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Sensitivity evaluation of rhodamine B hydrazide towards nitric oxide and its application for macrophage cells imaging

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ABSTRACT

A colorless and non-fluorescent rhodamine derivative, rhodamine B hydrazide (RH), is applied to detect nitric oxide and form fluorescent rhodamine B (RB). The reaction mechanism of RH with NO is proposed in this study. The probe shows good stability over a broad pH range (pH > 4). Furthermore, fluorescence intensity of RH displays an excellent linearity to the NO concentration and the detection limit is as low as 20 nM. A 1000-fold fluorescence turn-on from a dark background was observed. Moreover, the selectivity study indicated that the fluorescence intensity increasing in the presence of NO was significantly higher than those of other reactive oxygen/nitrogen species. In exogenously generated NO detection study, clear intracellular red fluorescence was observed in the presence of S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, a kind of NO releasing agent). In endogenously generated NO detection study, increasing incubation time of RH with lipopolysaccharied (LPS) pre-treated cells could obtain a highly fluorescent cell image. These cell imaging results demonstrated that RH can efficiently penetrate into Raw 264.7 cells and be used for detection of exogenously and endogenously generated nitric oxide.

1. Introduction

Nitric oxide (NO) is a gaseous, paramagnetic and highly reactive radical molecule, which plays various physiological and pathological roles through rapidly reacting with other radicals or metalloproteins in the organisms. In the animal systems, NO at low concentration acts as a molecular signal transmitter in a variety of biological processes, such as the blood-pressure regulation of the cardiovascular system, the antitumor activity of the immune systems and the neurotransmission of the central and peripheral nervous systems [1–9]. On the other hand, high concentration of NO can promote the generation of reactive nitrogen species (RNS) [3,6,10], which could degrade lipids, proteins and DNA, with or without the assistance by different kinds of reactive oxygen species (ROS). Since NO is presently regarded as a vital bioregulatory molecule, quantitative assay of the NO concentration either in vitro or in vivo is crucial for understanding the NO related physiological processes. In order to comprehend the major functions of NO in biological systems, a number of methods such as chemiluminescence [11], colorimetry [12–15], electron paramagnetic resonance [16–18], electrochemistry [19–21], and fluorimetry [22–33] have been developed for NO detection. Compared to other NO detection methods, fluorimetry, which monitor the presence of NO by using a fluorescence probe, has been considered as a favorable way to instantly detect NO with minimal invasiveness, high sensitivity, and high spatial and temporal resolution. This method provides a valuable tool to understand the versatile roles of NO in the biological system.

Typically, the NO detection mechanism is based on PeT (photoinduced electron transfer) effect. The fluorescence of the NO-sensitive modulator can be quenched by a PeT mechanism in the excited state. After reacting with NO or the NO oxidized products, the PeT fluorescence quenching property is suppressed and the fluorescence of the probe is restored. The most common PeT sensing systems comprise an electron-rich *o*-diamino aromatic moiety, which can react with NO under aerobic condition to produce fluorescent triazole derivatives [22,23,30]. However, there are some undesired handicaps, such as complicated and low-yield synthetic procedures and blank fluorescence, existing in these kinds of NO probes.

Rhodamine dyes are extensively used as fluorescent probes because of their high photo-stability, high fluorescence quantum yield, high absorption coefficient and broad fluorescence

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in the visible region of electromagnetic spectrum [34]. A spiro form derivative of rhodamine B (2-amino-3′,6′-bis(diethylamino)-2,3-dihydrospiro[iso-indole-1,9′-xanthene]-3-one, RH), which is totally colorless with no absorptions in the visible region, behaves as a peroxynitrite ($ONOO^-$) probe when encounters with peroxynitrite and form the fluorescent rhodamine B-like compound [35]. RH also reacts with nitrite (NO_2^-) at acidic condition (pH < 4) and forms a rhodamine derivative with absorption at 561 nm. Importantly, it can also react with NO released by NOC-7 (3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine) at neutral pH to give a fluorescence at 581 nm [36]. However, the detailed study relative to the NO detection has not been reported yet. The primary objective of the present study is to investigate the potential application of RH for NO quantification and in vitro detection.

In this study, the reaction product of RH with NO under aerobic conditions, RB, is identified and a possible reaction mechanism is proposed. For realizing the level of fluorescence enhancement, the fluorescent properties of RH and RB were studied and compared. In order to confirm the stability, the fluorescence intensities of RH and RB in various pH conditions were measured. To comprehend the NO detecting ability, time- and concentration-dependent studies were investigated. The correlation between fluorescence intensity and NO concentration was also examined. In addition, the selectivity of RH for NO against other reactive oxygen and nitrogen species was investigated. Furthermore, in vitro fluorescence images were performed.

2. Experimental

2.1. Materials

All chemicals were obtained from commercial suppliers, and used as received without further purification. Rhodamine B base, hydrogen peroxide, and lipopolysaccharied (LPS) were purchased from Sigma–Aldrich. Hydrazine, sodium nitrite ($NaNO_2$), and sodium nitrate ($NaNO_3$) were purchased from Showa. Angeli's salt ($Na_2N_2O_3$), S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), and peroxynitrite (as a solution in 0.3 M NaOH) were purchased from Cayman Chemical Company. Peroxynitrite was stored at −80 °C and quantified by UV–vis spectroscopy immediately prior to use. 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, free acid, Ultrapure Bioreagent) was purchased from MP Biomedicals. Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) was purchased from J. T. Baker. Dulbecco's Modified Eagle Medium (DMEM) and sodium pyruvate were purchased from Cellgro. Fetal bovine serum (FBS, 10%) was purchased from HyClone. The $NO_{(g)}$ (10% NO/90% N_2) was purchased from SanFu. It was passed through an Ascarite II column to remove higher nitrogen oxides before use [37]. De-ionized water was produced from Milli-Q reagent water purification system. Acetonitrile was distilled over calcium hydride (CaH_2) under nitrogen. Ethanol was distilled over Mg/I_2 and subsequently dried over 0.3 nm molecular sieves.

