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LANZHOU INSTITUTE OF CHEMICAL PHYSICS
Chinese Academy of Sciences

Dear referees:

We would like to submit the enclosed manuscript entitled "A rapid response "Turn–On" fluorescent probe for nitroreductase detection and its application in hypoxic tumor cell imaging" by **Jian Xu**, **Shaobo Sun**, **Qian Li**, **Ying Yue**, **Yingdong Li** and **Shijun Shao**, which we wish to be considered for publication in ***Analyst***.

In this paper, a novel "Turn-ON" fluorescent probe **1** was developed and applied for rapid and convenient detection of nitroreductase as well as the hypoxic status of tumor cells. This probe was constructed by introducing 5-nitrofuran as a reacting moiety to the quaternarized 4-pyridinyl-substituted BODIPY dye through a C-N bond and showed high selectivity for nitroreductase against other biorelevant interferences. Due to the rapid response time (within 5 min), favorable stability and water-solubility, the probe was applied to monitor the hypoxic status of A549 tumor cells under physiological conditions. Moreover, as a biocompatible molecule, the probe has also been used for real-time and quantitative determination of nitroreductase produced by *Escherichia coli*. Therefore, it is hopeful to utilize this novel method in the biomedical research fields, such as imaging of disease-relevant hypoxia and detection of pathogenic microorganism.

The authors claim that none of the material in the paper has been published or is under consideration for publication elsewhere.

We look forward to your appreciation and positive response earnestly.

With best wishes,

LANZHOU INSTITUTE OF CHEMICAL PHYSICS
Chinese Academy of Sciences

Sincerely yours

Shijun Shao,

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Dear Prof. Mao,

We are grateful to your and other referees' critical comments and thoughtful suggestions. We feel lucky that our manuscript (AN-ART-09-2014-001671) went to these referees as the valuable comments from them helped us with the improvement of our manuscript. Please do forward our heartfelt thanks to these experts.

After careful thinking, we are very thankful and willing to accept your suggestion to resubmit an appropriately revised manuscript to Analyst. Based on these comments and suggestions, we have made careful revisions on the original manuscript and ESI. All the revised and added sections have been marked with red in the revised manuscript and ESI. In addition, we have carefully considered the suggestion of the reviewers and below you will find our point-by-point responses to the referees' comments.

We are very grateful for your guidance to our manuscript. If you have any question about the resubmitted manuscript, please do not hesitate to contact me.

Best wishes,

Sincerely yours,

Shijun Shao

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Responses to referees:

Referee 1:

The authors reported a BODIPY-based FL probe for the detection of nitrreductase and for imaging the hypoxic status of tumor cells. The probe has satisfatory properties for detection of nitroreductase in biological systems, especially the fast response time (within 5 min). Moreover, the manuscript is well-written, the related background and literature research is convincing, and the data is properly presented. Thus, I recommend the manuscript to be published in analyst.

Answer: Thanks for your critical comments.

Referee 2:

This paper reports on a novel fluorescent probe for the detection of nitroreductase and hypoxic states in tumor cells. The probe has a high sensitivity and a fast turn-on rate, in contrast with many previously established probes.

Overall, I found the paper to be well-organized and the work to be high quality. The conclusions are fully justified by the arguments put forth in the paper. From a technical standpoint, I feel that the paper is ready for publication. However, there were a few minor grammatical errors and poor wording that detracts slightly from the content of the paper. As it stands, the paper needs thorough editing (presumably with the aid of the editorial staff) before it can be published.

Answer: We are very grateful for the reviewers' guidance to our manuscript. We have made a carefully check and corrected grammatical errors and wording in revised manuscript. Revised sections were marked with red.

Referee 3:

Re: Manuscript AN-ART-09-2014-001671, A rapid response "Turn-On" fluorescent probe for nitroreductase detection and its application in hypoxic tumor cell imaging.

The authors describe the synthesis of a new chemical probe for detecting nitroreductase, a chemical which may be useful for hypoxic tumor cell imaging. They

demonstrate the use of this new probe for detecting nitroreductase in two in vitro experiments, one with eukaryotic tumor cells, and one with E. Coli cells.

(1) The paper is easy to follow, and the grammar of the paper is very good. However, the paper contains some flaws. In particular, for a leading journal, such as The Analyst, it is necessary to better explain the advantage of this probe versus other, existing probes. As the authors point out in the second paragraph of the introduction, several fluorescent nitroreductase probes already exist. They say that these other probes exhibit long response times, but how long? And high detection limit, but how high? I do not wish to dig through the references. The authors need to point out the current state of the art and then explain how their new probe is better.

Answer to (1):

Thanks for your valuable questions. According to the referee's recommendation, the recent reported fluorescent probes for nitroreductase (NTR) have been outlined in the table below, which has been added to supplementary materials as **Table S1**. We have added proper descriptions in the revised manuscript, marked with red.

Fluorescent NTR probe	Response time (min)	Linear range (ng/mL)	Detection limit (ng/mL)	Reference
Resorufin based probe	30	15-300	0.27	*Anal. Chem.* 2013, 85, 3926-3932.
Resorufin based probe	20	5-300	0.1	*Chem. Commun.* 2013, 49, 5859-5861.
1,8-naphthalimide based probe	10	Not mentioned	Not mentioned	*Org. Lett.* 2011, 13, 928-931.
Tricarbocyanine based probe	15	3000-13000	77	*Chem. Commun.* 2013, 49, 2554-2556.
Coumarin based probe	120	Not mentioned	Not mentioned	*Biosens. Bioelectron.* 2011, 26, 3511-3516.
BODIPY based probe	5	100-1000	9.6	this work

After comparation of existing probes, we can see that our new probe indeed displayed some desired properties such as rapid response time (within 5 min) and

relative low detection limit of 9.6 ng/mL. The rapid response time was mainly due to the introduction of a quaternarized 4-pyridine moiety as a leaving group, which significantly increased the kinetics of the cleavage reaction and was favorable for cell and microorganism detection in the complex biological systems.

(2) They might also mention photoacoustic imaging, which can image hypoxia directly in vivo, without the need for exogenous probes. How does the author's technology fit in with these advances in biomedical imaging? The biosensors community already has several methods for detecting E. Coli. The introduction does not explain how this chemical probe advances the detection of E. Coli relative to other technologies.

Answer to (2):

We are grateful to your valuable suggestions. Photoacoustic imaging is a non-invasive imaging method which allows structural, functional and molecular imaging. Whereas, we cannot do research on photoacoustic imaging due to the restriction of experiment conditions and equipments.

In our work, we developed a novel "Turn-ON" fluorescent probe for rapid and convenient detection of nitroreductase as well as the hypoxic status of tumor cells. As one of the real biological applications, our experiments indicated that the new probe can be effectively used for real-time and quantitative determination of nitroreductase produced by *E. coli*. Compared to other methods for detecting *E. Coli*, the chemical probe has been successfully used as a microbial growth indicator for real-time and dynamic monitoring of the growth process of *E. coli* which can produce nitroreductase. Corresponding descriptions have been added in the revised manuscript, marked with red.

(3) A big sticking point for me with this paper is the authors' poor characterization of the detection limit. Looking at figure 2, the lowest concentration that they appear to have tested was 100 ng/mL, yet they claim a detection limit of 9.6 ng/mL (not 9.5, not

9.7). That is absurd. You cannot just extrapolate the slope of the calibration curve down to zero and see where it crosses the noise baseline. The signal will not necessarily surpass the noise baseline at that concentration. Other things besides statistics, like chemistry, can get in the way. You have to do the measurements!

Answer to (3):

Thanks for your valuable questions. The detection limit of 9.6 ng/mL was measured and obtained according the frequently-used method described below (*Org. Lett.* 2008, 10, 1481-1484; *Org. Biomol. Chem.* 2008, 6, 3038-3040).

The detection limit was calculated based on the fluorescence titration. In the absence of nitroreductase, the fluorescence emission spectrum of probe **1** was measured by 11 times and the standard deviation of blank measurement was achieved. To gain the slop, the fluorescence intensity at 520 nm was plotted to the concentration of nitroreductase. So the detection limit was calculated with the following equation:

Detection limit = $3\boldsymbol{\sigma}/\mathbf{k}$

Where $\boldsymbol{\sigma}$ is the standard deviation of blank measurement, **k** is the slop between the fluorescence intensity versus nitroreductase concentration.

The determination method of detection limit has been added to the revised manuscript and revised sections were marked with red.

A rapid response ‘‘Turn–On’’ fluorescent probe for nitroreductase detection and its application in hypoxic tumor cell imaging

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Abstract: A novel ‘‘Turn-On’’ fluorescent probe, quaternarized 4-pyridinyl-substituted BODIPY dye by incorporating 5-nitrofuran moiety, was developed and applied for imaging the hypoxic status of tumor cells by the indirect detection of nitroreductase. The design was based on a nitroreductase-catalyzed reduction of the nitrofuran moiety in the presence of reduced nicotinamide adenine dinucleotide (NADH) as an electron donor and followed by the 1,6-rearrangement-elimination and the release of free 4-pyridinyl -substituted BODIPY dye **2**. This probe displayed desired properties such as high specificity, “Turn-On” fluorescence response with suitable sensitivity, appreciable water solubility and rapid response time (within 5 min). Moreover, as a biocompatible molecule,

the probe has been successfully applied for imaging the hypoxic status of tumor cells (e.g. A549 cells) and especially used for real-time determination of nitroreductase produced by *Escherichia coli*. Therefore, it is hopeful to apply this novel method in the biomedical research fields, such as imaging of disease-relevant hypoxia and detection of pathogenic microorganism.

