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PII: S1386-1425(17)30540-1
DOI: doi: 10.1016/j.saa.2017.06.065
Reference: SAA 15281

To appear in: *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*

Received date: 28 March 2017
Revised date: 23 June 2017
Accepted date: 30 June 2017

Please cite this article as: Gaoping Xu, Yonghe Tang, Yanyan Ma, An Xu, Weiying Lin , A new aggregation-induced emission fluorescent probe for rapid detection of nitroreductase and its application in living cells, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* (2017), doi: 10.1016/j.saa.2017.06.065

A new aggregation-induced emission fluorescent probe for rapid detection of nitroreductase and its application in living cells

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Abstract

The biological activity of nitroreductase (NTR) is closely related to biological hypoxia status in organisms. The development of effective methods for monitoring the activity of NTR is of great significance for medical diagnosis and tumor research. Toward this goal, we have developed a new aggregation-induced emission (AIE) fluorescence NTR probe **TPE-HY** used the tetraphenylethene as the fluorophore, and used the nitro group as the NTR recognition site. The probe **TPE-HY** has many excellent properties, including rapid response, AIE characteristics, high sensitivity and selectivity, and low cytotoxicity. Importantly, the probe **TPE-HY** is successfully applied to monitor endogenous NTR in living HeLa cells.

Keywords

Fluorescent Probe; Nitroreductase; Aggregation-Induced Emission; Tetraphenylethene; Hypoxia

1. Introduction

Hypoxia, caused by the lack of oxygen supply [1], is associated with a variety of diseases, such as, cardiac ischemia [2-4], stroke [5], inflammatory diseases [6, 7], and solid tumors [8, 9]. Especially, inadequate supply of oxygen (≤ 4.4 %) is an important characteristic of solid tumors.

Medical research showed that the hypoxic status of solid tumors is directly related to tumor progression, metastasis, drug resistance, and resistance to treatment [10, 11]. Therefore, the development of new methods for monitoring the hypoxic conditions in cancer cells and body is of great significance for medical diagnosis and tumor research.

At present, there are many detection methods for oxygen level reported, such as nuclear magnetic resonance (NMR) [12, 13], clark electrode [14], immunostaining [15, 16], positron emission tomography imaging [17, 18], most of which offer high sensitivity and selectivity. However, those detection methods can't guarantee the integrity of the sample. In the past decade, fluorescence imaging as a powerful tool to monitor biomolecules in biological systems has been widely concerned. Fluorescent probes have many obvious advantages, including good selectivity, high sensitivity, as well as real-time imaging [19-22]. The recent studies indicated that the biological activity of nitroreductase (NTR) is directly related to the hypoxic status in the living cells [23-26]. Therefore, to monitor the activity of the NTR can reflect the oxygen content of the organism. Under hypoxic condition, in the presence of NTR, the nitro group is reduced to amino moiety through three steps. The nitro group first can be reduced to a nitroso group, then to a hydroxylamine moiety, finally to an amino group. So far, there is a large number of fluorescent NTR probes have been reported [23-31]. However, the response time of most fluorescent probes for NTR more than 20 min, it is more desirable to develop the fluorescent NTR probes with rapid response for NTR.

The aggregation-induced emission (AIE) fluorophores have been exploited to serve as novel optical materials and probes for biosensing and imaging applications [32]. AIE fluorophores have high emission efficiency when their molecules are aggregated. However, conventional organic fluorophores often give strong emission in dilute solution, but their emission is quenched in the aggregated state or in concentrated solution. A number of AIE-based probes have been reported [33], but AIE fluorescent probe for NTR detection has not been reported so far.

In this work, we have designed and synthesized a new AIE fluorescent probe **TPE-HY** for detecting NTR. The fluorescent probe has many obvious advantages, such as, fast response (about 5 min), high selectivity, and high sensitivity to NTR. In addition, we successfully apply the new probe **TPE-HY** for monitoring NTR in living cells.