2.2. Instruments

Flash column chromatography was performed on reverse phase C18 silica gel (25–40 μm). ^1H and ^{13}C NMR spectra were obtained on a Varian VXR-300 spectrometer at 300 MHz and 75 MHz, and were referenced to the internal ^1H and ^{13}C solvent peaks. ESI-MS spectra were recorded on a Micromass Q-Tof mass spectrometer. UV–visible absorption spectra were recorded on a Hitachi U-3000 spectrophotometer in 1 cm path length quartz cuvette with a volume of 3.5 mL. Fluorescence spectroscopic studies were acquired on a Hitachi F-7000 spectrophotometer. Bright-field and fluorescence images were recorded on a fluorescence microscope (IX71, Olympus) equipped with a 100 W mercury lamp, B-2A filters (excitation filter: 545–585 nm; emission filter: >610 nm), and a color CCD camera system.

2.3. Preparation of rhodamine B hydrazide (RH)

A modification of the synthetic procedure of Tong et al. [38] was employed. To 7.2 g of rhodamine B base dissolved in 180 mL of absolute ethanol, hydrazine (18 mL) was added dropwisely over 1 h with vigorous stirring under N_2 at room temperature. The stirred mixture was placed in an oil bath and sequentially heated to reflux for 7 h. The color of the solution changed from dark red to light orange. The solution was then cooled and the solvent was removed on a rotary evaporator. The residue was added 1 M HCl (300 mL) and adjusted the solution to pH 9.5 by 1 M NaOH. The resulting precipitate was filtered and washed 5 times with 10 mL de-ionized water. After removing the solvent in vacuo, the analytically pure product (4.8 g, 67%) was obtained after recrystallization from CH_3CN/H_2O (1/1, v/v).

ESI-MS, *m*/*z* 457.1 ($[M+H]^+$), M^+ calculated 456.2.

^1H NMR (DMSO-d_6) δ = 7.76 (m, 1H, ArH), 7.46 (m, 2H, ArH), 6.98 (m, 1H, ArH), 6.37–6.32 (d, 6H, xanthene-H), 4.27 (s, 2H, NH_2), 3.32 (q, 8H, NCH_2CH_3), 1.07 (t, 12H, NCH_2CH_3); ^{13}C NMR (DMSO-d_6) δ = 12.46, 43.7, 64.76, 97.42, 105.46, 107.79, 122.16, 123.51, 127.72, 128.12, 129.63, 132.39, 148.11, 151.9, 153.04, 165.29.

2.4. Reaction of RH with NO

To a 50 mL of RH (1.0 g, 2.2 mmol) solution in dry acetonitrile, 10% NO gas was added under aerobic condition at room temperature. The color of the solution immediately changed from colorless to deep pink. Then the solvent was removed on a rotary evaporator. The residue was purified by a column chromatography with the reverse phase C18 silica gel as stationary phase and the $MeOH/H_2O$ (1/1, v/v) solution as eluent. A pink product (0.8 g, 82%) was obtained after drying under vacuo.

ESI-MS, *m*/*z* 443.0 ($[M+H]^+$), M^+ calculated 442.2.

^1H NMR (DMSO-d_6) δ = 8.23 (d, 1H, ArH), 7.87 (m, 2H, ArH), 7.48 (d, 1H, ArH), 7.10 (d, 2H, ArH), 7.02 (m, 4H, ArH), 3.32 (q, 8H, NCH_2CH_3), 1.07 (t, 12H, NCH_2CH_3); ^{13}C NMR (DMSO-d_6) δ = 12.4, 45.2, 95.8, 112.8, 114.4, 130.1, 130.3, 130.9, 132.6, 155.0, 157.0, 166.2.

2.5. Preparation of NO stock solutions

The preparation of NO and its stock solutions were carried out according to the reported methods [39,40]. NO can be generated by slowly dropping 2.0 M $H_2SO_{4(aq)}$ into a glass flask containing saturated $NaNO_2$ water solution. Since O_2 will rapidly oxidize NO to form NO_2, all apparatus was carefully degassed with argon for 30 min to remove O_2. The forming gas was passed through a 30% NaOH solution twice and water once to trap NO_2 generated from the reaction of NO with traces of O_2. To produce a saturated NO solution (1.8 mM, at 20 °C) as a stock solution, 10 mL deoxygenated de-ionized water was bubbled with NO for 30 min and kept under NO atmosphere until being used. Stock solutions were freshly prepared for each experiment and stored in a glass flask with a rubber septum.

2.6. Fluorometric analysis

Fluorescence spectra were recorded by using a Hitachi F-7000 spectrophotometer. The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 500 V. Spectra were routinely acquired at 25.0 ± 0.1 °C in quartz cuvettes with a

volume of 3.5 mL. All of the fluorescence spectra were measured at 583 nm with excitation wavelength at 510 nm.

2.7. Stability studies

Solutions of 50 μM RH (or RB) in 100 mM HEPES with 20% CH_3CN in the pH range of 4–12 were prepared by mixing 1 mL of 250 μM solutions of RH (or RB) in CH_3CN with 4 mL of 100 mM HEPES whose pH values were adjusted by hydrogen chloride and sodium hydroxide. The fluorescence intensities of RH (or RB) were measured after 2 h mixing.

2.8. Selectivity studies

Nitric oxide: To a cuvette containing 3 mL RH solution (50 μM, in 100 mM HEPES with 20% CH_3CN at pH 7.4) was added 416 μL NO stock solution (1.8 mM) under aerobic condition.

Nitrate: To a cuvette containing 3 mL RH solution (50 μM, in 100 mM HEPES with 20% CH_3CN at pH 7.4) was added 50 μL $NaNO_{3(aq)}$ (30 mM) under aerobic condition.

Nitrite: To a cuvette containing 3 mL RH solution (50 μM, in 100 mM HEPES with 20% CH_3CN at pH 7.4) was added 50 μL $NaNO_{2(aq)}$ (30 mM) under aerobic condition.

Hydrogen peroxide: To a cuvette containing 3 mL RH solution (50 μM, in 100 mM HEPES with 20% CH_3CN at pH 7.4) was added a 2.0 μL aliquot of 30% $H_2O_{2(aq)}$ under aerobic condition. All of the solutions were prepared at 25.0 ± 0.1 °C.

Angeli's salt (*HNO donor*): To a gas-tight cuvette containing 1.8 mg Angeli's salt was added 3 mL RH (50 μM, in 100 mM HEPES with 20% CH_3CN at pH 7.4) under anaerobic condition.

