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Reversible Off–On Fluorescence Probe for Hypoxia and Imaging of Hypoxia–Normoxia Cycles in Live Cells

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W *Web-Enhanced Feature* *S* *Supporting Information*

ABSTRACT: We report a fully reversible off–on fluorescence probe for hypoxia. The design employs QSY-21 as a Förster resonance energy transfer (FRET) acceptor and cyanine dye Cy5 as a FRET donor, based on our finding that QSY-21 undergoes one-electron bioreduction to the radical under hypoxia, with an absorbance decrease at 660 nm. At that point, FRET can no longer occur, and the dye becomes strongly fluorescent. Upon recovery of normoxia, the radical is immediately reoxidized to QSY-21, with loss of fluorescence due to restoration of FRET. We show that this probe, RHyCy5, can monitor repeated hypoxia–normoxia cycles in live cells.

Hypoxia is a feature of various diseases, including tumors,[1] cardiovascular diseases,[2] and stroke.[3] So far, various approaches have been used to selectively detect hypoxic regions by utilizing immunostaining,[4,5] PET imaging,[6,7] phosphorescence imaging,[8,9] magnetic resonance imaging,[10,11] and fluorescence imaging.[12−15] Among them, fluorescence imaging offers various advantages, including high sensitivity and ease of use.

Hypoxia induces two distinctive biological responses, i.e., stabilization of hypoxia-inducible factor-1 (HIF-1)[16] and accelerated bioreductive reaction.[17] The latter has been the preferred target for the design of hypoxia-sensitive imaging probes because bioreductive reactions proceed efficiently under hypoxia, while they do not proceed under normoxia. Several hypoxia-sensitive fluorescence probes, whose fluorescence intensity increases upon bioreduction under hypoxia, have been developed, employing a nitro group,[12,13] quinone group,[14] or azo group[15] as the hypoxia-sensing moiety. However, the structural change of the functional group to the "fluorescence on" state is irreversible in these probes. In other words, once these probes become strongly fluorescent in a hypoxic region, they remain strongly fluorescent even after the region returns to normoxia.

Recently, it was reported that cycling hypoxia, i.e., repeated cycles of hypoxia–reoxygenation, induces increased HIF-1 activity of tumor cells, and this phenomenon is associated with a decreased success rate of radiotherapy or chemotherapy.[18] The mechanism of HIF-1 stabilization is unclear, although production of reactive oxygen species (ROS) is considered to be involved. However, no method was available to visualize the dynamics of cycling hypoxia in real time. Therefore, we set out to develop a reversible fluorescence probe for hypoxia.

Existing redox-sensitive reversible fluorescence probes, such as nitric oxide,[19] ROS,[20,21] and peroxynitrite[22] probes, detect oxidative stress but not reductive stress, so a key requirement for a reversible hypoxia probe is the choice of a suitable detector moiety.

We decided to employ the Förster resonance energy transfer (FRET) mechanism for our probe design. Namely, we required a dye that shows a reversible spectral change between its reduced and oxidized forms for use as a FRET acceptor (Figure 1). We examined several candidates (Supporting Information Figure S1). 2-Aminoanthraquinone and malachite green were selected as candidates because it is reported that quinones and malachite green can be enzymatically reduced,[23,24] and QSY-21 was selected because of the similarity of its chemical structure to that of malachite green. A solution of each compound was incubated with rat liver microsomes, which contain various reductases, at 37 °C in air (~20% pO_2; normoxia) or in an argon atmosphere (~0.1% pO_2; hypoxia), and the changes of the absorption spectra were measured. QSY-21 showed a spectral change which suggested that it might be suitable as a hypoxia-sensing moiety for a reversible fluorescence probe (Figure 2).

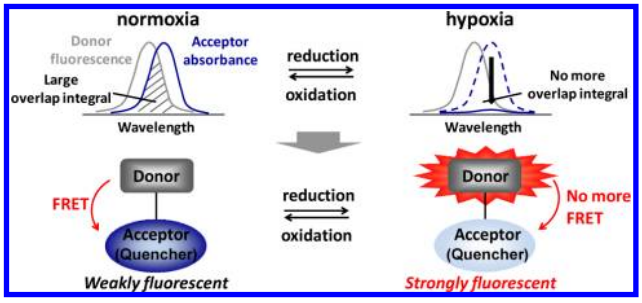

Figure 1. Design strategy for a reversible fluorescence probe for hypoxia.

