Anal. Chem. **2009,** *81,* 9239–9246

A Noninvasive Thin Film Sensor for Monitoring Oxygen Tension during in Vitro Cell Culture

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Oxygen tension in mammalian cell culture can profoundly affect cellular differentiation, viability, and proliferation. However, precise measurement of dissolved oxygen in real time remains difficult. We report a new noninvasive sensor that can accurately measure oxygen concentration during cell culture while being compatible with live-cell imaging techniques such as fluorescence and phase contrast microscopy. The sensor is prepared by integrating the porphyrin dye, Pt(II) *meso*-tetrakis(pentafluorophenyl)porphine (PtTFPP) into polydimethylsiloxane (PDMS) thin films. Response of the sensor in the presence of oxygen can be characterized by the linear Stern−Volmer relationship with high sensitivity (K_{SV} = 584 ± 71 atm^{-1}). A multilayer sensor design, created by sandwiching the PtTFPP-PDMS with a layer of Teflon AF followed by a second PDMS layer, effectively mitigates against dye cytotoxicity while providing a substrate for cell attachment. Using this sensor, changes in oxygen tension could be monitored in real-time as attached cells proliferated. The oxygen tension was found to decrease due to oxygen consumption by the cells, and the data could be analyzed using Fick's law to obtain the per-cell oxygen consumption rate. This sensor is likely to enable new studies on the effects of dissolved oxygen on cellular behavior.

In vivo, oxygen levels vary across a wide spectrum. While atmospheric oxygen enters the lungs at an oxygen partial pressure of 21% (P_{O2} = 0.21 atm = 2.1 × 10^4 Pa), the oxygen level decreases as it circulates through the body, reaching a mean oxygen level in tissue of ∼3−5% (P_{O2} = 0.03−0.05 atm).[1,2] In addition, some cells experience a dynamic oxygen environment. For example, hepatocytes in vivo are exposed to gradients of oxygen[3,4] while immune cells encounter different oxygen levels as they migrate through different tissues.[5] The variations of oxygen level throughout the body can significantly impact disease and wound healing processes. For instance, low oxygen levels mark the onset of wound healing process, but prolonged hypoxia will eventually lead to nonhealing wounds.[6] An in vitro study demonstrated how low oxygen levels lead to vasoocclusion in sickle cell anemia.[7] Cancer cells that exist in hypoxic conditions are characterized as more virulent and more resistant to radiotherapy.[8]

Despite the significance of oxygen tension in vivo, conventional cell culture methods rarely specify oxygen levels and most in vitro cell cultures are still performed at atmospheric oxygen levels (P_{O2} = 0.21 atm). There are many reported examples where the disparity between in vivo and in vitro oxygen levels resulted in misleading experimental conclusions. For example, in vitro studies of stem cells have demonstrated greater proliferation[1] and reduced spontaneous differentiation[9] at physiological oxygen tension (P_{O2} = 0.05 atm), as well as oxygen tension-dependent differentiation pathways.[10] Similarly the oxygen tension during cell culture has been shown to alter the intracellular redox state of T cells[2] and to affect the response of lymphocytes toward HIV viral protein.[11] Hepatocyte cultures must be exposed to a gradient of oxygen instead of a uniform oxygen tension in order to mimic the in vivo compartmentalization of liver.[3,4] These findings point to the critical need to monitor and control oxygen tension in cell cultures.

During in vitro culture, oxygen is locally consumed by the cells and is replenished through equilibrium with the bulk culture medium. However, at high cell densities or long culture periods, depletion can lead to changes in bulk oxygen tension. Therefore, real time measurement strategies are needed to continuously monitor oxygen levels. Indeed, changes in the rate of oxygen consumption in cell culture can also provide a direct measurement of cellular metabolic activity. Traditionally, the Clark electrode

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(1) Csete, M. *Ann. N. Y. Acad. Sci.* **2005**, *1049*, 1−8.
(2) Atkuri, K. R.; Herzenberg, L. A.; Niemi, A. K.; Cowan, T.; Herzenberg, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4547–4552.
(3) Allen, J. W.; Bhatia, S. N. *Biotechnol. Bioeng.* **2003**, *82*, 253–262.
(4) Allen, J. W.; Khetani, S. R.; Bhatia, S. N. *Toxicol. Sci.* **2005**, *84*, 110–119.
(5) Sitkovsky, M.; Lukashev, D. *Nat. Rev. Immunol.* **2005**, *5*, 712–721.
(6) Malda, J.; Klein, T. J.; Upton, Z. *Tissue Eng.* **2007**, *13*, 2153–2162.
(7) Higgins, J. M.; Eddington, D. T.; Bhatia, S. N.; Mahadevan, L. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 20496–20500.
(8) Brown, J. M.; William, W. R. *Nat. Rev. Cancer* **2004**, *4*, 437–447.
(9) Ezashi, T.; Das, P.; Roberts, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 4783–4788.
(10) Lennon, D. P.; Edmison, J. M.; Caplan, A. I. *J. Cell. Physiol.* **2001**, *187*, 345–355.
(11) Sahaf, B.; Atkuri, K.; Heydari, K.; Malipatlolla, M.; Rappaport, J.; Regulier, E.; Herzenberg, L. A.; Herzenberg, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5111–5116.

10.1021/ac9013379 CCC: $40.75
Published on Web 10/27/2009

has been utilized to monitor oxygen.[12,13] However, since the electrode consumes oxygen during measurement, it can potentially alter the oxygen concentration in culture. Also, for real-time measurements, the electrode has to be placed inside the culture chamber, creating a source of contamination for the culture and complicating simultaneous imaging of the cells. Spectroscopic techniques can provide less invasive methods of measuring the oxygen tension, based on the quenching of phosphorescent molecules. For example, ruthenium(II) complexes loaded in polymeric beads[14] or on microplates[15,16] can be used for continuous oxygen measurements. Also, oxygen probes synthesized by conjugating an oxygen-sensitive dye to macromolecular carriers (e.g., PEG or BSA) have been used to measure oxygen levels in cell culture.[17] Previously, these approaches have been relatively incompatible with imaging, and these sensors have been standalone, rather than integrated. In addition, low oxygen sensitivity, significant photobleaching during long culture periods, and possible fouling of the probes by the surrounding culture medium have limited widespread implementation.

In this report we describe a thin film polymeric sensor for making sensitive real-time oxygen measurement that is compatible with phase contrast and fluorescence microscopy of cells. The principle of the sensor is based on the oxygen-dependent quenching of phosphorescence from a platinum porphyrin dye embedded within a gas-permeable film. We have developed a multilayer design that prevents leakage of dye into the cells and thereby eliminates issues related to dye cytotoxicity, while still maintaining high sensor sensitivity. To demonstrate the utility of the sensor, we have continuously monitored changes in oxygen tension during long-term cell culture experiments for NIH 3T3 mouse fibroblasts. The data were used to determine the per-cell rate of oxygen consumption for the case of the NIH 3T3 fibroblasts.

