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Spectrochimica Acta Part A 61 (2005) 1045–1049
SPECTROCHIMICA ACTA PART A
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Design, synthesis and characterization of a novel fluorescent probe for nitric oxide based on difluoroboradiaza-s-indacene fluorophore

Xian Zhang[a,*], Hua-shan Zhang[b]

[a] *School of Chemical Engineer and Pharmocy, Wuhan Institute of Chemical Technology, Wuhan, Hubei 430073, PR China*
[b] *Department of Chemistry, Wuhan University, Wuhan 430072, PR China*

Received 10 March 2004; accepted 14 May 2004

Abstract

Nitric oxide (NO) is a gaseous, free radical, which plays a role as an intracellular second messenger and a diffusible intercellular messenger. To obtain evidence for NO function in vivo, 1,3,5,7-tetramethyl-8-(4′-aminophenyl-*N*-(2′′-amino)-phenzyl)-difluoroboradiaza-*s*-indacene (TMAPABODIPY) was designed and synthesized as a fluorescent probe for nitric oxide, which features high photostability and no pH dependency over a wide pH range. The fluorescence of TMAPABODIPY itself is very strong. When TMAPABODIPY traps NO in the presence of dioxygen, the weak fluorescent triazole form is obtained, which offers the advantages of specificity, and sensitivity for direct detection of NO. The relationship was obtained between the quenching fluorescence intensity and NO concentration in the range 0.02–4.0 μmol l^{-1}. The detection limit is 5 nmol l^{-1} (S/N = 3). The proposed method has been used to monitor the release of NO from *S*-nitrosocysteine, a NO-releasing agent.

Keywords: 1,3,5,7-Tetramethyl-8-(4′-aminophenyl-*N*-(2′′-amino)-phenzyl)-difluoroboradiaza-*s*-indacene; Fluorescent probe; Nitric oxide

1. Introduction

Nitric oxide (NO) acts as a biological second messenger, and plays an important role in different functions of the human body. NO has different, concentration-dependent roles in many tissues, such as in the vasculature, immune system and neurotransmission [1–3]. In vivo and in vitro studies have indicated that NO at low concentration is an important molecule, essential for normal bone physiological, however, high levels of NO have been demonstrated to be inhibitory for bone and cartiage cell function [4–5]. To understand more clearly the role of NO in physiology, better methods are required to determine the exact concentration of NO that are beneficial or harmful for specific cell types. Moreover, it is necessary to get more accurate information on the cellular localization of NO production.

Techniques for the detection of NO based on chemiluminescence, or using electrochemical probes have been developed [6–8]. Although these techniques allow detection of NO production from tissues, they do not allow imaging of NO production at cellular level. Therefore, we set out to design molecular probes that would allow real time visualization of NO production within cells. For studies of intracellular concentrations of small molecules and ions, fluorescent probes are widely used and they also offered the most attractive prospect for this application as a non-invasive and potentially highly sensitive method.

Several fluorescent probes for NO were developed based on which aromatic vicinal diamines can react with NO in presence of dioxygen to produce the corresponding triazenes. They include 2,3-diaminonaphthalene and its derivate [9–10], diaminofluorescein [11], and diaminorhodamine [12]. These methods can obtain much higher sensitivity than other fluorescent methods. Diaminofluorescein and diaminorhodamine have been already used to image NO in cells. But the final detection of 2,3-diaminonaphthalene was performed in alkaline

* Corresponding author. Tel.: +86 27 87196017; fax: +86 27 87196017.
E-mail address: xianzhang9@263.net (X. Zhang).

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doi:10.1016/j.saa.2004.05.041

medium, which does not fit for biological sample. Furthermore, diaminofluorescein is unstable in strong light and can only be applied in a small pH range, while diaminorhodamine has relatively low sensitivity.