Keywords: "Turn-On" fluorescent probe; nitroreductase detection; hypoxic tumor cell imaging; A549 cells; *Escherichia coli*

1. Introduction

Hypoxia is usually observed in solid tumors and has been considered to be an important feature of various diseases, including cardiac ischemia [1,2], stroke [3] and inflammatory diseases [4]. Clinical research found that a hypoxic status of solid tumors has a positive correlation with tumor progression toward a more malignant phenotype with increased metastatic potential and resistance to treatment [5,6]. Consequently, it is of considerable clinical significance to develop novel methods for hypoxia detection. It is known that hypoxia can induce accelerated bioreductive reactions and result in excess expression of intracellular reductases, such as quinone reductase, azoreductase and nitroreductase (NTR) [7,8]. Among them, more attention was paid to the NTR as the most representative of reductases. Under hypoxic conditions, intracellular nitroaromatic compounds are well-characterized to be superior substrates for nitroreductase in the presence of reduced nicotinamide adenine dinucleotide (NADH) as an electron donor. Such an enzyme-catalyzed one-electron reduction has been recognized to be a useful

reaction for the design of a hypoxia targeting and imaging approach [9-11]. Moreover, as a kind of bacterial enzyme produced by *Escherichia coli*, nitroreductase has been considered to be useful for the removal of nitroaromatic pollutants [12-14]. Thus, it is of great importance to detect the specific level of nitroreductase produced by *Escherichia coli* and solving this problem would be helpful for better understanding the biodegradation efficiency of nitroaromatic compounds as well as the activation of nitrofuran prodrugs [15,16].

Recently, several fluorescent nitroreductase probes based on the enzymatic reduction of nitroaromatic compounds have been developed and some of them have been used for hypoxia-sensitive imaging in tumor cells [17-21]. For example, Ma et al. reported 5-nitrofuran substitued phenoxazine derivative as a novel fluorescent probe for monitoring nitroreductase activities [19] and Qian et al. synthesized an effective ratiometric fluorescent probe by attaching a *p*-nitrobenzyl moiety to naphthalimide for hypoxia detection [17]. However, most common fluorescent probes exhibit long response time [18,19] and high detection limit [18,21], which affected their application for nitroreductase detection in the complicated biosystem.

As their notable merits of high fluorescence quantum yield, high extinction coefficient, excellent photochemical stability and low biological toxicity, BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes have been widely used in many fields including fluorescent switching, chemosensors, laser dyes and especially as labeling reagents in proteins and DNA research [22-25]. Whereas, the hydrophobic fluorescent core has greatly affected their application in biochemical and living systems [26,27]. In order to improve water-solubility, a few BODIPY derivatives bearing a quaternarized pyridine

moiety in their *meso* position have been reported lately as functional dyes for various applications, which showed good solubility in water because of the cationic pyridinium unit on the head moiety [28-34]. It is noteworthy that, for the quaternarized 4-pyridinyl -substituted BODIPY derivative, fluorescence from the dye fragment was extensively quenched due to the onset of a light-induced charge-shift reaction [30,32].

Inspired by this finding, a novel "Turn-On" fluorescent probe **1** with better water-solubility was synthesized for the quantitative detection of nitroreductase in PBS buffer solution. The probe was designed by introducing a quaternized 4-pyridinium group to the fluorescent BODIPY moiety in order to increase water solubility as well as to enhance cell permeability. Meanwhile, a 5-nitrofuran moiety has been chosen as the reaction site, because the compounds containing nitrofuran are well-known substrates for nitroreductase [16,35]. The probe exhibited high selectivity for nitroreductase over other biological relevant species and the rapid response time (within 5 min), which facilitated its application in the complicated biological systems. Meanwhile, a good linearity with relative low detection limit of 9.6 ng/mL was obtained under the optimized reaction condition. Moreover, the enzyme reaction mechanism was further determinated by the ESI-MS and HPLC analysis. In view of the recommendable properties such as high specificity, "Turn-On" fluorescence response, appreciable solubility, rapid response time and well biocompatibility, the probe has been successfully applied for imaging the hypoxic status in A549 tumor cells, and especially for the real-time quantitative determination of nitroreductase produced by *Escherichia coli*, which can be applied for real-time and dynamic monitoring of the growth process of *Escherichia coli*.

2. Experimental

2.1. Materials and measurements

Nitroreductase (≥100 units/mg) from *Escherichia coli* and NADH were purchased from Sigma reagent company. The lyophilized powder of nitroreductase was dissolved in pure water, and the enzyme solution was frozen immediately at −20 °C for storage and allowed to thaw before use, which resulted in no change of the enzyme activity. All other chemicals were purchased from Sigma and Aladdin reagent company without further purification except especial instruction. All the organic solvents were of analytical grade. Water was purified by a Milli-Q system. Melting points were determined on a PHMKG 05 (Germany) apparatus. ^{1}H and ^{13}C NMR spectra were measured on a Varian INOVA 400M spectrometer. ESI mass spectra were recorded out on an Agilent 1100 series LC/MSO Trap of MS spectrometer.

All pH measurements were made with a Sartorius basic pH-Meter. UV-visible spectral studies were performed on a Perkin Elmer Lambda-35 UV-visible double beam scanning spectrophotometer. Solution fluorescence spectra were measured on a Perkin Elmer LS 55 scanning spectrofluorometer equipped with a Xenon flash lamp. Samples for absorption and fluorescence measurements were contained in 1cm×1cm quartz cuvettes. Fluorescence imaging experiments were performed on an Olympus Fluoview 1000 confocal laser scanning microscope with excitation at 488 nm.

*2.2. Synthesis of compound **2***

4-Pyridinecarboxaldehyde (9.0 mmol, 0.96 g) was stirred with 2,4-dimethylpyrrole

(19.4 mmol, 1.85 g) in deoxygenated CH_2Cl_2 (150 mL). One drop of TFA was added and the mixture was stirred overnight under N_2 at room temperature. The red solution was treated with TCBQ (9.0 mmol, 2.21 g), stirring was continued for 30 min followed by the addition of Et_3N (15 mL). After 15 min, $BF_3{\cdot}Et_2O$ (15 mL) was added at 0 °C, and the mixture was stirred at room temperature for further 3 h. After washing with saturated aqueous $NaHCO_3$, the organic phase was separated, dried with $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/ethyl acetate, 1/1, v/v, as eluent) to afford the desired compound **2** as a red powder (0.35 g, Yield 12%). m.p. 241–243 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ=8.75 (d, 2H, pyridine H), 7.28 (d, 2H, pyridine H), 5.99 (s, 2H, pyrrole H), 2.54 (s, 6H, CH_3), 1.38 (s, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.6, 121.8, 123.3, 130.3, 137.6, 142.6, 143.6, 150.6, 156.4. MS (ESI): Calcd for $C_{18}H_{18}BF_2N_3$: 325.2, found: m/z 326.2 $(M+H)^+$.

*2.3. Synthesis of probe **1***

Compound **2** (1.2 mmol, 0.40 g) and 2-(bromomethyl)-5-nitrofuran (2.4 mmol, 0.50g) were dissolved in toluene, and then the mixture was refluxed at 110 °C for 12 h. The residue was evaporated and purified by silica gel column chromatography (ethyl acetate/ methanol, 5/1, v/v, as eluent) to afford the desired compound **1** as a red powder (0.13 g, Yield 24%). m.p. 183–185 °C. ^{1}H NMR (CD_3CN, 400 MHz): δ=9.18 (d, 2H, ArH), 8.32 (d, 2H, ArH), 7.57 (d, 2H, furan H), 7.21 (d, 2H, furan H), 6.26 (s, 2H, pyrrole H), 3.34 (s, 2H, CH_2), 2.57 (s, 6H, CH_3), 1.48 (s, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.7, 15.5, 113.8, 117.3, 123.4, 124.4, 130.1, 134.3, 144.0, 147.1, 148.7, 151.0, 154.4, 158.7. MS

(ESI): Calcd for $[C_{23}H_{22}BF_2N_4O_3]^+$: 451.1753, found: m/z 451.1797 $(M)^+$.

2.4. Measurement of fluorescence quantum yields

For determination of the fluorescence quantum yields (Φ_{fl}), a Perkin Elmer LS 55 instrument was used with fluorescein in 0.1 M NaOH as a fluorescence standard. Fluorescence quantum yields (Φ_{fl}) were obtained with the following equation (F denotes fluorescence intensity at each wavelength and $\Sigma[F]$ was calculated by summation of fluorescence intensity).

$$\Phi_{fl}{}^{sample} = \Phi_{fl}{}^{standard}\, Abs^{standard}\, \Sigma[F^{sample}] / Abs^{sample}\, \Sigma[F^{standard}]$$

2.5. Determination of detection limit

The detection limit was calculated based on the fluorescence titration [36,37]. In the absence of nitroreductase, the fluorescence emission spectrum of probe **1** was measured by 11 times and the standard deviation of blank measurement was achieved. To gain the slope, the fluorescence intensity at 520 nm was plotted against the concentration of nitroreductase. So the detection limit was calculated with the following equation:

Detection limit = $3\boldsymbol{\sigma}/\mathbf{k}$

Where **σ** is the standard deviation of blank measurement, **k** is the slope between the fluorescence intensity versus nitroreductase concentration.

2.6. General procedure for nitroreductase detection

All spectroscopic measurements were performed in 10 mM phosphate buffer (pH 7.4)

according to the following procedure. In a 10 mL tube, 5 mL of PBS and 50 μL of 1 mM probe **1** were mixed, followed by addition of NADH (final concentration, 50 μM) and an appropriate volume of nitroreductase sample solution. The final volume was adjusted to 10 mL with PBS and the reaction solution was mixed rapidly. After incubation at 37 ℃ for 5 min in a thermostat, a 3 mL portion of the reaction solution was transferred to a quartz cell to measure the absorbance or fluorescence with $\lambda_{ex/em}$ = 470/520 nm and both excitation and emission slit widths of 10 nm. Meanwhile, a blank solution containing no nitroreductase (control) was prepared and measured under the same conditions for comparison.

2.7. Cell culture and fluorescence imaging of hypoxia

A549 cells (human epithelial lung carcinoma cells) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. A549 cells were cultured in Dulbecco's modified eagle media (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin−streptomycin in a humidified incubator under hypoxic (93% N_2, 2% O_2 and 5% CO_2) condition for different time (2, 4, 6 and 8 h) [38,39]. As a control group, A549 cells were incubated under normoxic [95% air and 5% CO_2 (i.e., 20% O_2)] condition. Before use, the adherent cells were washed with FBS-free DMEM. For fluorescence imaging, the cells were further incubated with 5 μM of probe **1** in FBS-free DMEM at 37 °C for 30 min under the respective conditions and then washed three times with PBS buffer (pH 7.4) to remove the free probe.