2. Experimental

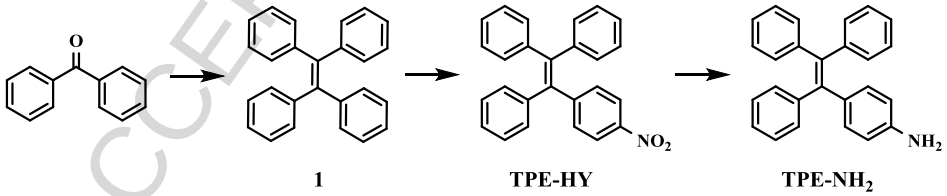
2.1. Materials and instruments

Unless otherwise specified, all reagents were mentioned in this work of analytical grade and directly used without further purification. The *Escherichia coli* nitroreductase (NTR) was purchased from *Sigma-Aldrich*. To keep the enzyme activity, NTR was dissolved into ultrapure water and stored immediately at -80 °C [34]. The *β*-Nicotinamide adenine dinucleotide disodium salt (NADH) was purchased from *J&K Scientific Ltd*.

The ^{1}H NMR and ^{13}C NMR spectra were collected on an AVANCE III 400 MHz Digital NMR spectrometer, and using tetramethylsilane (TMS) as internal; High resolution mass spectrometric (HRMS) analyses were measured by using a Finnigan MAT 95 XP spectrometer; UV-vis and fluorescence spectra were acquired on a Shimadzu UV-2700 Power spectrometer (with a 1 cm standard quartz cell), HITACHI F4600 fluorescence spectrophotometer (with a 1 cm standard quartz cell); The pH measurements were carried out with a Mettler-Toledo Delta 320 pH meter; The fluorescence imaging of living cells was performed through Nikon A1MP confocal microscopy.

2.2. Synthesis

The synthetic routes of compound **1**, **TPE-HY**, and **TPE-NH$_2$** were shown in Scheme 1. The detailed synthetic procedures were given below.



Scheme 1 Design strategy of the fluorescent probe **TPE-HY** and **TPE-NH$_2$**.

*2.2.1. Synthesis of 1,1,2,2-tetraphenylethene (**1**).*

Zinc powder (7.20 g, 0.11mol) was added in dry THF (80 mL) with rigorous magnetic stirring, and after the suspension was stirred at 0 °C for 15 min. $TiCl_4$ (6 mL, 0.055 mol) was injected the suspension slowly over a period of 30 min. Then the reaction system was refluxed for about 2 h. And a solution of benzophenone (5.00 g, 0.028 mol) in dry THF (20 ml) was injected slowly using

a syringe, and the reaction system was refluxed for 12 h. Then the reaction was quenched with 5 % aqueous solution of ammonium chloride ((w/v), 100mL), and extracted with ethylacetate. The organic extracts were combined, washed with water, and dried with $MgSO_4$. After filtration and the solvent was removed using a rotary evaporator. The crude product was re-crystallized from EtOH to afford a white solid compound **1** with a yield of 90%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.12 (m, 12H), 7.05 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.75, 140.99, 131.35, 127.67, 126.43; HR-MS calculated for $C_{26}H_{20}$ $[M+H]^+$ m/z 333.1638, found $[M+H]^+$ m/z 333.1651.

2.2.2. *Synthesis of (2-(4-nitrophenyl)ethene-1,1,2-triyl)tribenzene* ***(TPE-HY)***.

Compound **1** (3.32 mg, 10 mmol) was added in CH_2Cl_2 (30 mL). Then glacial acetic acid (1 mL) was slowly dropped into the solution at 0 °C with rigorous magnetic stirring. Maintaining the reaction temperature, concentrated HNO_3 (65 wt %, 0.72 mL) was slowly dropped into the solution within 30 min. After stirred at 0 °C for 30 min, the reaction was stirred at room temperature for another 6 h, and then poured into water. The formed precipitate was collected, and washed with water. The crude product was purified by flash chromatography on silica gel with petroleum ether/ethyl acetate (50:1, v/v) as the eluent to afford a yellow solid **TPE-HY** with a yield of 80%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.8 Hz, 2H), 7.20 (d, *J* = 8.8 Hz, 2H), 7.16 (m, 9H), 7.03 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.05, 145.98, 143.89, 142.74, 142.67, 142.54, 138.83, 132.09, 131.27, 131.26, 131.19, 128.12, 128.11, 127.82, 127.37, 127.13, 127.11, 123.04; HR-MS calculated for $C_{26}H_{19}NO_2$ $[M+H]^+$ m/z 378.1489, found $[M+H]^+$ m/z 378.1494.