Difluoroboradiaza-*s*-indacenes (boron-dipyrromethene, BODIPY®) as a fluorophore, has favourable photophysical and optoelectronic properties. Its derivatives have found numerous applications in biochemistry and molecular biology [13–15]. In previous work, we have synthesized 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-s-indacence [16] and 8-(3′,4′-diaminophenyl)-difluoroboradiaza-s-indacence [17]. Now, we newly designed and synthesized a fluorescent NO probe 1,3,5,7-tetramethyl-8-(4′-aminophenyl-N-(2′′-amino)-phenzyl)-difluoro-boradiaza-*s*-indacene (TMAPABODIPY), to detect NO in biological systems as a means to examine the physiological functions of NO. TMAPABODIPY itself has very strong fluorescence, due to two amines groups as electron-donating group. When it reacts with NO to produce the corresponding triazole, the fluorescence of the fluorophore is quenched. Based on this finding, a simple method with high sensitivity and selectivity for the detection of NO has been developed.

2. Experimental

2.1. Apparatus and reagents

Unless otherwise specified, all reagents were of analytical reagent grade from Shanghai Chemical Reagent Co. (Shanghai, China). 2,3-Dichloro-5,6-dicyanobenzo-quinone (DDQ) was purchased from Acros Organce Co. (Belgium). All solutions were prepared in doubly distilled water.

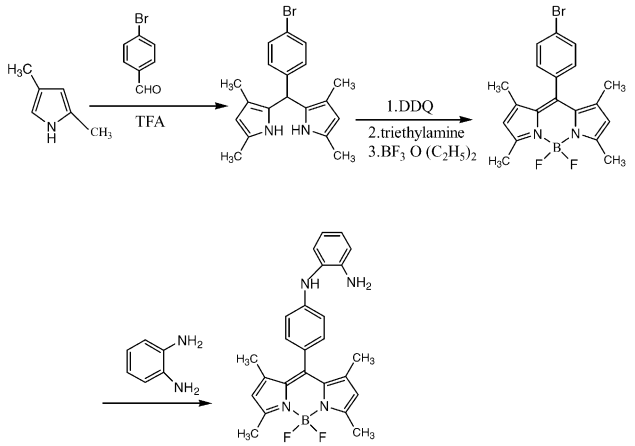
TMAPABODIPY was synthesized in our laboratory and its 0.2 mmol l^{-1} stock solution was prepared with ethanol, and stable for at least 1 month when stored in refrigerator. Working solution was appropriately diluted. KH_2PO_4–Na_2HPO_4 buffer was prepared by mixing 0.78 mmol l^{-1} KH_2PO_4 solution and 0.78 mmol l^{-1} Na_2HPO_4 solution to appropriate pH value.

Mass spectra were obtained by means of a VG ZAB-3F GC–MS instrument (Manchester, UK). FTIR spectra were obtained for the products in KBr disks by means of a Bruker (Karlsruhe, Germany) IFS48 instrument. Fluorescence spectra were recorded with a Shimadzu (Kyoto, Japan) RF-5000 spectrofluorimeter. Absorption spectra were recorded with a Shimadzu (Kyoto, Japan) UV-1601 spectrophotometer. pH was determined by means of a DF-801 accurate acidometer (Zhongshan University, Guangzhou, China).

2.2. Synthesis of TMAPABODIPY

The synthetic route employed to obtain the new dye is outlined in Scheme 1.

2,4-Dimethypyrrole was synthesized according to the literature [18].



Scheme 1. Synthetic scheme of TMAPABODIPY.

The mixture of 2,4-dimethylpyrrole 0.95 g (10 mmol) and *p*-bromo-benzaldehyde 0.92 g (5 mmol) in 30 ml CH_2Cl_2 was purged with N_2 for 30 min. Several drops trifluoroacetic acid was added to initiate the condensation. After 1.5 h, thin layer chromatography (TLC) silica (dichloromethane, CH_2Cl_2) showed that all of the aldehyde had been consumed. The reaction solution was then washed with 100 ml of 0.1 mol l^{-1} NaOH solutions and 200 ml of water, dried (Na_2SO_4) and filtered. The solvent was evaporated on a rotary evaporator. The resultant product was used immediately. The product was redissolved in 50 ml of toluene. 2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ) (1.0 g, 4.49 mmol) was added in the solution. After 10 min, triethylamine (7 ml) was added to the black reaction mixture followed immediately by BF_3-etherate (7 ml of neat BF_3-etherate). The mixture was stirred for 1.5 h, poured into water, and extracted with CH_2Cl_2. The CH_2Cl_2 solution was washed three times with 100 ml portions of water and dried (Na_2SO_4). The solvent was evaporated on a rotary evaporator.