Hydroxyl radical: To a gas-tight cuvette containing ferrous ammonium sulfate (6 mg, 15.3 μmol) and 2.0 μL aliquot of 30% $H_2O_{2(aq)}$ was added 3 mL RH solution (50 μM, in 100 mM HEPES with 20% CH_3CN at pH 7.4) under anaerobic condition.

Peroxynitrite: To a cuvette containing 3 mL RH solution (50 μM, in 100 mM HEPES with 20% CH_3CN at pH 7.4) was added a 400 μL aliquot of freshly thawed peroxynitrite solution (3.76 mM in 0.3 M NaOH, previously stored at −80 °C) under aerobic condition.

2.9. Cell viability studies

The cells were grown in 96-well plates at an initial density 10^5 cells per well for 24 h. Subsequently, the 5 μM of RH (2.5%, v/v DMSO) was incubated with Raw 264.7 macrophages for 2, 4, 8, and 12 h. After incubation, the supernatant was removed and the cells were washed three times with PBS buffer solution. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazoilum bromide (MTT) reduction assay. Briefly, MTT (20 μL, 5 mg mL^{-1}) assay was added to each well. After 4 h of incubation, each well was treated with dimethyl sulfoxide (100 μL) with pipetting. Absorption at 570 nm was measured on a plate reader. Each result was the average of three wells and the viability of untreated cells was assumed to be 100%. Cell viability (%) of Raw 264.7 macrophages at different intervals (2, 4, 8, and 12 h) was calculated.

2.10. Cell culture and in vitro cell imaging

Raw 264.7 murine macrophage is organized from tumor ascites induced by intraperitoneal injection of Abselon Leukemia Virus (A-MuLV) in male mouse. Raw 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, and 1% MEM nonessential amino acids at 37 °C in a humidified 5% CO_2 atmosphere. For imaging studies, Raw 264.7 murine macrophages were passed and plated into poly-D-lysine coated plates containing 2 mL of DMEM, and incubated at 37 °C with 5% CO_2. For exogenously generated nitric oxide detection studies, the cells were incubated with 5 μM RH (2.5%, v/v DMSO) for 4 h, and further co-incubated with DMEM containing 375 μM SNAP for 2 h before being subjected to fluorescence microscope. For endogenously generated nitric oxide detection studies, iNOS (inducible nitric oxide synthase) was induced in Raw 264.7 macrophages with 1 μg mL^{-1} of lipopolysaccharide (LPS) for 4 h, and the cells were then co-incubated with 5 μM RH (2.5%, v/v DMSO) for 2, 4, 6, and 8 h. Prior to imaging, the cells were washed three times with 1 mL of phosphate buffered saline (PBS) and then bathed in 2 mL of PBS. The fluorescence alterations were monitored by fluorescence microscope. For iNOS inhibition study, Raw 264.7 macrophages were sequentially treated with L-NNA (1 mM) for 1 h, LPS for 4 h, and RH for 8 h.

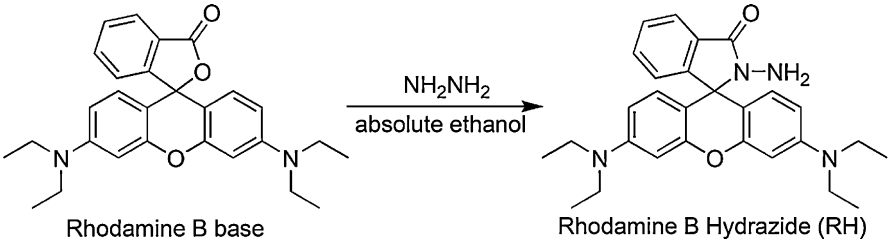
3. Results and discussion

RH was synthesized by the reaction of rhodamine B base with hydrazine followed by a reported procedure [38] with slight modifications (Scheme 1). In order to comprehend the compounds generated after reacting with NO, we dissolved the RH in acetonitrile and bubbled 10% NO gas under aerobic condition. After purification and identification, the final fluorescent product (RB) was obtained. A proposed mechanism for RH reacting with NO is shown in Scheme 2. Firstly, NO reacted with its oxidized product, NO_2, to form N_2O_3 under aerobic condition according to the following equations [41]:

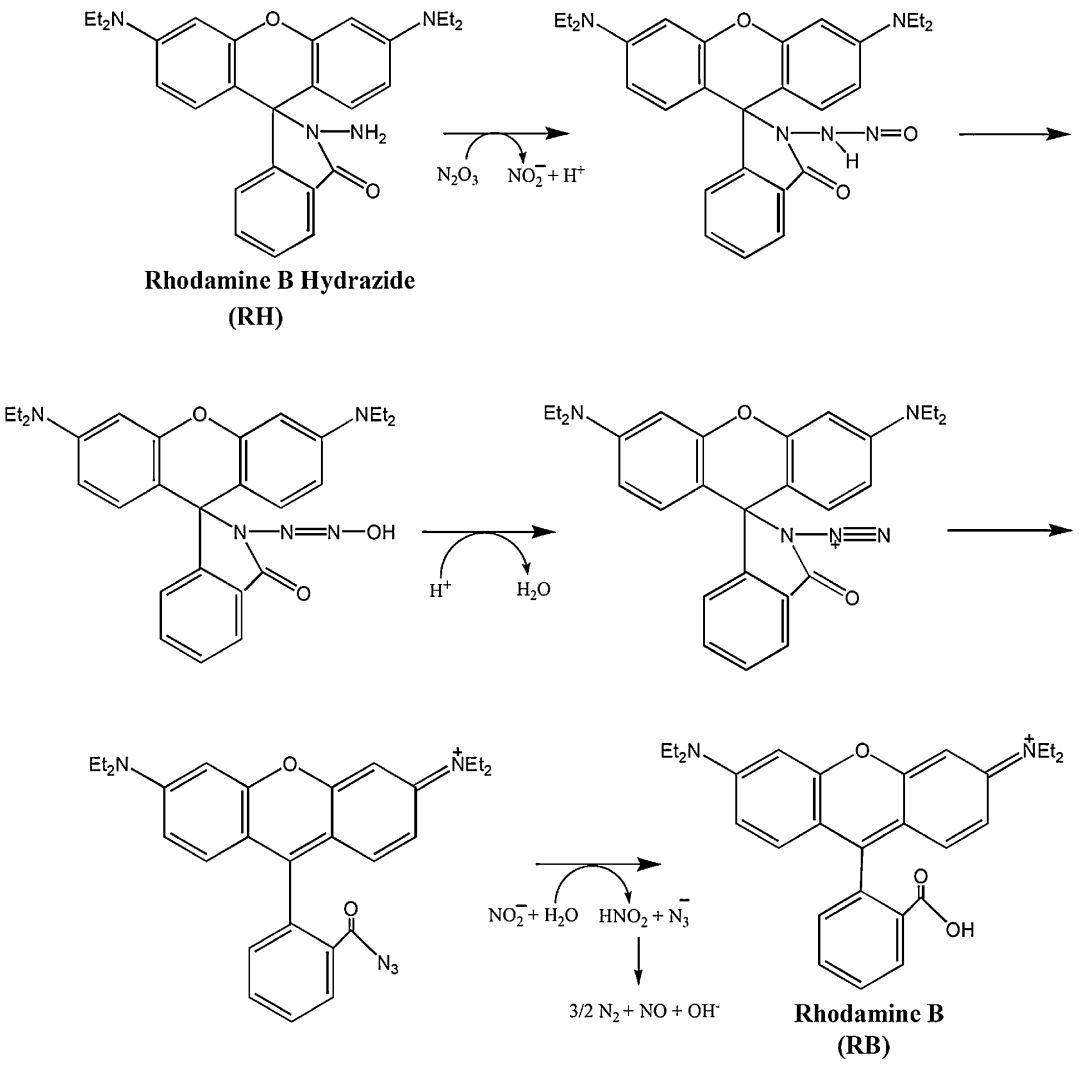
$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$NO_2 + NO \rightarrow N_2O_3 \quad (2)$$