2.2.3. *Synthesis of (2-(4- aminophenyl)ethene-1,1,2-triyl)tribenzene* ***(TPE-NH$_2$)***.

Tin(II) chloride (4.015 g, 21.2 mmol) was dissolved in concentrated HCl (36%, 3.5 mL), then the solution was added dropwise to a suspension of compound **TPE-HY** (1.55 g, 4.1 mmol) in ethanol (3 mL) with rigorous magnetic stirring. After reflux for 3 h, the reaction system was cooled to room temperature. The formed precipitate was collected, and washed with water. The crude product was purified by flash chromatography on silica gel with petroleum ether/ethyl acetate (50:1, v/v) as the eluent and re-crystallized from EtOH to afford a white solid **TPE-NH$_2$** with a yield of 51%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (m, 13H), 7.01 (m, 2H), 6.81 (d, *J* = 8.4

Hz, 2H), 6.46 (d, *J* = 8.4 Hz, 2H), 4.09 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.69, 144.36, 144.23, 144.19, 140.95, 139.34, 134.11, 132.52, 131.50, 131.44, 131.39, 127.69, 127.57, 127.54, 126.27, 126.09, 114.39; HR-MS calculated for $C_{26}H_{21}N$ $[M+H]^+$ m/z 348.1747, found $[M+H]^+$ m/z 348.1744.

*2.3. Preparation of Solutions of probe **TPE-HY***

The concentration of the probe stock solution was 1.0 mM in DMSO. The concentration of the NTR stock solution was 10.0 μg/mL in ultrapure water. The concentration of the NADH stock solution was 100 mM in ultrapure water. The concentration of analytes stock solutions were 100 mM prepared in the ultrapure water or DMSO.

For the test solution, the concentration of the probe was 5.0 μM and the concentration of the NADH was 300 μM in 10 mM PBS buffer (pH=7.4, 10 % DMSO). The concentration of analytes were different: biothiols, cations, anions (2.5 mM); reactive oxygen/nitrogen species (ROS/RNS) (100 μM). The resulting solution was incubated at 37 °C for 20 min before recording the spectra. Unless otherwise noted, for all the measurements, the excitation wavelength was 405 nm, the excitation slit widths were 5 nm, and the emission slit widths were 5 nm.

2.4. HeLa cells culture and hypoxic imaging

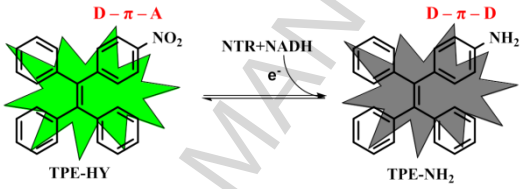
The requisite density HeLa cells were seeded into the imaging dish (35 mm glass-bottom culture dishes). In the next 12 hours, the cells were incubated under different oxygen level environments (the normoxic condition: 20 % O_2; and the hypoxic condition: 1 % O_2). Then the probe was added to the HeLa cells, further incubated for another 20 min under respective conditions. After washed with PBS buffer (pH=7.4) for three times, and the fluorescence images were acquired through a Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera. The fluorescence emission was collected at 500-550 nm upon excitation at 405 nm with a femtosecond pulse.