The phenylenediamine (1.2 g, 10 mmol) was dissolved in dry methanol (50 ml) and heated to reflux. The above obtained compound was slowly added and the mixture refluxed for 1 h. After cooling, the mixture was poured into ether (200 ml) and the hydrochloride salt precipitated and filtered off. The salt was dissolved in 20 ml of ethanol and 1 mol l^{-1} of NaOH solution was used to adjust the solution to the neutral. The mixture solution was extracted by ethyl acetate. The extracted solvent was evaporated on a rotary evaporator. The product was purified by silica gel chromatography with ethyl acetate and petroleum ether (3:1). TLC showed that the product purified had only one point. Mass spectra: *m/z:* 435(M^+ + 1). IR (KBr, ν (cm^{-1})): 3425, 3203 (–NH_2), 2924.0, 2853 (–CH_3), 1083.7 (B–F), 1654.0 (–C=N).

2.3. Preparation of NO solution and S-nitrosocysteine solution

NO solution was prepared according to the literatures [19,20] and working solution was appropriately diluted.

S-nitrosocysteine solution (0.15 mmol l^{-1}) was prepared as described by Field et al. [21]. The solution was diluted with methanol to the desired concentration.

2.4. Fluorometric analysis

Relative fluorescence quantum efficiencies were obtained by comparing the peak area under the corrected emission spectrum of the test sample at 492 nm excitation with that of a solution of fluorescein in 0.1 mol l^{-1} NaOH solution that has a quantum efficiency of 0.85 according to the literature [11]. The slit width was 1.5 nm for both excitation and emission.

2.5. Procedure

The 0.3 ml of 2×10^{-4} mol l^{-1} TMAPABODIPY solution and 2.0 ml of pH 7.4 sodium phosphate buffers were transferred to a 10 ml test-tube. Then a solution containing NO with a gas-tight syringe was added. The resulting solution was diluted to 5 ml with water and stood for 35 min at 40 °C. After 35 min, the solution was diluted to the mark with water. The relative fluorescence intensity was measured at 510.0 nm with excitation at 500.0 nm. The slit width for emission and excitation was both 5 nm.

2.6. Sample analysis procedure

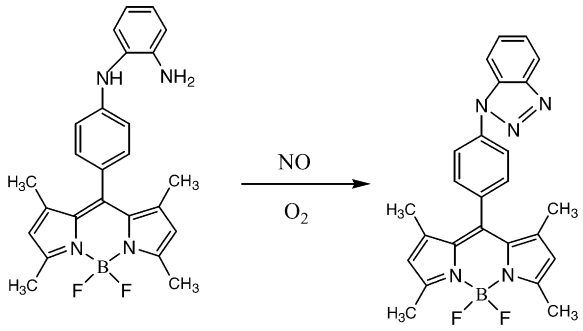
A series of 0.3 ml of 2.0×10^{-4} mol l^{-1} TMAPABODIPY solution was transferred to 10 ml test-tubes. Then 2.0 ml pH 7.4 sodium phosphate buffers and 0.5 ml of 0.15 mmol l^{-1} *S*-nitrosocysteine solutions were added, respectively. The resulting solutions were diluted to 5 ml with water and stood for different time at 40 °C. Every 5 min, the whole solution was diluted to the mark with water. The measure procedure is same to that above described.

3. Result and discussion

3.1. The spectra properties of TMAPABODIPY and its product

It was reported that *o*-phenylenediamine group react with NO in the presence of dioxygen to produce the corresponding triazole under neutral conditions [22]. Based on this, the reaction scheme of TMAPABODIPY with NO in the presence of dioxygen is shown in Scheme 2.

