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Real-Time Measurements of Dissolved Oxygen Inside Live Cells by Organically Modified Silicate Fluorescent Nanosensors

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Optical PEBBLE (probes encapsulated by biologically localized embedding) nanosensors have been developed for dissolved oxygen using organically modified silicate (ormosil) nanoparticles as a matrix. The ormosil nanoparticles are prepared via a sol−gel-based process, which includes the formation of core particles with phenyltrimethoxysilane as a precursor followed by the formation of a coating layer with methyltrimethoxysilane as a precursor. The average diameter of the resultant particles is 120 nm. These sensors incorporate the oxygen-sensitive platinum porphyrin dye as an indicator and an oxygen-insensitive dye as a reference for ratiometric intensity measurement. Two pairs of indicator dye and reference dye, respectively, platinum(II) octaethylporphine and 3,3′-dioctadecyloxacarbocyanine perchlorate, and platinum-(II) octaethylporphine ketone and octaethylporphine, were used. The sensors have excellent sensitivity with an overall quenching response of 97%, as well as excellent linearity of the Stern−Volmer plot ($r^2 = 0.999$) over the whole range of dissolved oxygen concentrations (0−43 ppm). In vitro intracellular changes of dissolved oxygen due to cell respiration were monitored, with gene gun injected PEBBLEs, in rat C6 glioma cells. A significant change was observed with a fluorescence ratio increase of up to 500% after 1 h, for nine different sets of cells, which corresponds to a 90% reduction in terms of dissolved oxygen concentration. These results clearly show the validity of the delivery method for intracellular studies of PEBBLE sensors, as well as the high sensitivity, which is needed to achieve real-time measurements of intracellular dissolved oxygen concentration.

Oxygen is one of the key metabolites in aerobic systems, and the measurement of dissolved oxygen is of major importance in medical, industrial, and environmental applications. Recent interest in the methods for measuring dissolved oxygen concentration has been focused mainly on optical sensors, due to their advantages over conventional amperometric electrodes in that they are faster, do not consume oxygen, and are not easily poisoned.[1−4] The principle behind the operation of these sensors is the reduction in luminescent intensity as a consequence of oxygen quenching of the emitting state. The sensor optode, either in typical film type or in recent nanoparticle type (probes encapsulated by biologically localized embedding (PEBBLE) sensor), consists of dye entrapped in a polymer with a high permeability to oxygen. While sensitivity depends only on the rate of quenching of the excited states for the free dye, sensitivity of the polymer-based sensor depends on both quenching rate and the matrix characteristics. Many published reports have described that the same sensing dye has different response behavior depending on the properties of the matrixes such as density, viscosity, and hydrophobicity.[5−8] Therefore, intrinsic properties of both dye and matrix need to be considered for the design of optical sensors. To maximize the performance of the oxygen sensor, dyes with long unquenched state lifetimes and polymers with high oxygen permeabilities should be utilized.

For the sensing dye, most work in this area has focused on the ruthenium complex,[2,3,9−11] Ru(II)−tris(4,7-diphenyl-1,10-phenanthroline)$^{2+}$ ($[Ru(dpp)_3]^{2+}$), which has a long excited lifetime (5.3 μs) and a high luminescence quantum yield (∼30%) and is readily quenched by oxygen.[12] To produce more sensitive oxygen sensors, longer-lived, luminescent oxygen-sensitive dyes must be used. Moreover, a longer excitation and emission wavelength is preferred in order to increase the signal-to background ratio, especially for cellular or in vivo measurements,

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because of reduced background light scattering and autofluorescence from cells in the red and near-IR regions. The phosphorescent porphyrins of platinum, platinum(II) octaethylporphine (PtOEP) or platinum(II) octaethylporphine ketone (PtOEPK), have the desirable features of longer lifetimes (0.06−0.09 ms), convenient excitation and emission wavelengths with large Stokes shifts (100−170 nm), and reasonable luminescence quantum yields (10−50%).[13] However, only a few studies on sensors containing platinum porphyrin dyes for the measurement of dissolved oxygen have been reported so far,[14−16] while many have reported on their superior sensitivity in gaseous oxygen sensing.[5−7,13,17]

As to the matrix, an oxygen-permeable polymer with a low diffusion barrier for oxygen is desired. Among many published reports, sol−gel-derived glass has been proven to be a promising matrix for oxygen sensing, due to its many useful properties, such as high oxygen permeability, mechanical and chemical stability, and optical clarity. We note that the oxygen-quenching behavior in water is different from what has been observed in gas phase. The number density of oxygen molecules in air-saturated water is quite lower than that of air, under ambient conditions, and the dissolved oxygen has a lower diffusion coefficient compared with the gaseous oxygen. These factors result in a lower quenching sensitivity and a wider nonlinear response toward dissolved oxygen, compared to gaseous oxygen.[18] Some sol−gel-based works using ruthenium dye have shown that sensor response for dissolved oxygen is greatly enhanced by the use of the organically modified silicates (ormosil) matrix.[3,9,18,19] Ormosils are organic−inorganic hybrid materials in which organic fragments are built into silicon oxide networks. The precursor for ormosil usually contains an alkyl-substituted silicon alkoxide, structurally acting as a network modifier that terminates the silicate networks and provides the desired properties for a range of applications. The introduction of alkyl group increases the hydrophobicity and porosity of the matrix, resulting in an improved linearity of the sensor as well as an improved sensitivity.

