Fluorescent Probes

DOI: 10.1002/ange.201107557

Ratiometric Molecular Sensor for Monitoring Oxygen Levels in Living Cells**

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Oxygen (dioxygen) is one of the key metabolites in aerobic organisms.[1] In cellular respiration, oxygen plays a major role as the terminal acceptor of the electron transport chain and oxidative phosphorylation. Oxygen deprivation (hypoxia) is connected with various diseases and occurs in tumor microenvironments.[2] It is thus essential to quantify oxygen levels in biological cells and tissues to understand cellular function (dysfunction) and to assess tumor pathophysiology during drug delivery in cancer therapies.

We recently demonstrated that phosphorescent iridium(III) complexes can be used as optical sensors for visualizing the oxygen levels in biological cells and tissues.[3] We used a red-emitting iridium complex [$(btp)_2Ir(acac)$] (BTP; bis(2-(2′-benzothienyl)-pyridinato-$N,C^{3'}$)iridium(acetylacetonate)) for optical imaging, because this compound has a moderately long emission lifetime (6.3 μs) and a high quantum yield (0.31) in deaerated hexane. The red phosphorescence of BTP is significantly quenched by dissolved oxygen in solution. Similar quenching by oxygen has also been observed for living cells. Emission images of HeLa (human cervical cancer) cells were brighter, when cells were cultured with BTP at low oxygen pressures, and thus the images reflected the cellular oxygen levels.

In addition to intensity measurements, emission lifetime measurements are generally required to quantify oxygen levels in cells and tissues.[4] As an alternative method, ratiometric oxygen sensors, which do not require specialized instrumentation for measuring emission lifetimes, are suitable for general measurements and will be beneficial for cell biologists and medical scientists. Ratiometric oxygen sensors developed recently for biological imaging are nanoparticle-based optical sensors consisting of a phosphorescent dye encapsulated inside a polymer or semiconductor nanocrystal.[5–8] Nanoparticles typically exhibit higher brightness and better photostability than molecular dyes, because they have more chromophores per particle and have a protective matrix. On the other hand, small-molecule sensors have the advantages of greater affinity to biological cells and of affecting living cells less. Moreover, chemical modifications can improve the physicochemical and optical properties of molecular sensors.

Herein, we report a novel ratiometric molecular sensor for monitoring oxygen levels in living cells and tissues. Figure 1 schematically depicts the design concept. The probe consists of an oxygen-insensitive fluorophore and an oxygen-sensitive phosphor, which are connected by a rigid linker. Ideally, ratiometric oxygen probes will have the following properties: 1) fluorescence and phosphorescence exhibit good spectral separation, and only phosphorescence exhibits oxygen quenching; 2) reverse energy transfer does not occur from the phosphor to the fluorophore; 3) electron transfer quenching does not occur between the fluorophore and the phosphor; 4) the emission properties of both the fluorophore and the phosphor are independent of the ambient physical conditions (e.g., pH value[9] and the polarities[3] of the medium) and of biological substances; and 5) a sufficiently high intracellular uptake efficiency. To satisfy these requirements, we selected coumarin 343 (C343) as the fluorescent group and the iridium complex BTP as the phosphorescent group. These luminophores were connected by a tetraproline linker to spatially separate these groups. Scheme 1 shows the molecular structures of C343-Pro_4-BTP together with those of reference compounds. When C343-Pro_4-BTP is excited at 405 nm, the excitation photons are mainly absorbed by the C343 moiety, and the electronic energy is partially transferred from C343 to the BTP moiety

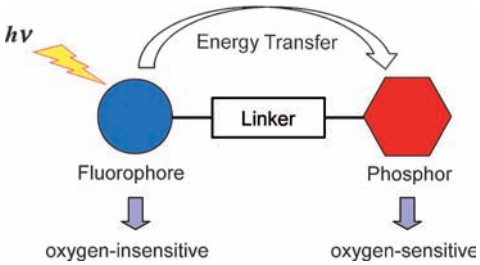

Figure 1. Design concept of a ratiometric molecular probe for sensing oxygen levels in cells and tissues.

