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# Development of 4-hydrazinyl-7-nitrobenzofurazan as a fluorogenic probe for detecting malondialdehyde in biological samples

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ABSTRACT

Malondialdehyde (MDA) is a critical product of polyunsaturated fatty acid peroxidation and represents a common biomarker of oxidative stress. In this study, we have developed a fluorogenic MDA probe, 4-hydrazinyl-7-nitrobenzofurazan (NBDH). The reaction between NBDH and MDA in acidic conditions yields a highly fluorescent product, 4,5-dihydro-1H-pyrazole derivative (NBD-P1). The MDA detection range of our probe NBDH falls between 0.1 and 20 μM, and the corresponding detection limit amounts to 7.2 nM (3δ). NBDH also demonstrates a high selectivity toward MDA over other aldehydes and biologically relevant molecules, and proves to be an excellent MDA assay in biological fluids.



## 1. Introduction

Abnormal oxidative stress is related to many critical diseases, including diabetes [1], cancers [2], and neurodegenerative conditions [3]. Under oxidative stress, polyunsaturated fatty acids could be attacked by reactive oxygen species to form lipid hydroperoxides. These lipid hydroperoxides readily give rise to several secondary products. Among these products, malondialdehyde (MDA) is the principal product of polyunsaturated fatty acid peroxidation and represents a typical biomarker of oxidative stress. It has thus attracted considerable research interests. Recent studies suggest that MDA is toxic, potentially mutagenic, and atherogenic due to its high reactivity with other biomolecules, such as proteins [4,5] and nucleic acids [6–8]. Alteration of MDA levels in the living organism often reflects pathological changes [9], which have been verified in various types of illnesses, such as leukemia [10], diabetes [1], cancer [2], cardiovascular disease [11], age-related macular degeneration [12], and liver disease [13]. Therefore, it is highly important to develop analytical methods that can quickly, sensitively, and selectively detect MDA in biological systems.

Currently, 2-thiobarbituric acid (TBA) assay has been widely used to detect MDA. The condensation product formed between TBA and MDA can be analyzed via spectrophotometric or fluorimetric measurements [14–16], or high-performance liquid chromatography (HPLC) [17–19]. However, TBA based methods have a low analytical specificity and often overestimate MDA levels in human urine. Subsequently, many other derivatization reagents have been developed, such as 2,4,6-trichlorophenylhydrazine [20], 2,4-dini-trophenylhydrazine(DNPH) [21–23], diaminonaphthalene (DAN) [24], and dansylhydrazine [25]. In general, the accuracy of these methods relies on effective product separations, which require rather complicated and time-consuming derivatization procedures. Besides, most of these methods are based on UV–vis detection, lacking sufficient sensitivity to measure small amounts of MDA. In contrast, fluorescence-based detection techniques offer high sensitivity, excellent selectivity and simplicity. These techniques have thus attracted considerable attention. However, few fluorescent probes for MDA detection are available till today [26,27]. It is thus imperative to develop fluorescent probes that can rapidly response to MDA.

In this study, we report 4-hydrazinyl-7-nitrobenzofurazan (NBDH) as a new fluorescent probe for the selective detection of MDA. NBDH is essentially non-fluorescent in neutral aqueous solution. However, under acidic conditions, the terminal amine group on the hydrazine moiety of NBDH condenses with MDA to produce the corresponding hydrazone (**1**), which subsequently triggers intramolecular cyclization between an aromatic secondary amine and an aldehyde group to afford a 4,5-dihydro-1H-pyrazole derivative (NBD-P1) (Scheme 1). The resulted NBD-P1 is highly fluorescent over a broad pH range (tested from pH 2.2 to 9.2) in aqueous

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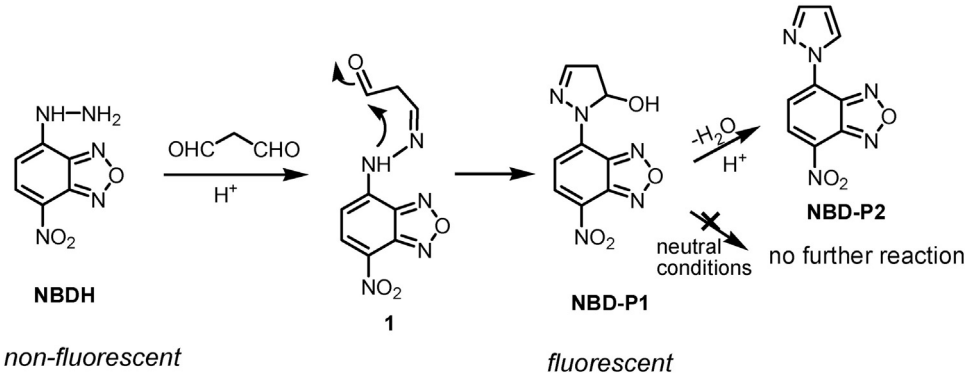


**Scheme 1.** Proposed reaction mechanism of NBDH with MDA.

solution. Moreover, the fluorescence intensity of this reaction system at 555 nm increases linearly with MDA concentration over a large dynamic range from 0.1 to 20 μM. NBD-P1 also demonstrates good selectivity towards MDA over aldehydes and ketones in neutral solution, making it a highly suited MDA assay in biological fluids.