While most polymer-based sensors have been made of film on planar surfaces, or on tips of optical fibers, a new kind of sensor made of nanoparticles, the PEBBLE sensor, was developed in this laboratory. PEBBLE sensors encapsulate the dyes inside an inert nanoparticle, to achieve noninvasive cellular measurements, by avoiding both physical and chemical interferences between sensor and sample. A sol−gel silica oxygen PEBBLE sensor previously developed by our group showed an improved sensitivity and linearity comparable to ormosil film-type sensors, indicating that PEBBLE sensors have the advantage of negligible matrix effects, due to their very small sizes.[2]

To further increase the sensitivity and linear response range of the oxygen sensors, we have developed an oxygen PEBBLE sensor with platinum porphyrins rather than a ruthenium complex dye, entrapped in ormosil nanoparticles. In this paper, we describe the preparation of ormosil PEBBLEs and their characterization, stability, and applications to real-time oxygen analysis inside rat C6 glioma cells. The measurement by PEBBLE sensors was done ratiometrically, with a second internal standard fluorophore with emission at a different wavelength, to compensate for the signal changes due to fluctuations in light intensity, detector sensitivity, and light scattering.

EXPERIMENTAL SECTION

Reagents. Phenyltrimethoxysilane (PTMS), methyltrimethoxysilane (MTMS), and ammonia were obtained from Aldrich (Milwaukee, WI) and used without further purification. PtOEP and octaethylporphine (OEP) were purchased from Frontier Scientific, Inc. (Logan, UT). 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO) was purchased from Molecular Probes (Eugene, OR). PtOEPK was synthesized according to a method in the literature.[20,21] The chemicals for synthesis of PtOEPK, including osmium tetroxide (very toxic), anhydrous ether, dichloromethane (99% HPLC), pyridine, benzonitrile, anhydrous sodium acetate, and platinum chloride, were obtained from Aldrich (Milwaukee, WI). (3-(*N*-morpholino)propanesulfonic acid) sodium salt (MOPS) was purchased from Sigma (St. Louis, MO). All solutions were prepared from 18-MΩ water purified by a Barnstead 1 Thermolyne Nanopure II system.

Gases. O_2 (99.6%, extra dry grade), air (dry grade), and N_2 (99.998%, prepurified) were obtained from Cryogenic Gases (Detroit, MI). Nitric oxide/nitrogen gas mixture (5.03% NO, certified) and nitric oxide (99%, CP grade) were obtained from Matheson Tri-Gas.

PEBBLE Preparation. To a 50-mL round-bottomed flask, in a water bath kept at 60 °C on a Corning pc-351 hot plate stirrer, 31 mL of deionized water and 38 μL of HNO_3 were added and the solution was stirred until the temperature of the reaction mixture reached to 60 °C. A 0.1-mL sample of PTMS was then added to the flask, and the resultant mixture was stirred at full speed. After 20 min, 6 mL of ammonium hydroxide was added at once. The resultant solution was kept stirring for additional 1−2 h or until the solution became milky. A mixture of sensing dye and reference dye in a proper solvent and 0.2 mL of MTMS were added into the reaction mixture, which was kept stirred for additional 1 h. The dye solution mixtures used were as follows: for PtOEP oxygen PEBBLEs, 0.5 mL of PtOEP (1.1 mg/mL in THF) and 120 μL of DiO (0.5 mg/mL in 5:2 ethanol/THF mixture); and for PtOEPK PEBBLEs, 0.4 mg of OEP and 1.2 mg of PtOEPK in 1.6 mL of 5:4 THF/ethanol mixture. The resulting PEBBLEs were suction filtered through a Fisherbrand glass microanalysis vacuum filter holder with a 0.1-μm Osmonics/MSI Magna Nylon membrane filter. The PEBBLEs were rinsed three times with water and then resuspended in a water/ethanol 1:2 mixture, sonicated for 5 min, then filtered through a 0.02-μm Whatman Anodisc filter membrane, and allowed to air-dry.

Optical Apparatus for Ratiometric Sensor Measurements. Fluorescence spectra from PEBBLE sensors were mostly obtained

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on an Olympus inverted fluorescence microscope, IMT-II (Lake Success, NY), using Nikon 50-mm *f* /1.8 camera lenses to project the image into an Acton 150-mm spectrograph (Acton, MA) with spectra read on a Princeton Instruments, liquid nitrogen-cooled, 1024 × 256 CCD array (Trenton, NJ). The PEBBLEs were excited using a mercury arc lamp (Opti Quip, Highland Mills, NY) with the excitation and emission light filtered by a filter cube unit. We have used an Olympus blue dichroic mirror unit (module IMT2-DMB, excitation wavelength 350−500 nm, and long-pass emission filter 515 nm) for PtOEP PEBBLEs and an Omega filter cube (excitation filter 570DF20, dichroic mirror 590DRLP, and long-pass emission filter 600ALP) for PtOEPK PEBBLEs. For the leaching experiment, measurements were taken with a 570-nm excitation light on a FluoroMax-3 spectrofluorometer with slits set to 5 and 2 nm for emission and excitation, respectively. Images of PEBBLE-loaded cells were obtained using a Perkin-Elmer Ultra View confocal microscope system equipped with an Ar−Kr laser, Omnichrome Series 43 (Melles Griot Inc., Irvine, CA). Data on intracellular PEBBLE response and function were acquired in 3-D and in real time using software-controlled multichannel filter wheels and a piezoelectric-driven *z* stage with the UltraView software package. The 568-nm line was used for excitation of PtOEPK PEBBLEs. The emission filter for OEP fluorescence was a band-pass filter at 620 nm with 60-nm bandwidth and that for PtOEPK fluorescence was a band-pass filter at 750 nm with bandwidth 70 nm.