Subsequently, N_2O_3 nitrosylated the hydrazide amino group of RH, and then the amine was converted to diazonium group. In the next step, the 5-membered ring was opened and an azide intermediate was formed. Finally, the azide intermediate was converted into rhodamine B. The absorption and fluorescence spectra of RH and RB were shown in Fig. 1. RH showed a weak absorption at 320 nm and no absorptions between 450 nm and 650 nm, whereas an intense absorption of RB at 560 nm existed. In addition, an obvious



Scheme 1. The synthetic scheme of RH.



Scheme 2. Proposed mechanism of reacting RH with NO under aerobic condition.

fluorescence was observed in the fluorescence spectrum of RB, but RH had no fluorescence.

3.1. Stability study of RH and RB

Since the stability of probes in various pH conditions is concerned for the biological applicability, the effect of pH values on the fluorescence enhancement was measured in the pH range of 4–12, and the results were shown in Fig. 2. There was no fluorescence observed for RH at pH > 4 indicating that the spirocyclic moiety can be retained at physiological pH. When the pH values were below 4, the RH solutions only showed slight fluorescence and change to light pink. The increase of fluorescence was observed owing to a ring-opening reaction of the 5-membered ring of RH at

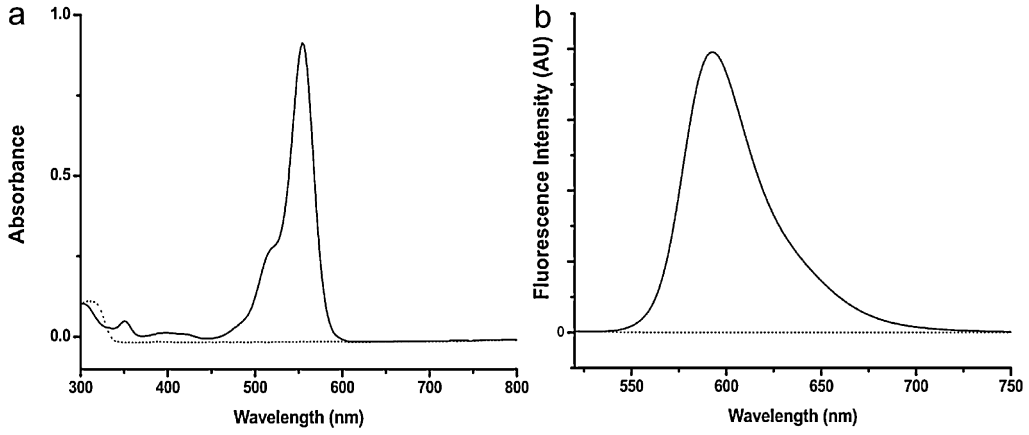


Fig. 1. (a) The absorption spectra of RH (dash line) and RB (solid line) and (b) the fluorescence spectra of RH (dash line) and RB (solid line).

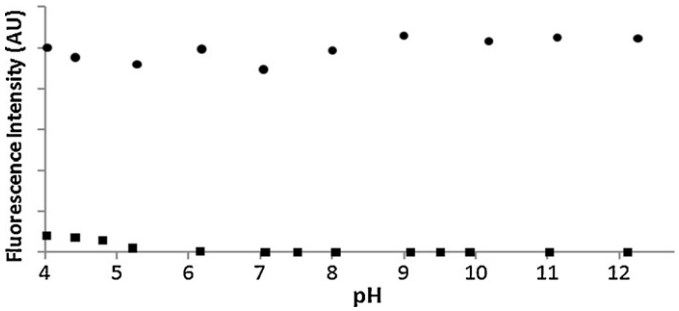


Fig. 2. The pH dependence of the fluorescence intensity of 50 μM RH (■) and RB (●) in 100 mM HEPES buffer with 20% CH_3CN at 25.0 ± 0.1 °C. The fluorescence intensities were detected at 583 nm with excitation at 510 nm.

pH < 4 [42,43]. In contrast, there were almost no fluorescence intensities changes in RB in the pH range of 4–12 as shown in Fig. 2. In other words, the fluorescence intensities of RH and RB were pH-independent at pH > 4, which demonstrates a broad pH range for using RH as a NO probe.