3. Results and discussion

*3.1. Design and synthesis of the probe **TPE-HY***

The tetraphenylethene fluorophore has many advantages, including aggregation-induced

emission, high photochemical stability, and high emission efficiency [32-33], and has been reported as intermediate product in several research areas [35-39]. We designed a new NTR fluorescent probe **TPE-HY** employed nitro group as the responding site for NTR as it could be easily reduced by NTR to the amine moiety. With the introduction of nitro group (electron donating group), the electronic structure of the probe became "D-π-A". The probe emitted strong fluorescence emission because of the intramolecular charge transfer (ICT). When treated with NTR, via a series of one-electron reduction processes, the nitro group of the probe would be reduced to the amino moiety, and the electronic structure of the probe became "D-π-D", results in the excitation wavelength of the reduction product (**TPE-NH$_2$**) becoming shorter than that of probe **TPE-HY**. The fluorescence signal couldn't be observed (Scheme 2). The new probe **TPE-HY** was fully characterized by ^{1}H NMR, ^{13}C NMR and HRMS and used as an AIE organic fluorescent probe for NTR detection for the first time.



Scheme 2. Proposed reaction mechanism of **TPE-HY** with NTR.

*3.2. Photophysical properties of the probe **CoNO$_2$-HY***

Due to the tetraphenylethene fluorophore was the typical representative aggregation induced emission dye, we first tested the fluorescence intensity changes of **TPE-HY** and **TPE-NH$_2$** in DMSO/water mixtures with different fractions of water (f_w). As shown in Fig. S1, the solution of **TPE-HY** in DMSO (contain 1 vol % water) were almost non-fluorescent. With the increase of water content, the fluorescence intensity increased gradually. However, the fluorescence intensity still weak until 80 vol % water added. At the 90 vol % water content, a large fluorescence signal observed. The compound **TPE-NH$_2$** showed the same fluorescence intensity changes. As shown in Fig. S2, with the increase of water content, the fluorescence intensity increased gradually, but the fluorescence intensity of the compound **TPE-NH$_2$** was excitated by the 405 nm excitation light was very low. By contrast, the compound **TPE-NH$_2$** was excitated by the 340 nm excitation light exhibited strong fluorescent changes. These studies demonstrated that **TPE-HY**

and **TPE-NH_2** have the aggregation induced emission (AIE) characteristics, and slso confirmed that the reaction mechanism of the probe **TPE-HY** with NTR as proposed we expected in Scheme 2.

In the titration experiments, as expected, the free probe **TPE-HY** displayed a strong fluorescent emission at 525 nm. However, when incubated with NTR, the fluorescence intensities of the probe **TPE-HY** significantly decline. As shown in the Fig. 1, with the increase of concentration of NTR, the fluorescence intensities of the probe **TPE-HY** gradually reduced. When 5 μM probe **TPE-HY** incubated with 10.0 μg/mL NTR for 20 min, it was about 4.7-fold decrease in fluorescence is detected. Based on the titration experiments, the detection limit of the probe for NTR was calculated to be 11.5 ng/mL with the assay conditions as shown in Fig. S3. Similar to the emission spectrum, the absorption spectrum of the probe also changed significantly. As shown in the Fig. S4, with the increase of the introduction of the concentration of NTR (0-10 μg/mL), the absorption value of the probe at about 340 nm gradually reduced. These studies demonstrated that the probe **TPE-HY** could detect the basal level of NTR in the living samples.

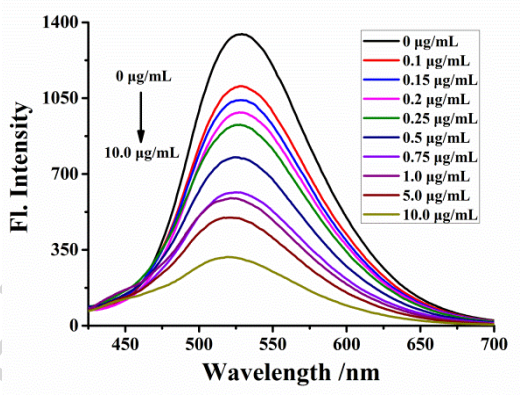


Fig. 1. The fluorescence response of the probe **TPE-HY** to NTR at the varied concentrations in PBS buffer (pH 7.4, 5 % DMSO). λ_{ex} = 405 nm. The spectra were recorded upon treatment of the probe (5.0 μM) with NTR (0- 10.0μg/mL) in the presence of NADH (300 μM) for 20 min.