Scanning Electron Microscope (SEM) Imaging. The PEBBLEs were dispersed in water and sonicated for 10 min to prevent aggregation of particles. Then a drop of the PEBBLE solution was placed on the SEM specimen mount (aluminum) and dried gradually at room temperature. The sample was then sputter coated with gold, and the SEM images were taken on the Phillips XL30 SEM.

PEBBLE Calibration and Reversibility. Sensor calibration was made in a glass gastight chamber with gas inlet and outlet ports. A 4-mL sample of 0.2 mg/mL PEBBLE solution was placed in the chamber, and the chamber is closed. The introduction of gas and ventilation were made by inserting a 4-in. hypodermic needle into the solution through the septum-sealed inlet port and by inserting a 1.5-in. needle through the septum-sealed outlet port, respectively. The excitation light was introduced through the inverted microscope objective lens from underneath the chamber. Different dissolved oxygen concentrations were obtained by flowing, from the gas blender into the PEBBLE solution, a gas mixture of predetermined nitrogen and oxygen concentrations. The spectra of PEBBLE solution were taken three to four times at each different oxygen concentration, and the peak ratios were measured and averaged. The peak ratio (R) is the fluorescence intensity of the indicator dye divided by the fluorescence intensity of the reference dye. All measurements were performed at a constant temperature of 22 ± 0.5 °C. The oxygen concentrations in air-saturated and oxygen-saturated solutions were calculated to be 9.0 and 42.8 ppm at 22 °C, respectively. This calculation was based on the solubility equation of oxygen in water.[22] The Stern−Volmer curve was constructed for calibration by plotting the ratio of peak ratio at oxygen-free condition (R_0) to the peak ratio at different oxygen concentrations (R) versus calculated dissolved oxygen concentration. The calibration study was repeated with fresh PEBBLE solution at least once to ensure the reproducibility of the results.

The reversibility of the PEBBLE sensor response to dissolved oxygen was monitored using the same setup for the calibration over 60 min by alternate purging of air, nitrogen, and oxygen gases.

PEBBLE Leaching. A leaching test was performed by preparing 1 mg/mL PEBBLE solution in MOPS buffer (100 mM, pH 7.2) and monitoring the fluorescence of filtrate over 6 days. The PEBBLE solution was stirred in the Amicon Ultra filtration setup with a 5000-kDa membrane filter. At various time intervals, 3 mL of filtrate was collected, analyzed, and put back into the Amicon.

Photostability. A photobleaching test of the PEBBLEs was performed. A 1 mg/mL PEBBLE suspension in ethanol/water mixture was prepared, and a drop of the solution is placed and air-dried on a glass microslide. The sample was continuously illuminated by the mercury lamp with the filter cube as described before. The light intensity at the sample location after passing the filter cube was 20 μW. A spectrum was acquired every 30 s over a 20-min period.

Cell Culture. Two types of cells were used for the studies. Rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium containing 400 mg/L D-glucose, 2 mM L-glutamine, 20% fetal bovine serum, 0.3% penicillin, streptomycin, and neomycin and incubated at 37 °C in a 5% CO_2 environment. Cells were subcultured 2 days prior to experiments and plated at a density of 100 000 cells/plate on uncoated 22-mm glass coverslips in 35-mm culture dishes. Virus transformed mouse, alveolar (lung) macrophage cells (MHS) were cultured in RPMI-1640 medium containing 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4500 mg of glucose/L, 1500 mg of sodium bicarbonate/L, 10% fetal bovine serum, 0.3% penicillin, streptomycin, and neomycin and incubated at 37 °C in a 5% CO_2 environment. Cells were subcultured 4 days prior to experiments and plated at a density of 100 000 cells/plate on uncoated 22-mm glass coverslips in 35-mm culture dishes. To prevent contamination after PEBBLE experimentation. the plated cells were not returned to the cell culture incubators.

PEBBLE Delivery and Intracellular Measurements. PEBBLEs were delivered into the intracellular environment using a BioRad (Hercules, CA) Biolistic PDS-1000/He Gene Gun system. A thin film of ormosil oxygen PEBBLEs suspended in water was dried on the target membrane. The cells (on glass coverslip) were removed from the culture medium and rinsed with 37 °C 1× Hanks buffered salt solution and placed in a microscope cell. The microscope cell was placed in the gene gun chamber and 15-in.Hg vacuum was applied to the chamber. The PEBBLEs were delivered successfully using a firing pressure of 900−1000 psi with the cell culture placed 45 mm below the carrier disk. Following PEBBLE delivery, the cells were rinsed three times with 37 °C Hanks 1× buffered salt solution and allowed 30 min of recovery, in a nonsterile incubator, before experimentation in the same solution. For experimentation, the cells (on glass coverslip) were transferred to a gastight microscope chamber (inner volume ~21 mL) with 1 mL of the same buffered solution. Cells in a gastight