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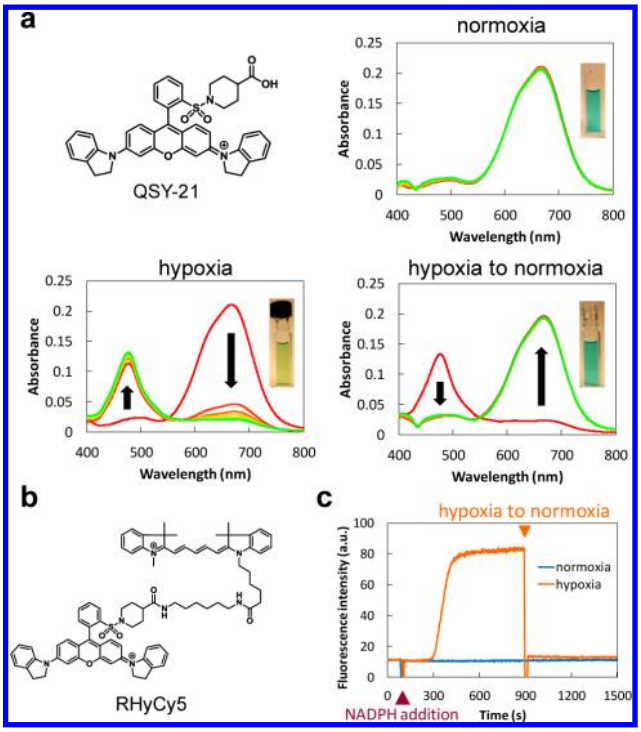

Figure 2. *In vitro* assay of QSY-21 and RHyCy5. (a) Chemical structure of QSY-21 and changes of the absorption spectrum of 5 μM QSY-21 under normoxia, hypoxia, and hypoxia-to-normoxia transition. Spectra were measured every 5 min for 30 min at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) containing rat liver microsomes (0.25 mg protein/mL), 50 μM NADPH as a coenzyme, and 0.5% DMF as a cosolvent. Red lines show the initial and subsequent spectra (some are overlapping), and green lines show the final spectra at 30 min. (b) Chemical structure of RHyCy5. (c) Time-dependent changes of fluorescence intensity of 1 μM RHyCy5 under normoxic and hypoxic conditions. Data were measured at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) containing rat liver microsomes (0.25 mg protein/mL), 50 μM NADPH as a coenzyme, and 0.1% DMF as a cosolvent. NADPH addition: NADPH was added at the point indicated by the arrowhead. The transition of hypoxia to normoxia was achieved by exposing the hypoxic solution to air. Ex/Em = 650/670 nm.

We found that QSY-21,[25] a dark quencher of fluorescence in the red to near-infrared (NIR) region, was enzymatically reduced under hypoxia. Concomitantly, the absorbance at 660 nm decreased and the absorbance at 470 nm increased (Figure 2a). No spectral change was observed under normoxia. Moreover, the absorbance at 660 nm recovered and the absorbance at 470 nm decreased within 1 min after exposure of the hypoxic solution to air (Video 1). Based on these characteristics of QSY-21, we designed and synthesized a novel reversible fluorescence probe for hypoxia, RHyCy5 (Reversible Hypoxia Cy5 Probe) (Figure 2b). RHyCy5 showed extremely weak fluorescence under normoxia (Figure S2, Table S1) that increased 7- to 8-fold (Φ_{fl} = 0.002−0.022) under hypoxia and then rapidly decreased to the initial level after exposure to air and subsequently remained unchanged (Figure 2c, Table S2). Thus, RHyCy5 works as a reversible fluorescence probe for hypoxia.