3.2. Time- and concentration-dependence of RH reacting with NO

To investigate the NO detection ability, the time- and concentration-dependent reactions between RH and NO were carried out and the results were shown in Fig. 3. To 50 μM RH solutions (in 100 mM HEPES with 20% CH_3CN at pH 7.4) were added to 0.2, 0.4, 0.6, 0.8, and 1.0 equiv. of NO stock solution (1.8 mM) at 25.0 ± 0.1 °C under aerobic condition. The fluorescence intensity at 583 nm increased with time and reached a plateau after 25 min as shown in Fig. 3(a). Furthermore, the fluorescence intensity at 583 nm was enhanced with the increasing equivalent of $NO_{(aq)}$, as shown in Fig. 3(b). From Fig. 3(c), an excellent linear correlation (R^2 = 0.998) between the fluorescence intensity and the NO concentration could be observed. For RH, the limit of detection (LOD) is the concentration of NO at which the fluorescence signal equals to the blank value plus three-fold of standard deviation, as shown in the following equation [44]:

$$\mathrm{LOD} = X_{\mathrm{bi}} + 3S_{\mathrm{bi}}$$

where X_{bi} is the mean fluorescence signal of the blank and S_{bi} is the standard deviation of the blank. This fluorescence signal was calculated and compared with the linear correlation to obtain the LOD with a value of 20 nM. These results indicated that RH had a potential ability for quantitative NO measurement.

3.3. Selectivity

The selectivity of RH for NO against other reactive oxygen and nitrogen species requires investigations. The fluorescence intensities of RH reacted with a series of possible competitive reactive oxygen or nitrogen species up to 100-fold excess for H_2O_2, NO_3^-, NO_2^-, HNO, and OH•, and 10-fold excess for $ONOO^-$ were measured and the results were shown in Fig. 4. After reacting with 100 equiv. of H_2O_2, NO_3^-, NO_2^-, and HNO, the enhancements of fluorescence intensities were significantly lower than that of NO (994-fold). For the comparison of OH• and NO, the concentration of OH• was 20-fold higher than that of NO, but the fluorescence intensity enhancement of OH• was only 32% of the enhancement of NO. Compared to the result of NO, the fluorescence intensity enhancement of 10 equiv. peroxynitrite was around 81% of the enhancement from reacting with 5 equiv. of NO. The detection is affected when nitric oxide and peroxynitrite simultaneously exist. However, it is well-known that peroxynitrite is formed from the reaction of nitric oxide and superoxide. The formation of peroxynitrite is inhibited by an effective superoxide scavenging enzyme, superoxide dismutase (SOD), in biological environment [45].

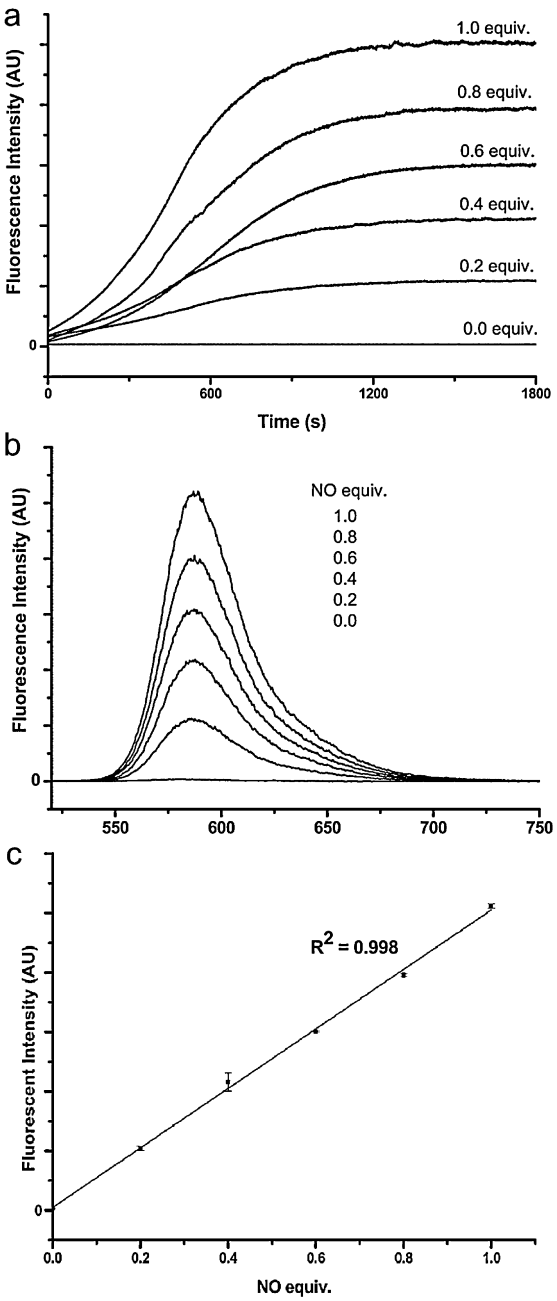


Fig. 3. (a) Time-dependent fluorescence spectra obtained when various equivalents of $NO_{(aq)}$ were added to a 50 μM RH solution (in 100 mM HEPES with 20% CH_3CN at pH 7.4) at 25.0 ± 0.1 °C (λ_{ex} = 510 nm, λ_{em} = 583 nm); (b) concentration-dependent fluorescence spectra based on the intensities at 25 min as shown in (a); and (c) fluorescence intensities upon various equivalents of $NO_{(aq)}$ based on (b).

3.4. Fluorescence imaging of NO in living cells

In order to estimate the exogenously and endogenously generated NO detection ability of RH in living cells, a suitable concentration of RH is required. There was no significant change in cell viability after the Raw 264.7 macrophages incubated with 5 μM RH (2.5%, v/v DMSO) for 12 h, as shown in Fig. 5. According to these results, the following cell imaging studies were performed in the presence of 5 μM RH. For exogenously generated NO detection study, after the cells were incubated with RH for 4 h, the

