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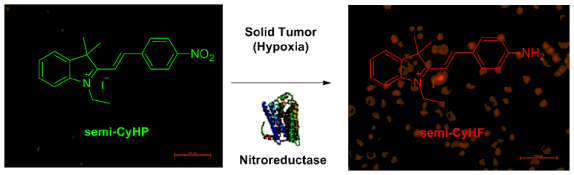

A selective turn-on fluorescent probe based on semi-cyanine for the detection of nitroreductase (NTR) and hypoxia was designed and synthesized.

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A Highly Selective Turn-on Fluorescent Probe Based on Semi-Cyanine for Detection of Nitroreductase and Hypoxic Tumor Cell Imaging

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Based on a semi-cyanine fluorophore, a selective turn-on fluorescent probe semi-CyHP for the detection of nitroreductase (NTR) and hypoxia was designed and synthesized. It can be activated by NTR and to restore the pull-push electronic systems of semi-CyHF, which strongly fluoresces at 556 nm. Besides, the investigation of the hypoxic tumor cell imaging with semi-CyHP was significant with minimal endogenous interference.

Nitroreductases are a family of evolutionarily related proteins, which participate in the reduction of those containing the nitro functional group compounds by using flavin monoucleotide as a cofactor[1-2]. NTR has been used not only to activate nitrofuran antibiotics but also to eliminate pervasive nitroaromatic pollutants[3-6]. Moreover, decrease of oxygen levels in tumor cells is often accompanied by the apparent increase of endogenous nitroreductase activity[7-8]. However, hypoxia is a common feature of solid tumors and renders tumor cells higher resistance towards therapy by preventing the proper metabolism of various anticancer drugs[9-10]. Hypoxia results in reductive stress and over expression of nitroreductase (NTR), azoreductase and quinone reductase[11-12]. And it is believed that the technique of tumor targeting is of significance for the diagnosis and treatment of cancer[13-15]. Therefore, the detection of hypoxia and NTR has been a feasible tool for the diagnosis of tumor cells and warrants robust detection for biological and environmental studies.

Various approaches for detection of NTR and hypoxia are available, among which fluorescence probe has attracted much attention because of its great temporal and spatial sampling capability as well as high sensitivity[7-8, 16-19]. Nitroaromatics are readily metabolized by NTR in a stepwise reduction pathway by cellular nitroreductase under hypoxic condition[20]. Probe with pimonidazole as a hypoxia marker for nitroreductase was reported by Nagasawa *et al*[21]. And based on that azo group has excellent response to hypoxia, the first near-infrared fluorescent probe was developed by Nagano *et al*[22].

Cyanines are a facile family of fluorescent dyes. Depending on the length of polymethine backbone, the entire spectrum from IR to UV may be covered. Cyanines have been extensively used in biomedical imaging. There are few developments in the structural modifications of cyanines, which achieves the diversity and novelty of chemical probes. Because of the unique nature of indole group, the main component of cyanines, it is usually covalently linked to other existed fluorophores[23-24], which increases the conjugated system and results in the improvement of its spectroscopic properties. Doron *et al* developed a new paradigm for generation of novel class of Turn-ON NIR cyanine-based probes[25-26]. A distinctive change of π-electronic system leads to generation of a cyanine dye with strong NIR fluorescence[27]. Although previous cyanine-based fluorescent probes for nitroreductase and hypoxia are of satisfactory by using nitroimidazole or *p*-nitrobenzyl as the receptor, the receptor is not involved in the composition of the fluorophore[16, 18]. Herein, we describe a novel cyanine-based Turn-ON fluorescent probe (**semi-CyHP**) for detection of NTR and hypoxia imaging and the corresponding reduction product **semi-CyHF** was achieved by organic synthesis for the spectroscopic studies in solution.