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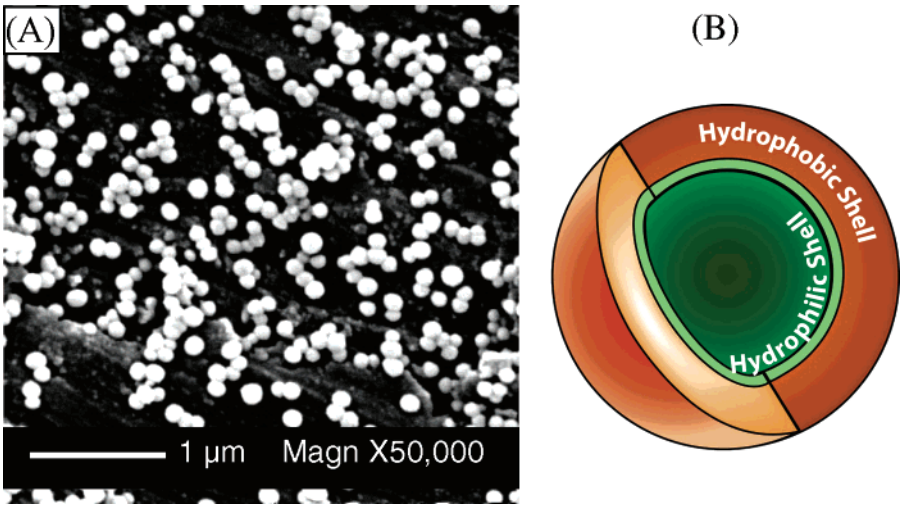

Figure 1. (A) SEM image of typical ormosil PEBBLEs containing platinum porphyrin dyes. The average diameter of the particles is 120 nm. (B) Schematic diagram of ormosil PEBBLE.

chamber are then placed on the IMT-II Olympus inverted fluorescent microscope. For monitoring the response under air-, N_2-, and O_2-saturated cells, each gas was blown through the solution at a very low rate (5 mL/min) for 10 min before taking the spectra. For monitoring the response to cell respiration, the spectra of PEBBLEs were first taken while the cells were still open to air and then the chamber containing cells was sealed. The spectra of PEBBLEs in cells were then taken periodically during the course of the experiment. Cell viability was evident from cell respiration.

Table 1. Ormosil Particle Size with PTMS Concentration and Hydrolysis Time at 60 °C

av diameter (nm)	PTMS concn (M)	hydrolysis time (min)
800	0.06	0.5
575	0.06	2
300	0.06	4
280	0.03	20
150	0.02	20
100	0.01	20

RESULTS AND DISCUSSION

Ormosil PEBBLE Formation. *Ormosil Nanoparticles.* Ormosil nanoparticles were synthesized using two precursors, PTMS and MTMS. First, PTMS-based nanoparticles are formed through a two-step method based on the sol−gel process:[23] In the first step, the hydrolysis of PTMS was performed under acidic condition. In the second step, the condensation of the silane progressed under basic condition. The average size of the particles by this method was reported to range from 300 to 800 nm.[23] The oxygen PEBBLE sensors have been developed mainly for intracellular or in vivo oxygen sensing. Attempts to reduce the PEBBLE size were made since the smaller the particles are, the shorter the response time is and the less biological damage will be caused by the introduction of PEBBLEs into the cells for intracellular study. Hydrolysis time in the first step and monomer concentration are key reaction parameters for size control. A successful reduction of the size of particles was achieved by reducing the PTMS monomer concentration and increasing hydrolysis time. Table 1 compares the size of particles at different PTMS concentrations and hydrolysis times at 60 °C. These PTMS-based nanoparticles have poor solubility in aqueous solution, which makes sensor calibration to dissolved oxygen in water as well as potential in vivo application difficult. Therefore, another ormosil layer, using the less hydrophobic MTMS precursor, is coated over the PTMS-based nanoparticles to increase suspension in aqueous solution. The formation of the initial core particles and of the coating layer proceeded in the same solution.

Dye Incorporation. For typical PEBBLE sensor preparation, dyes are first dissolved in a suitable solvent and usually added into the reaction mixture at the beginning of synthesis, to achieve homogeneous incorporation of dyes into the nanoparticles. As the PTMS-based particle formation utilizes phase separation (silane/water), the organic solvent, at the early stage, affects the formation of initial particles. In addition, PTMS-based nanoparticles are soluble in organic solvents. Both the sensing dyes and reference dyes used in this study are hydrophobic and dissolve in organic solvents but not in water. To avoid any interference from the dye solution (hydrophobic dyes and organic solvent) on the initial particle formation, these dye solutions were added after the formation of PTMS-based particles was completed, but right before MTMS was added. The morphology of the dye-incorporated MTMS/PTMS nanoparticles was determined by SEM. Figure 1A shows typical monodisperse spherical ormosil PEBBLEs with average diameter of 120 nm.