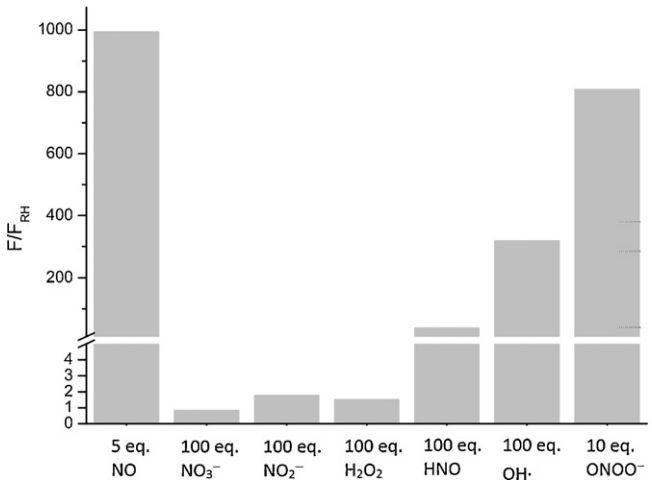


Fig. 4. Specificity of RH for NO over other reactive nitrogen and oxygen species. Fluorescence response of RH were determined in the absence (F_{RH}) and presence (F) of following substances: 5 equiv. NO, 100 equiv. NO_3^-, NO_2^-, H_2O_2, HNO (Angeli's salt), $OH^•$, and 10 equiv. $ONOO^-$. All data were measured after 1 h addition reactive nitrogen/oxygen species at 25.0 ± 0.1 °C.

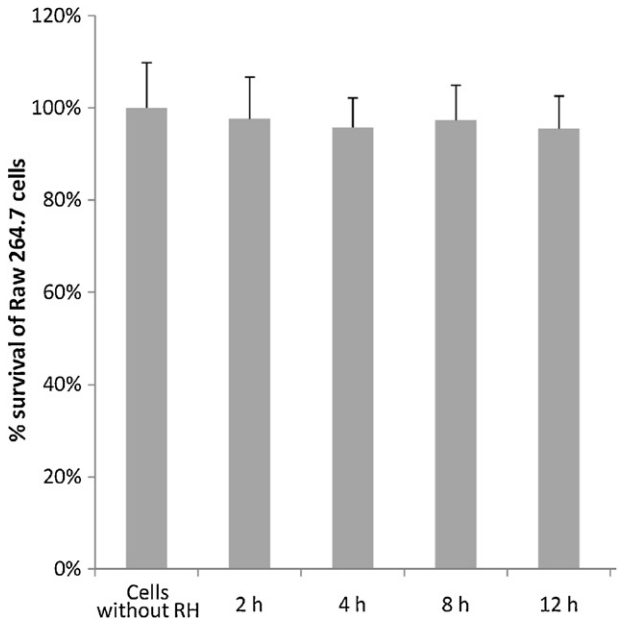


Fig. 5. Cell viability with different incubation time (5 μM RH in 2.5%, v/v DMSO). Left to right: Raw 264.7 cells without RH and incubated with RH for 2, 4, 8, 12, and 24 h, respectively.

RH-deposited cells were further co-incubated with SNAP for another 2 h. The bright-field and fluorescence images of the cells in the absence and presence of SNAP were shown in Fig. 6. Clear red intracellular fluorescence was observed in the presence of SNAP. It indicates that the RH can permeate through the cell membrane into the cells and can be used for in vitro fluorescence imaging of NO.

The ability of RH for detecting NO, which was produced in Raw 264.7 macrophages under physiological conditions, was investigated. In this cell line iNOS can be activated by lipopolysaccharide (LPS) to produce NO [46]. For endogenously generated NO detection study, Raw 264.7 macrophages were incubated with LPS for 4 h. The western blotting results indicated that the expression level of iNOS was increased after stimulated by LPS, as shown in Fig. 7(a). The LPS pre-stimulated cells were further incubated with the RH for 2, 4, 6, and 8 h and the fluorescence images are shown in Fig. 7(b). The cells that were stimulated by LPS showed fluorescence enhancement over 8 h, however, the cells that were incubated with the RH for 12 h in the absence of LPS stimulating did not display any

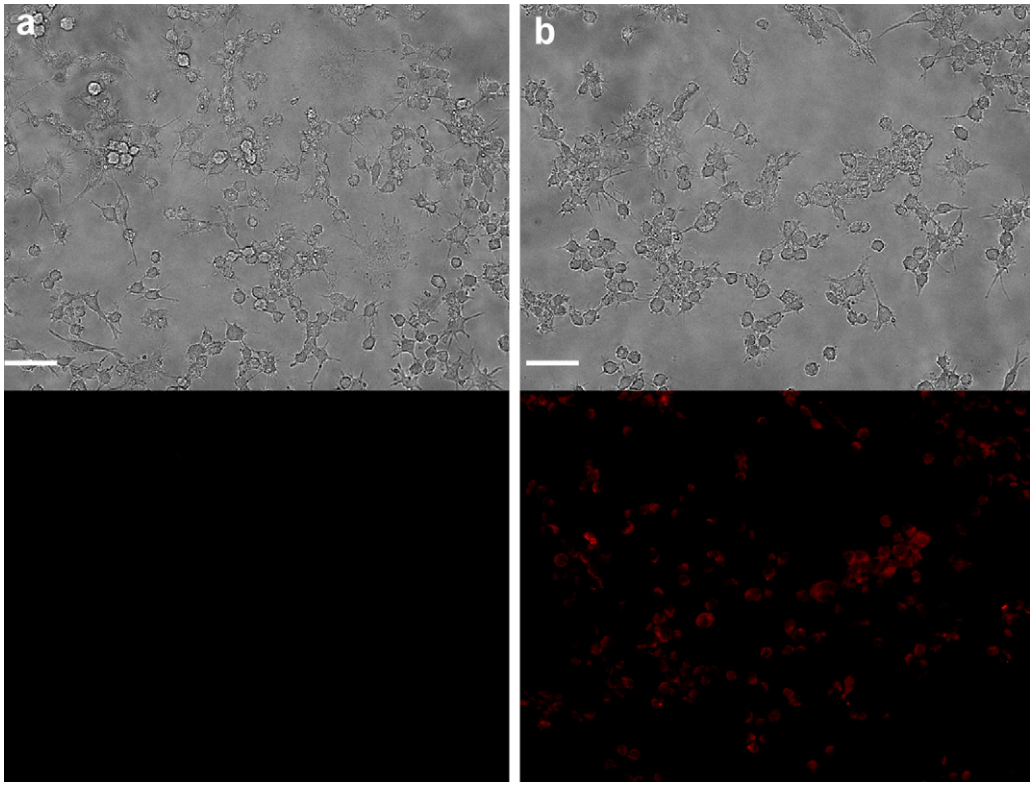


Fig. 6. Exogenously generated NO detection in Raw 264.7 macrophage cells by RH. Bright-field (up) and fluorescence (down) images of the RH-deposited Raw 264.7 cells in the absence (a) and presence (b) of SNAP. The scale bar represents 50 μm.