The *p*-nitrophenyl group can be conveniently converted into *p*-aminophenyl by nitroreductase or under hypoxic condition. Therefore, our strategy for the construction of this hypoxia probe

semi-CyHP relies on tethering the *p*-nitrophenyl group to the indole moiety via an alkenyl linkage (Scheme 1). The *p*-nitrophenyl group is not only a receptor for NTR but also an excellent fluorescence-quench group[28]. The electron-withdrawing nitro group inhibits the ICT effect and results in a weak fluorescence emission. Upon reduction, the amino group of **semi-CyHF** will reconstruct the electronic push-pull system and the fluorescence emission will be restored (Fig. 1).

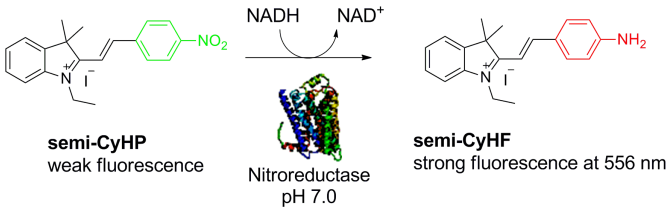
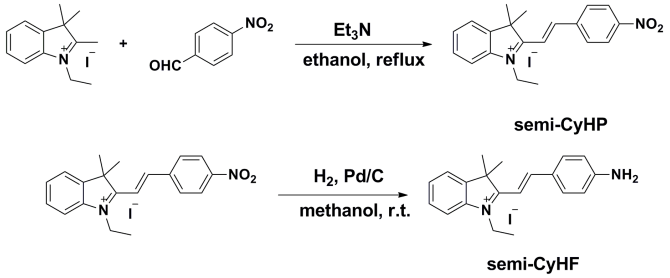


Fig. 1. Proposed detection mechanism of **semi-CyHP**.

The probe **semi-CyHP** was readily synthesized by condensing *p*-nitrobenzaldehyde and 1,2,3-trimethyl-3-methyl-benzo indole iodized salt. The bioreduction product **semi-CyHF** was also synthesized by reduction of **semi-CyHP** (Scheme 1).



Scheme 1. The synthetic route of **semi-CyHP** and **semi-CyHF**.

Next, we studied their spectral properties in chemical, enzymatic and cell media. Spectroscopic evaluation of **semi-CyHP** and **semi-CyHF** was carried out under physiological conditions at 37 ^{o}C in PBS buffer (pH = 7.0, 0.01 M) with 1% DMSO as co-solvent (Fig. S1). The probe **semi-CyHP** shows a maximal absorption at 385 nm. The reductive **semi-CyHF** has a strong absorption peak at 490 nm and a strong fluorescence peak at 556 nm. When **semi-CyHP** was incubated with the NTR, a drastic enhancement of fluorescence intensity at 556 nm was observed (Fig. S2).

Then, the assay of the probe **semi-CyHP** toward the reduction of NTR was performed as described previously[7, 19]. The fluorescence of the solution of probe **semi-CyHP** (10 μM) was undetectable when excited at around 490 nm. After that 17.5 μg mL^{-1} of NTR was added, an apparent fluorescence enhancement at around 556 nm was observed (Fig. 2). It can be drawn that the reduction of the probe **semi-CyHP** was underway and the reductive product **semi-CyHF** was formed as expected. It was estimated that ca. 95% of **semi-CyHP** was converted to **semi-CyHF** (Fig. S3). The drastic color change concomitant to reduction of **semi-CyHP** was easily noticed to naked eyes. It is also found that the fluorescence enhancement and maximal fluorescence intensity (around 556 nm) are connected to the initial concentrations of **semi-CyHP** (1-20 μM) (Fig. S4).