2.8. Real-time detection of nitroreductase produced by Escherichia coli

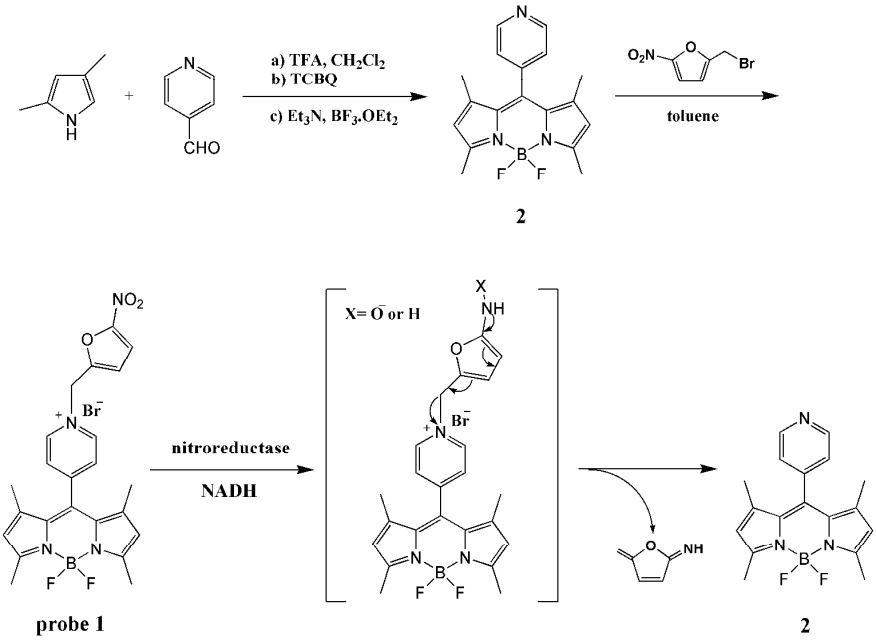
Escherichia coli (DH5α) was received from the laboratory of Prof. H. Liu at Lanzhou University. The Luria-Bertani (LB) culture medium was prepared by dissolving 10 g bacto-tryptone, 5 g bactoyeast extract and 10 g NaCl in 1 L water, followed by adjusting the pH to 7.4 with 1 M of NaOH. The culture medium was autoclaved prior to use. For determining nitroreductase generated by *Escherichia coli* (DH5α), the bacteria was first grown at 37 °C in LB culture media (pH 7.4) for 24 h. Then, the bacterial colonies were harvested using a sterile swab and inoculated into 100 mL of fresh LB culture media with an OD_{600} of 0.045. An appropriate volume (typically 5 mL) of the 100 mL LB culture media was taken and then pre-incubated at 37 °C in a rotary shaker (200 r/min) for different periods of time (0–6 h). After addition of 25 μL of probe **1** (final concentration: 5 μM) and then reaction for 5 min, both absorbance and fluorescence of the reaction solutions were measured.

3. Results and discussions

*3.1. Spectroscopic properties of probe **1** and its response to nitroreductase*

The probe was designed by introducing 5-nitrofuran as a reacting moiety to the quaternarized 4-pyridinyl-substituted BODIPY dye through a C-N bond (Scheme 1). As expected, probe **1** showed weak fluorescence due to the photo-induced electron transfer (PET) process [30,32] and such an extremely low background signal was rather favorable to affording high detection sensitivity for nitroreductase. Reaction of **1** with nitroreductase in the presence of NADH caused the reduction of the 5-nitrofuran moiety, followed by the

1,6-rearrangement elimination reaction and thereby the release of 4-pyridyl substituted BODIPY dye **2** (Scheme 1). As a result, a "Turn-on" fluorescent response was obtained, which leads to the development of a highly sensitive and selective method for monitoring nitroreductase activity in biological systems.



Scheme 1. Synthesis of probe **1** and its reaction with nitroreductase.

The spectroscopic evaluation of probe **1** and its response to nitroreductase were carried out under physiological conditions at 37 °C in PBS buffer (pH=7.4, 10 mM). Probe **1** exhibited a maximum absorbance at 508 nm and its reaction with nitroreductase induced a blue shift in the absorption band to 500 nm (Fig. S1 in ESI†), which was reasonably attributed to the enzyme-triggered cleavage reaction of probe **1** to release BODIPY fluorophore **2**. In the fluorescence emission spectrum, probe **1** featured a very weak emission at 520 nm, with a quantum yield of Φ_{fl} = 0.003. This low background signal was

due to the quaternization of 4-pyridine group of BODIPY and was quite desirable for sensitive detection [28-30]. However, treatment of probe **1** with nitroreductase triggered a dramatic increase of fluorescence intensity at 520 nm and correspondingly an obvious bright green-colored fluorescence was clearly observed (Fig. S2 in ESI† and inset of Fig. 1). 20-fold increase in its fluorescence intensity suggested that probe **1** was one of the most sensitive probes for the detection of nitroreductase in bionic systems [40,41]. Moreover, both the absorption and fluorescence spectra from the reaction system resembled those of BODIPY fluorophore **2**, supporting the fact that the enzyme-triggered cleavage reaction caused the release of free BODIPY fluorophore **2** (Fig. S1 in ESI†).

3.2. Time response and kinetic parameters

The time response curves of the nitroreductase-catalyzed reaction at varied concentrations were depicted in Fig. 1, which indicated that higher concentrations of nitroreductase resulted in faster cleavage reaction and stronger fluorescence intensity. For nitroreductase of no more than 5 μg/mL, the fluorescence increase could promptly become saturated in about 5 min. The rapid response time was mainly due to the introduction of a quaternarized 4-pyridine moiety as a leaving group, which significantly increased the kinetics of the cleavage reaction and was favorable for cell and microorganism detection in the complex biological systems [42,43]. The performance characteristic of rapid response for nitroreductase detection was superior to some recent reported fluorescent probes (Table S1 in ESI†). In contrast, the fluorescence of probe **1** in the absence of nitroreductase almost kept unchanged during the same period of time which implied that

the probe was stable in the detection system.

We next investigated the kinetic parameters for the enzymatic cleavage reaction of probe **1**. The Lineweaver-Burk plot of $1/V$ (V represents the initial reaction rate) versus the reciprocal of the probe concentration was established (Fig. S3 in ESI†). By fitting the data with the Michaelis–Menten equation, the corresponding Michaelis constant (K_m) and maximum of initial reaction rate (V_{max}) for the enzyme-activated reaction were determined to be 36.29 μM and 0.059 μM/s, respectively, which was comparable to the values reported previously [44,45].

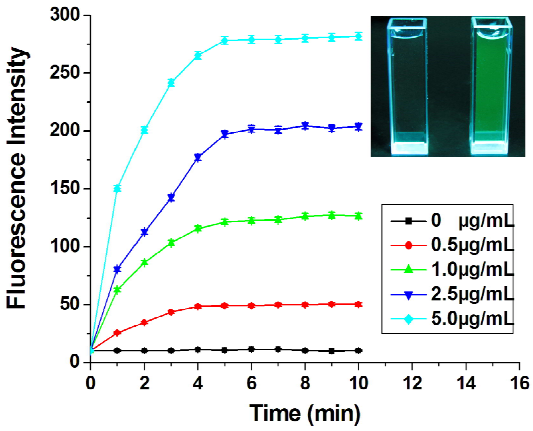


Fig. 1. A plot of fluorescence intensity of probe **1** (5 μM) vs. the reaction time in the presence of varied concentrations of nitroreductase (from bottom to top): 0 (control), 0.5, 1.0, 2.5 and 5.0 μg/ mL. Inset: Fluorescence photograph of probe **1** (5 μM) upon incubation with nitroreductase (2.5 μg/ mL) for 5min. All measurements were acquired at 37°C in 10 mM PBS, pH 7.4, with excitation at 470 nm.

3.3. Selectivity and stability test

The selectivity of the reaction was evaluated by treatment of various potential interfering species, such as inorganic salts (NaCl, KCl, $CaCl_2$, $MgCl_2$), biothiols (cysteine, glutathione and dithiothreitol), glutamic acid, arginine, vitamin C, vitamin B6, reactive

oxygen species (H_2O_2, HClO), glucose and human serum albumin (HSA) in parallel under the same conditions. As shown in Fig. 2, the probe exhibited high selectivity for nitroreductase over other species tested, which may be attributed to the specific reduction of the substrate (5-nitrofuran) by the enzyme. Particularly, the addition of *Escherichia coli* solution also triggers significant fluorescence enhancement with clear green fluorescence (A3, inset of Fig. 2). This result was mainly ascribed to the generation of nitroreductase by *Escherichia coli* and using this method, it is hopeful to develop a novel technique for rapid detection of certain bacteria.

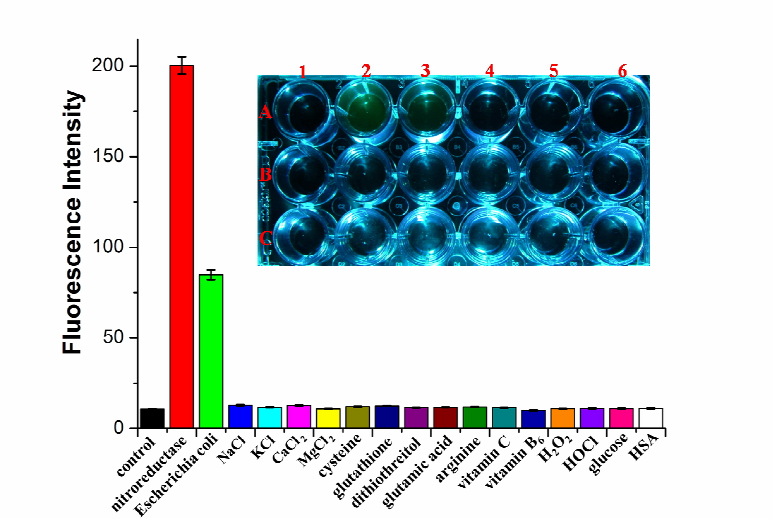


Fig. 2. Fluorescence responses of probe **1** (5 μM) in the presence of NADH (50 μM) to various species and corresponding fluorescence photograph (inset): A1 (control, probe **1** + NADH), A2 (nitroreductase, 2.5 μg/mL), A3 (*Escherichia coli,* OD_{600} =1.0), A4 (NaCl, 10 mM), A5 (KCl, 10 mM), A6 ($CaCl_2$, 2.5 mM), B1 ($MgCl_2$, 2.5 mM), B2 (cysteine, 1 mM), B3 (glutathione, 1mM), B4 (dithiothreitol, 1 mM), B5 (glutamic acid, 1 mM), B6 (arginine, 1 mM), C1 (vitamin C, 1 mM), C2 (vitamin B6, 1 mM), C3 (H_2O_2, 10μM), C4 (HClO, 10μM), C5 (glucose, 1 mM), C6 (HSA, 100 μM). The results are the mean ± standard deviation of three separate measurements, $\lambda_{ex}/_{em}$ = 470/520 nm.