The fluorescence spectra of TMAPABODIPY and its reactive product with NO in the presence of dioxygen, the triazole (TMAPABODIPY-T) are shown in Fig. 1. The fluorescence of TMAPABODIPY itself is very strong at $\lambda_{ex}/\lambda_{em}$ = 500/510 nm. For its stoke shift is very small, the fluorescence quantum efficiency is high. The fluorescence was decreased greatly by addition of NO solution, which indicated TMAPABODIPY reacted with NO in the presence



Scheme 2. Reaction of TMAPABODIPY with NO.

of dioxygen to form a new compound, TMAPABODIPY-T. The fluorescence maximum excitation and emission wavelengths of TMAPABODIPY-T were at 508 and 520 nm, with a red shift in comparison with TMAPABODIPY, exhibiting the extension of the conjugated system compared to TMAPABODIPY (Fig. 2).

The phenomenon of TMAPABODIPY reacted with NO is the same to that of 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)-difluoroboradiaza-*s*-indacence with NO. In

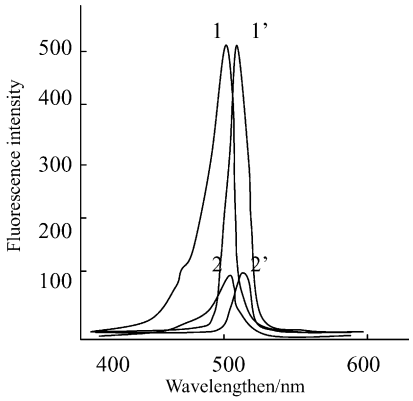


Fig. 1. Fluorescence spectra of TMAPABODIPY and TMAPABODIPY-T $C_{TMAPABODIPY}$ = 1.00 × 10^{-6} mol l^{-1}, C_{NO} = 2.00 × 10^{-6} mol l^{-1}, pH 7.4, 1 is excitation spectrum of TMAPABODIPY at 510 nm emission wavelength, 2 is excitation spectrum of TMAPABODIPY-T at 520 nm emission wavelength, 1′ is emission spectrum of TMAPABODIPY at 500 nm excitation wavelength, 2′ is emission spectrum of TMAPABODIPY-T at 508 nm excitation wavelength,. The slit of excitation and emission are both 5 nm.

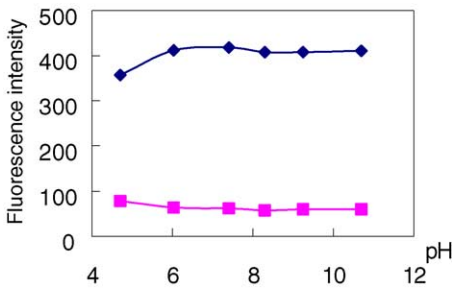


Fig. 2. Effect of pH on the fluorescence intensity of TMAPABODIPY and TMAPABODIPY-T, $C_{TMAPABODIPY}$ = 6.0 × 10^{-6} mol l^{-1}, C_{NO} = 2.00 × 10^{-5} mol l^{-1}, (♦) TMAPABODIPY, (■) TMAPABODIPY-T.

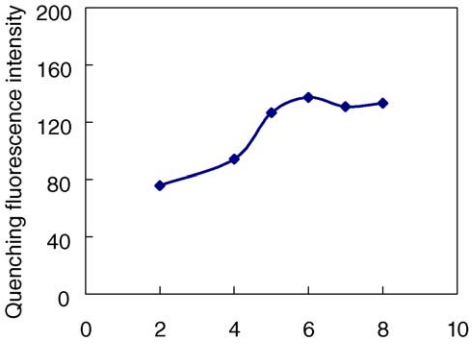


Fig. 3. Effect of TMAPABODIPY concentration (C_{NO} = 2.00 × 10^{-6} mol l^{-1}, pH 7.40, reaction time: 30 min, reaction temperature: 30 °C).

our previous literature, we have explained this phenomenon in details [17].