We note that the ormosil nanoparticles synthesized by the above procedure can be loaded with hydrophilic dyes as well as hydrophobic dyes. This is somewhat unexpected as the organically modified silica matrix is more hydrophobic than plain silica matrix. However, water-soluble hydrophilic Texas Red sulfonyl chloride was tried as a reference dye. When added in the initial acidic reaction mixture, it was successfully incorporated into the PEBBLEs, without any leaching into the aqueous suspension. The ormosil particles used for the present study seem to have a hydrophilic inner shell and hydrophobic outer shell, which can be utilized for effective loading of both hydrophobic and hydrophilic dyes into the particles (see schematic diagram in Figure

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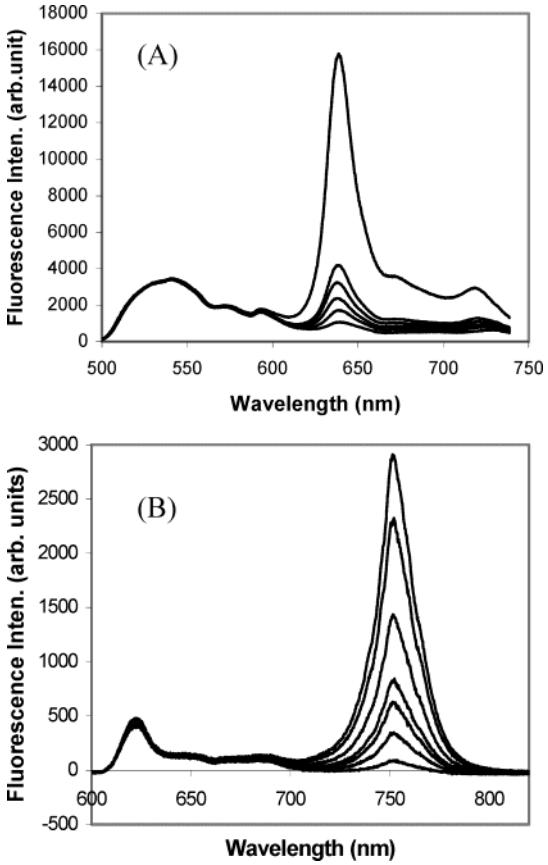

Figure 2. Fluorescent spectra of two different ormosil PEBBLE sensors suspended in water. For both PEBBLEs, the peak on the left is the fluorescence emission from the reference dye while that on the right is the emission from sensing dye. (A) PtOEP/DiO ormosil PEBBLEs: The calculated DO (dissolved oxygen) concentrations for each spectrum are 0.0 (nitrogen saturated), 0.5, 0.9, 1.8, 9.0 (air-saturated), and 43 ppm (oxygen-saturated) from top to bottom lines. (B) PtOEPK/OEP ormosil PEBBLEs: The calculated DO concentrations for each spectrum are 0.0 (nitrogen saturated), 0.5, 1.8, 3.6, 5.4, 9.0 (air-saturated), and 43 ppm (oxygen-saturated) from top to bottom lines.

1B). This attractive feature provides for a more versatile utilization of these particles as PEBBLE sensors, compared to other nanoparticles.

Oxygen PEBBLE Response and Calibration. The fluorescence emission spectra of PtOEP/DiO PEBBLEs and PtOEPK/OEP PEBBLEs in different dissolved oxygen (DO) concentrations are shown in Figure 2. The sensor response was determined from the ratio (R) of the fluorescence intensity maximum of sensing dye to that of reference dye (I_{PtOEP}/I_{DiO} or I_{PtOEPK}/I_{OEP}). The sensor sensitivity can be expressed by the overall quenching response to DO (Q_{DO}), which is defined by

$$Q_{DO} = (R_{N_2} - R_{O_2})/R_{N_2} \times 100$$

where R_{N_2} is the fluorescence intensity ratio in fully deoxygenated water and R_{O_2} is that in fully oxygenated water.

The quenching response of polymer-based oxygen sensors also depends on the polymer matrix. The measured values of Q_{DO} for both PtOEP and PtOEPK PEBBLEs are 97%, which is the highest among those reported so far (See Table 2).

Table 2. Properties of Dissolved Oxygen Sensors

probe	sensor type	Q_{DO} (%)	ref
$[(Ru(dpp)_3]^{2+}$	silica film	30	18
$[(Ru(dpp)_3]^{2+}$	ormosil film	56−80	3
$[(Ru(dpp)_3]^{2+}$	silica nanoparticle	80	2
PtOEP	ormosil nanoparticle	97	this work
PtOEPK	ormosil nanoparticle	97	this work

The spectra show that the response of PtOEP PEBBLEs is more sensitive than that of PtOEPK at a low concentration of dissolved oxygen. By changing from nitrogen-saturated solution (DO 0 ppm) to air-saturated solution (DO 9.0 ppm), the ratio of the peaks was reduced by a factor of 14 for PtOEP PEBBLEs and by a factor of 9 for PtOEPK PEBBLEs. Such lowered intensity of PtOEP PEBBLEs under air-saturated conditions may become an obstacle to obtaining good data with high signal-to-background noise ratio (S/N), especially for in vitro or in vivo experiments. It should be noted that there is always a tradeoff between the sensitivity of sensors to the oxygen concentration and the S/N limitations, because the luminescence intensity of the sensors is rather low when the luminescence quenching is very strong. Moreover, the PtOEP dye has lower excitation and emission wavelengths than the PtOEPK dye, which is disadvantageous for contemplated in vitro or in vivo applications. Therefore, further research efforts were focused on the PtOEPK PEBBLEs.

