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RESEARCH PAPER

A Near-Infrared Fluorescent Probe for Detection of Nitroxyl in Living Cells

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Abstract: Nitroxyl (HNO), the one-electron reduced and protonated congener of nitric oxide (NO), has been demonstrated with excellent bio-pharmacological effects in cardiovascular disorder treatment, which is distinctive from that of NO. Despite its high reactivity, the accurate detection of HNO is a challenging issue. To resolve this problem, in this work, a near-infrared (NIR) metal-free fluorescent probe, ER-JN, was designed and synthesized for the detection of intracellular HNO concentration in simulated physiological conditions and living cells. The probe was consisted of two moieties, the BF_2-chelated tetraarylazadipyrromethane fluorophore (aza-BODIPY) and the HNO recognition unit, diphenylphosphinobenzoyl group. The probe was purified by silica column chromatography eluting with CH_2Cl_2 to obtain the green solid product with a yield of 28%. The as-prepared probe exhibited high sensitivity, good selectivity and low cytotoxicity, and was applied to fluorescent bio-imaging of HNO in simulated physiological conditions. When used in detection of HNO, quantum yield of the probe increased from 0.01 to 0.35. The linear range was 0–50 μM, with the detection limit of 0.03 μM (S/N = 3). With confocal laser scanning microscope imaging analysis, the probe could be used to detect HNO concentration in living cells. Furthermore, the results of flow cytometry confirmed that the probe could be employed for the qualitative and quantitative detection of intracellular HNO level. In this work, we found that probe ER-JN could not only detect HNO in aqueous solution and in living cells, but also target endoplasmic reticulum. We anticipate that ER-JN will provide experimental bases in studying physiological and pathological functions of HNO in cells, *in vitro* and *in vivo*.

Key Words: Fluorescent probe; Nitroxyl; Cell analysis; Near-infrared bioimaging

1 Introduction

Nitroxyl (HNO) is the one-electron reduced and protonated form of nitric oxide (NO), and displays distinct biological and pharmacological activities compared with NO. Due to its potential pharmacological activities, it has caused wide attention in recent years. HNO displays unique biological effects in the treatment of cardiovascular disorders such as angina and heart failure[1]. Angeli's salt (AS) (a HNO donor) is a potent vasodilator both *in vitro* and *in vivo*, and can elicit vasorelaxation in isolated large conduit, small resistance arteries and intact coronary arteries[2,3]. Moreover, HNO elicits distinct actions on myocardial contractile function that are not common to NO. Its cardiac effects are mediated by cardiac sarcoplasmic ryanodine receptors, which can effectively avoid the issue of nitroglycerin tolerance[4], and may provide a potential treatment for cardiovascular diseases. Despite more and more evidences showing the pharmacological importance of HNO, the understanding of endogenous HNO action mechanism is hampered by the lack of effective detection methods because HNO is unstable, and promptly dimerizes and dehydrates to nitrous oxide (N_2O)[1,2,5].

Many techniques are available for detecting HNO, including electrochemistry, electron paramagnetic resonance

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(EPR), colorimetry and chemiluminescence method, etc[6,7]. Compared with these techniques, fluorescent probe based methods have the advantages of high resolution, high sensitivity, and noninvasive damage to biological specimens[8,9]. Nevertheless, few fluorescent probes for HNO detection were reported, and were mostly based on the reduction of Cu^{2+} [10–12]. These fluorescent probes were susceptible to interferences from cellular reductants such as glutathione (GSH) and ascorbate. Moreover, the emission wavelengths of these reported probes were mainly located in UV-visible region. Because many organisms emit fluorescence under UV-visible light, the detection of biological samples using these probes might be interfered severely by the background autofluorescence. While the excitation/emission wavelengths of near-infrared (NIR) probes are located in 650–900 nm, which can minimize photo damage and avoid noise from background autofluorescence[13,14]. Our group was committed to the development of probes for detecting RNS, RSS and ROS[13–19]. Recently, we reported two fluorescent probes (Lyso-JN and Cyto-JN) for detecting HNO[20,21], which were used to test the change of cellular HNO level and applied to the fluorescent bio-imaging of HNO in mice. In this work, a near-infrared (NIR) metal-free fluorescent probe, ER-JN, was synthesized for the detection of intracellular HNO level in simulated physiological conditions and living cells. It was found that probe ER-JN could be used to detect HNO concentration in living cells with high sensitivity and low cytotoxicity.