The kinetic profiles of the probe **TPE-HY** (10 μM) treated with the varied concentrations of NTR (0-10.0 μg/mL) were shown in Fig 2. The probe **TPE-HY** untreated with NTR, the fluorescence intensity almost had no change at 525 nm. However, a fast fluorescence reduced phenomenon was noted when the incubated with NTR in PBS buffer, and the signal reached an equilibrium state after about 5 min. These results suggested that the rapid fluorescence response of

the probe may make it suitable for detection of NTR.

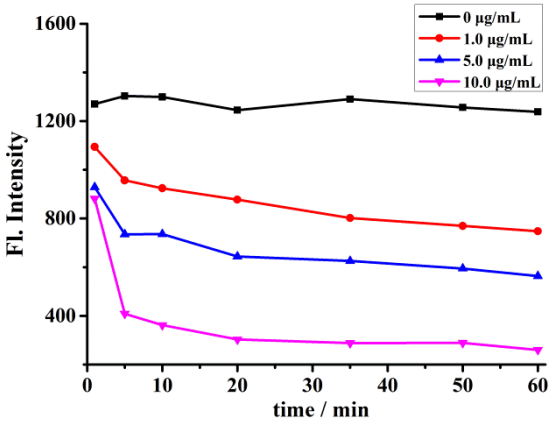


Fig. 2. Reaction-time profiles of the probe **TPE-HY** (5.0 μM) treated with the varied concentrations of NTR in the presence of NADH (300 μM). The fluorescence intensities at 525 nm are continuously monitored at time intervals for 60 min in PBS buffer (pH 7.4, 5 % DMSO).

In order to confirm the reaction mechanism, the reaction system was characterized by the HR-MS analyses. 50 μM probe **TPE-HY** was reacted with 10.0 μg/mL NTR in the presence of 300 μM NADH at 37 °C for 20 min, and then the reaction solution was investigated and determined by HR-MS analysis. The HRMS showed a peak at m/z= 348.1750 $[M+H]^+$, which attributing to compound **TPE-NH$_2$** (calcd. for $C_{26}H_{21}N$ 348.1747 $[M+H]^+$) (Fig. S5). The result was in agreement with the proposed mechanism in Scheme 2.

The high selectivity for target molecules is one of the important properties of fluorescent probes for the complex biological application. In order to better illustrate the selectivity of the probe, the probe **TPE-HY** was treated with various biologically relevant species, including cations, anions, reactive oxygen/nitrogen species (ROS/RNS), and biothiols, to investigate the selectivity. As shown in the Fig. 3, the probe **TPE-HY** incubated with the other analytes triggered almost non-fluorescence change. By contrast, when the probe treated with NTR for 20 min, there was a decrease of approximately 4.7-fold as compared with that of the probe **TPE-HY**. Similar to the emission spectrum, the absorption spectrum of the probe showed the same phenomenon. The probe treated with the other analytes, the absorption spectrum of the probe barely changed. By contrast, when NTR was introduced, the absorption value of the probe at 340 nm decreased

significantly (Fig. S6). These results showed that the probe **TPE-HY** was highly selective for NTR over other tested analytes, suggesting that the probe is favourable for investigation of NTR in biological systems.