Next, we investigated the product formed upon bioreduction of RHyCy5 under hypoxia. We initially considered that leuco QSY-21, the two-electron reduction product of QSY-21, might be produced by the bioreduction of QSY-21 and would have no absorbance in the visible region (Figure S3). However, the reduced form of QSY-21 showed an absorbance peak at 470 nm in the visible region, as shown in Figure 2a. It is reported that rhodamine dyes, whose absorbance lies in the range of 491−564 nm, can be reversibly photoreduced by thiols to form stable radicals in the absence of O_2.[26] These radicals have absorbance at shorter wavelength than that of rhodamine dyes themselves, but still in the visible region, i.e., 386−432 nm. In addition, triphenylmethyl radical, the first organic radical, discovered by Gomberg,[27] is reported to be stable because the free electron is delocalized and the radical center is shielded by three phenyl groups, and this radical also has absorption at around 330 nm.[28] So, we thought that a radical might have been formed from QSY-21 by the bioreduction.

Electron spin resonance (ESR) measurement showed that an ESR signal (g = 2.0032, indicating carbon center radical[29]) appeared after bioreduction of QSY-21 under hypoxia but was not observed under normoxia (Figures 3 and S4). The same

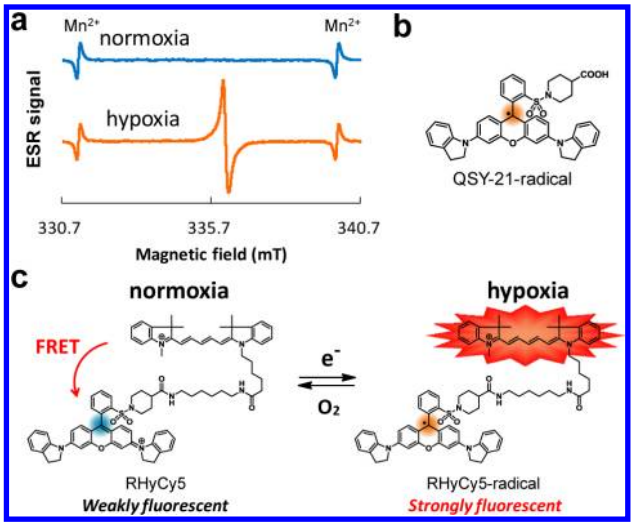

Figure 3. Investigation of the mechanism of the fluorescence increase of RHyCy5 by bioreduction under hypoxia. (a) ESR spectra of QSY-21 under normoxia or hypoxia were measured in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5% DMF as a cosolvent. The solution contained rat liver microsomes (0.25 mg protein/mL) and 50 μM NADPH as a coenzyme. Mn^{2+} was used as an internal standard. (b) Proposed structure of the radical form of QSY-21 generated upon bioreduction. (c) Proposed mechanism of reversible detection of hypoxia by RHyCy5.

ESR signal was also detected upon addition of dithionite, a chemical reducing agent (Figure S5a). Further, addition of dithionite to QSY-21 in potassium phosphate buffer (pH 7.4) resulted in the same change in the absorption spectrum as that seen in Figure 2a (Figure S5b). Upon addition of dithionite to RHyCy5 in potassium phosphate buffer (pH 7.4), the fluorescence intensity increased 7- to 8-fold (Φ_{fl} = 0.002−0.021), as was the case in the bioreduction shown in Figure 2c (Figure S5c, Table S2).

Moreover, leuco QSY-21 was not oxidized to QSY-21 under normoxia for at least 1 h (Figure S6). Based on these results, it is considered that the radical form of QSY-21, i.e., the one-electron reduction product of QSY-21 shown in Figure 3b, is produced by bioreduction under hypoxia. Further, after bioreduction of RHyCy5 under normoxia or hypoxia, the solution was analyzed by HPLC (Figure S7). In the case of hypoxia, the sample was subsequently exposed to air. Almost no

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peak at 650 nm was observed in the sample solutions incubated under normoxia and hypoxia, except for that of RHyCy5. Thus, we propose that the fluorescence off−on mechanism of this reversible fluorescence probe is as shown in Figure 3c. The immediate fluorescence decrease of RHyCy5 upon transition from hypoxia to normoxia can be attributed to the rapid oxidation of the radical form of RHyCy5 in the air. In general, the fluorescence of fluorophores linked to radicals is known to be quenched owing to electron transfer, but it has been reported that the fluorescence of Cy5 conjugated to nitric oxide, which is a radical, via a 2- or 5-methylene linker is not quenched.[30] The separation between Cy5 and QSY-21 in RHyCy5 is larger than five methylenes, and this is probably why the fluorescence of the radical form of RHyCy5 was not quenched.