The oxygen-quenching process in a homogeneous system is described by the linear Stern−Volmer equation:

$$I_o/I = K_{SV}^{conc}[O_2] = K_{SV}^{gas}pO_2$$

where K_{SV}^{conc} and K_{SV}^{gas} are the Stern−Volmer constants expressed in terms of solution oxygen concentration and gas-phase partial pressure.

However, nonlinear Stern−Volmer plots have been observed for many polymer film-based sensors,[7,8,24] due to matrix effects, such as oxygen permeability or microheterogeneity of the local environment of the dye molecules in the sensing layer. Any nonlinearity of response is a major drawback in the practical application of the sensors.

Figure 3 shows the Stern−Volmer plot of the fluorescence intensity ratios, versus dissolved oxygen concentrations, for the ratiometric ormosil PtOEPK PEBBLEs. The solid line represents the best linear fits generated by the least-squares method. The oxygen ormosil PEBBLE sensors have a nearly straight line in the Stern−Volmer plot over the whole range of oxygen concentration (0% free oxygen to 100% oxygen saturated), indicating negligible matrix heterogeneity effects. Such complete linearity has only been observed before for "naked" molecular probes. The oxygen silica PEBBLE sensors previously developed in this laboratory showed a narrower linearity range.[2]

The high Q_{DO} and linearity in the Stern−Volmer plot over the whole range of dissolved oxygen concentrations may result from the structure and hydrophobic nature of the ormosil nanoparticles used in this study. PTMS has a phenyl group, which does not

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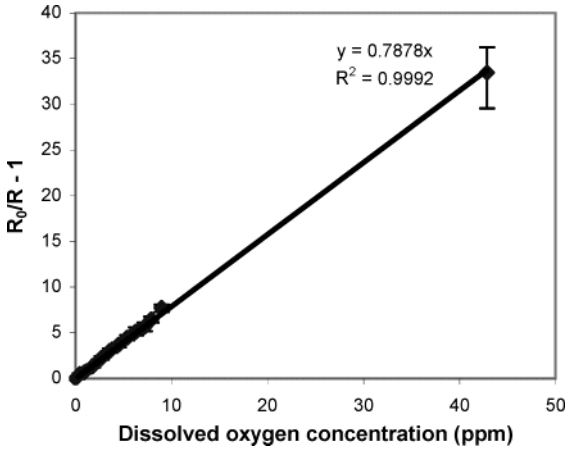

Figure 3. Stern−Volmer plot of relative fluorescence intensity ratios for ratiometric ormosil PtOEPK PEBBLEs. R_0 is the ratio between the fluorescent intensities of PtOEPK and OEP in the absence of oxygen, and R is the ratio at a given oxygen concentration.

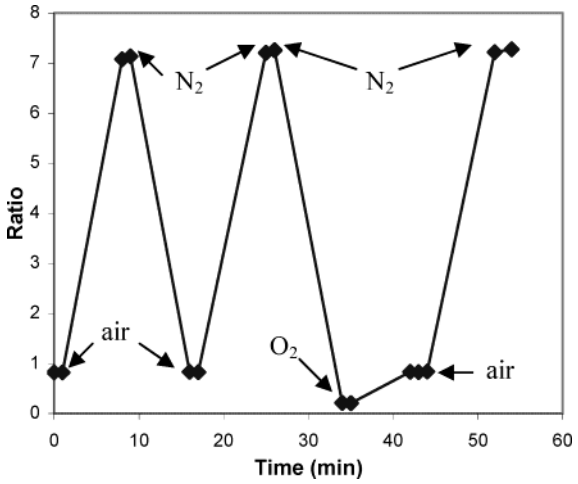

Figure 4. Reversibility of PEBBLE sensor response to dissolved oxygen. The fluorescence ratio measured was plotted vs the experimental time.

undergo hydrolysis throughout the sol−gel process. The bulky phenyl group inside the structure provides higher porosity and hydrophobicity than the methyl or ethyl group containing ormosil reported previously. As the pore can serve as an oxygen channel to the sensing dye molecules entrapped in the matrix, and as oxygen has a higher affinity for hydrophobic matrixes, the ormosil matrix in the current study leads to an increase in the sensitivity of sensors to DO, as well as to an improvement in the linear response. Moreover, the small size and sphericity of the PEBBLEs reduce the needed depth of penetration of oxygen into the matrix and provide a larger surface-to-volume ratio, compared to the film-type sensors, resulting in a larger linear range in the Stern−Volmer plot.

Leaching Test. Over 6 days, no appreciable signals were observed in the filtrate spectra at the emission wavelengths of the two dyes. This indicates that there was no detectable leaching of dye molecules out of the ormosil matrix. This is expected due to the hydrophobic nature of both the matrix and the entrapped dyes.

Photostability and Storage Stability. Under illumination of the mercury lamp light, the overall change in the peak ratio was 24% over 20 min. The half-life, $t_{1/2}$, of OEP under continuous illumination was 9 min and that of PtOEPK was >20 min. In our biological applications, typical exposure times per measurement consisted of only 100-ms exposure times and the number of measurements for each set of cellular experiments in this report was 2−7. As the total exposure time was less than 1 s, the data presented here were not affected by the photodegradation of the dyes inside the matrix. Furthermore, the photostability is limited by the reference dye, so the introduction of a more stable reference dye would further improve the sensor photostability.

The ormosil PEBBLE sensors displayed excellent storage stability. No changes in spectral or quenching characteristics were observed for dry PEBBLE sensors over 12 months in the absence of light, at room temperature. An aqueous suspension of PEBBLE sensors showed no change in response after 12-month storage in the refrigerator.