The effects of pH and temperature on the reaction system were investigated (Fig. S4 in ESI†), which revealed that probe **1** functioned well under physiological conditions (about pH 7 and 37 °C). Besides, probe **1** alone was stable at various pH and temperature, which was favourable for its application in the complicated biosystems.

3.4. Emission titration experiments and proposed reaction mechanism

The detailed emission titration experiments of probe **1** with various concentrations of nitroreductase were also carried out under the optimized conditions (reaction at 37 °C for 5 min in 10 mM PBS of pH 7.4 in the presence of 50 μM NADH). As shown in Fig. 3, the fluorescence intensity was increased with increasing nitroreductase concentration, and a good linearity was obtained in the concentration range of 0.1–1.0 μg/mL nitroreductase, with a detection limit of 9.6 ng/mL.

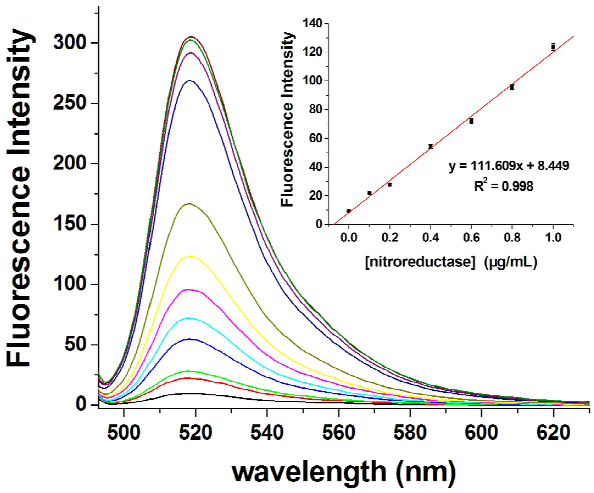


Fig. 3. Fluorescence response of probe **1** (5 μM) in the presence of NADH (50 μM) to nitroreductase at varied concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 μg/ mL). Inset: Relationship between the concentration of nitroreductase and the fluorescence intensity of the reaction mixture. All measurements were acquired at 37 °C in 10 mM PBS, pH 7.4, with excitation at 470 nm.

To confirm the sensing mechanism, the reaction of probe **1** with nitroreductase was conducted using ESI-MS and HPLC analysis. The ESI-MS spectrum of the reaction solution of probe **1** with nitroreductase showed a major peak at m/z = 326.1400 $[M+H]^+$, which was characterized as compound **2** (Fig. S5 in ESI†). Meanwhile, 4-pyridinyl BODIPY dye **2** was further verified as a major final product by HPLC analysis (Fig. S6 in ESI†). After reaction with nitroreductase for 5 min, the peak at 3.79 min representing probe **1** decreased markedly, concomitant with the emergence of a new peak at 14.00 min for compound **2**. Therefore, all these data indicated that the reaction most likely undergoes the proposed mechanism as shown in Scheme 1.

*3.5. Fluorescence imaging of hypoxia in A549 cells by probe **1***

Further, probe **1** was applied for fluorescence imaging of hypoxia conditions in tumor cells. A549 cells (human epithelial lung carcinoma cells) were selected because they are known to express nitroreductase [17,19,39]. In our experiments, A549 cells were first grown at 37 °C under normoxic (20% O_2) for 8 h and hypoxic (2% O_2) conditions for different time (2, 4, 6 and 8 h) and then incubated with probe **1** for 30 min under the respective conditions. As shown in Fig. 4A, A549 cells treated with probe **1** under 8 h normoxic condition showed negligible intracellular background fluorescence. However, the cells treated with probe **1** under hypoxic conditions produced a striking bright-green fluorescence and the fluorescence intensity was enhanced dramatically with the time increasing. These results demonstrated that the intracellular produced nitroreductase was in direct proportion to the incubation time under hypoxic condition and probe **1** can be

used for indicating the extent of relative hypoxia in living cells. In addition, both brightfield measurements and nuclear staining with Hoechst 33342 [46,47] showed that the cells were viable throughout the imaging experiments (Fig. 4B).

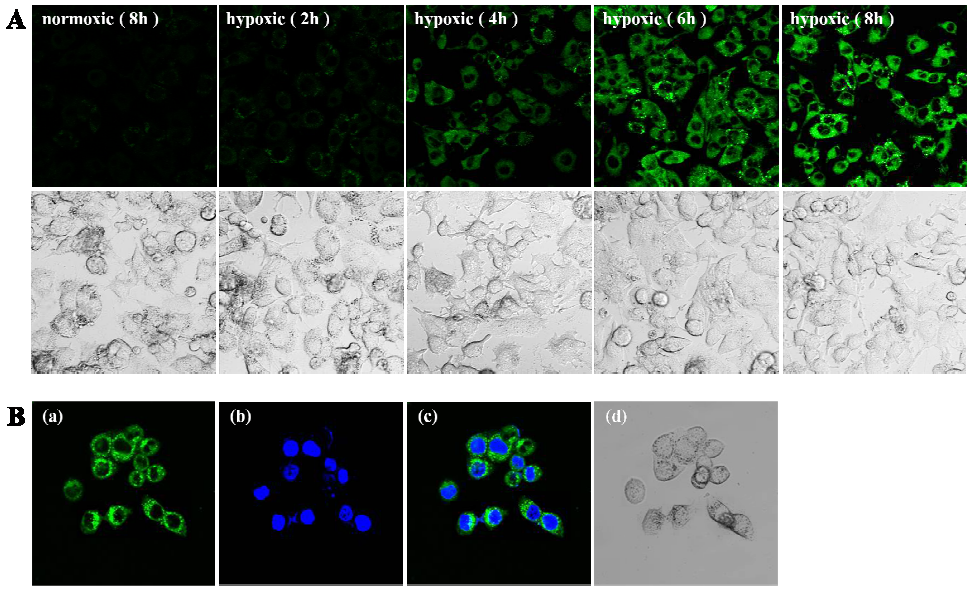


Fig. 4. (A) Confocal fluorescence images of A549 cells under normoxic (20% O_2) for 8 h and hypoxic (2% O_2) conditions for different time (2, 4, 6 and 8 h). A549 cells pretreated at various O_2 levels were incubated with 10 μM probe **1** for 30 min. The differential interference contrast (DIC) images of the corresponding samples are shown below. (B) confocal fluorescence images of A549 cells under hypoxic (2% O_2) conditions for 6 h and then (a) imaged with 10 μM probe **1** for 30 min, (b) imaged with 1 μM Hoechst 33342 for 20 min, (c) overlay of (a) and (b), (d) bright-field image.

3.6. Real-time monitoring of nitroreductase produced by Escherichia coli

The cell experiments confirmed probe **1** can serve as a biocompatible probe in live biological systems, which encouraged us to evaluate the feasibility for the application of probe **1** to detect nitroreductase generated by *Escherichia coli*. In this experiment, *Escherichia coli* (DH5α) was grown at 37 °C for 12 h in Luria-Bertani (LB) culture media

(pH 7.4) [48-50]. Then, the bacterial colonies were inoculated into 100 mL of fresh LB culture media with an OD_{600} of 0.045. An appropriate volume (typically 5 mL) of the 100 mL culture media was taken and then pre-incubated at 37 °C in a rotary shaker for different periods of time (0–6 h). After reaction with probe **1** (5 μM) for 5 min, both fluorescence and absorbance of the reaction solutions were measured.

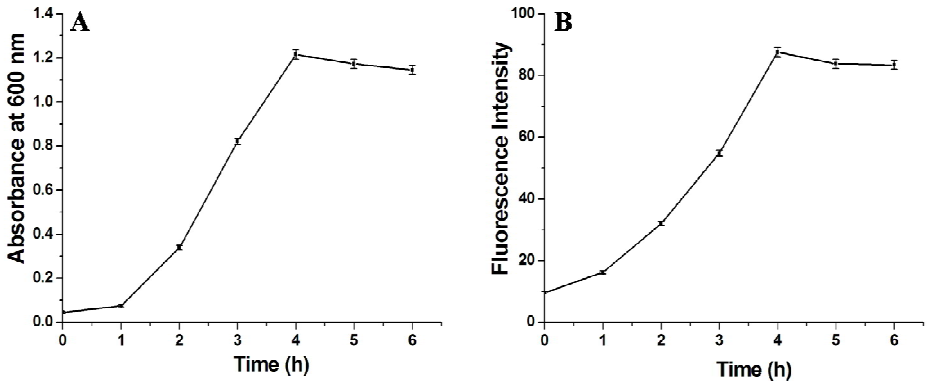


Fig. 5. (A) Absorbance change of *Escherichia coli* solution (LB culture media, pH=7.4) with increased incubation time. (B) Fluorescence change of probe **1** (5 μM) reacting with *Escherichia coli* which was pre-incubated in the LB culture media (pH 7.4) for different periods of time (0, 1, 2, 3, 4, 5 and 6 h), $\lambda_{ex}/_{em}$ = 470/520 nm. In these experiments, *Escherichia coli* has an initial OD_{600} of 0.045 and the data are the mean ± standard deviation of three separate measurements.