Finally, we applied RHyCy5 to living cells and investigated whether this probe can reversibly detect repeated cycles of hypoxia−normoxia in live cells. It has been shown that a pO_2 gradient can be formed by putting a thin cover glass on top of cells to prevent O_2 diffusion from above.[31] We used this method to evaluate the suitability of RHyCy5 for real-time imaging of hypoxia. Cycles of hypoxia−normoxia were established by repeatedly placing a cover glass over the cells and removing it. RHyCy5 was loaded into A549 cells, human breast cancer cells, by the incubation of cells with RHyCy5 in Dulbecco's modified Eagle's medium (DMEM) for 1 h. Almost no fluorescence was observed under normoxia (Figure 4a).

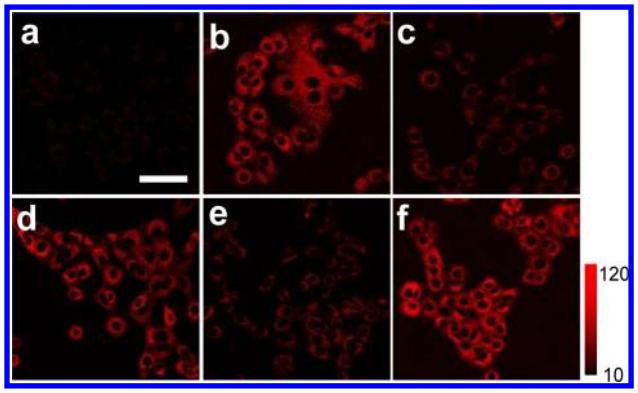

Figure 4. Fluorescence confocal microscopic images of A549 cells loaded with 1 μM RHyCy5 and exposed to cycles of normoxia−hypoxia. (a) Cells were incubated under normoxia for 1 h in DMEM containing 1 μM RHyCy5, then washed with PBS (pH 7.4), and placed in fresh DMEM. (b) The cells were incubated under a cover glass (hypoxic condition) for 1 h after (a). (c) The cover glass was removed and incubation was continued for 10 min under 5% CO_2 in air. (d) Cells were incubated under a cover glass for 1 h after (c). (e) The cover glass was removed and incubation was continued for 10 min under 5% CO_2 in air. (f) The cells were incubated under a cover glass for 1 h after (e). Scale bar: 50 μm.

After a cover glass had been placed on the cells for 1 h (hypoxic condition), the fluorescence intensity of the cells was dramatically increased (Figure 4b). When the cover glass was removed and the cells were further incubated under normoxia for 10 min, the fluorescence intensity rapidly decreased (Figure 4c). Replacement of the cover glass again resulted in an increase of the fluorescence intensity (Figure 4d). The same changes of fluorescence intensity of the cells were observed when the normoxia−hypoxia cycle was repeated once more (Figure 4e,f). On the other hand, no fluorescence intensity change was observed when cells were incubated under normoxia (air) for at least 3 h (Figure S8). Thus, this fluorescence probe could visualize repeated hypoxia−normoxia cycles in live cells. To our knowledge, this is the first reversible fluorescence probe that is able to detect repeated hypoxia−normoxia cycles by utilizing bioreductase activity under hypoxia.

In summary, we found that QSY-21 is reversibly bioreduced under hypoxia, and we identified the product of the bioreduction as the radical form of QSY-21, which undergoes rapid oxidation upon exposure to air. We utilized these findings to design and synthesize a reversible fluorescence probe, RHyCy5, based on the FRET mechanism by using QSY-21 as a FRET acceptor and Cy5 as a FRET donor. This probe could successfully detect repeated cycles of hypoxia−normoxia in live cells. We anticipate that this probe will be a valuable tool for exploring the response of organisms to hypoxia.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full experimental procedures, characterization data for all compounds, spectral properties of cyanine dyes and QSY-21 derivatives, compounds as candidates for a hypoxia-sensing moiety other than QSY-21, ESR spectra, HPLC analyses, and fluorescence imaging under normoxia. This material is available free of charge via the Internet at http://pubs.acs.org.

Ⓦ Web-Enhanced Feature

Video 1, showing exposure of a hypoxic solution of QSY-21 to air, is available in the online versions.

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Notes

The authors declare no competing financial interest.

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