EXPERIMENTAL SECTION

Cell Culture. Human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, VA) (certain commercial products are identified in order to adequately specify the experimental procedure; this does not imply endorsement or recommendation by NIST) were cultured in F12K Medium (ATCC), supplemented with 0.1 mg/mL heparins (Sigma, St. Louis, MO), 0.03 mg/mL Endothelial Cell Growth Supplement (Sigma), and 10% (v/v) fetal bovine serum. NIH 3T3 mouse fibroblasts (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagles Medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% (v/v) fetal bovine serum and the nonessential amino acids and glutamine (4 mmol/L). Both cell lines were maintained in humidified air balanced with 5% (v/v) CO_2 at 37 °C. Unless otherwise stated, cells were maintained inside the incubator until experiments began.

Cell Staining. Live/Dead Viability/Cytotoxicity Kit (L-3224) assay and Hoechst 33342 stain (both from Invitrogen Corporation, Carlsbad, CA) were performed according to the manufacturer's direction. For the Live/Dead assay, calcein AM (4 mmol/L) and ethidium homodimer-1 (2 mmol/L) were diluted in culture medium to get a final concentration of 1 μmol/L and 4 μmol/L, respectively. Cells were washed with phosphate-buffered saline (PBS) twice, and the stain solution was added. Cells and stain were then placed in the incubator for 30 min before being imaged. For nuclear staining with Hoechst 33242, the dye was dissolved in DMEM and an equivalent volume of dye solution was added to each cell culture well resulting in a concentration of 2 μg/mL Hoechst 33342. Cells were imaged without rinsing after 15 min of incubation with the staining solution.

Sensor Preparation. Two oxygen sensor compositions were explored in this study. The first sensor consisted of the porphyrin dye Pt(II) *meso*-tetrakis(pentafluorophenyl)porphine (PtTFPP, Frontier Scientific Inc., Logan, Utah) dissolved in toluene and thoroughly mixed with a 10:1 ratio of polydimethylsiloxane prepolymer:curing agent solution (PDMS, Slygard 184; Dow-Corning, Midland, MI) and spin-coated onto 18 mm glass coverslips. Toluene was allowed to evaporate overnight while the polymer cured. The final PtTFPP sensor film had a dye concentration of 1 mmol/L and was 66 μm thick. The second composition consisted of a three-layer sensor (PtTFPP-Tef-PDMS) prepared by spin-coating Teflon AF (Dow Chemical) onto the PtTFPP sensor films described previously and allowing solvents to evaporate overnight. Finally, an additional film of PDMS without dye was spin -coated and cured on top of the Teflon. All sensor formulation covered the entire surface of the coverslip with an area of 2.54 cm^2. The final thickness of the PtTFPP-Tef-PDMS film was approximately 150 μm. Sensor thickness was measured using an optical profiler (WYKO NT100 DMEM; Veeco, Tucson, AZ). Sensors were stored in the dark and used within 2 weeks of being made; new sensors were used for each experiment. The fabrication strategy (spin-coating sequential sensor layers) could be rapidly adapted to a variety of sensor geometries. This sensor formulation was not optimized but was used consistently throughout this work. Ongoing efforts focus on sensor design optimization.

Sensor Calibration. PtTFPP sensor and PtTFPP-Tef-PDMS films were placed inside a multiwell dish modified to allow continuous gas flow (0%, 5%, 10%, 15%, or 21% O_2 in N_2; Scotts Specialty Gas; Plumsteadville, PA) through the dish headspace. Oxygen flux through the dish material (polystyrene) was minimal ($D_{O2\ in\ PS} \approx 10^{-7}$ cm^2s^{-1}) and was ignored.[18] While controlling the headspace composition, the PtTFPP emission intensity was captured using a 10× (0.3NA) objective on an inverted microscope (Zeiss Axiovert 200, Thornwood, NJ) with the focus placed on the surface of the sensor (Figure 1a). The films were illuminated with an X-Cite metal halide light source (EXFO, Ontario, Canada) at 546 ± 6 nm, and the emission was captured using a 580 nm dichroic, a Color IEEE-1394 camera (Scion Corporation, Frederick, MD) with an integration time of 70 ms, and a long pass emission filter with a 590 nm cutoff.

All images were analyzed using NIH ImageJ (http://rsbweb.nih.gov/ij/) software. Blank PDMS films (without PtTFPP) spun to the same thickness as the oxygen sensors were also imaged

(12) Mamchaoui, K.; Saumon, G. *Am. J. Physiol.* **2000**, *278*, L858–L863.
(13) Tokuda, Y.; Crane, S.; Yamaguchi, Y.; Zhou, L.; Falanga, V. *J. Cell. Physiol.* **2000**, *182*, 414–420.
(14) Kuang, Y. N.; Walt, D. R. *Biotechnol. Bioeng.* **2007**, *96*, 318–325.
(15) Guarino, R. D.; Dike, L. E.; Haq, T. A.; Rowley, J. A.; Pitner, J. B.; Timmins, M. R. *Biotechnol. Bioeng.* **2004**, *86*, 775–787.
(16) Wang, W. J.; Upshaw, L.; Strong, D. M.; Robertson, R. P.; Reems, J. A. *J. Endocrinol.* **2005**, *185*, 445–455.
(17) O'Donovan, C.; Hynes, J.; Yashunski, D.; Papkovsky, D. B. *J. Mater. Chem.* **2005**, *15*, 2946–2951.
(18) Arain, S.; Weiss, S.; Heinzle, E.; John, G. T.; Krause, C.; Klimant, I. *Biotechnol. Bioeng.* **2005**, *90*, 271–280.

