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Hypoxia-Sensitive Fluorescent Probes for *in Vivo* Real-Time Fluorescence Imaging of Acute Ischemia

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Abstract: Based on the findings that the azo functional group has excellent properties as the hypoxia-sensor moiety, we developed hypoxia-sensitive near-infrared fluorescent probes in which a large fluorescence increase is triggered by the cleavage of an azo bond. The probes were used for fluorescence imaging of hypoxic cells and real-time monitoring of ischemia in the liver and kidney of live mice.

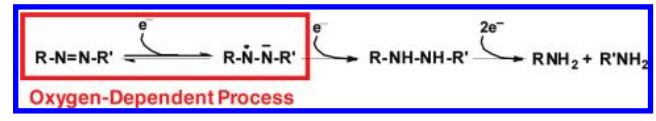
Hypoxia, which is caused by an inadequate oxygen supply, is a feature of various diseases, including cancer, cardiopathy, ischemia, and vascular diseases.[1] Therefore, hypoxia-specific molecular probes would be useful as diagnostic agents. Hypoxia-selective probes developed for positron emission tomography (PET),[2] fluorescence imaging, immunostaining,[3] and phosphorescence imaging[4,5] exploit the properties of two key intracellular responses caused by hypoxia. One is stabilization of hypoxia inducible factor-1 (HIF-1), and the other is the imbalance of cellular redox states leading to an increase in the reducing equivalent.

One of the earliest cellular responses is stabilization of HIF-1, a transcription factor of genes encoding a series of proteins involved in glycolytic metabolism and neovasculization. Since the stabilization is easily reversed by oxygenation through activation of a prolyl hydroxylase-domain protein (PHD) and ubiquitin-proteasome system, *in vivo* imaging of hypoxia has been achieved by using a fusion protein of the HIF-1α-binding domain called HRE with appropriate reporter proteins.[6,7] However, it is very difficult to apply this method in living animals due to the need for genetic modification.

A decrease in oxygen causes an increase in reductive stress. Indeed many small molecule-based probes to detect hypoxia take advantage of this phenomenon, and most probes employ nitroaromatic or quinone derivatives as hypoxia-sensitive moieties.[8−11] But the fluorescent probes previously developed are inadequate for *in vivo* imaging of ischemia, because of the low sensitivity response to hypoxia and/or use of short wavelengths for photoemission that potentially renders living cells and tissues vulnerable. We speculated that the poor reactivity in hypoxic reduction might be due to the probes being poor substrates for reductases. Nitroaromatic groups or quinone groups have very low LUMO energy, so that fluorescence quenching occurs *via* electron transfer, which is known as a photoinduced electron transfer (PeT) mechanism.[12] Generally, to effectively quench the fluorescence, electron-withdrawing groups such as nitroaromatics have to be placed in the vicinity. At the same time, to effectively serve as a substrate for reductases, however, the same groups have to be rather distant from the fluorophore moiety to prevent interference with the enzymatic activity.

To fulfill seemingly two opposing requirements, we employed Förster resonance energy transfer (FRET). FRET occurs efficiently when the distance between donor and acceptor is within 10 nm. With this distance between the fluorophore moiety and hypoxia-sensitive moiety, the fluorophore moiety should have little effect on the enzymatic reactivity. The FRET efficiency can be altered either by changing the distance to the FRET donor or by changing the overlap integral between the emission spectrum of the FRET donor and the absorption spectrum of the FRET acceptor.[13,14] In order to change the distance or overlap integral, the hypoxia-sensitive moiety should act as a linker between the FRET donor and acceptor or as the acceptor itself. However, considering the structure of nitroaromatic or quinone groups, hypoxic reduction of nitro groups does not appear to induce a drastic change in the distance or absorption, so it is likely to be difficult to develop hypoxia-sensitive fluorescent probes based on nitro aromatic groups. Therefore, we focused on an azo structure as a hypoxia-sensitive alternative to nitroaromatic or quinone moieties.

Scheme 1



Generally, azobenzene derivatives are reduced stepwise by various reductases to aniline derivatives (Scheme 1).[15] Through a series of reactions, the azo bond is cleaved and the distinctive absorption of the azo moiety is lost. In other words, a drastic change in distance and overlap integral becomes possible by using azo compounds. In the case of nitroaromatics, the formation of nitroanion radical compounds in the first step of reduction is reversible. This step is also strongly influenced by oxygen, and when the reaction system is abundant in oxygen, back oxidation readily occurs; thus, reduction is completed.[16] Azobenzene derivatives look like "oxidized amines", similar to nitroaromatics, which leads us to hypothesize that the reduction of azobenzene would also be strongly influenced by the oxygen concentration.