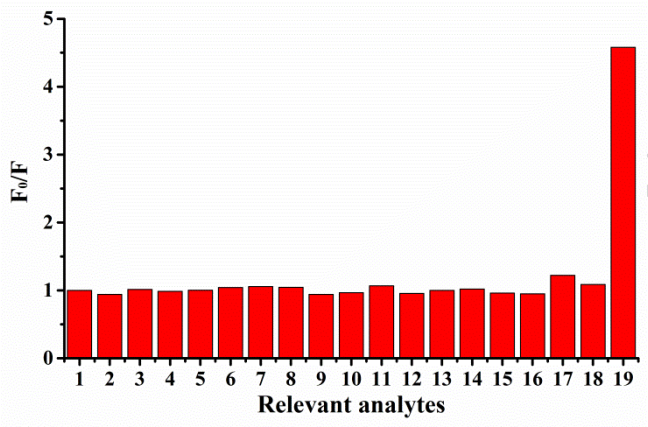


Fig. 3. Fluorescence changes (F_0/F) of the probe **TPE-HY** (5 μM) treated with various relevant analytes. The data are obtained after the incubation of the probe **TPE-HY** with the analytes for 20 min in the presence of NADH (300 μM). Legend: 1) free probe; 2) $NaNO_2$; 3) $CaCl_2$; 4) $FeSO_4$; 5) KI; 6) KNO_3; 7) $MgCl_2$; 8) NaBr; 9) $ZnCl_2$; 10) $NaHSO_3$; 11) Na_2S; 12) Cys; 13) GSH; 14) Hcy; 15) H_2O_2; 16) NaClO; 17) *tert*-butyl hydroperoxide; 18) *di-tert*-butylperoxide; 19) NTR.

The solution of pH and temperature have great influence on the enzyme activity. We also investigated the fluorescent changes of the probe **TPE-HY** with or without NTR in different pH PBS buffer and at different temperature. As shown in Fig. S7 and S8, the fluorescence intensities of the free probe **TPE-HY** have produced subtle change over a wide pH range of 5.0-8.0 and a wide temperature range of 20-40 °C, suggesting that the probe **TPE-HY** was stable in this pH range and this temperature range. Obviously, with the introduction of NTR, a significant fluorescence signal decrease was observed, especially at physiological pH 7.4 and physiological temperature 37 °C, suggesting that the probe has the potential value for biological applications. These results reveal that the probe **TPE-HY** functions well under physiological conditions (about pH 7.4 and 37 °C).

Furthermore, the photo-stability of probe **TPE-HY** also was surveyed. The probe was excited at 365 nm using a UV lamp, the fluorescent intensity displayed almost no change (Fig. S9),

suggesting that the probe is highly stable and may have potential usefulness in living system.

3.3. Fluorescence imaging of NTR in the living cells

Encouraged by the excellent properties of the probe, including fast response, working appropriately at physiological pH and temperature, high sensitivity and selectivity for NTR, and excellent photo-stability, the fluorescence imaging experiments were carried out in living cells.

As imaging reagents, the probe **TPE-HY** should have low biological toxicity. The cytotoxicity of the probe **TPE-HY** for HeLa cells was carried out. As shown in the Fig. S10, the standard MTT assay indicated that the probe **TPE-HY** (0-20 μM) has no remarkable cytotoxicity to the HeLa cells, meaning that the probe can be used for the bio-imaging.

For the endogenous NTR imaging experiments, as the anoxic condition would improve the NTR biological activity, the HeLa cells were incubated under different oxygen contents (the normoxic condition: 20 % O_2; the hypoxic condition: 1 % O_2) for 12 h. Then the probe **TPE-HY** (5 μM) was added to the HeLa cells under the original condition for another 20 min, respectively. As shown in the Fig. 4, only the living cells displayed non-fluorescence (Fig. 4e, 4f). At the normoxic condition, the HeLa cells incubated with probe showed strong fluorescence (Fig. 4g). And as we expected, at hypoxic condition, weak fluorescence signal was detected in the HeLa cells treated with the probe **TPE-HY** (Fig. 4h). These results demonstrated that the probe **TPE-HY** has good cell-membrane permeability and is responsive to intracellular NTR. The probe **TPE-HY** is the first AIE NTR fluorescent probe and can be used to the activity of NTR in living cells.