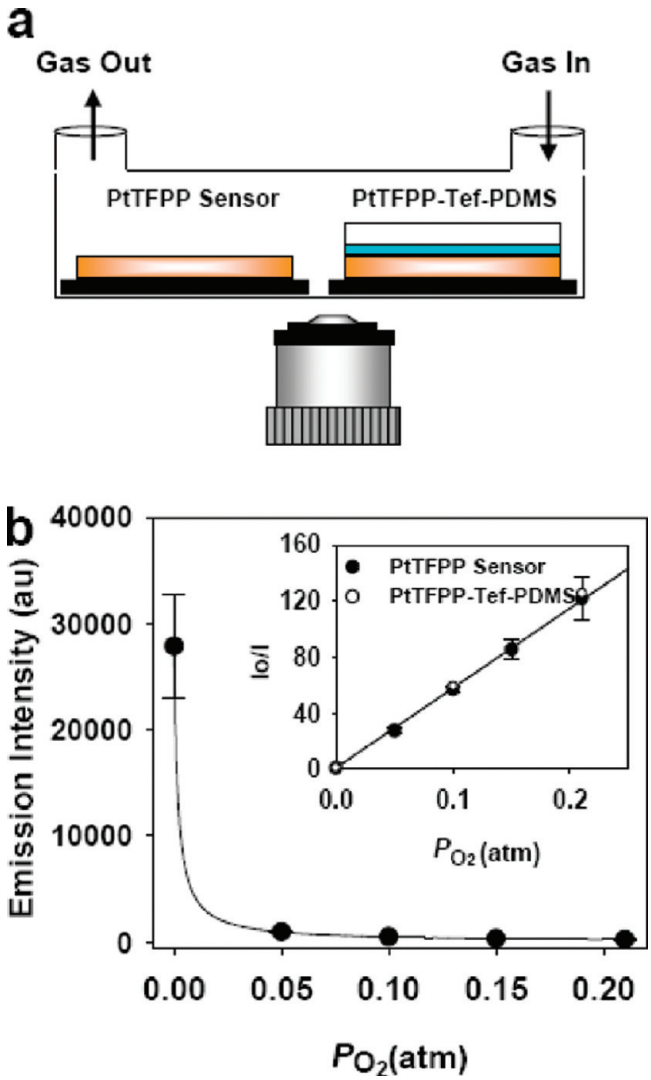

Figure 1. Gas-phase calibration of the thin film PtTFPP and PtTFPP-Tef-PDMS oxygen sensors. The experimental setup for acquiring P_{O2} measurements on an inverted microscope at controlled gas compositions is shown schematically (a). The phosphorescent oxygen sensors exhibited significant quenching in the presence of oxygen (b) and followed the Stern−Volmer equation (inset) with high linearity and sensitivity (K_{SV} = 584 ± 71 atm^{-1}). Black circles represent PtTFPP sensors (n = 4), and open circles represent PtTFPP-Tef-PDMS (n = 10) films. Data points indicate the mean values from the sensor films with standard deviations given by the vertical error bars; solid lines indicate the best fit to the data using the Stern−Volmer relationship.

to account for background intensity. These intensity values were subtracted from all sensor images in order to obtain the actual intensity from the dye itself. To determine the sensor response, K_{SV} was calculated from a linear regression using the Stern−Volmer equation. The reported mean K_{SV} and standard deviation were determined from 29 positions on 14 different sensors. To measure the dissolved oxygen in solution, culture medium without phenol red was added on top of the oxygen sensor, and gas was introduced as described above to control the headspace composition.

Sensor Preparation for Cell Culture. The oxygen sensor was bonded to the inside of a 12-well cell culture plate with 0.5 μL of PDMS. Prior to cell seeding, the sensor was cleaned with 70% ethanol and rinsed with PBS. Bovine fibronectin solution (Sigma, St. Louis, MO) was diluted with PBS to 25 μg/mL and added to the culture well for 1 h to facilitate cell attachment. The fibronectin solution was aspirated and freshly trypsinized cells (HUVEC or NIH 3T3) were subsequently seeded on the fibronectin-coated sensor.

Cytoxoticity Study. NIH 3T3 cells were seeded at 60 cells/mm^2 on fibronectin treated films and allowed to attach overnight in 1 mL of phenol red free culture medium to minimize background fluorescence. Cells were exposed to fluorescent excitation light with an integration time of 70 ms every 15 min for 3.5 h. Live/Dead cell assays were performed as described above on PDMS, PtTFPP sensors, and PtTFPP-Tef-PDMS and five positions were selected and imaged, with all conditions evaluated in triplicate. Live cells were imaged and counted with an excitation wavelength of 470 nm and emission of 540 nm. Dead cells were imaged and counted using a filter cube with excitation at 546 nm and emission at 590 nm. Total viable cells were reported by dividing the number of live cells over the total cell count for each position.

O_2 Measurement in Cell Culture. NIH 3T3 or HUVEC cells were seeded on sensors prepared for cell culture and allowed to attach overnight in 1 mL of culture medium without phenol red. Cells were placed on an inverted Axiovert 200 M microscope (Zeiss, Thornwood, NJ) with an automated stage (Ludl, Hawthorone, NY) and maintained at 37 °C in a microscope incubator (In Vivo Scientific) attached to the microscope.

In initial experiments, the P_{O2} in the headspace above the cell culture medium was controlled as described in the sensor calibration section. For subsequent experiments during extended cell culture, cells were maintained on the microscope throughout the experiment. The culture headspace was maintained with humidified 5% CO_2 in balanced air (In Vivo Scientific) and maintained at 37 °C as described above. Cells were cultured in 3 mL of culture medium without phenol red. Image capture began immediately after seeding. Prior to the start of each experiment, three positions were selected in each well. All experiments were performed in triplicate; control wells contained medium without cells. Under these conditions, the doubling time of NIH 3T3 cells was ≈20 h.

RESULTS AND DISCUSSION

Sensor Calibration in Gas and Liquid. The phosphorescent platinum porphyrin dye Pt(II) *meso*-tetrakis(pentafluorophenyl)porphine (PtTFPP) has been used previously to measure the partial pressure of oxygen because of its compatibility with common polymers and a lack of photobleaching.[19−21] In the current study, PtTFPP was dissolved in a gas-permeable PDMS polymer matrix that was spin-coated to form thin film oxygen sensors (this sensor formulation is referred to as PtTFPP sensor). This thin film geometry allowed rapid diffusion of oxygen throughout the dye-filled matrix. As expected, the phosphorescence from PtTFPP sensor was significantly quenched in the

(19) Puklin, E.; Carlson, B.; Gouin, S.; Costin, C.; Green, E.; Ponomarev, S.; Tanji, H.; Gouterman, M. *J. Appl. Polym. Sci.* **2000**, *77*, 2795–2804.
(20) Papkovsky, D. B.; O'Riordan, T. C. *J. Fluoresc.* **2005**, *15*, 569–584.
(21) Lee, S. K.; Okura, I. *Anal. Commun.* **1997**, *34*, 185–188.

presence of oxygen gas (Figure 1b). The quenching response followed the Stern−Volmer equation:[22]

$$\frac{I_0}{I} = 1 + K_{SV} \times P_{O2} \quad (1)$$

where I is the phosphorescence intensity, I_0 is the intensity in the absence of oxygen, K_{SV} is the Stern−Volmer constant, and P_{O2} is the partial pressure of oxygen in the gas phase. Thus, a plot of I_0/I vs P_{O2} follows a straight line (Figure 1b). From the slope, $K_{SV} = 584 \pm 71$ atm^{-1} (mean and standard deviation of 14 different films). As expected, the sensor response was less reproducible at higher oxygen levels where significant quenching results in low emission intensity. This is evident in the relative large standard deviation at atmospheric oxygen conditions. However, the K_{SV} value for this oxygen sensor was much higher than values reported for other dyes, yielding a sensor with greater sensitivity than previously reported formulations.[15,23−25]

In a second sensor formulation, thin layers of Teflon AF and PDMS without dye were spin-coated on top of the PtTFPP sensor (the resulting sensor is denoted as PtTFPP-Tef-PDMS). The presence of the thin Teflon layer did not alter the response of PtTFPP-Tef-PDMS sensor toward oxygen compared to the PtTFPP sensors (Figure 1b, open circles) but did sequester the dye away from the sensor surface. For both sensor formulations, equilibrium was faster than the time required to change the headspace gas composition (<1 min), reflecting the high permeability of both Teflon AF (1500 barrer) and PDMS (800 barrer).[26,27]