PEBBLE Reversibility. The ratio of the fluorescence maximum of PtOEPK to that of OEP was analyzed after alternate purging of the suspended aqueous sensor solution with air, N_2, and O_2 for ~7 min at a 50 mL/min flow rate. The sensors showed complete recovery each time that the sensing environments were changed between air-, N_2-, and O_2-saturated sensor solutions, as can be seen in Figure 4.

Specificity of the PtOEPK PEBBLE Sensor. The oxygen sensing of the PEBBLE sensor is based on the quenching of the PtOEPK dyes, which may be quenched by other reactive species. In fact, a film-type oxygen sensor containing PtOEP had shown that its response to oxygen was affected by the presence of nitrogen oxides (NO_x) in the gas phase.[25] We have tested the interference of dissolved NO and dissolved CO with the oxygen sensing of the PEBBLEs.

NO Interference. The interference due to NO was tested by purging the PEBBLE suspension first with nitrogen, second with NO, and then with nitrogen again. The purging procedure was repeated to check the reversibility of the PEBBLE response to NO. Two types of NO were used: 99% NO and 5.03% NO in nitrogen. The test solution was prepared in buffer solution (Hanks 1×) to avoid any possible pH change due to NO. The peak intensity of PtOEPK changed with NO but that of OEP remained unchanged. The overall quenching responses to the dissolved species were calculated as in Table 3:

The PEBBLE response to oxygen is affected by the presence of NO. However, the PEBBLE response to NO is much less sensitive than that to oxygen. Considering the physiological concentration of NO (1 nM to 1 μM, i.e., 0.03 to 30 ppb),[26] NO interference should not affect the intracellular oxygen measurement by the PtOEPK oxygen PEBBLE sensor. In addition, the ratio of fluorescent maximums of indicator and reference dyes changed back to the previously measured value under each gas-saturated environment after repeated purging cycles. This indicates that the PEBBLE response recovered completely after exposure to NO. This is different from the PtOEPK film-type sensor results by Papkovsky et al., which showed irreversible degradation of dye molecules after exposure to gaseous nitrogen oxides.[27]

(25) Papkovsky D. B. *Sens. Actuators, B* **1995**, *29*, 213−218.
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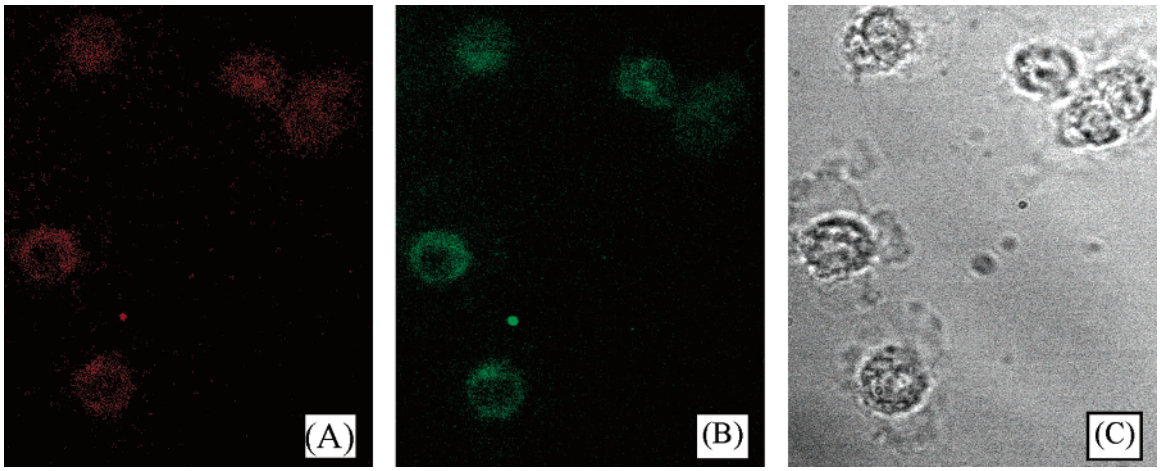

Figure 5. Confocal images of live macrophage cells injected with PtOEPK ormosil oxygen PEBBLEs. The excitation wavelength was 568 nm, and the magnification 60× was used for all three images. (A) OEP fluorescence of PEBBLEs inside cell (620-nm emission band-pass filter). (B) PtOEPK fluorescence of PEBBLEs inside cell (750-nm emission band-pass filter). (C) Bright-field image of cell.

Table 3. Overall Quenching Response of the PEBBLE Sensor

	NO, 99%	NO, 5%	O_2	air
calcd dissolved NO concn (ppm)[a]	60	3	0	0
calcd dissolved O_2 concn (ppm)[a]	0	0	43	9
Q_{DX} (%)	73	18	97	86

[a] The calculation was based on the solubility equation of gas from ref 22.

CO Interference. The CO interference was tested in the same way as for NO interference. The ratio of two fluorescent dyes did not change by purging with CO gas, indicating no interference.

Intracellular Study. Confocal microscopy was used to determine the localization of the PEBBLE sensors after gene gun delivery. Figure 5 shows the confocal images of the PEBBLEs delivered into alveolar macrophage cells by gene gun. The image indicates that the PEBBLE sensors are localized within the cell boundaries.