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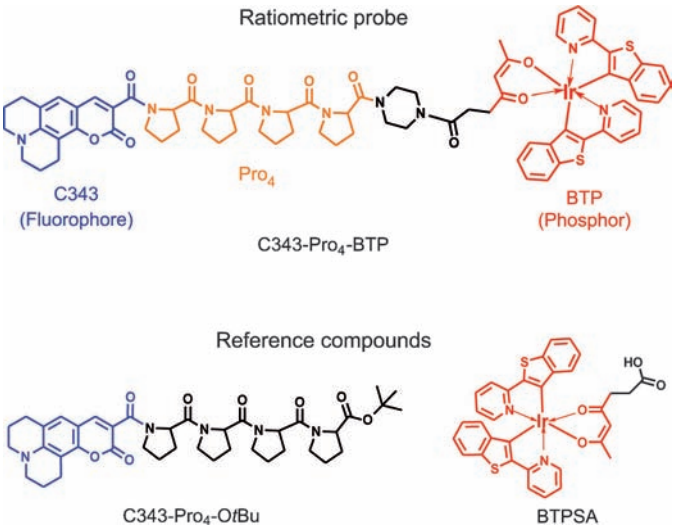
[**] S.T. was financially supported by a Grant-in-Aid (Development of Advanced Measurement and Analysis Systems) from the Japan Science and Technology Agency and a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 21655024). T.Y. was financially supported by a Grant-in-Aid from the Sumitomo Foundation.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201107557.



Scheme 1. Structures of the ratiometric probe C343-Pro_4-BTP and its reference compounds C343-Pro_4-O*t*Bu and BTPSA.

by singlet energy transfer, resulting in both blue fluorescence of C343 and red phosphorescence of BTP (Figure 1).

We first examined the spectral properties of C343-Pro_4-BTP and the reference compounds, C343-Pro_4-O*t*Bu and BTPSA. From the absorption spectra of the reference compounds in acetonitrile (Figure S1a in the Supporting Information), it was found that the absorption peak that is due to C343 appears at about 405 nm and that at this wavelength the absorption coefficient of BTP ($\varepsilon = 3600\ dm^3\,mol^{-1}\,cm^{-1}$) is much smaller than that ($\varepsilon = 25\,800\ dm^3\,mol^{-1}\,cm^{-1}$) of C343. The absorption spectrum of C343-Pro_4-BTP agreed well with that of a 1:1 mixture of the reference compounds (Figure S1b in the Supporting Information), thus indicating that electronic interactions between C343 and BTP chromophores in C343-Pro_4-BTP are negligibly small in the ground state. We then measured the emission spectrum of C343-Pro_4-BTP in CH_3CN (Figure 2a). As expected, under Ar purging both the C343 fluorescence and BTP phosphorescence exhibit comparable intensities, whereas under aerated conditions the phosphorescence of

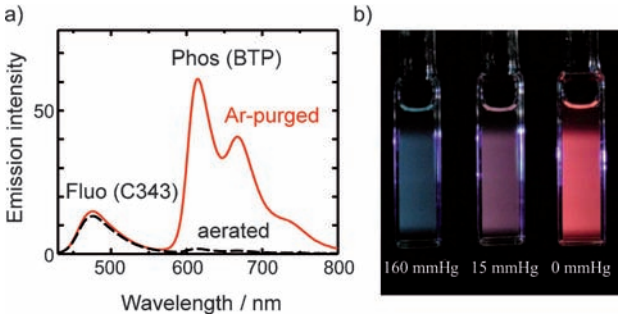


Figure 2. a) Emission spectra of C343-Pro_4-BTP in CH_3CN under aerated and Ar-purged conditions ($\lambda_{exc} = 405$ nm), and b) emission images of C343-Pro_4-BTP in CH_3CN under different oxygen pressures; the images were obtained by irradiation with UV light using a black light ($\lambda_{exc} = 365$ nm).