We first evaluated the reduction potential of representative nitroaromatic compounds, such as nitroimidazole and nitrofuran,

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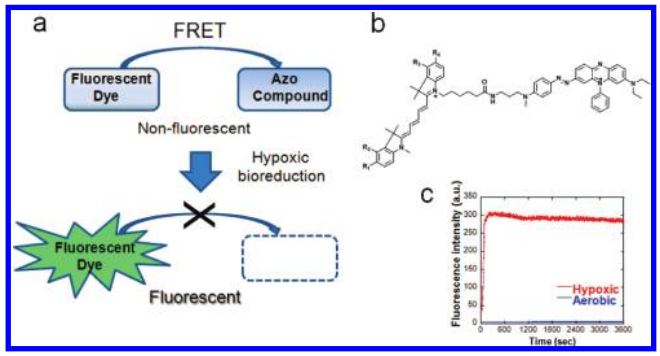

Figure 1. (a) Design strategy of QCys. (b) Chemical structure of QCys. QCy5 ($R_1 = R_2 = R_3 = R_4 = H$), QCy5.25 ($R_1 = R_2 =$ CH═CH, $R_3 = R_4 = H$), QCy5.5 ($R_1 = R_2 = R_3 = R_4 =$ CH═CH). (c) Time-dependent change of the fluorescence intensity of 1 μM QCy5 in the presence of rat liver microsomes (50 μL/3 mL) under hypoxic (red line) or normoxic (blue line) conditions. Measurements were performed in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent and 50 μM NADPH as an electron donor. Excitation and emission wavelengths were 650 and 670 nm.

and azobenzene derivatives, such as methyl red and disperse red, by means of cyclic voltammetry (Table S1). The reduction potentials of azobenzene derivatives are nearly equal to those of nitroaromatic compounds; in other words, azobenzene derivatives are reduced as efficiently as nitroaromatic compounds. Thus azobenzene derivatives appear to be good candidates for hypoxia-sensitive moieties.

In order to develop fluorescent probes for *in vivo* imaging, it is desirable that the fluorophore has near-infrared (NIR) absorption and emission since tissue penetration is best in that wavelength range. However, most of the azobenzene derivatives have absorptions and emissions in the visible region. We, therefore, focused on the Black Hole Quencher (BHQ) series. Among them, BHQ-3 has a very wide absorption from the visible to NIR and can quench NIR emissions. To examine the oxygen sensitivity of BHQ-3, we conducted an enzymatic assay of BHQ-3 using rat liver microsomes, which contain diverse metabolic enzymes. Under hypoxia, but not under normoxia, the absorption of BHQ-3 disappeared within 10 min (Scheme 1 and Figure S1). Hence we concluded that BHQ-3 is an excellent candidate for a hypoxia-sensitive moiety. We then designed and synthesized hypoxia-sensitive fluorescent probes combining an NIR fluorophore and BHQ-3. Under normoxic conditions, BHQ-3 is not reduced and the probes are nonfluorescent owing to the FRET mechanism. Under hypoxic conditions, BHQ-3 is readily reduced and loses its absorption, so FRET efficiency is decreased and the probes become fluorescent (Figure 1a). A great advantage of this design is that various probes with a wide range of absorption and emission wavelengths can be produced by simply changing the combination of fluorophores and quenchers.

We chose dicarbocyanines as candidate NIR fluorophores. First, we synthesized three dicarbocyanines (Schemes S1 and S2) and confirmed the stability of them in the presence of reductases under hypoxic conditions. The absorption spectra of all the dyes showed essentially no change under aerobic or hypoxic conditions, indicating that the dyes are stable (Figure S2). Therefore, we adopted this design strategy and synthesized three hypoxia-sensitive probes (QCy5, QCy5.25, QCy5.5) which have different absorption characteristics (Scheme S3, Figures 1b and S3, Table S2). All the probes had extremely weak fluorescence, and the emission could hardly be detected. After enzymatic reactions under hypoxic conditions, a 50- to 100-fold fluorescence increase was observed, and the fluorescence intensity reached a plateau within 10 min (Figures 1c and S4). On the other hand, under normoxic conditions, no fluorescence increase occurred. Furthermore, fluorescence intensities of QCys were insensitive to the pH change between 4.0 and 11.0. These results indicated that QCys could detect hypoxia selectively and rapidly, being superior to known fluorescent probes, in these respects.

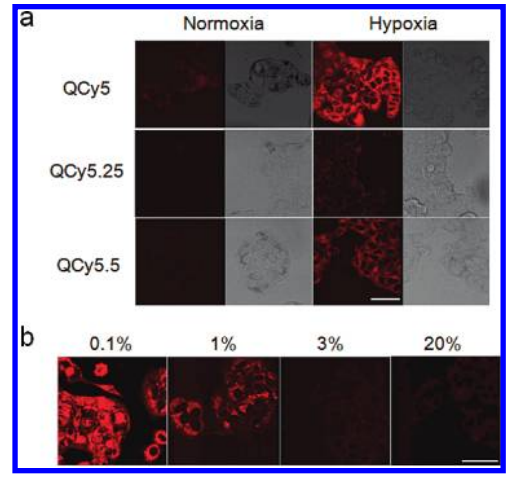

Figure 2. Oxygen-dependent fluorescence increase of QCys inside living MCF-7 cells, and oxygen dependency of QCy5 fluorescence. (a) Confocal laser scanning microscopy (left) or white light image (right) of 1 μM QCy5, QCy5.25, QCy5.5 loaded MCF-7 cells incubated under hypoxic (less than 0.1% O_2) or normoxic (20% O_2) conditions for 6 h. The QCys emission was obtained using excitation at 650 nm (QCy5) or 670 nm (QCy5.25 and QCy5.5). Scale bar, 50 μm. (b) Oxygen dependency of QCy5 fluorescence. MCF-7 cells loaded with 1 μM QCy5 were incubated at various oxygen levels. Scale bar, 50 μm.