To verify the responsiveness of the oxygen PEBBLE nanosensors to intracellular oxygen concentration, the spectra of PEBBLEs inserted inside rat C6 glioma cells in Hanks buffer solutions were taken by purging with gas in the order of air, nitrogen, and oxygen (see Figure 6). As can be seen, the PEBBLE sensors maintain their spectral characteristics and sensitivity after gene gun delivery into the cells.

The measurements of intracellular oxygen concentrations were repeated with additional sets of adhered cells injected with oxygen PEBBLE sensors. The average ratio (R) was measured to be 1.01, which corresponds to a dissolved oxygen concentration of 7.8 ppm, based on the calibration curve given in Figure 3. The measured intracellular oxygen value is comparable with previously reported values.[2,28]

After each measurement of oxygen concentration under open air, the gastight chamber containing the cells was closed to shut off the air supply. A series of spectra were taken over 1 h. As live

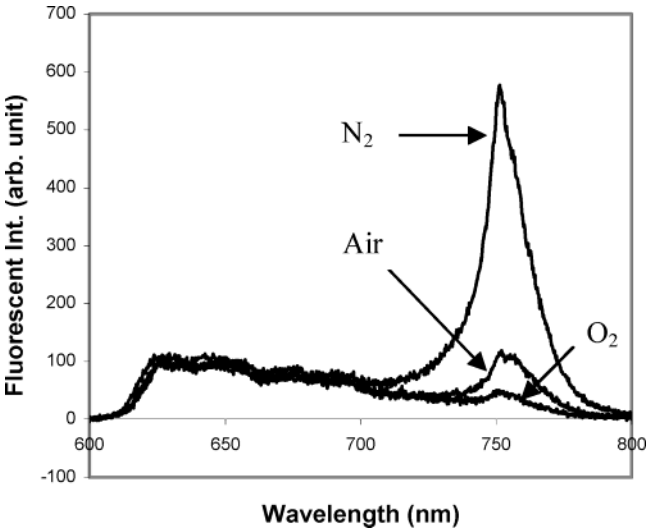

Figure 6. Fluorescence spectra of typical PEBBLE sensors inserted inside rat C6 glioma cells in air-, nitrogen-, and oxygen-saturated buffer solutions.

rat C6 glioma cells breathe and consume the oxygen, the observed change would result from cell respiration and hence provide evidence of the viability of the cells after gene gun shooting, as well as offer real time measurements of intracellular oxygen concentrations. We note that the concentration measured by the PEBBLE sensors is the one localized in the cells, since the PEBBLEs are located only in the cells. Figure 7 shows a typical profile obtained by monitoring the change in peak ratio (i.e., dissolved oxygen concentration) due to the respiration of the cells confined in a container. A signigicant increase in the peak ratio was observed, with ratio increases of 50−500% after 1 h, for nine sets of cells, which corresponds to 30−90% reductions in terms of oxygen concentration. The variation in the ratios may be attributed to the number of cells on each glass slide and the condition of the cells. This result clearly indicates the validity of the delivery method for intracellular studies of PEBBLE sensors and the high sensitivity of the developed PEBBLEs, sufficient to achieve real-time measurements of the intracellular dissolved oxygen concentration.

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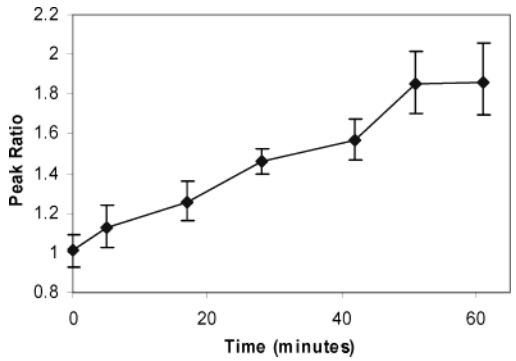

Figure 7. Typical peak ratio profile of the oxygen PEBBLE sensors in C6 glioma cells in a closed container. The ratio of indicator to reference dye increases with time, indicating a decrease of the dissolved oxygen concentration, due to respiration.

CONCLUSIONS

This paper presents the description of a ratiometric, highly linear, and highly sensitive dissolved oxygen PEBBLE sensor. The sensor consists of ormosil nanoparticles loaded with platinum porphyrin dyes, two of the most oxygen-sensitive dyes, and proper reference dyes. The highly permeable structure and the hydrophobic nature of the ormosil nanoparticles, as well as their small size, result in a superb overall quenching response to dissolved oxygen and a linear response over the whole range, from 0 to 100% oxygen-saturated water. Compared to the previously developed oxygen PEBBLE sensor in this laboratory, this PEBBLE sensor has a higher sensitivity and a wider linearity as well as longer excitation and emission wavelengths, resulting in reduced background noise for cellular measurement. These PEBBLE sensors have excellent reversibility and stability to leaching and long-term storage. The photostability of the sensors was limited by the photostability of the reference dye but was sufficient to perform cellular measurements. A real-time monitoring of changes in the dissolved oxygen due to cell respiration in a sealed chamber was made by gene gun delivered PEBBLEs. The intensity ratio changed as cells consumed the oxygen, demonstrating cell viability after PEBBLE delivery and excellent intracellular response of the sensors to dissolved oxygen. This sensor is being used now for simultaneous intracellular measurements of oxygen and glucose.

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