Previous oxygen sensors based on the quenching of ruthenium complexes exhibited lower K_{SV} values ($0.2 - 40$ atm^{-1}) and were confounded by issues of dye aggregation.[15,23−25] Those dyes were relatively polar and ionic, leading to low solubility and aggregation in nonpolar polymer matrices (e.g., PDMS). Dye aggregation has been associated with high variability in oxygen response, nonlinear Stern−Volmer plots, and uneven background emission that limits integration.[24,25] In comparison with previous dyes, the PtTFPP dye was relatively nonpolar and readily dissolved in the PDMS polymer matrix without dye aggregation or phase separation.[25] As a result, the PtTFPP-based sensors exhibited a homogeneous intensity and its response to P_{O2} levels followed the Stern−Volmer relationship. Additionally, no significant photobleaching effects were observed even during continuous illumination for >3600 s (Supporting Information, Figure S-1).

Next, the PtTFPP sensor was placed at the bottom of a multiwell dish filled with cell culture medium. The oxygen partial pressure within the multiwell dish headspace was decreased in steps. After each step, the amount of dissolved oxygen in solution gradually decreased as it equilibrated with the controlled headspace composition. The rapid PtTFPP sensor equilibration (<1 min) allowed this transient behavior in solution to be monitored in real time (Figure 2).

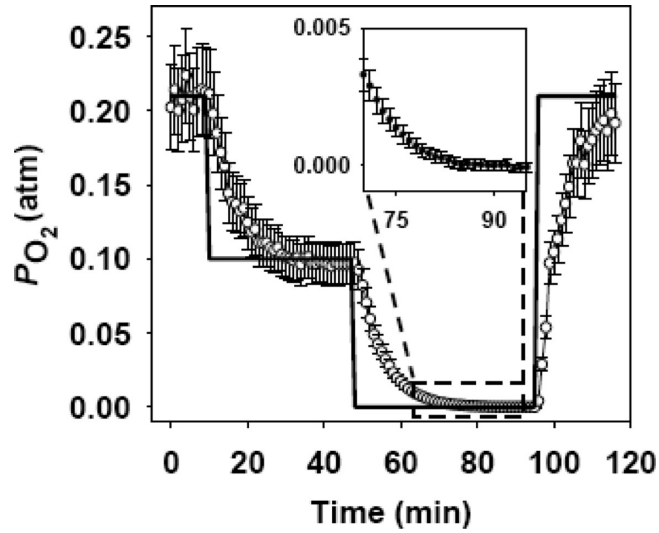

Figure 2. Performance of the oxygen sensor in cell culture medium. The oxygen tension in solution was measured (open circles) following step changes in the headspace gas composition (solid trace). The inset shows that the oxygen level in solution eventually decreased to 0 atm (within error) when pure N_2 was present in the headspace. Vertical error bars indicated the standard deviation in K_{sv} and I_0 used to calculate P_{O2}.

The sensor calibration yielded a measurement of gas-phase oxygen partial pressure even though it is the dissolved oxygen within the PDMS that actually quenches the PtTFPP dye. This calibration assumes that the sensor equilibrates rapidly with the adjacent medium so that the oxygen concentration in the PDMS is always proportional to the P_{O2} in the gas phase. For solution-phase measurements, the rapid sensor equilibrium ensures accurate measurement of the dissolved oxygen in solution, and the calculated oxygen level reports the gas-phase P_{O2} (in atm) that would yield an identical oxygen concentration (in mol/L) at equilibrium. This was verified in Figure 2 when the solution was allowed to equilibrate, and the resulting oxygen measurement matched the controlled headspace composition. These units are interchangeable using Henry's Law Constant for oxygen in water ($H_{O2\ in\ H2O} = 933$ L·atm/mol at 37 °C).[28] The gas-phase P_{O2} was reported here even for aqueous phase measurements as is conventional for reporting oxygen tension.

Sensor Cytotoxicity Studies. Cell viability assays were performed with NIH 3T3 cells to identify any potential cytotoxic effects with the sensors. All sensor films were coated with fibronectin to facilitate cell attachment. In the absence of illumination, cells proliferated as well on the PtTFPP-based sensors as on polystyrene culture dishes (Supporting Information; Figure S-2). However, upon exposure to phosphorescent excitation (546 nm for 0.07 s every 15 min for 3.5 h), cells on PtTFPP sensors were observed by phase microscopy to round up and detach (Figure 3a). A Live/Dead assay utilizing calcein AM to stain live cells and ethidium homodimer to stain dead cells confirmed significant phototoxicity for the PtTFPP sensor (Figure 3b,c). Cells attached to PDMS without PtTFPP proliferated normally, and no

(22) Lakowicz, J. R. *Principles of Fluorescence Sepctroscopy*; Plenum Press: New York, 1983.
(23) Bukowski, R. M.; Ciriminna, R.; Pagliaro, M.; Bright, F. V. *Anal. Chem.* **2005**, *77*, 2670–2672.
(24) Bukowski, R. M.; Davenport, M. D.; Titus, A. H.; Bright, F. V. *Appl. Spectrosc.* **2006**, *60*, 951–957.
(25) Bedlek-Anslow, J. M.; Hubner, J. P.; Carroll, B. F.; Schanze, K. S. *Langmuir* **2000**, *16*, 9137–9141.
(26) Pinnau, I.; Toy, L. G. *J. Membr. Sci.* **1996**, *109*, 125–133.
(27) Merkel, T. C.; Bondar, V. I.; Nagai, K.; Freeman, B. D.; Pinnau, I. *J. Polym. Sci., Part B: Polym. Phys.* **2000**, *38*, 415–434.
(28) Geankoplis, C. J. *Transport Process and Unit Operations*, 3rd ed.; Prentice-Hall Inc.: Englewood Cliffs, NJ, 1993.