BTP is almost entirely quenched. It can also be seen that the fluorescence and phosphorescence exhibit reasonably good spectral separation, which is favorable for ratiometric measurements.[10] Figure 2b shows the emission images obtained by irradiation of C343-Pro_4-BTP in CH_3CN with UV light (365 nm). Under degassing (0 mmHg), the ratiometric probe exhibits red emission, because the BTP phosphorescence dominates over fluorescence, whereas under aerated conditions (160 mmHg), phosphorescence is almost completely quenched, and the emission appears blue owing to C343 fluorescence. Under an intermediate oxygen pressure (15 mmHg), the emission appears violet, as expected.

To determine the relaxation mechanism of electronically excited C343-Pro_4-BTP, we measured the emission lifetimes and quantum yields.[11] Table S1 in the Supporting Information lists the photophysical parameters obtained for C343-Pro_4-BTP and the reference compounds. The photorelaxation mechanism of C343-Pro_4-BTP can be explained in terms of the energy state diagram shown in Figure 3, which was

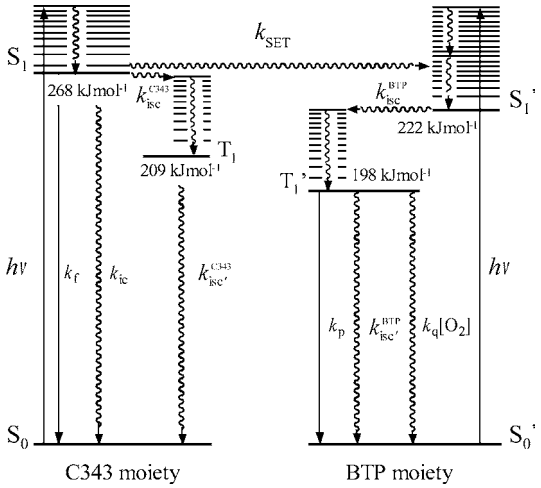


Figure 3. Energy state diagram and relaxation processes of the excited singlet (S_1) and triplet (T_1) states of C343-Pro_4-BTP. k_{isc}, k_f, k_{ic}, k_{SET}, k_p, and k_q are the rate constants for the processes intersystem crossing, fluorescence, internal conversion, singlet energy transfer, phosphorescence, and quenching, respectively.

constructed from spectral data for C343-Pro_4-BTP. The lowest excited singlet and triplet levels of the C343 and the BTP moieties are depicted separately, because the electronic interactions between these chromophores are sufficiently small in the ground state. The photorelaxation processes involved in this dyad are shown with their rate constants. The fluorescence decay profile of C343-Pro_4-BTP monitored at 480 nm could be fitted by two exponential functions with lifetimes of 65 (48%) and 260 ps (52%; Table S1 in the Supporting Information). From these lifetimes and the fluorescence lifetime of 4.1 ns of the reference compound C343-Pro_4-O*t*Bu, the efficiency of Förster resonance energy transfer from the excited singlet-state of C343 to BTP in the ground state was estimated to be 98% for the shorter-lifetime component and 94% for the longer-lifetime component. We assume that fluorescence quenching for both components is

entirely due to energy transfer. The observation of two lifetime components suggests the presence of two different conformers for C343-Pro_4-BTP. As a possible explanation, the oligoprolines are known to adopt two helical conformations,[12] a type I *cis* helix and a type II *trans* helix, which have different distances for the two chromophores. By using the two fluorescence lifetimes in Table S1and by assuming the Förster mechanism for energy transfer,[13] the energy transfer rate constants were estimated to be 1.5×10^{10} and $0.36\times10^{10}\ s^{-1}$, and the distances between C343 and BTP chromophores were calculated to be 19 and 24 Å (see the Supporting Information). In contrast, the phosphorescence lifetime (5.9 μs) of the BTP moiety under degassing was almost equal to that (5.6 μs) of the reference compound BTPSA. This finding demonstrates that quenching that is due to reverse energy transfer and/or electron transfer does not occur in the excited triplet state.