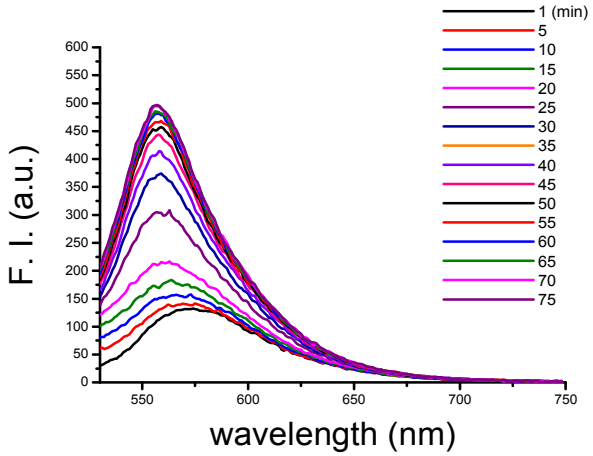


Fig. 2. Fluorescent spectra of **semi-CyHP** to NTR. **semi-CyHP** (10.0 μM) was cultured with 17.5 μg/mL NTR and NADH (500.0 μM). The fluorescent intensity data were collected after certain time intervals as indicated in the figure with excitation at 490 nm. Silt: 10, 10 nm.

Additionally, the fluorescence enhancement and maximal fluorescence intensity of the **semi-CyHP** (10 μM) varied according to the exposed doses of NTR. A linear relationship between the probe and NTR was established at 2.5-17.5 μg mL^{-1} and the detection limit was deduced to be 40 ng mL^{-1} within 30 min (Fig. 3). Because of its sensitivity for detection of NTR, it implied the potential for application in biological systems.

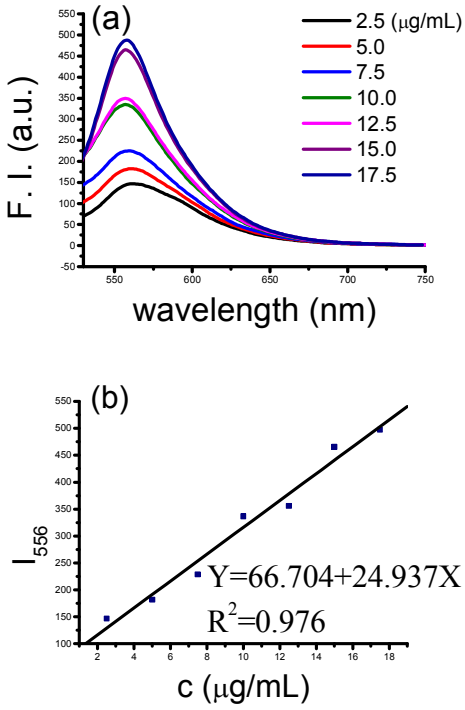


Fig. 3. Fluorescence spectra of **semi-CyHP** with different concentrations of nitroreductase. (a) **semi-CyBP** (10 μM) was cultured with different concentration

of nitroreductase and 50 equiv. NADH. (b) A linear correlation between fluorescent response and concentrations of nitroreductase. Excitation wavelength was at 490 nm. Silt: 10, 10 nm.

For further application in biological system, the selectivity of **semi-CyHP** toward other biological reducing agents was under investigation. Thiols have been suggested to be the electron providers for reductive activation of various hypoxia and prodrug[29-31]. Therefore, the probe **semi-CyHP** was treated with biorelevant thiols, such as homocysteine (Hcy), glutathione (GSH), cysteine (Cys), dithiothreitol (DTT) and *β*-Nicotinamide adenine dinucleotide (NADH). Under normal physiological condition, the concentration of reduced biological reductants is far lower than 1 mM. As shown in Fig. 4, incubation with biological thiols (2 mM) did not induce any noticeable signal modulation. And the selectivity was also tested in the mixture of NTR with these thiols, the fluorescence intensity of **semi-CyHF** increased slightly, even after reaction for 90 min under the same condition. Also, the impact of NADH or NTR on the probe was undergone respectively (Fig. S5). The remarkable fluorescence emission with 85-fold enhancement at around 556 nm was observed by incubation of **semi-CyHP** with NADH (50 equiv) and NTR.