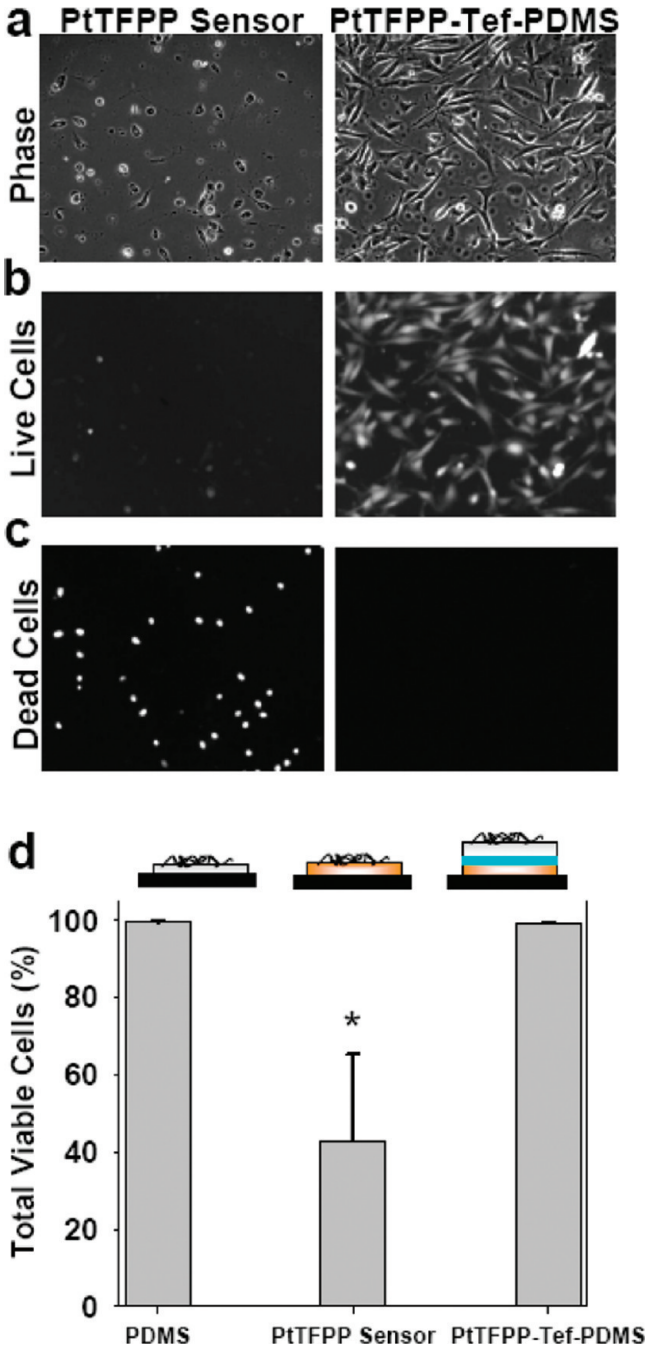

Figure 3. Phototoxicity of sensor formulations. NIH 3T3 cells were cultured on three surfaces (PDMS, PtTFPP sensor, and PtTFPP-Tef-PDMS) exposed to 70 ms exposures of 546 nm light every 15 min for 3.5 h and evaluated by phase contrast imaging (a) as well as by Live/Dead assays (b, c). Significant phototoxicity was observed on the PtTFPP sensor as compared to PDMS without dye (Supporting Information; Figure S-3), but the three-layer sensor mitigated the phototoxic effects. The percentage of viable cells was quantified for each substrate (d) and a two-tail *t* test was used to compare each sensor formulation to the PDMS without dye (* indicates $p < 0.05$). Columns and error bars indicate mean and standard deviation from triplicate experiments.

phototoxicity was observed (Supporting Information; Figure S-3). The excited PtTFPP has been shown previously to produce reactive oxygen species (ROS) when quenched by oxygen,[29] The short lifetime of these reactive species should preclude their diffusion out of the sensor after illumination. However, the PtTFPP dye itself may escape from the PtTFPP sensors and partition into the cell membranes, leading to ROS generation locally and the observed phototoxicity. In the second sensor formulation, PtTFPP-Tef-PDMS, phototoxicity was mitigated. Here a thin Teflon AF layer was added on top of the PtTFPP sensor, followed by a second PDMS layer without dye. We hypothesize that while oxygen diffuses easily through the Teflon layer, the PtTFPP would be effectively blocked due to the low diffusivity of larger molecules through Teflon AF.[30] The second PDMS layer was added to facilitate protein adsorption and cell attachment. Cells cultured on the PtTFPP-Tef-PDMS sensor (Figure 3a) exhibited similar morphology to cells on just PDMS (Figure S-3). Quantification of the Live/Dead assay (Figure 3d) confirmed that the phototoxicity was completely mitigated with the PtTFPP-Tef-PDMS formulation.

All of the sensor films (including the nontoxic PtTFPP-Tef-PDMS formulation) were compatible with common microscope methods, allowing oxygen sensing and microscopy to be performed simultaneously. Using phase microscopy, cell motility as well as filopodia movement were easily observed through time-lapsed imaging. Likewise, the fluorescence from cells stained with calcein AM and ethidium homodimer in the Live/Dead assay was easily visualized. Even though the emission wavelengths of the PtTFPP and the ethidium homodimer overlapped, the PtTFPP dye was homogeneous throughout the sensor film while the cell stain was localized in the fixed cells, enabling facile discrimination (Supporting Information; Figure S-4). It should be noted that such microscopic evaluations on cells would have been difficult or impossible to perform with previous oxygen sensor designs but were easily accomplished using the current thin film sensors.

Monitoring O_2 During Live Cell Culture. NIH 3T3 and HUVEC cells were seeded at very low densities on separate PtTFPP-Tef-PDMS sensors and allowed to attach and spread overnight. Subsequently, the P_{O2} in the cell culture was monitored following step changes in the head space gas composition to verify that oxygen measurements could be made during active cell culture. All culture medium was replaced prior to the start of the experiment to ensure that the P_{O2} levels in all chambers were 0.21 atm (ambient) when measurements began. The measured P_{O2} levels in solution equilibrated gradually to the composition of the head space following each step change (Figure 4a). The P_{O2} measurements for wells containing cells at low densities were not statistically different from the control wells that did not contain cells. This data confirmed that oxygen measurements could be made in the presence of cells and indicated that at low cell densities, the P_{O2} levels were not affected by the cells.

When cells were plated at high densities, however, cellular consumption of oxygen led to significant changes in solution oxygen tension (Figure 4b). Culture medium was again replaced at the start of the experiement ($t = 0$), but in this case, P_{O2} in the presence of cells quickly dropped to a level significantly lower than that in the control well without cells (from 0.21 to 0.16 atm over the first 50 min). Subsequently, the P_{O2} in the cell culture responded to the step changes in the headspace gas composition and exhibited time constants that were similar to

(29) Khalil, G. E.; Chang, A.; Gouterman, M.; Callis, J. B.; Dalton, L. R.; Turro, N. J.; Jockusch, S. *Rev. Sci. Instrum.* **2005**, *76*, 054101.
(30) Murphy, B.; Kirwan, P.; McLoughlin, P. *Anal. Bioanal. Chem.* **2003**, *377*, 195–202.