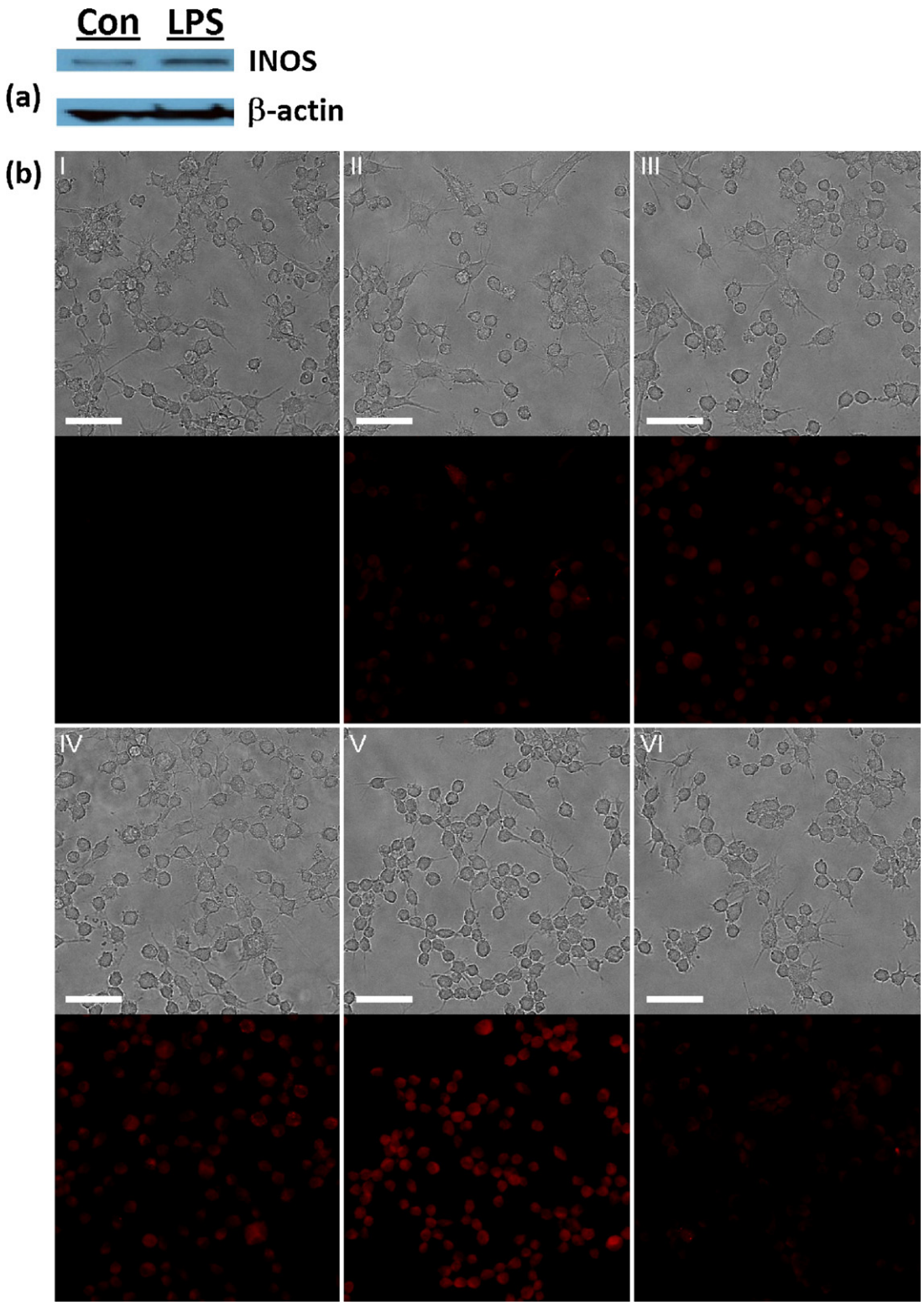


Fig. 7. Endogenously generated NO detection in Raw 264.7 macrophage cells by RH. (a) The expression of iNOS in the absence and presence of stimulation with LPS for 4 h. Left lane: cells without LPS stimulation; right lane: cells with LPS stimulation. (b) RH (5 μM) incubation with cells for (I) 12 h without LPS pre-stimulation; (II) 2 h with LPS (1 μg mL^{-1}) pre-stimulated for 4 h; (III) 4 h with LPS (1 μg mL^{-1}) pre-stimulated for 4 h; (IV) 6 h with LPS (1 μg mL^{-1}) pre-stimulated for 4 h; (V) 8 h with LPS (1 μg mL^{-1}) pre-stimulated for 4 h; and (VI) Raw 264.7 cells sequentially treated with L-NNA (1 mM) for 1 h, LPS for 4 h, and RH for 8 h. The scale bar represents 50 μm.

fluorescence. Furthermore, the weaker fluorescence response in the presence of the iNOS inhibitor L-N^{G}-Nitroaginine (L-NNA) pinpoints NO as being responsible for the fluorescence change. These results indicated that RH can be used to detect exogenously and endogenously generated nitric oxide in cells.

4. Conclusion

In summary, a turn-on fluorogenic probe (RH) for NO is proposed in this study. A great linearity relation between the fluorescence intensity and NO concentration provides the possibility of

quantitative measurement through fluorometric method. A wide range of pH-independent stability allows RH having high stability at pH > 4. The results of fluorescence imaging of monitoring the exogenously and endogenously generated NO suggest that it will be widely useful for the detection of NO in biological system.