Fig. 5A showed the growth of *Escherichia coli* with time. As can be seen, *Escherichia coli* was in their logarithmic growth phase during the time from 1 h to 4 h and reached a plateau beyond 4 h [51,52]. Meanwhile, the fluorescence of the reaction solutions was increased with increasing pre-incubation time of *Escherichia coli* (Fig. 5B), which may be ascribed to that the generation of more nitroreductase leads to the release of more 4-pyridinyl BODIPY dye **2**. The formation of compound **2** in the LB culture media was also verified by electrospray ionization mass spectral analysis (m/z = 326.1407 $[M+H]^+$,

Fig. S7 in ESI†). Moreover, using the standard curve established above (inset of Fig. 3), the concentration of nitroreductase produced by *Escherichia coli* at various time has been quantitatively determined (Table S2 in ESI†), which shows that probe **1** is suited for real-time and quantitative determination of nitroreductase in biosystems. Notablely, the growth curve of *Escherichia coli* (Fig. 5A) was approximately consistent with the fluorescence change curve of Fig. 5B, suggesting that probe **1** not only can be used for quantitative determination of nitroreductase produced by *Escherichia coli* but also can be served as a microbial growth indicator for real-time and dynamic monitoring of the growth process of *Escherichia coli*.

4. Conclusions

In summary, we have presented a novel "Turn-ON" fluorescent probe for rapid and convenient detection of nitroreductase as well as for imaging the hypoxic status of tumor cells. This probe showed high selectivity for nitroreductase against other biorelevant interferences, which was ascribed to the enzyme-catalyzed reduction of the nitrofuran moiety, followed by the 1,6-rearrangement-elimination and the release of free dye **2**. Due to the fast response time (within 5 min), low detection limit, favorable stability and water-solubility, the probe was applied for monitoring the hypoxic status of A549 tumor cells under physiological conditions. Furthermore, the probe has been successfully used for real-time and quantitative determination of nitroreductase produced by *Escherichia coli*. Therefore, it is hopeful to utilize the novel fluorescent probe for investigating the effects of nitroreductase in the biomedical research fields.

Acknowledgements

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References

1. J. H. Crawford, T. S. Isbell, Z. Huang, S. Shiva, B. K. Chacko, A. N. Schechter, V. M. Darley-Usmar, J. D. Kerby, J. D. Lang and D. Kraus, *Blood*, 2006, **107**, 566-574.
2. M. T. Gladwin, N. J. Raat, S. Shiva, C. Dezfulian, N. Hogg, D. B. Kim-Shapiro and R. P. Patel, *Am. J. Physiol-Heart. C.*, 2006, **291**, 2026-2035.
3. Y. Tang, A. Lu, B. J. Aronow, K. R. Wagner and F. R. Sharp, *Eur. J. Neurosci.*, 2002, **15**, 1937-1952.
4. C. Murdoch, M. Muthana and C. E. Lewis, *J. Immunol.*, 2005, **175**, 6257-6263.
5. M. Höckel, K. Schlenger, B. Aral, M. Mitze, U. Schäffer and P. Vaupel, *Cancer Res.*, 1996, **56**, 4509-4515.
6. E. K. Rofstad, H. Rasmussen, K. Galappathi, B. Mathiesen, K. Nilsen and B. A. Graff, *Cancer Res.*, 2002, **62**, 1847-1853.
7. H. Komatsu, H. Harada, K. Tanabe, M. Hiraoka and S.-i. Nishimoto, *MedChemComm*, 2010, **1**, 50-53.
8. K. Kiyose, K. Hanaoka, D. Oushiki, T. Nakamura, M. Kajimura, M. Suematsu, H. Nishimatsu, T. Yamane, T. Terai and Y. Hirata, *J. Am. Chem. Soc.*, 2010, **132**, 15846-15848.
9. N. A. Helsby, G. J. Atwell, S. Yang, B. D. Palmer, R. F. Anderson, S. M. Pullen, D. M. Ferry, A. Hogg, W. R. Wilson and W. A. Denny, *J. Med. Chem.*, 2004, **47**, 3295-3307.
10. Y. Hitomi, T. Takeyasu, T. Funabiki and M. Kodera, *Anal. Chem.*, 2011, **83**, 9213-9216.
11. J. Napp, T. Behnke, L. Fischer, C. Wurth, M. Wottawa, M. Katschinski, F. Alves, U. Resch-Genger and M. Schaferling, *Anal. Chem.*, 2011, **83**, 9039-9046.
12. J. C. Spain, *Annu. Rev. Microbiol.*, 1995, **49**, 523-555.
13. P. Van Dillewijn, A. Caballero, J. A. Paz, M. M. González-Pérez, J. M. Oliva and J. L. Ramos, *Environ. Sci. Technol.*, 2007, **41**, 1378-1383.

14. I. Solyanikova, B. Baskunov, M. Baboshin, A. Saralov and L. Golovleva, *Appl. Biochem. Microbiol.*, 2012, **48**, 21-27.

15. P. R. Race, A. L. Lovering, R. M. Green, A. Ossor, S. A. White, P. F. Searle, C. J. Wrighton and E. I. Hyde, *J. Biol. Chem.*, 2005, **280**, 13256-13264.

16. J. Whiteway, P. Koziarz, J. Veall, N. Sandhu, P. Kumar, B. Hoecher and I. Lambert, *J. Bacteriol.*, 1998, **180**, 5529-5539.

17. L. Cui, Y. Zhong, W. Zhu, Y. Xu, Q. Du, X. Wang, X. Qian and Y. Xiao, *Org. Lett.*, 2011, **13**, 928-931.

18. H.-C. Huang, K.-L. Wang, S.-T. Huang, H.-Y. Lin and C.-M. Lin, *Biosens. Bioelectron.*, 2011, **26**, 3511-3516.

19. Z. Li, X. Li, X. Gao, Y. Zhang, W. Shi and H. Ma, *Anal. Chem.*, 2013, **85**, 3926-3932.

20. K. Okuda, Y. Okabe, T. Kadonosono, T. Ueno, B. G. Youssif, S. Kizaka-Kondoh and H. Nagasawa, *Bioconjugate Chem.*, 2012, **23**, 324-329.

21. K. Xu, F. Wang, X. Pan, R. Liu, J. Ma, F. Kong and B. Tang, *Chem. Commun.*, 2013, **49**, 2554-2556.

22. A. Loudet and K. Burgess, *Chem. Rev.*, 2007, **107**, 4891-4932.

23. G. Ulrich, R. Ziessel and A. Harriman, *Angew. Chem. Int. Edit.*, 2008, **47**, 1184-1201.

24. J.-S. Lee, N.-y. Kang, Y. K. Kim, A. Samanta, S. Feng, H. K. Kim, M. Vendrell, J. H. Park and Y.-T. Chang, *J. Am. Chem. Soc.*, 2009, **131**, 10077-10082.

25. L.-Y. Niu, Y.-S. Guan, Y.-Z. Chen, L.-Z. Wu, C.-H. Tung and Q.-Z. Yang, *J. Am. Chem. Soc.*, 2012, **134**, 18928-18931.

26. S. Zhu, J. Zhang, G. Vegesna, F.-T. Luo, S. A. Green and H. Liu, *Org. Lett.*, 2010, **13**, 438-441.

27. L. Li, J. Han, B. Nguyen and K. Burgess, *J. Org. Chem.*, 2008, **73**, 1963-1970.

28. M. A. Alamiry, A. Harriman, L. J. Mallon, G. Ulrich and R. Ziessel, *Eur. J. Org. Chem.*, 2008, 2774-2782.

29. G. Ulrich and R. Ziessel, *J. Org. Chem.*, 2004, **69**, 2070-2083.

30. A. Harriman, L. J. Mallon, G. Ulrich and R. Ziessel, *ChemPhysChem*, 2007, **8**, 1207-1214.

31. D. Wang, R. Miyamoto, Y. Shiraishi and T. Hirai, *Langmuir*, 2009, **25**, 13176-13182.

32. J. Xu, Q. Li, Y. Yue, Y. Guo and S. Shao, *Biosens. Bioelectron.*, 2014, **56**, 58-63.

33. S. Zhang, T. Wu, J. Fan, Z. Li, N. Jiang, J. Wang, B. Dou, S. Sun, F. Song and X. Peng, *Org. Biomol. Chem.*, 2013, **11**, 555-558.

34. N. Jiang, J. Fan, T. Liu, J. Cao, B. Qiao, J. Wang, P. Gao and X. Peng, *Chem. Commun.*, 2013, **49**,

10620-10622.

35. N. Zhang, C. Tan, P. Cai, P. Zhang, Y. Zhao and Y. Jiang, *Chem. Commun.*, 2009, 3216-3218.

36. M. Zhu, M. J. Yuan, X. F. Liu, J. L. Xu, J. Lv, C. S. Huang, H. B. Liu, Y. Y. Li, S. Wang and D. B. Zhu, *Org. Lett.*, 2008, **10**, 1481-1484.

37. H. T. Niu, D. D. Su, X. L. Jiang, W. Z. Yang, Z. M. Yin, J. Q. He and J. P. Cheng, *Org. Biomol. Chem.,* 2008, **6**, 3038-3040.

38. E. Nakata, Y. Yukimachi, H. Kariyazono, S. Im, C. Abe, Y. Uto, H. Maezawa, T. Hashimoto, Y. Okamoto and H. Hori, *Bioorg. Med. Chem.*, 2009, **17**, 6952-6958.

39. S. Takahashi, W. Piao, Y. Matsumura, T. Komatsu, T. Ueno, T. Terai, T. Kamachi, M. Kohno, T. Nagano and K. Hanaoka, *J. Am. Chem. Soc.*, 2012, **134**, 19588-19591.