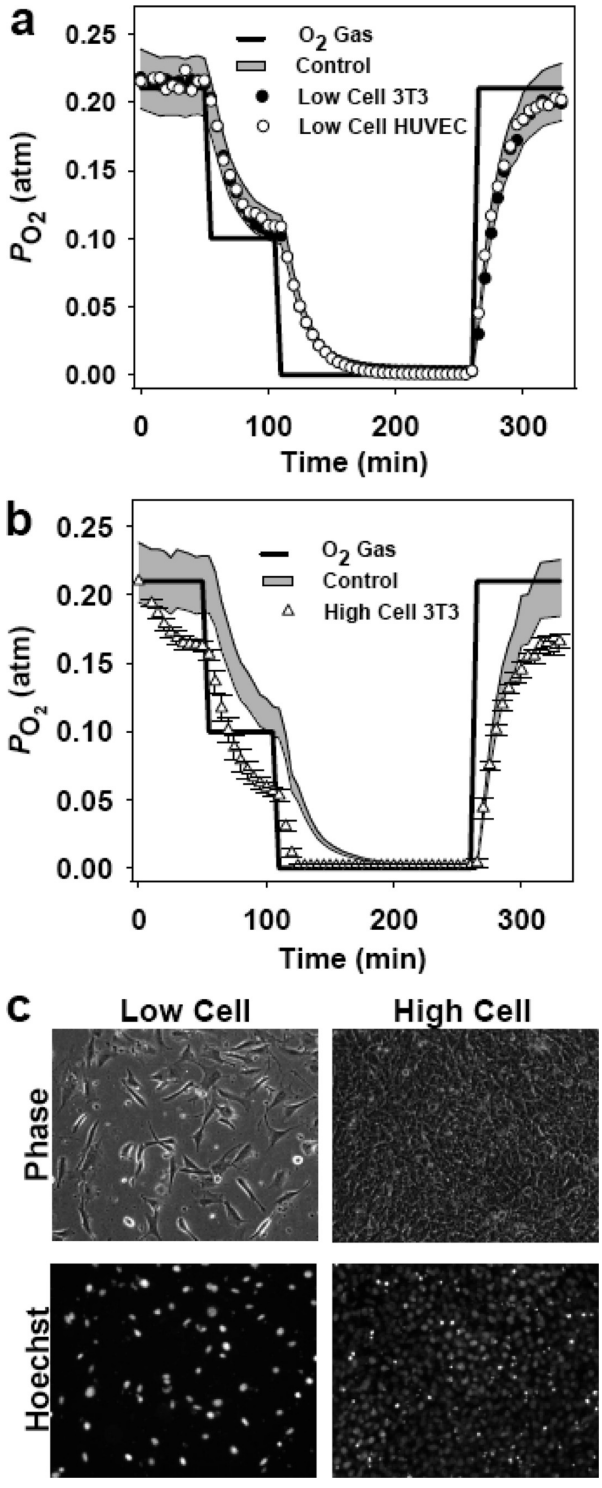

Figure 4. Oxygen measurements in the presence of live mammalian cells. At low cell seeding density (60 cells/mm^2) of HUVEC (open circles) or NIH 3T3 (black circles), the P_{O2} measured in culture was not statistically different from the control well (shaded region) that contained no cells (a). At high cell densities (3100 cells/mm^2, open triangles), the measured P_{O2} was significantly lower than the control throughout the experiment, indicating oxygen consumption by the cells (b). Representative phase contrast and Hoechst nuclear stained fluorescent microscopy images of NIH 3T3 cells on the PtTFPP-Tef-PDMS oxygen sensor are shown at low and high densities (c). The shaded region in a and b shows the 95% confidence interval envelope based on the uncertainty in measuring K_{SV} and I_0. Data points and error bars in b represent means and standard deviations from triplicate experiments.

those observed in the low-density cultures or in the control well. However, the measured values of P_{O2} in the high-density culture remained significantly lower than that in the headspace or the measured P_{O2} in the control wells. For the confluent cell monolayer evaluated, no spatial variation in P_{O2} was observed across the field of view (1 mm^2). Representative phase and nuclear-stained fluorescence microscopy images allowed the different cell densities evaluated here to be observed (Figure 4c,d). These images further demonstrated the facile integration of live-cell microscopy with this oxygen sensing strategy.

During normal metabolism in culture, cells consume oxygen continuously, and the depleted oxygen is replaced diffusively from the surrounding culture medium, the culture flask head space, or the cell culture plate material. At low cell densities (Figure 4a), small amounts of oxygen were consumed and diffusion was sufficient to replace the consumed oxygen, giving similar results to the control. However, at higher cell densities (Figure 4b), the greater number of cells consumed considerably more oxygen with a significant decrease in the local oxygen concentration compared to the control. In this case, oxygen supplied by diffusion was only able to keep up with oxygen consumption once a relatively steep concentration gradient emerged between the culture substrate and the solution surface (e.g., 0.16 to 0.21 atm at 50 min). Once established, this gradient was maintained throughout the experiment. For example, when the headspace P_{O2} was brought to 0.1 atm, the measured P_{O2} in the high density culture dropped to 0.05 atm. Without the direct measurements shown here, the local oxygen tension could only be roughly inferred by making assumptions about per-cell rates of oxygen consumption. Further, any changes in metabolic activity that affect the rate of oxygen consumption (e.g., in response to a novel drug treatment) would go unmonitored.

For short observation times, the rate of oxygen consumption was constant and a stable concentration gradient was observed. However, over longer observation times, cellular proliferation increases the number of cells present as well as the rate of total oxygen consumed. These effects were observed by measuring P_{O2} immediately following the addition of suspended cells and repeating the measurement every hour for 72 h (Figure 5). As cells sedimented and attached to the bottom of the culture dish and then proliferated over the course of the experiment, the P_{O2} in solution decreased monotonically. Initially, between 0−8 h, the measured P_{O2} decreased rapidly in all cell cultures, and the steepness of the decrease correlated with the cell density. This time frame correlated with the time required for attachment of cells to the sensor surface, and this decrease in P_{O2} was attributed to the initial formation of a stable concentration gradient between the attached cells on the sensor and the surface of the culture medium. Subsequently, P_{O2} in culture continued to drop, until it leveled off when approaching the limiting value of $P_{O2} = 0$ atm. The above P_{O2} response is consistent with constant cell proliferation followed by insufficient supply of oxygen by diffusion alone.

This experiment depicted the dynamic oxygen tension that can occur during long-term cell culture. For the highest cell seeding density (3100 cells/mm^2), the local oxygen tension after 24 h of culture was essentially zero. Thereafter, cell consumption