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References

[1] R.M.J. Palmer, A.G. Ferrige, S. Moncada, Nature 327 (1987) 524–526.
[2] A.R. Butler, D.L.H. Williams, Chem. Soc. Rev. 22 (1993) 233–241.
[3] F. Murad, Angew. Chem. Int. Ed. 38 (1999) 1856–1868.
[4] R.F. Furchgott, Angew. Chem. Int. Ed. 38 (1999) 1870–1880.
[5] L.J. Ignarro, Angew. Chem. Int. Ed. 38 (1999) 1882–1892.
[6] J.M. Zimmet, J.M. Hare, Circulation 114 (2006) 1531–1544.
[7] D.A. Wink, Y. Vodovotz, J. Laval, F.D. Laval, M.W.J.B. Mitchell, Carcinogenesis 19 (1998) 711–721.
[8] B. Mayer, Nitric Oxide, Springer, Berlin, 2000.
[9] L.J. Ignarro, Nitric Oxide Biology and Pathobiology, Academic Press, San Diego, CA, 2000.
[10] F.L.M. Ricciardolo, P.J. Sterk, B. Gaston, G. Folkerts, Physiol. Rev. 84 (2004) 731–765.
[11] J.F. Brien, B.E. McLaughlin, K. Nakatsu, G.S. Marks, Methods Enzymol. 268 (1996) 83–92.
[12] L.C. Green, D.A. Wagner, J.S. Glogowski, P.L.J.S. Wishnok, S.R. Tannenbaum, Anal. Biochem. 126 (1982) 131–138.
[13] R.W. Nims, J.F. Darbyshire, J.E. Saavedra, D. Christodoulou, I. Hanbauer, G.W. Cox, M.B. Grisham, F. Laval, J.A. Cook, M.C. Krishna, D.A. Wink, Methods 7 (1995) 48–54.
[14] L.A. Ridnour, J.E. Sim, M.A. Hayward, D.A. Wink, S.M. Martin, G.R. Buettner, D.R. Spitz, Anal. Biochem. 281 (2000) 223–229.
[15] F. Brown, N.J. Finnerty, F.B. Bolger, J. Millar, J.P. Lowry, Anal. Bioanal. Chem. 381 (2005) 964–971.
[16] H. Kosaka, M. Watanabe, H. Yoshihara, N. Harada, T. Shiga, Biochem. Biophys. Res. Commun. 184 (1992) 1119–1124.
[17] Y. Katayama, N. Soh, M. Maeda, ChemPhysChem 2 (2001) 655–661.
[18] T. Nagano, T. Yoshimura, Chem. Rev. 102 (2002) 1235–1270.
[19] T. Malinski, Z. Taha, Nature 358 (1992) 676–678.
[20] K. Ichimori, I.H. Shida, M. Fukahori, H. Nakazawa, E. Murakami, Rev. Sci. Instrum. 65 (1994) 2714–2717.
[21] F. Bedioui, N. Villeneuve, Electroanalysis 15 (2003) 5–18.
[22] H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Anal. Chem. 70 (1998) 2446–2453.
[23] H. Kojima, M. Hirotani, N. Nakatsubo, K. Kikuchi, Y. Urano, T. Higuchi, Y. Hirata, T. Nagano, Anal. Chem. 73 (2001) 1967–1973.
[24] H. Kojima, Y. Urano, K. Kikuchi, T. Higuchi, Y. Hirata, T. Nagano, Angew. Chem. Int. Ed. 38 (1999) 3209–3212.
[25] K.J. Franz, N. Singh, S.J. Lippard, Angew. Chem. Int. Ed. 39 (2000) 2120–2122.
[26] N. Soh, Y. Katayama, M. Meada, Analyst 126 (2001) 564–566.
[27] Y. Gabe, Y. Urano, K. Kikuchi, H. Kojima, T. Nagano, J. Am. Chem. Soc. 126 (2004) 3357–3367.
[28] M.H. Lim, D. Xu, S.J. Lippard, Nat. Chem. Biol. 2 (2006) 375–380.
[29] E.W. Miller, C.J. Chang, Curr. Opin. Chem. Biol. 11 (2007) 620–625.
[30] E. Sasaki, H. Kojima, H. Nishimatsu, Y. Urano, K. Kikuchi, Y. Hirata, T. Nagano, J. Am. Chem. Soc. 127 (2005) 3684–3685.
[31] H. Zheng, G.Q. Shang, S.Y. Yang, X. Gao, J.G. Xu, Org. Lett. 10 (2008) 2357–2360.
[32] Z.J. Tonzetich, L.E. McQuade, S.J. Lippard, Inorg. Chem. 49 (2010) 6338–6348.
[33] Y. Yang, S.K. Seidlits, M.M. Adams, V.M. Lynch, C.E. Schmidt, E.V. Anslyn, J.B. Shear, J. Am. Chem. Soc. 132 (2010) 13114–13116.
[34] M. Beija, C.A.M. Afonso, J.M.G. Martinho, Chem. Soc. Rev. 38 (2009) 2410–2433.
[35] X.F. Yang, X.Q. Guo, Y.B. Zhao, Talanta 57 (2002) 883–890.
[36] T. Rieth, K. Sasamoto, Anal. Commun. 35 (1998) 195–197.
[37] C.F. Works, C.J. Jocher, G.D. Bart, X. Bu, P.C. Ford, Inorg. Chem. 41 (2002) 3728–3739.
[38] Y. Xiang, A. Tong, P. Jin, Y. Ju, Org. Lett. 8 (2006) 2863–2866.
[39] J. Ouyang, H. Hong, C. Shen, Y. Zhao, C.G. Ouyang, L. Dong, J.U. Zhu, Z.J. Guo, K. Zeng, J.G. Chen, C.Y. Zhang, J.F. Zhang, Free Radic. Biol. Med. 45 (2008) 1426–1436.
[40] K.J. Huang, H. Wang, M. Ma, X. Zhang, H.S. Zhang, Nitric Oxide 16 (2007) 36–43.
[41] L.J. Ignarro, J.M. Fukuto, J.M. Griscavage, N.E. Rogers, R.E. Byrns, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 8103–8107.
[42] Y. Zhao, X.B. Zhang, Z.X. Han, L. Qiao, C.Y. Li, L.X. Jian, G.L. Shen, R.Q. Yu, Anal. Chem. 81 (2009) 7022–7030.
[43] S. Kang, S. Kim, Y.-K. Yang, S. Bae, J. Tae, Tetrahedron Lett. 50 (2009) 2010–2012.
[44] EURACHEM, The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics, LGC, United Kingdom, 1998.
[45] P. Pacher, J.S. Beckman, L. Liaudet, Physiol. Rev. 87 (2007) 315–424.
[46] E.M. Conner, M.B. Grisham, Methods 7 (1995) 3–13.