Fig. 3 shows the effect of pH on the fluorescence intensity of TMAPABODIPY and its product. The fluorescence intensity of TMAPABODIPY is stable above pH 4. While under pH 4, it greatly decreases. Maybe in strong acidic condition, amine groups of TMAPABODIPY are protonated and make fluorescence intensity decrease. The triazole is stable from pH 2 to 12 for 12 h.

3.2. Optimization of reaction conditions of TMAPABODIPY with NO

The conditions for the reaction of TMAPABODIPY were optimized. Several factors influencing the reaction were studied, including reagent concentration, amount and pH range of KH_2PO_4–Na_2HPO_4 buffer, reaction time and temperature.

TMAPABODIPY concentration affected the quenching fluorescence intensity due to TMAPABODIPY itself having strong the fluorescence. Fig. 3 shows the effect of TMAPABODIPY concentration on the quenching fluorescence intensity. The maximum quenching fluorescence intensity was obtained at range of TMAPABODIPY concentrations of 5.0–8.0 μmol l^{-1}. We used 6.0 μmol l^{-1} of TMAPABODIPY as optimal condition.

The effect of the buffer pH on the quenching fluorescence intensity was study by using KH_2PO_4–Na_2HPO_4 buffer from pH 4.7 to 12. It was found that the quenching fluorescence intensity kept stable at pH 7.4–12. In order to fit biological sample, pH 7.4 was chosen for the determination of NO (Fig. 4).

The variation of the buffer volume from 1.0 to 3.5 ml had the effect shown in Fig. 5. When 2.0 ml of KH_2PO_4–Na_2HPO_4 buffer was added, the quenching fluorescence intensity reached the maximum. Therefore, 2.0 ml of was used in the procedure.

Temperatures and times are two critical factors for the reaction. The effects of varied times and temperatures on the reaction of TMAPABODIPY with NO in the presence of dioxygen are demonstrated in Fig. 6. Both high and low temperature gave the weaker quenching fluorescence intensity compared with 40 °C. At a high temperature (50 °C), the fluorescence of TMAPABODIPY was decreased, while the fluorescence of the triazole was unchanged. So the quenching fluorescence intensity decreased. It was found that the reaction was much faster at high temperature (40–50 °C) compared with low temperature (30 °C). However, the reaction at high temperatures (40–50 °C) got to balance more quickly than at low temperature (30 °C). At 40 °C, the quenching fluorescence intensity was almost unchanged after 30 min of the reaction. Therefore, the reaction was performed at 40 °C for 35 min. After formation, the product was stable for at least 12 h.

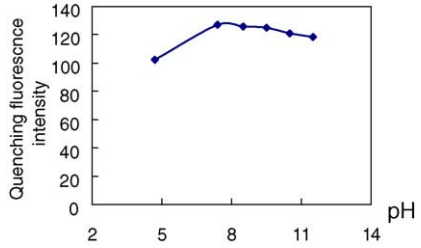


Fig. 4. The pH effect of the final determination ($C_{TMAPABODIPY}$ = 6.0 × 10^{-5} mol l^{-1}, C_{NO} = 2.00 × 10^{-6} mol l^{-1}, reaction time: 30 min, reaction temperature: 30 °C).

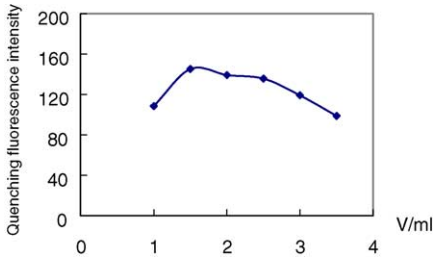


Fig. 5. Effect of buffer volume ($C_{TMAPABODIPY}$ = 6.0 × 10^{-5} mol l^{-1}, C_{NO} = 2.00 × 10^{-6} mol l^{-1}, pH 7.40, reaction time: 30 min, reaction temperature: 30 °C).