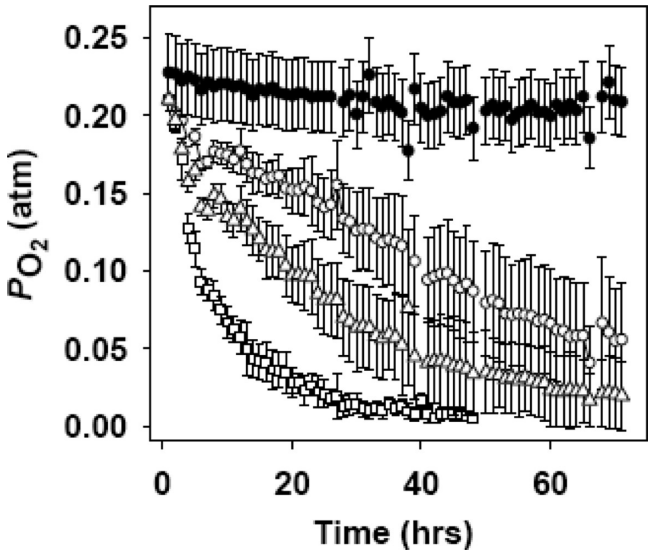

Figure 5. Changes in measured P_{O2} during long-term cell culture. NIH 3T3 mouse fibroblasts were seeded at three densities: 790 cells/mm^2, 1580 cells/mm^2, 3100 cells/mm^2. PtTFPP-Tef-PDMS sensors were used to measure the P_{O2} throughout three days of culture. In each case, the solution oxygen tension decreased over time relative to a control well with no cells (black circles) due to proliferation and cellular oxygen consumption. As cells proliferated over several days, oxygen tension continued to drop. Data points indicate mean P_{O2} measurements from triplicate experiments with the standard deviation shown by error bars. The difference between each seeding density was statistically significant (two factor ANOVA, $p < 0.05$).

of oxygen was limited by the rate of diffusion of oxygen from solution. Further, the specific oxygen tension was very dependent on the number of cells present. At the lower seeding densities (790 cells/cm^2), the oxygen level dropped throughout culture but remained above 0.05 atm even after 72 h. The direct oxygen measurements shown here provided critical information regarding the state of the changing culture conditions throughout the long-term culture.

When P_{O2} in culture after cells attached was compared between seeding densities, it was evident that the decrease in oxygen tension correlated with the cell density in culture (Figure 6a). The linearity of the relationship between P_{O2} and cell density indicated that the net oxygen consumption in each case was dependent only on the total number of oxygen consuming cells. Since the cells were evenly distributed across the bottom of the well, a spatially uniform oxygen concentration gradient was assumed between the cells and the top surface of the culture medium. The oxygen flux (J_{O2}) that resulted was analyzed using the one-dimensional steady state solution to Fick's Law:[12,15]

$$J_{O2} = \frac{D\Delta P_{O2}}{h} \qquad (2)$$

where D is the diffusivity of oxygen in aqueous solution (3.3×10^{-5} cm^2/s),[12] ΔP_{O2} is the difference in oxygen partial pressure between the cell microenvironment and the culture medium surface, and h is the height of the culture medium. Using eq 2, the P_{O2} measurements were used to calculate oxygen flux

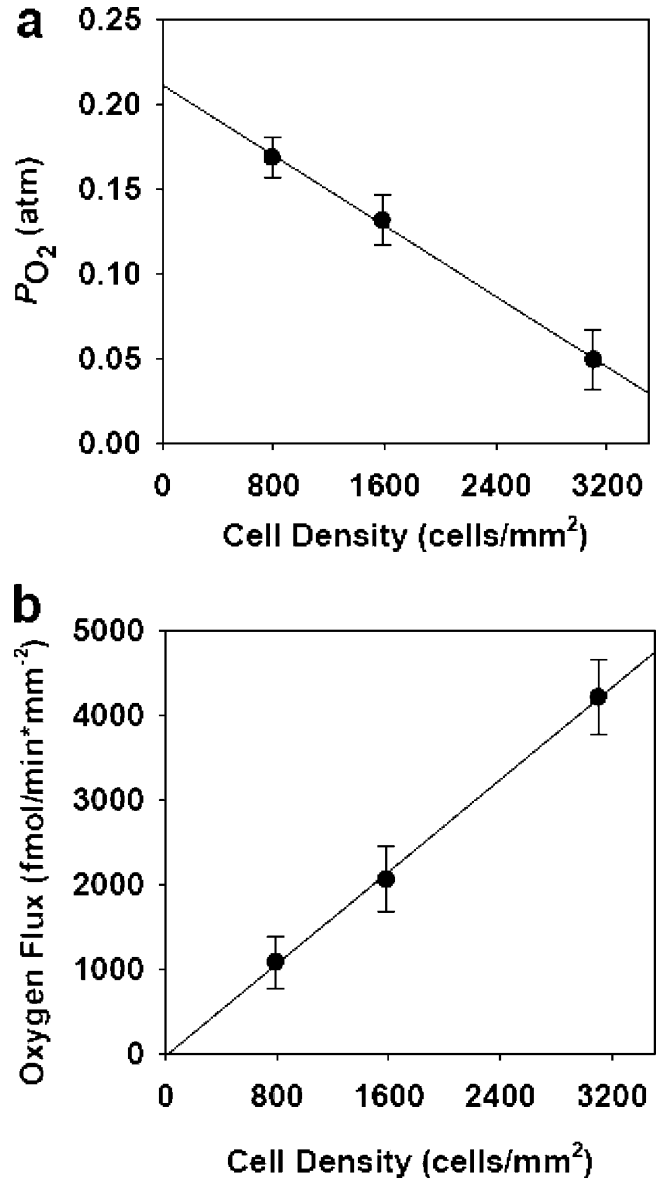

Figure 6. Effect of cell density on P_{O2} and oxygen consumption. Measured P_{O2} at 12 h postseeding was plotted against the cell density (a). Fick's law was used to calculate the oxygen flux at each cell density (b). The per cell rate of oxygen consumption determined from the linear regression was 1.38 ± 0.04 fmol·min^{-1}·cell^{-1} for the NIH 3T3 mouse fibroblasts. Data points and error bars indicate the mean values and standard deviations of triplicate experiments; solid traces are linear regressions to the data.

as a function of cell seeding density (Figure 6b). The slope of this data is the per-cell rate of oxygen consumption, and the linear relationship shows that this rate is independent of cell density for the conditions explored here. From Figure 6b, the per-cell rate of oxygen consumption for NIH 3T3 mouse fibroblasts is 1.38 ± 0.04 fmol·min^{-1}·cell^{-1}. This value is similar to rates of oxygen consumption reported for other cell types.[15]

CONCLUSION

We have developed a new oxygen sensor to quantify changes in oxygen tension during in vitro cell culture. The sensor is based on the oxygen-dependent quenching of the phosphorescent porphyrin dye, PtTFPP, which is incorporated into a gas-perme-

able thin polymer film. A multilayer sensor design, with a first layer of the PtTFPP dye in PDMS, a second layer of Teflon AF, and a third (top) layer of PDMS with no dye, was found to be effective for culturing adherent cells and mitigating phototoxicity from the PtTFPP dye. A major advantage of the current thin film sensor is that it allows facile integration of oxygen measurements with both phase contrast and fluorescence microscopy to characterize and quantify cell viability, motility, and cell count. Compared to previous oxygen sensors which had problems associated with photodegradation and lack of sensitivity, the current thin film sensor has high sensitivity ($K_{SV} = 584$ atm^{-1}) and negligible photobleaching. In situ measurements of P_{O2} during cell culture revealed significant drops in local oxygen tension for high cell densities. Further, long time cultures revealed dynamic changes in P_{O2} as cells proliferated. The rate of per-cell oxygen consumption calculated here for NIH 3T3 fibroblasts was consistent with reported values for other cell types. The method reported here to measure oxygen tension in situ during cell culture will enable the effects of local oxygen levels on cell behavior to be determined and provide the needed measurements for controlling this important component of the cellular microenvironment.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 19, 2009. Accepted September 15, 2009.