Next, we applied the probes to living cells and investigated whether they could detect intracellular hypoxia. The three probes were loaded into MCF-7 breast cancer cells and incubated under normoxic conditions in a CO_2 incubator (20% O_2, 5% CO_2) or hypoxic conditions generated with an AnaeroPack (Mitsubishi Gas Corp.) (>0.1%O_2, 5% CO_2) for 6 h. Each probe showed strong fluorescence only in cells incubated under hypoxic conditions. These results indicated that QCys could detect hypoxia inside living cells (Figure 2a). We next investigated whether QCy5 could distinguish oxygen concentrations inside cells; i.e., MCF-7 cells loaded with 1 μM QCy5 were incubated under various oxygen concentrations (20%, 10%, 3%, 1%, 0.1%) for 6 h, and fluorescence images were obtained. In this experiment, strong fluorescence was detected from the cells incubated at 1% oxygen or less. Furthermore, the fluorescence intensity of the cells under 0.1% oxygen was twice that of the cells under 1% oxygen. These results indicated that QCy5 fluorescence was oxygen concentration dependent (Figures 2b and S5). We also examined other cell lines and obtained similar data. Hence, QCy5 could visualize the hypoxic status of a wide variety of cell lines. Interestingly, the oxygen sensitivity varied from cell line to cell line, presumably reflecting the origin of each cell line (Figure S5).

We next examined whether QCy5 can detect hypoxia in the intact animal *in vivo* using an ischemia model of the mouse liver. At 30 min after an intravenous injection of QCy5 (0.5 mg/kg), the portal vein was ligated to induce ischemia. Immediately after the ligation, the fluorescence intensity rapidly increased, suggesting that the uptake of QCy5 by hepatocytes occurred. Results indicate that QCy5 was readily reduced in the ischemic liver, but not in the normal liver. Thus, we achieved real-time imaging of liver ischemia using QCy5 (Figures S6 and S7, Supporting Video 1).

Next, we examined ischemic imaging at the whole-body level. A solution of QCy5 (0.5 mg/kg) was administered to mice by intravenous injection, and the portal vein and renal vein were ligated. Just after ligation, the fluorescence intensity of each organ

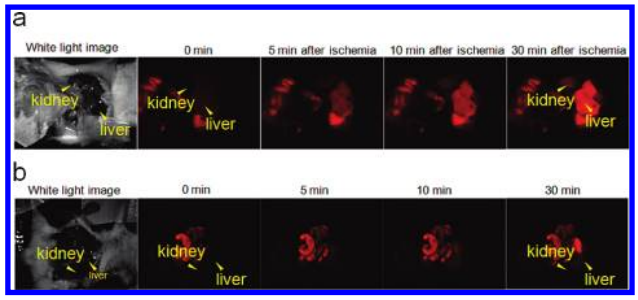

Figure 3. (a) Fluorescence images of living mouse after injection of QCy5 followed by vessel ligation as described in the text. Fluorescence images were obtained for 30 min, with excitation at 620 nm and emission at 680 nm. (b) Fluorescence images of living mouse after injection of QCy5 without vessel ligation.

rapidly increased, and after 30 min, the fluorescence intensity was saturated. The fluorescence intensity of these organs in nontreated mice did not change (Figures 3 and S8, Supporting Video 2). In this experiment, we could detect a fluorescence increase within 1 min, which means that real-time monitoring of the ischemic organs was achieved with QCy5. Because acute ischemia of the liver or kidney is often problematic in transplant surgery,[17] these probes have the potential to become powerful tools for clinical use. To our knowledge, QCys are the first fluorescent probes suitable for real-time imaging of acute ischemia in living animals.

In summary, we identified azobenzene derivatives as a novel type of hypoxia-sensitive moiety and developed a series of NIR fluorescent probes, QCys, for detecting hypoxia. These probes could distinguish hypoxia *in vitro* and were also suitable for fluorescence imaging of ischemic organs in live mice. The *in vivo* fluorescence response was rapid, occurring within 1 min after vessel ligation. These probes should be suitable for many biological applications, such as real-time monitoring of cerebral infarction.

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Supporting Information Available: Full experimental procedures, characterization data for all compounds, spectral properties of cyanine dyes and QCys, and the pH profiles of QCys. This material is available free of charge via the Internet at http://pubs.acs.org.

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