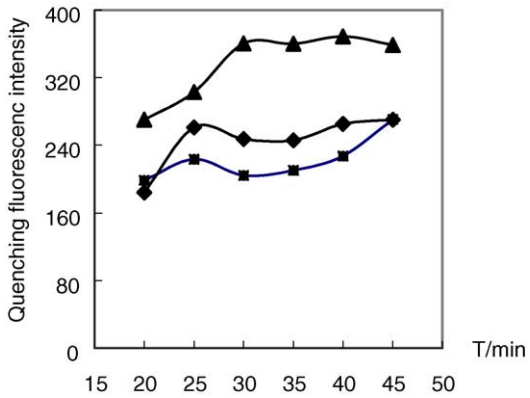


Fig. 6. Effect of reaction and temperature ($C_{TMAPABODIPY}$ = 6.0 × 10^{-5} mol l^{-1}, C_{NO} = 2.00 × 10^{-6} mol l^{-1}, pH 7.40, (■) 30 °C, (▲) 40 °C, (♦) 50 °C).

Table 1
Effect of foreign ions

Foreign ion	Tolerance ($\mu g\,ml^{-1}$)	Foreign ion	Tolerance ($\mu g\,ml^{-1}$)
Ca^{2+}	100	Fe^{3+}	2[a]
Mg^{2+}	10	CO_3^{2-}	20
Al^{3+}	20	NO_3^-	100
Zn^{2+}	40	BSA	20
SO_4^{2-}	40	NO_2^-	2

[a] In the presence of 0.01 mg ml^{-1} EDTA.

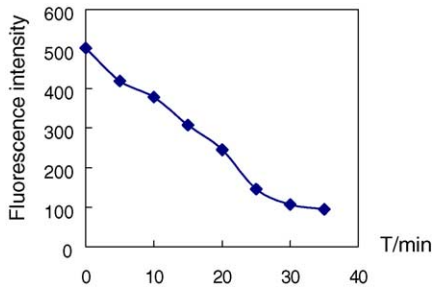


Fig. 7. The time course of the decrease of the fluorescence intensity at 510 nm of the fluorescence probe ($C_{TMAPABODIPY}$ = 6.0 × 10^{-5} mol l^{-1}). A spontaneous NO-releasing reagent, *S*-nitrosocysteine was added to probe solution at a final concentration of 7.5 μmol l^{-1} at 0 min.

3.3. Linearity, sensitivity and precision

According to the proposed method, the calibration curve was obtained in the NO concentration range of 0.02–4.0 μmol l^{-1}. The detection limit was found to be 5 nmol l^{-1} with a signal-to-noise ratio of 3. The linear regression equation and the coefficient are $Y = 300.91X - 10.78$, where Y is the fluorescence quenching intensity, X is NO concentration (μmol l^{-1}), r = 0.9978. The relative standard deviation for 10 replication measurement of 0.2 μmol l^{-1} NO concentration is 2.52%.

3.4. Effect of foreign ions

Using 2.0 μmol l^{-1} of nitric oxide, under the chosen conditions, the interference from foreign ions was investigated. The tolerance limit of foreign ions was taken as the maximum amount causing an error $\leq \pm 5\%$ in the fluorescence of the sample. The results are shown in Table 1. From the result it was observed that most of the ions studied did not interfere in the determination of NO in biological specimen.

3.5. Sample analysis

S-nitrosocysteine is thermodynamically and photolytically unstable compounds, which is a spontaneous NO-releasing agent. Fig. 7 showed the time course of the decrease of the fluorescence intensity at 510 nm the probe. When *S*-nitrosocysteine was added to the probe solution, the fluorescence at 510 nm decreased gradually. The time course of the fluorescence decrease corresponded to that of NO production from *S*-nitrosocysteine. This finding indicates that the fluorescence decrease was caused by TMAPABODIPY reaction with NO.

The sensitivity of this method is enough for the determination of NO in biological body. The half-life of NO in the physiological body is around 5–15 s, and NO releasing is a continuous procedure. Therefore, the proposed method can monitor NO releasing off line. But the proposed method needs reaction time much longer, it cannot determinate NO releasing in situ for a relatively long time needed.

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