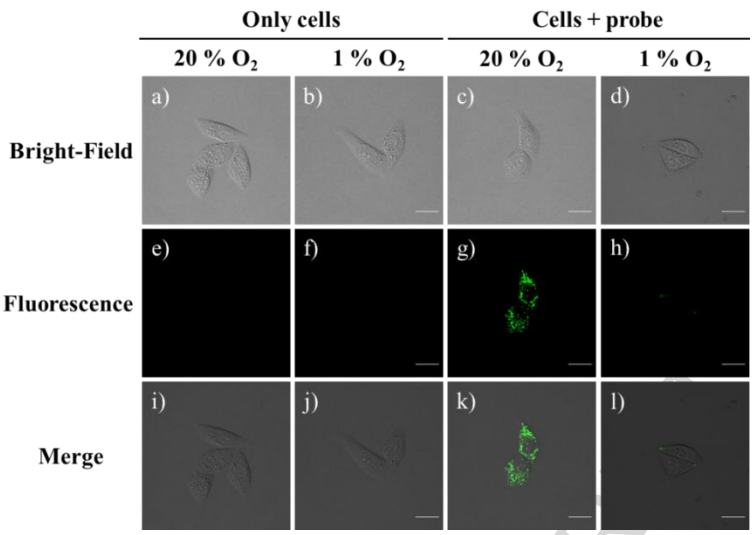


Fig. 4. Fluorescence imaging of NTR in the living HeLa cells under normoxic (20% O_2) and hypoxic (1% O_2) conditions incubated with the probe **TPE-HY** for 20 min. a-d) Bright-field image of the HeLa cells under normoxic and hypoxic conditions; e-h) Fluorescence image of a)-d); i-l) The merge of the bright-field image of and a)-d) and e-h). Scale bar: 20 μm.

4. Conclusion

In summary, we have designed and synthesized a new aggregation induced emission (AIE) fluorescent probe **TPE-HY** for detecting NTR for the first time. The probe used tetraphenylethene, the classic AIE dye, as the fluorophore, and used the nitro group as the NTR recognition site. With the introduction of NTR, the nitro group of the probe would be reduced to the amino moiety, the electronic structure of the probe had been changed, and then the ICT process was suppressed. The probe **TPE-HY** had many excellent properties, such as, aggregation induced emission (AIE) characteristics, rapid response, high sensitivity and selectivity to NTR. More importantly, the probe was successfully applied to monitor NTR in living HeLa cells. Further applications of the probe for the biological functions, pathological roles and disorders of NTR were under progress.

Acknowledgements

This work was financially supported by NSFC (21472067, 21672083), Taishan Scholar Foundation (TS 201511041), and the startup fund of the University of Jinan (309-10004).

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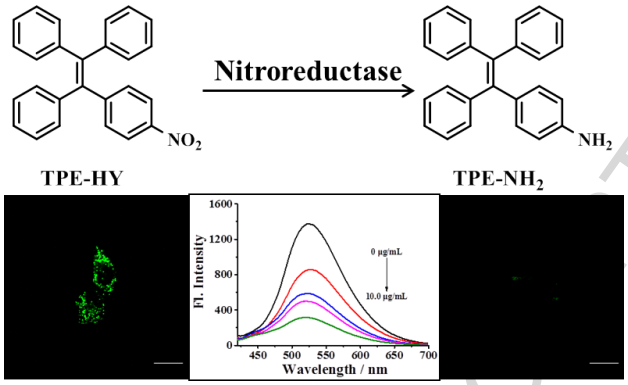
Graphical abstract



A new fluorescent quenched probe for nitroreductase (**TPE-HY**) was engineered for monitoring nitroreductase in the living cells.

Highlights

1) A new aggregation induced emission (AIE) fluorescent probe **TPE-HY** of detecting nitroreductase has designed and synthesized for the first time.
2) The probe **TPE-HY** is a new fluorescent nitroreductase probe, with high selectivity and sensitivity for nitroreductase.
3) When the probe **TPE-HY** treated with NTR in PBS solution, a fast fluorescence reduce phenomenon is noted, and the signal reached equilibrium state at about 5 min.
4) Fluorescence imaging shows that the probe **TPE-HY** is membrane-permeable and suitable for detecting endogenous NTR in living cells.