AC9013379

Supporting Information

A Non-Invasive Thin Film Sensor for Monitoring Oxygen Tension During *In Vitro* Cell Culture

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Additional Sensor Calibration Experiments

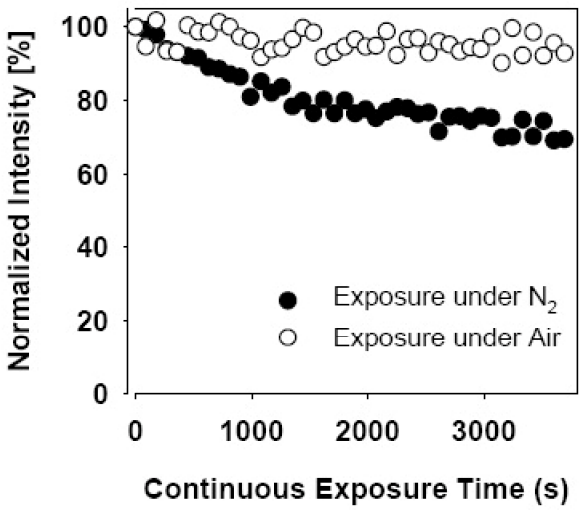

Figure S-1. Photodegradation of oxygen sensor under continuous light exposure. PtTFPP sensor under continuous exposure of phosphorescent excitation for 3600 seconds in Air (open circles) showed negligible photobleaching effects and in pure N_2 (closed circles) showed a reduction of intensity by 30%. Images were taken every 90s. All images were normalized against the first image of the data set. During P_{O_2} measurements, only a 70ms exposure was required. Even for the longest oxygen measurement experiments, the total integrated sensing time was less than 10 s.

Additional Proliferation Experiment

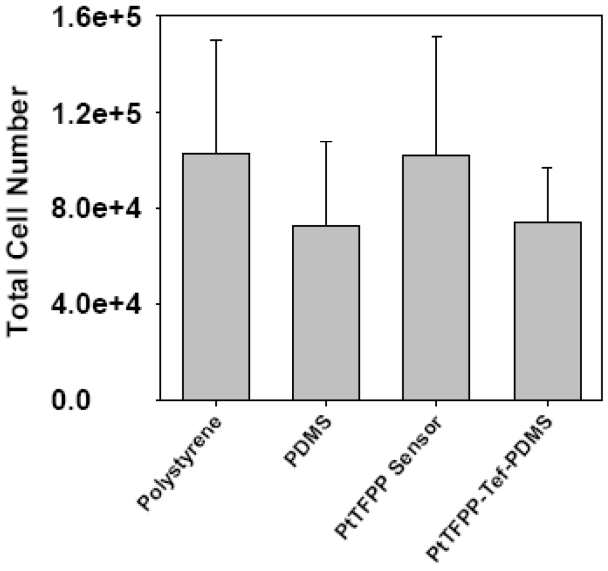

Figure S-2. Proliferation of cells on various substrates. NIH 3T3 mouse fibroblasts were seeded (60 cells/mm^2) on 4 different fibronectin coated surfaces (polystyrene, PDMS films, PtTFPP sensor and PtTFPP-Tef-PDMS sensor) and allowed to proliferate for 70 hrs in the incubator (in darkness). Cells were subsequently trypsinized and counted. No statistically significant difference was observed between the different surfaces. Error bars depict the mean and standard deviation from triplicate experiments. No statistically significant difference was observed between the different surfaces ($p = 0.72$).

Additional Cytotoxicity Studies

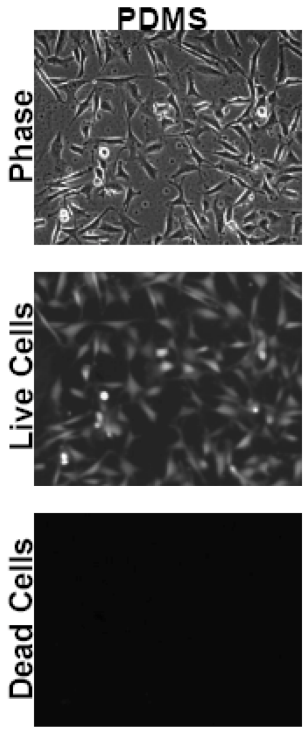

Figure S-3. Phototoxicity for cells grown on PDMS. Representative phase contrast images and fluorescently labeled Live/Dead Assay images of NIH 3T3 fibroblasts grown on fibronectin coated PDMS film and exposed to 70 ms exposures of 546 nm light every 15 min for 3.5 hrs. These data provide a control set for phototoxicity evaluation of the oxygen sensor formulation (See Figure 3).

Additional Images

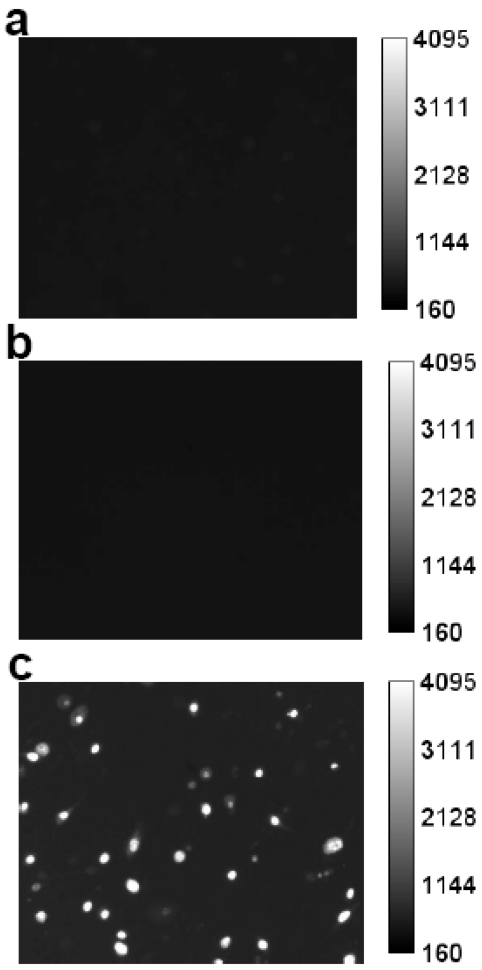

Figure S-4. Integration of oxygen sensor and fluorescent cell stains. Representative fluorescent image of blank PDMS films showed minimal background emission (a). The PtTFPP sensor without cells showed an emission that was homogeneous in intensity throughout the field of view (b). On the same senosro formuation, dead cells (NIH 3T3 fibroblasts) stained with ethidium homodimer generated local bright spots that were clearly visible over the PtTFPP emission (c) even though the emission wavelengths of the film and stain overlapped.