RuPhen
Q_{DO} (%)	98	84	99	76
$N_{2 sat.}$ τ (μsec)	4.8	0.92	4.15	2.02
$Air_{sat.}$ τ (μsec)	1.7	0.46	1.06	1.16
$O_{2 sat.}$ τ (μsec)	0.07	0.14	0.05	0.48

4.3 Oxygen Sensing in the Presence of Salts and Heavy Metal Ions

Ru complex based PEBBLEs and free ("naked") dye solutions were examined with interference quenchers known as possible PL intensity and lifetime quenchers[5]. Saturated solutions of $Hg(CO_2CH_3)_2$, $AgNO_3$ and KI were added to the above solutions, while the mixed solutions were kept under nitrogen-saturated environment. The results of PL intensity-based measurements are summarized in Table 3. Dissolved dyes exhibit near complete or partial quenching of the emission, in contrast to the response of the dye immobilized in the PEBBLE's polyacrylamide matrix. The PEBBLE's capability of oxygen sensing is not affected at all by the presence of other quenchers, such as heavy ions (e.g. Hg^+ and Ag^+), which presumably do not permeate the PEBBLE.

Table 3: PL intensity quenching parameters of free dyes and PEBBLEs as a result of adding saturated solutions of salts. All the solutions were kept under gas-saturated nitrogen environment.

Saturated solutions added to the sample	PL intensity quenching of free dyes		PL intensity quenching of **PEBBLEs**	
	RuDPP	RuPhen	RuDPP	RuPhen
Hg Acetate	23%	98%	0%	0%
Ag Nitrate	75%	80%	0%	0%
K Iodide	72%	10%	0%	0%

5. SUMMARY

Nano-optodes containing RuDPP and RuPhen immobilized within a polyacrylamide matrix have been utilized for the phase sensitive detection of dissolved oxygen. The very bright luminescence emitted from the PEBBLEs dispersed in water solution is not quenched by a variety of heavy ions. The nano-optodes show high performance very similar to that of the free dye, in terms of dynamic range, quenching ratio and response time. On the other hand, in contrast to the free dyes, they show no interference by heavy ions. Such miniature sensors thus have many advantages over micrometer-sized optical fiber sensors on one hand,, and over free ("naked") dye indicator on the other, for precise intra-cellular analyte detection[19]. Hence, the combination of PEBBLE delivery to the living cell[19] and phase modulation fluorometry, will enable the non-invasive oxygen nano-optode to provide intra-cellular lifetime-based chemical images.

6. ACKNOWLEDGMENTS

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