We performed oxygen quenching experiments on C343-Pro_4-BTP incorporated in lipid bilayer membranes, because this lipophilic probe is expected to be localized in plasma or organelle membranes in cellular measurements. The lipid bilayer of dimyristoyl-phosphatidylcholine (DMPC) was used as a model membrane. Figure 4a shows the change in the emission spectra of C343-Pro_4-BTP (1 μM) partitioned into DMPC (1 mM) liposome membrane in tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (pH 7.0), when the oxygen partial pressure was increased from 0 to 160 mmHg (i.e., from degassed to aerated conditions). Figure 4a clearly shows that the fluorescence spectrum is almost completely independent of the oxygen partial pressure, whereas the phosphorescence intensity decreases remarkably with increasing oxygen partial pressure. Using the data in Figure 4a, we quantitatively analyzed oxygen quenching. The ratios of the phosphorescence intensity to that of the fluorescence in the absence and presence of oxygen are defined as $R_I^0=(I_p^0/I_f^0)$ and $R_I=(I_p/I_f)$, respectively. When, the fluorescence quenching that is due to oxygen is assumed to be negligible, the following equation can be derived based on the Stern–Volmer equation:[13]

$$\frac{R_I^0}{R_I}=1+K_{SV}pO_2 \qquad (1)$$

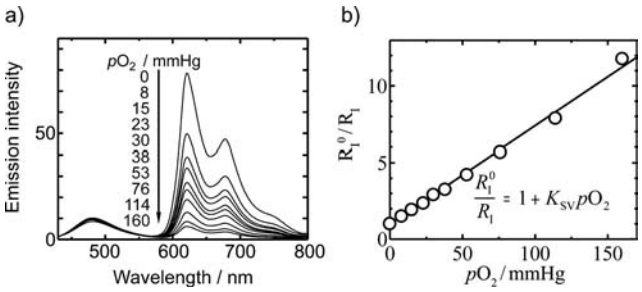


Figure 4. a) Emission spectra of C343-Pro_4-BTP (1 μM) in the presence of DMPC (1 mM) in Tris/HCl buffer at 35 °C under different oxygen partial pressures; $\lambda_{exc}=405$ nm. b) Plots of R_I^0/R_I as a function of oxygen pressure.

where K_{sv} is the Stern–Volmer constant and pO_2 is the oxygen partial pressure. There is a linear relation between R_I^0/R_I and pO_2, as shown in Figure 4b. From the slope of the straight line, the constant K_{sv} was determined to be $6.4\times10^{-2}\ mmHg^{-1}$. By using the K_{sv} value, the oxygen partial pressure in liposome membranes can be determined from ratiometric measurements.

To confirm the reliability of our ratiometric measurements, we performed similar oxygen quenching experiments based on lifetime measurements of the BTP phosphorescence in the DMPC membrane. The K_{sv} value obtained from the lifetime measurements was $6.4\times10^{-2}\ mmHg^{-1}$, which is equal to that ($6.4\times10^{-2}\ mmHg^{-1}$) obtained from our ratiometric measurements (Figure S2 in the Supporting Information), that is, the static quenching was not observed under sufficiently low concentrations of C343-Pro_4-BTP. These results demonstrate that C343-Pro_4-BTP can be used as a ratiometric probe for measuring oxygen levels in membranes.

We then used our ratiometric probe to examine cellular oxygen levels in HeLa cells. HeLa cells were cultured under 20% or 2.5% O_2 concentrations for 24 h at 37 °C. C343-Pro_4-BTP was added to the medium at a final concentration of 2 μM and cells were incubated for two hours after washing with phosphate-buffered saline (PBS) under 20% or 2.5% O_2 concentrations. The excitation wavelength was 400–410 nm. Figure 5a shows the emission images of the live cells observed in the wavelength range from 460 to 510 nm. This wavelength range corresponds to the fluorescence wavelength of C343. The fluorescence intensities from the live cells are almost the same under 20% and 2.5% O_2 conditions. In contrast, the emission image taken at wavelengths longer than 610 nm (Figure 5b), which corresponds to the phosphorescence wavelength of BTP, gives a brighter image under 2.5% O_2 conditions than under 20% O_2 conditions. The cellular uptake of C343-Pro_4-BTP is currently too low to perform quantitative oxygen measurements, although we observed oxygen quenching in the HeLa cells. Increase of lipophilicity in C343-Pro_4-BTP is probably related to lower cellular uptake of C343-