40. H. C. Huang, K. L. Wang, S. T. Huang, H. Y. Lin and C. M. Lin, *Biosens. Bioelectron.*, 2011, **26**, 3511-3516.

41. K. Xu, F. Wang, X. Pan, R. Liu, J. Ma, F. Kong and B. Tang, *Chem. Commun.*, 2013, **49**, 2554-2556.

42. Y. Bao, B. Liu, H. Wang, J. Tian and R. Bai, *Chem. Commun.*, 2011, **47**, 3957-3959.

43. M. Zheng, H. Tan, Z. Xie, L. Zhang, X. Jing and Z. Sun, *ACS Appl. Mater. Inter.*, 2013, **5**, 1078-1083.

44. Z. Naal, J.-H. Park, S. Bernhard, J. Shapleigh, C. Batt and H. Abruna, *Anal. Chem.*, 2002, **74**, 140-148.

45. H. Zhang, L. Qi, J. Qiao and L. Mao, *Anal. Chim. Acta*, 2011, **691**, 103-109.

46. S. C. Dodani, S. C. Leary, P. A. Cobine, D. R. Winge and C. J. Chang, *J. Am. Chem. Soc.*, 2011, **133**, 8606-8616.

47. B. C. Dickinson and C. J. Chang, *J. Am. Chem. Soc.*, 2008, **130**, 9638-9639.

48. Z. Li, X. Gao, W. Shi, X. Li and H. Ma, *Chem. Commun.*, 2013, **49**, 5859-5861.

49. G. Sezonov, D. Joseleau-Petit and R. D'Ari, *J. Bacteriol.*, 2007, **189**, 8746-8749.

50. M. D. Roldan, E. Perez-Reinado, F. Castillo and C. Moreno-Vivian, *FEMS Microbiol. Rev.*, 2008, **32**, 474-500.

51. A. M. Sánchez, G. N. Bennett and K.-Y. San, *J. Biotechnol.*, 2005, **117**, 395-405.

52. S. J. Berríos-Rivera, G. N. Bennett and K.-Y. San, *Metab. Eng.*, 2002, **4**, 217-229.

Supporting Information

A rapid response ‘‘Turn–On’’ fluorescent probe for nitroreductase detection and its application in hypoxic tumor cell imaging

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Table of Contents:

1. Absorption and emission spectra of probe 1 toward nitroreductase.

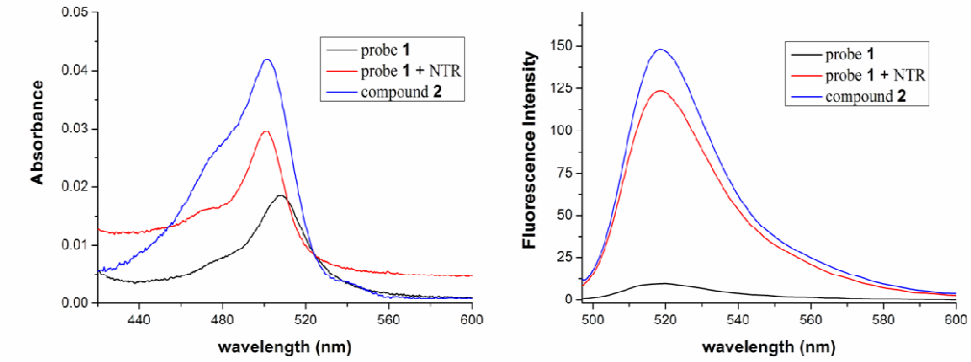

Fig. S1. UV-vis absorption spectra and fluorescent emission spectra of probe **1** (5 μM, black line), compound **2** (5 μM, blue line) and the reaction mixture (red line) of probe **1** (5 μM) with nitroreductase (2.5 μg/ mL), in the presence of 50 μM NADH for 5 min. All measurements were acquired at 37 °C in 10 mM PBS, pH 7.4, with excitation at 470 nm.

2. Time response of probe 1 to nitroreductase.

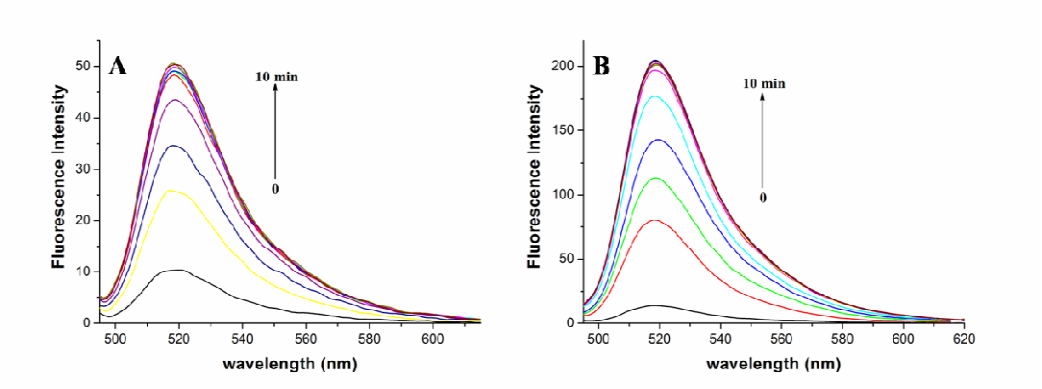

Fig. S2. Fluorescence turn-on response of probe **1** (5 μM) to (A) 0.5 μg/ mL nitroreductase and (B) 2.5 μg/ mL nitroreductase in the presence of 50 μM NADH at 37 °C. Spectra shown were acquired before nitroreductase addition and 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min after nitroreductase was added. All measurements were acquired at 37 °C in 10 mM PBS, pH 7.4, with excitation at 470 nm.

3. Comparison of various fluorescent probes for NTR detection.

Table S1

Comparison of various fluorescent probes for nitroreductase detection.

Fluorescent NTR probe	Response time (min)	Linear range (ng/mL)	Detection limit (ng/mL)	Reference
Resorufin based probe	30	15-300	0.27	*Anal. Chem.* 2013, 85, 3926-3932.
Resorufin based probe	20	5-300	0.1	*Chem. Commun.* 2013, 49, 5859-5861.
1,8-naphthalimide based probe	10	Not mentioned	Not mentioned	*Org. Lett.* 2011, 13, 928-931.
Tricarbocyanine based probe	15	3000-13000	77	*Chem. Commun.* 2013, 49, 2554-2556.
Coumarin based probe	120	Not mentioned	Not mentioned	*Biosens. Bioelectron.* 2011, 26, 3511-3516.
BODIPY based probe	5	100-1000	9.6	this work

4. Lineweaver-Burk plot for the enzyme-catalyzed reaction.

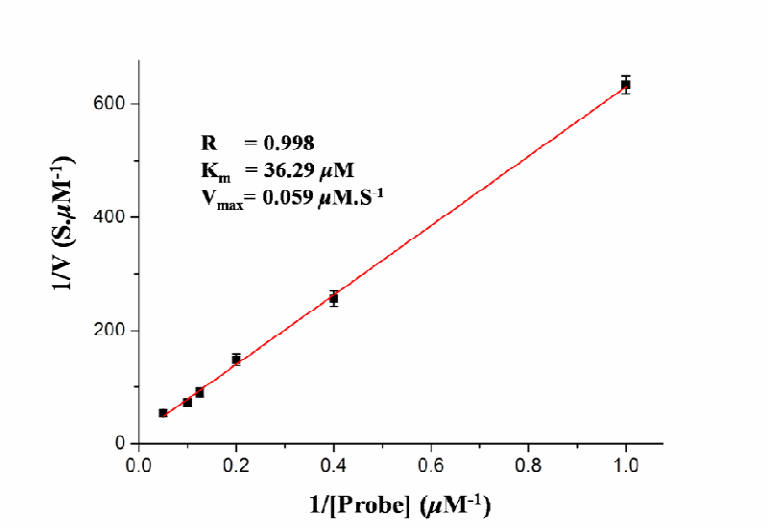

Fig. S3. Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V_{max}$ [probe] / (K_m + [probe]), where V is the reaction rate, [probe] is the probe concentration (substrate), and K_m is the Michaelis constant. Conditions: 0.50 μg/mL nitroreductase, 50 μM NADH, 1 - 20 μM of probe **1**, $\lambda_{ex}/_{em}$ = 470/520 nm. Reaction at each probe concentration was repeated three times, and the error bars represent standard deviations. Points were fitted using a linear regression model (correlation coefficient R = 0.998).

5. Effects of pH and temperature on the reaction system.

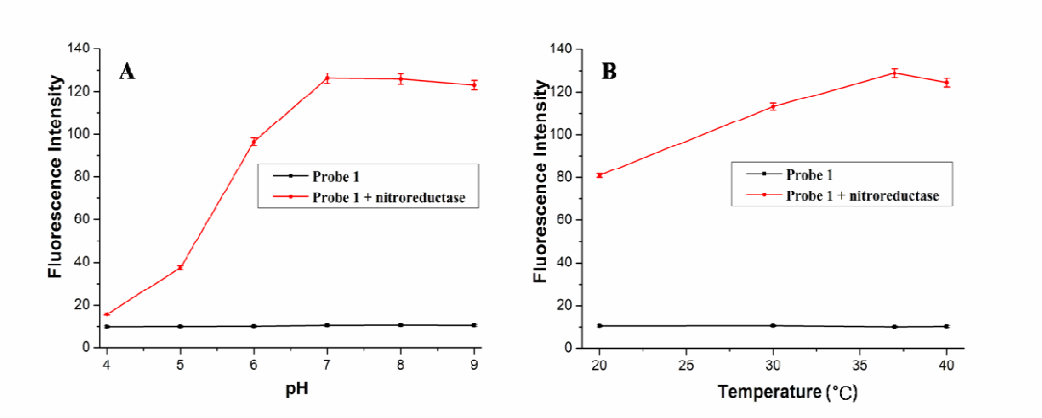

Fig. S4. Effects of (A) pH and (B) temperature on the fluorescence of probe **1** (5 μM) reacting with nitroreductase (1 μg/mL) in the presence of 50 μM NADH. All measurements were acquired in 10 mM PBS, pH 7.4, with excitation at 470 nm. Every data point was the mean of three measurements. The error bars are the standard deviation.