## 2. Experimental

### 2.1. Materials and instruments

4-Chloro-7-nitrobenzofurazan (NBD-Cl) and hydrazine hydrate (98%) were obtained from J&K (Beijing, China). 1,1,3,3-Tetraethoxypropane (TEP) was obtained from Macklin (Shanghai, China). All other chemicals and solvents used were of analytical grades. Double distilled water was used throughout the experiments. MDA solution was prepared from TEP accordingly to previously reported procedures [28], and its concentration was determined by its absorbance at 267 nm ($\varepsilon_{267} = 3.18 \times 10^4\ M^{-1}\ cm^{-1}$) [29].

The fluorescence spectra and relative fluorescence intensities were measured with a Shimadzu RF-5301 fluorescence spectrometer. Absorption spectra were recorded using a Shimadzu UV-2550 spectrophotometer. High-resolution mass spectra were collected using a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics Corp., USA) in electrospray ionization (ESI) mode. $^1$H and $^{13}$C NMR spectra were recorded on an INOVA-400 spectrometer (Varian Unity) with reference to solvent signals. pH was measured with a Sartorius PB–10 pH meter.

### 2.2. Synthesis of 4-Hydrazinyl-7-nitrobenzofurazan (NBDH)

NBDH was prepared according to previously reported procedures (Scheme S1) [30]. To a solution of NBD-Cl (50 mg, 0.25 mmol) in $CHCl_3$ (25 mL), a 1% hydrazine solution (0.26 mL hydrazine in 25 mL $CH_3OH$) was added. The mixture was then stirred at room temperature for 1 h. After completion of the reaction, the resulting precipitate was filtered off and dried under vacuum to give NBDH as a yellow-brown solid (52 mg, yield: 90.4%.). $^1$H NMR (400 MHz, $D_2O$): 6.93 (d, $J$ = 10.0 Hz, 1H), 6.29 (d, $J$ = 12.0 Hz, 1H). HRMS (ESI): $m/z$ cacld for $C_6H_5N_5O_3Na^+$ $[M+Na]^+$ 218.0290; found: 218.0285.

### 2.3. Synthesis of 4-(5-hydroxy-4,5-dihydro-1H-pyrazol-1-yl)-7-nitrobenzofurazan (NBD-P1)

To a solution of NBDH (23 mg, 0.10 mmol) in $CH_3CN$, MDA solution (4.67 mM, 25 mL) was added at room temperature. The resulting solution was stirred at room temperature for 5 min and then quickly extracted with ethyl acetate (3 × 20 mL). The organic layer was collected, dried with anhydrous $MgSO_4$, and evaporated to dryness to afford the crude product, which was further purified by flash chromatography using $CH_2Cl_2$ as eluent to afford NBD-P1 (6.4 mg, yield: 25.5%). $^1$H NMR (400 MHz, $CD_3CN$): $\delta$ 8.54 (d, $J$ = 8.8 Hz, 1H), 7.51 (s, 1H), 7.13 (d, $J$ = 8.8 Hz, 1H), 6.51 (t, $J$ = 5.8 Hz, 1H), 4.83 (s, 1H), 3.38 (dd, $J_1$ = 7.2 Hz, $J_2$ = 20.4 Hz, 1H), 3.00 (d, $J$ = 19.2 Hz, 1H). HRMS (ESI): $m/z$ cacld for $C_9H_7N_5O_4Na^+$ $[M+Na]^+$ 272.0396; found: 272.0390.

### 2.4. Synthesis of 4-(1H-pyrazole-1-yl)-7-nitrobenzofurazan (NBD-P2)

NBDH (23 mg, 0.1 mmol) was dissolved in 25 mL of acetonitrile, and then to the solution was added 25 mL of MDA solution (4.67 mM). The mixture was stirred at room temperature for 30 min and then extracted with ethyl acetate (3 × 20 mL). The organic layer was combined, dried with anhydrous $MgSO_4$, and evaporated to dryness. The resulting residue was purified by flash chromatography using $CH_2Cl_2$ as eluent to give NBD-P2 (10.3 mg, yield: 43.9%). $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 9.05 (s, 1H), 8.65 (d, $J$ = 8.4 Hz, 1H), 8.24 (d, $J$ = 8.4 Hz, 1H), 7.94 (s, 1H), 6.69 (s, 1H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 145.34, 144.91, 144.58, 134.56, 133.77, 133.02, 132.81, 116.59, 111.42. HRMS (ESI): $m/z$ cacld for $C_9H_5N_5O_3Na^+$ $[M+Na]^+$ 254.0290; found: 254.0285.

### 2.5. General procedures for spectral measurements

The stock solution of NBDH (1.0 mM) was prepared in deionized water. In a test tube, 50 μL of NBDH (1.0 mM) stock solution was added and then diluted to 4 mL with 0.01 M HCl, followed by the addition of different concentrations of MDA. The resulting solution was well-mixed and kept at room temperature for 6 min, and then 1.0 mL of phosphate buffer (0.2 M, pH 7.4) was added. Absorption and fluorescence measurements were performed by transferring the solution into a 1-cm quartz cell. Meanwhile, a blank solution containing no MDA was prepared and measured under identical conditions for comparison.

### 2.6. Quantum chemical calculations

Density functional theory (DFT) and time-dependent (TD)-DFT calculations were performed using *Gaussian 09* [31]. Geometry optimizations in the ground state ($S_0$) and the first excited singlet state ($S_1$) were performed in water, employing CAM-B3LYP functional [32] in combination with the 6-31 + G(d,p) basis set [33]. Solvent effects were taken into account using the IEFPCM model. Frequency checks were carried out after each geometry optimiza-