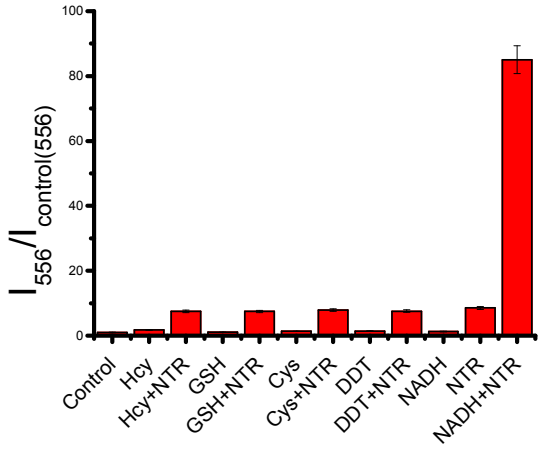


Fig. 4. Fluorescence response of **semi-CyHP** to the biological reductants. Control: the free probe **semi-CyHP**. Fluorescence response of **semi-CyHP** (10 μM) treated with various biological thiol reductants (2 mM) in PBS buffer (0.01M, pH 7.0 with 1% DMSO) at 37 °C. The fluorescent intensity data were collected after reaction for 90 min at around 556 nm divided by the initial fluorescence emission at around 556 nm. Fluorescence excitation was at 490 nm. Silt: 10, 10 nm.

These results suggested that those thiols employed in the experiments exhibited no interferences and our probe **semi-CyHP** has potentials for selectively monitoring NTR under physiological conditions.

The MTT assay showed that **semi-CyHP** exhibited little cytotoxicity to A549 cell at 0-10 μM (Fig. S6). A549 cells were incubated with **semi-CyHP** (5 μM) under hypoxic and normal conditions, respectively, at 37 °C for 7 h and then washed 3 times with PBS (pH 7.0) and treated with 5 μM **semi-CyHP** in FBS-free DMEM for 1 h. The changes of fluorescence intensity were measured using an inverted fluorescence microscope (Fig. 5).

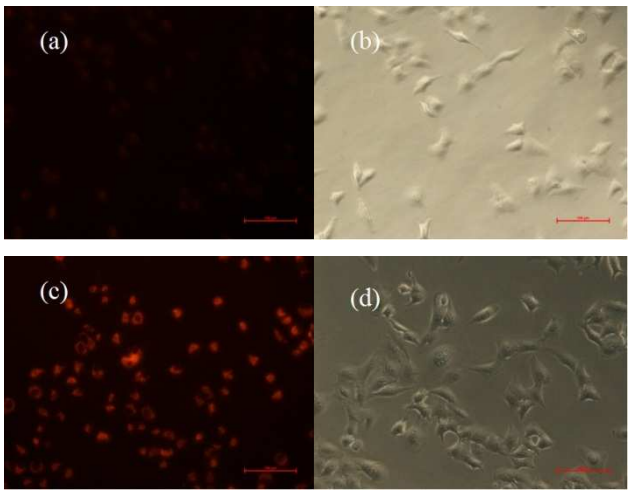


Fig. 5. Fluorescence and brightfield microphotographs of A549 cells incubated with 5 μM of fluorescent probe at 37 °C. The top row was taken at aerobic condition (a and b, 75% N_2, 5% CO_2, 20% O_2). The down row was taken at hypoxic condition (c and d, 94% N_2, 5% CO_2, 1% O_2). All cells incubated with 5 μM probe for 1 h. (b) and (d) were taken in bright field; (a) and (c) were taken in optical windows (green filter, between 540 and 580 nm). Scale bar: 100 μm.

It was obvious that A549 cells treated with the probe **semi-CyHP** under normoxic conditions showed nearly no fluorescence enhancement (Fig. 5a). Conversely, lots of drastic fluorescence spots appeared within the cells with the same reagents incubated under hypoxic conditions (Fig. 5c). These results clearly demonstrated that **semi-CyHP** is capable of in vitro imaging of hypoxia in solid tumors.

In summary, we have developed a novel selective and sensitive fluorescent probe **semi-CyHP** for the detection of NTR and hypoxia. The probe was activated by reaction with NTR and NADH under physiological conditions, to form the reduction product **semi-CyHF**, leading to a 85 fold fluorescence emission enhancement at ca. 556 nm. And its potentials for imaging applications were exhibited with hypoxic A549 cells.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental section, NMR and MS copies of new compounds and Fig. S1. See DOI: 10.1039/c000000x/

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