6. HRMS proof for the sensing mechanism.

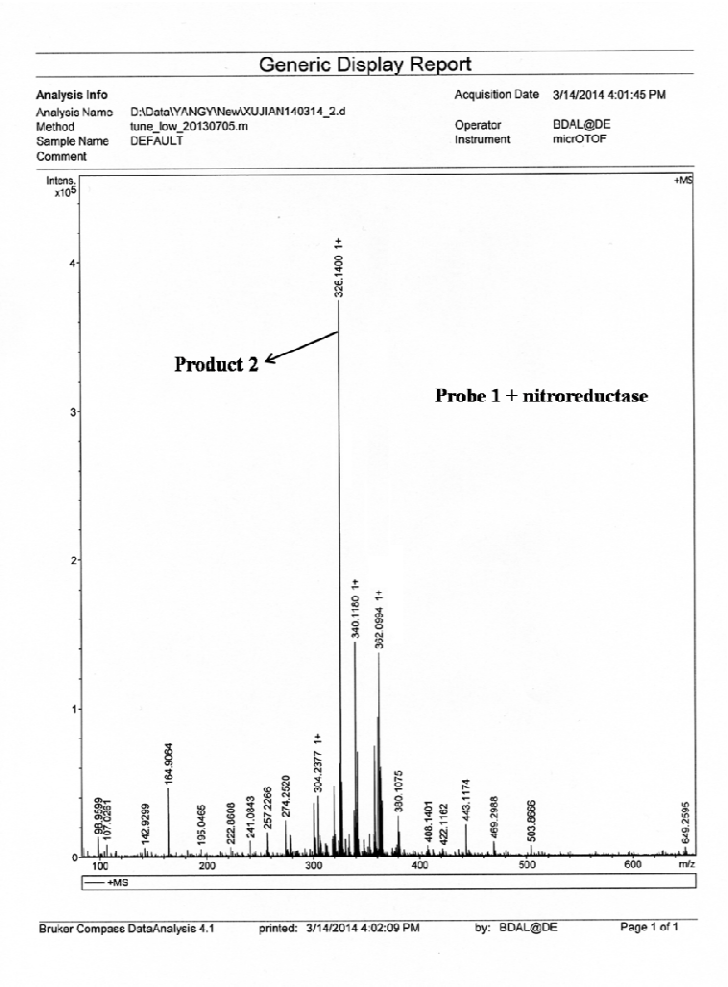

Fig. S5. HRMS spectra of the reaction solution of probe **1** (100 μM) with nitroreductase (5 μg/ mL).

7. HPLC analysis for the reaction system.

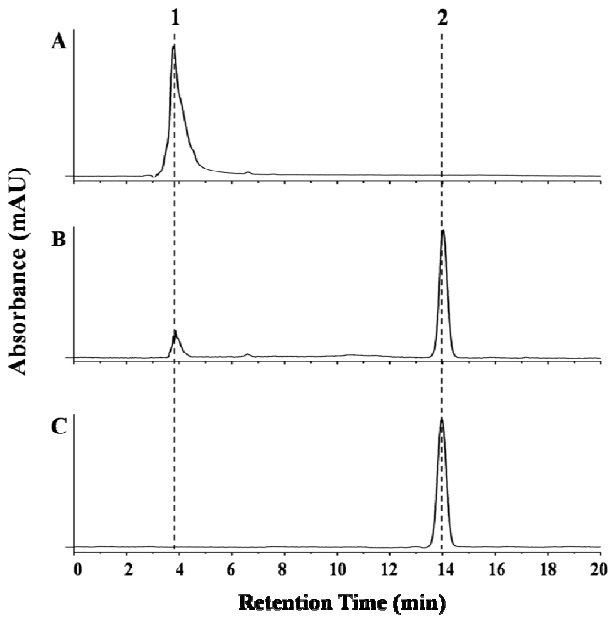

Fig. S6. Chromatograms of different reaction systems. (A) 100 μM probe **1**; (B) the reaction products of 100 μM probe **1** with 5 μg/ mL nitroreductase in the presence of 500 μM NADH for 5 min; (C) 100 μM 4-pyridinyl BODIPY **2**. The assignments of the peaks: (1) 3.79 min, probe **1**; (2) 14.00 min, 4-pyridinyl BODIPY **2**. Mobile phase: methanol–water, 70:30 (v/v). Detection: UV-vis (500 nm) detector. Flow rate: 1mL/min. T: 20 °C. Injection volume: 10 μL.

8. HRMS spectra of the reaction of probe 1 with *Escherichia coli.*

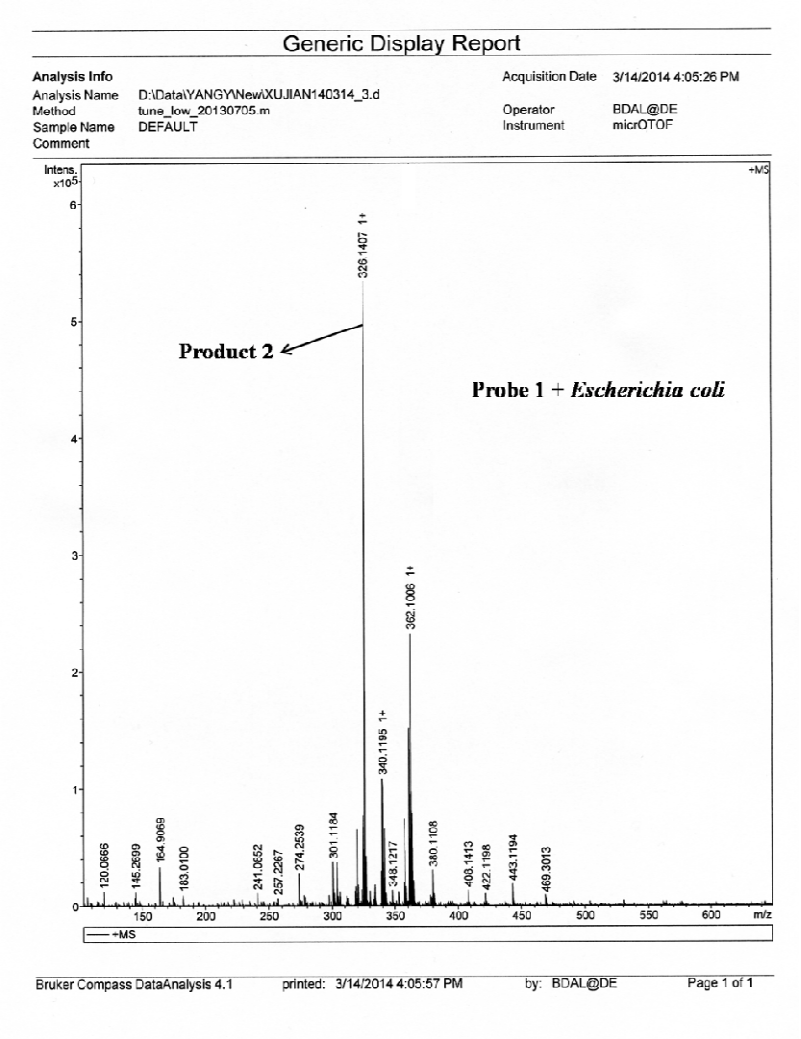

Fig. S7. HRMS spectra of the reaction solution of probe **1** (100 μM) with *Escherichia coli* (OD_{600} =1.0).

9. Real-time detection of nitroreductase produced by *Escherichia coli*

Table S2

Real-time detection of nitroreductase produced by *Escherichia coli* (DH5α) with an initial OD_{600} of 0.045.

Growth time (h)	0	1	2	3	4	5	6
Concentration of nitroreductase in the culture media[a] (μg/mL)	0.01± 0.001	0.07± 0.003	0.212± 0.004	0.415± 0.007	0.709± 0.011	0.675± 0.009	0.672± 0.008

[a] Mean of three determinations ± standard deviation.

10. ^{1}H NMR, ^{13}C NMR and MS spectra of compound 1-2.

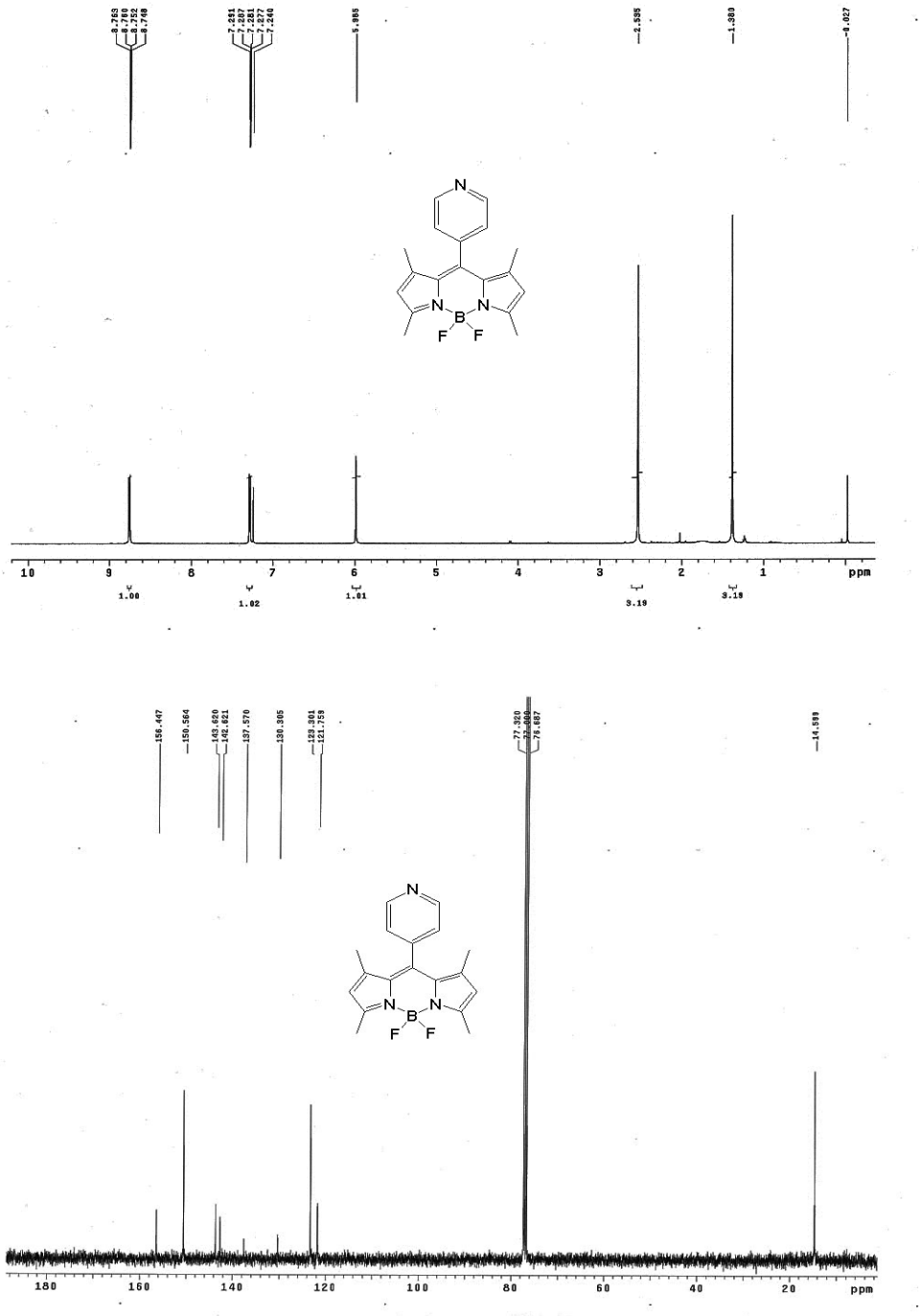

Fig. S8. ^{1}H NMR and ^{13}C NMR spectra of compound **2** in $CDCl_3$.