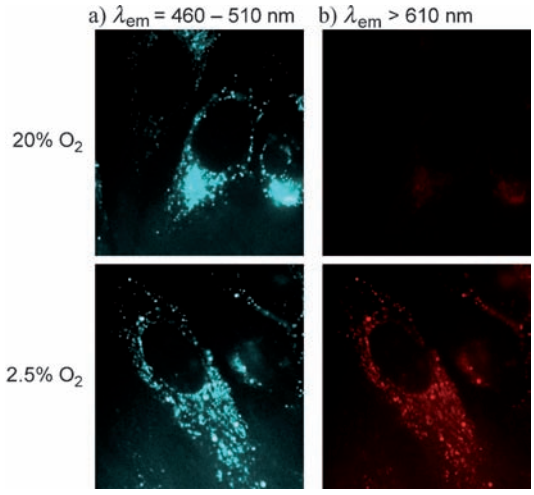


Figure 5. Luminescence images ($\lambda_{exc}=400$–410 nm) of HeLa cells incubated under 20% and 2.5% O_2 conditions observed at emission wavelenghts of a) 460–510 nm and b) above 610 nm.

Pro_4-BTP compared to that of BTP. We are currently modifying the proline linker of C343-Pro_4-BTP by incorporating cationic amino acid residues such as amino proline to enhance its delivery to cells.

In summary, we have developed a small-molecule oxygen sensor (C343-Pro_4-BTP) that uses coumarin 343 as an oxygen-insensitive fluorophore and the iridium complex BTP as an oxygen-sensitive phosphor. The ratio of the fluorescence and phosphorescence of C343-Pro_4-BTP exhibited a quantitative dependence on the oxygen concentrations in a DMPC membrane as well as in solution. Oxygen quenching experiments on C343-Pro_4-BTP in HeLa cells demonstrated the potential of our molecular probe for quantitative mapping of local oxygen levels in living cells and tissues.

Please note: Minor changes have been made to this manuscript since its publication in *Angewandte Chemie* Early View. The Editor.

Received: October 26, 2011
Revised: December 29, 2011
Published online: February 17, 2012

Keywords: biosensors · energy transfer · fluorescent probes · iridium · phosphorescence

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Note added in proof (March 12, 2012): Similar systems with either a C343 donor or a polyproline spacer have been reported by Vinogradov and co-workers in their studies on Förster resonance energy transfer (FRET) enhanced phosphorescent oxygen sensors: R. P. Brinas, T. Troxler, R. M. Hochstrasser, S. A. Vinogradov, *J. Am. Chem. Soc.* **2005**, *127*, 11851 – 11862; O. S. Finikova, T. Troxler, A. Senes, W. F. DeGrado, R. M. Hochstrasser, S. A. Vinogradov, *J. Phys. Chem. A* **2007**, *111*, 6977 – 6990; O. S. Finikova, A. Y. Lebedev, A. Aprelev, T. Troxler, F. Gao, C. Garnacho, S. Muro, R. M. Hochstrasser, S. A. Vinogradov, *ChemPhysChem* **2008**, *9*, 1673 – 1679; A. Y. Lebedev, T. Troxler, S. A. Vinogradov, *J. Porphyr. Phthalocyanines* **2008**, *12*, 1261 – 1269; S. Sakadzic, E. Roussakis, M. A. Yaseen, E. T. Mandeville, V. J. Srinivasan, K. Arai, S. Ruvinskaya, A. Devor, E. H. Lo, S. A. Vinogradov, D. A. Boas, *Nat. Methods* **2010**, *7*, 755 – 759; J. Lecoq, A. Parpaleix, E. Roussakis, M. Ducros, Y. G. Houssen, S. A. Vinogradov, S. Charpak, *Nat. Med.* **2010**, *17*, 893 – 898.
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