2 Experimental

2.1 Instruments and reagents

All pH measurements were performed with a pH-3C digital pH meter (Shanghai Lei Ci Device Works, Shanghai, China). Fluorescence spectra were measured on FluoroMax-4 Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were obtained on NANO Drop 2000c UV-visible spectrophotometer (Thermo Fisher Scientific, USA). Fluorescence imaging of cells was performed on an Olympus Laser Scanning Confocal Microscope (FV1000, Olympus, Japan) at 60 × magnifications. Intracellular fluorescence detection was carried out on a flow cytometry (Aria, BD, USA) with excitation at 633 nm and emission in the range of 750–810 nm. Mouse leukaemic monocyte macrophage cell line (RAW264.7) was obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Ultrapure water was used throughout. DMEM and trypsin were purchased from Gibco (Grand Island, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich.

2.2 Synthesis of probe ER-JN

As shown in Fig.1, under argon atmosphere, a solution of Aza-BODIPY (52.9 mg, 0.1 mmol) in CH_2Cl_2 (50 mL) was treated with 2-(diphenylphosphino) benzoic acid (61.2 mg, 0.2 mmol), 4-dimethylaminopyridine (DMAP, 24.4 mg, 0.2 mmol) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC, 19.2 mg, 0.1 mmol). The mixture was stirred at room temperature for 24 h. The course of the reaction was monitored by thin layer chromatography (TLC). Then coarse product was neutralized with saturated NaBr solution, and partitioned with CH_2Cl_2 and H_2O. Then the solvent was evaporated under reduced pressure, and the obtained residue was purified by column chromatography. ER-JN was obtained as a green solid with a yield of 28%. ^{1}H NMR (500 MHz, DMSO-D_6) δ (ppm): 8.31–8.01 (m, 1H), 7.53–7.40 (m, 6H), 7.25–7.23 (m, 23H), 7.16 (m, 2H), 6.98–6.94 (m, 2H), 4.05 (m, 2H), 1.98 (m, 2H), 1.26–0.98 (m, 37H). ^{13}C NMR (125 MHz, DMSO-D_6) δ (ppm): 165.02, 162.82, 157.01, 152.12, 137.63, 137.54, 134.31, 134.14, 133.97, 133.63, 131.84, 131.66, 131.00, 129.96, 129.77, 129.63, 129.52, 125.39, 129.31, 129.26, 129.21, 129.16, 122.40, 121.27, 116.72, 60.22, 30.01, 21.02, 14.55. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): –4.05. LC-MS (ESI^-): $C_{71}H_{75}BF_2N_3O_3P$ calcd. 1097. 56, found 1097. 57.

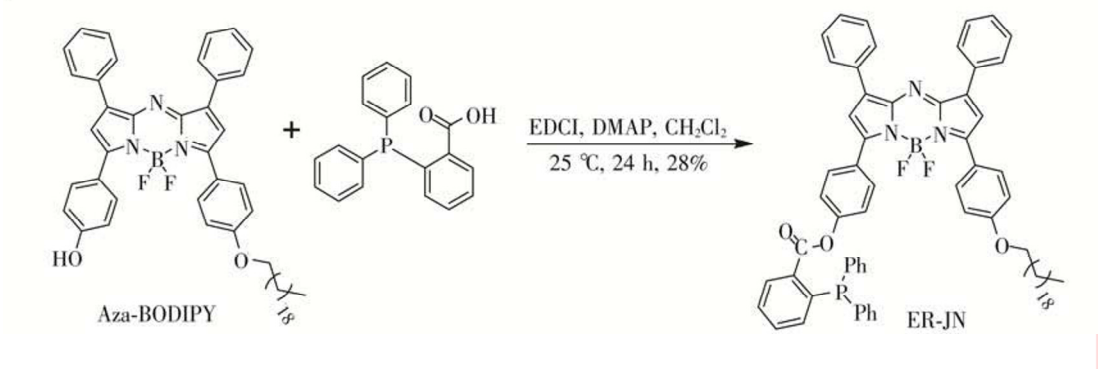

Fig.1 Synthetic route for probe ER-JN