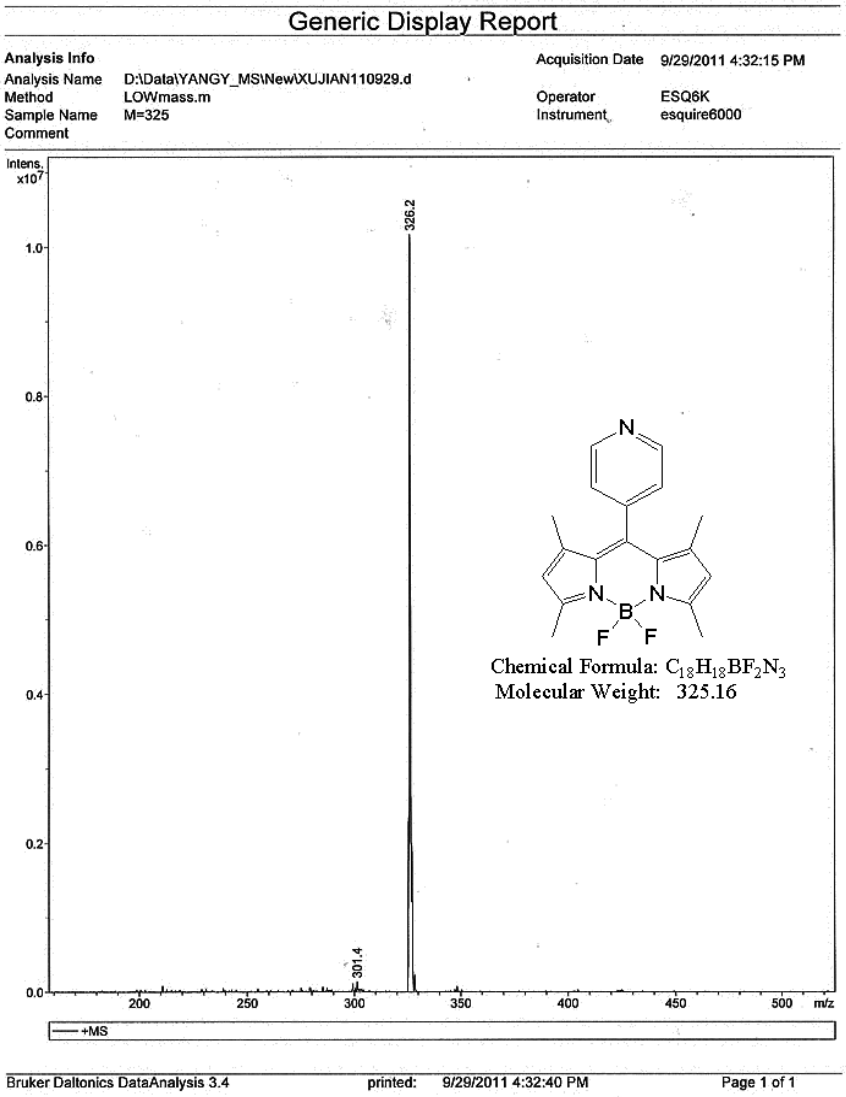

Fig. S9. ESI-MS spectra of compound **2**.

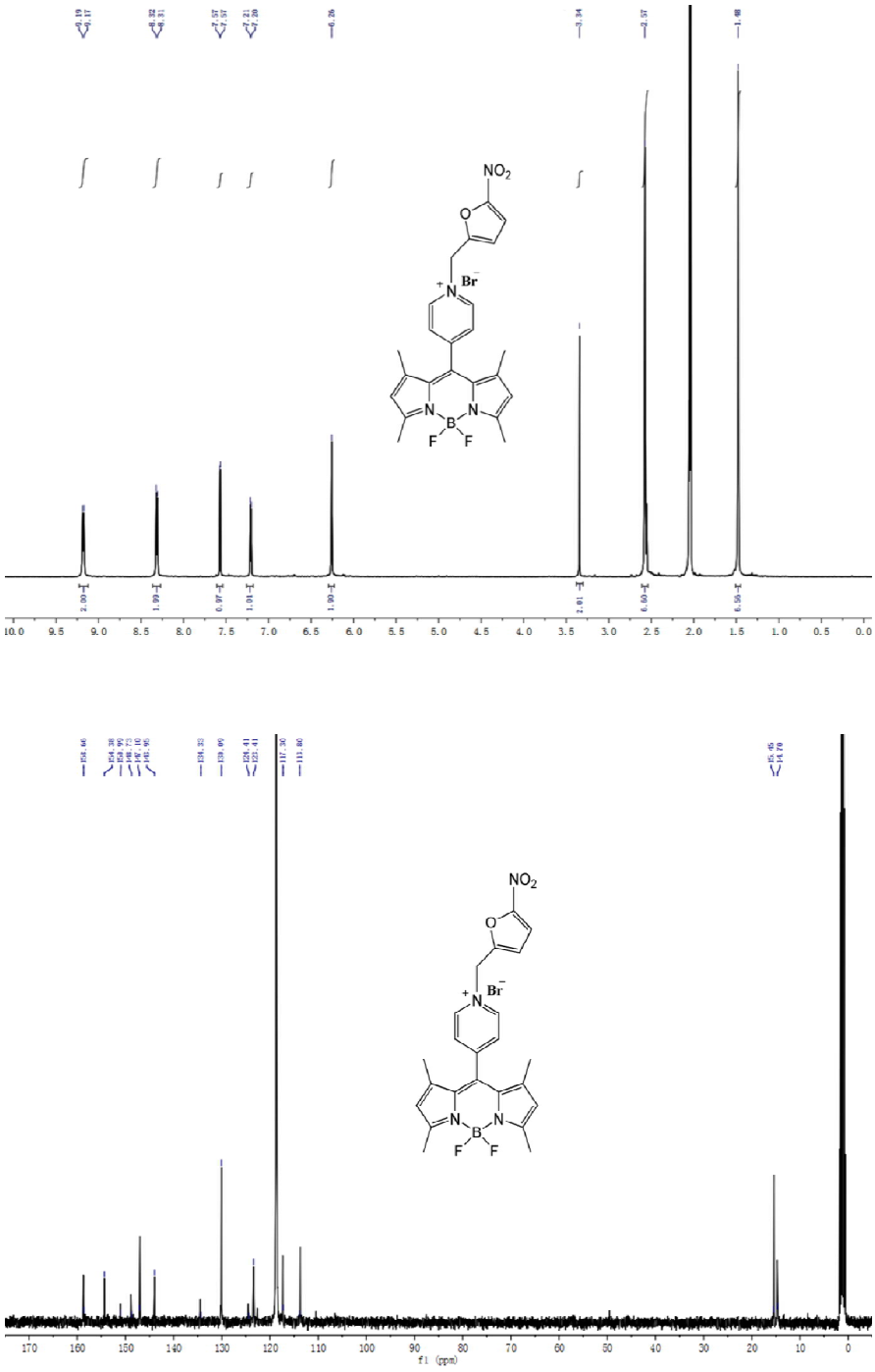

Fig. S10. ^{1}H NMR and ^{13}C NMR spectra of probe **1** in CD_3CN.

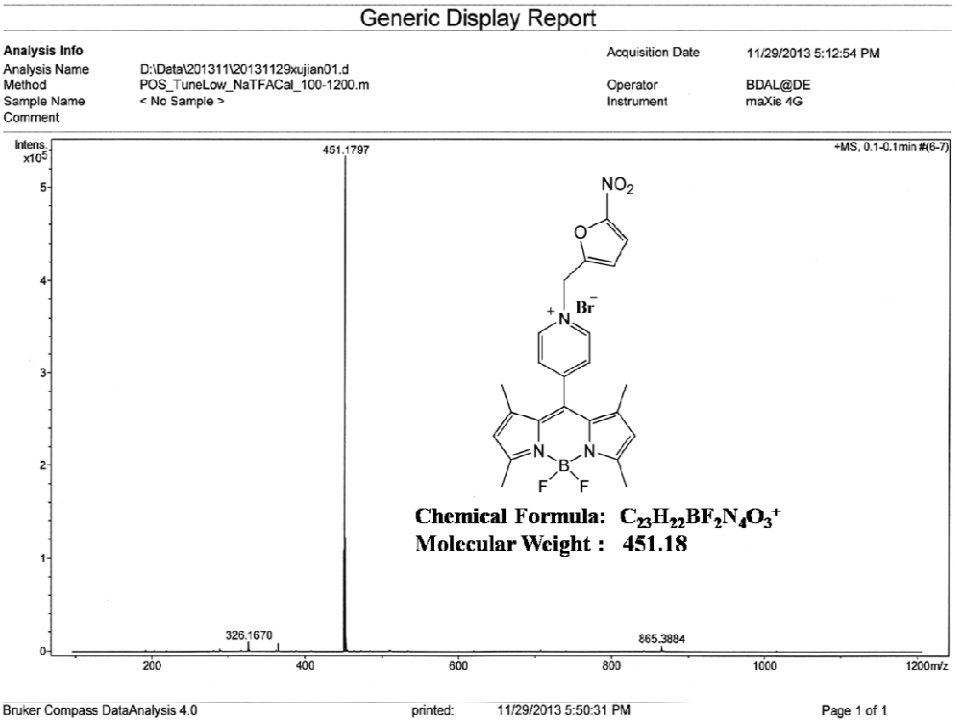

Fig. S11. ESI-MS spectra of probe **1**.