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ORIGINAL PAPER

Bioimaging of Peroxynitrite in MCF-7 Cells by a New Fluorescent Probe Rhodamine B Phenyl Hydrazide

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Abstract Peroxynitrite is a potent oxidizing and nitrating agent which has detrimental effects on cells by altering the structure and function of biomolecules present within. A fluorescent probe rhodamine B phenyl hydrazide (RBPH) has been proposed for peroxynitrite ($ONOO^-$) imaging in MCF-7 cells based on its oxidation property, which converts RBPH to pink colored and highly fluorescent rhodamine B. The fluorescence emission intensity of the rhodamine B produced in the above process is linearly related to the concentration of peroxynitrite. The method obeys Beer's law in the concentration range 2–20 nM and the detection limit has been found to be 1.4 nM. The possible reaction mechanism of peroxynitrite with RBPH to produce rhodamine B has been discussed with spectroscopic evidence. The Probe is selective to the peroxynitrite in the pH range 6–8 which is near physiological pH. Fluorescence microscopic studies suggest that the probe is cell permeable and hence peroxynitrite was imaged in MCF-7 cells.

Keywords Peroxynitrite · Rhodamine B phenyl hydrazide · Fluorimetry · Bioimaging · MCF-7 cells

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Introduction

Reactive nitrogen species such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), oxygen species such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are usually involved in several physiological and pathological processes [1]. Peroxynitrite is a potent nitrating species formed in macrophages, neurons, endothelial cells and platelets by the fast reaction ($\sim 1 \times 10^{10}\ M^{-1}\ s^{-1}$) of nitric oxide (NO) and superoxide anion [2]. Peroxynitrite is a short lived species with a biological half-life lesser than 0.1 s [3]. It can interact with wide range of molecules in cells, including DNA, proteins and modify them [4, 5]. Peroxynitrite possesses high affinity for tyrosine residues in proteins and 3-nitrotyrosine serves as an indicator of peroxynitrite mediated protein modification [6]. Protein nitration has been observed in a series of diseases like Parkinsons, Alzheimers, Huntingtons, Atherosclerosis and Hypertension [7–11]. At lower concentration they behave as signaling molecules but at higher concentration they induce cell damage [12]. Detection and quantification of peroxynitrite localized in a cell is a challenging task which is needed to be addressed. As peroxynitrite is produced only in trace amounts there has to be a sensitive technique to detect the same. Fluorescence bioimaging plays a pivotal role in such crippling conditions which is found to be highly sensitive and safe compared to other techniques. In recent years, various methods have been tried to detect peroxynitrite such as fluorescence spectrometry, UV–Vis spectrometry, chemiluminescence, electrochemical methods, enzymatic methods and immunohistochemistry [13–20]. But fluorescent methods find significant among all other methods for in vivo and in vitro monitoring of various oxidants, especially the xanthene derivatives due to their attractive spectroscopic properties like longer emission wavelengths, high quantum yields and their water solubility which are the desirable properties that can be utilized in the

biological systems study [21–24]. These xanthene derivatives are colorless and non fluorescent in their spiro closed forms but highly fluorescent and colored (either green or pink) in their corresponding open forms [25, 26]. This property has been utilized extensively for sensing and monitoring both biological analytes as well as metal ions and anions by converting the spiro closed forms (non fluorescent) into spiro open forms (fluorescent) by the analytes involving various strategies like specific bond cleavage followed by hydrolysis, oxidizing the reduced forms, by coordination leading to ring opening [27–29]. Some of the fluorescein derivatives have been recently reported for bioimaging of peroxynitrite and these probes are found to posses short excitation wavelengths compared to that of rhodamine derivatives [14, 30–32]. The probes possess longer excitation wavelength found to be advantageous for bioimaging due to low background fluorescence of them and also longer wavelength UV excitation leads to less toxicity. So the efforts are made to synthesize a new fluorescent probe which has longer excitation and emission wavelengths to image peroxynitrite in biological cells.

A new rhodamine derivative called rhodamine B phenyl hydrazide (RBPH) bearing an active spirolactam group to sense peroxynitrite in vivo as well as in vitro systems has been proposed. This probe is very sensitive due to the presence of active phenyl hydarazone group which is susceptible to oxidation by free radicals compared to hydrazine groups and reduced forms of the dyes as in case of other reported xanthene derivatives [13–15]. The proposed probe is selective to the peroxynitrite in the near physiological pH and can be utilized for sensing peroxynitrite. No other oxidants like OCl^-, H_2O_2 and ClO_4^- as well as metal ions like Cu^{2+} and Hg^{2+} induce the spectroscopic changes in the probe in this pH range studied.

Materials and Methods

Materials

Peroxynitrite was synthesized as per the reported literature [33]. Rhodamine B hydrazide was purchased from Sigma-Aldrich. Phenyl hydrazide and Acetonitrile were purchased from the SD fine Chem. Ltd., Mumbai. Stock solution of RBPH (1.0 mM) was prepared by dissolving 0.532 g in acetonitrile—water mixture (%v/v). 0.1 M of KH_2PO_4 and Na_2HPO_4 solutions were used for the buffer solution preparation of pH 8. Robinson buffer solutions of pH3–12 were prepared using 0.04 M H_3BO_3, 0.04 H_3PO_4, 0.04 M CH_3COOH and 0.2 M NaOH. All the reagents were of analar grade and double distilled water was used throughout. MCF-7 cell lines were procured from National Centre for Cell Sciences, Pune, India.

Apparatus

Absorbance measurements were made using a Shimadzu Scanning Spectrophotometer (model UV-3101PC) with 1 cm quartz cuvettes. All the fluorescence measurements were measured using Ocean optics (USA) spectrofluorimeter with 1 cm quartz cuvettes. All pH measurements were carried out using a Control Dynamics digital pH meter (model APX 175). NMR spectra were recorded using a Bruker-400 MHz Spectrometer with chemical shifts reported as parts per million (ppm in $CDCl_3$, TMS as internal standard). Mass spectral data was obtained using a Thermo Finnigin DecaQXP Mass Spectrometer. Fluorescence imaging of MCF-7 cells were performed with an Olympus FluoView FV1000 laser scanning microscope with 20 times magnification.

Synthesis of RBPH

In a 250 mL round bottom flask, 2.4 g rhodamine B (4.8 mM) was dissolved in 100 mL of absolute ethanol. Then 20 mL (200 mM) of phenyl hydrazine was added and the reaction mixture was refluxed for 4 h. The solvent was evaporated under reduced pressure. The obtained solid was dissolved in 50 ml of dichloromethane and washed several times with saturated $NaHCO_3$ solution. The organic phase was dried over anhydrous $NaHSO_4$. Then the solution was filtered and the filtrate obtained was evaporated to dryness. The obtained solid was washed with acetonitrile and dried under vacuum. RBPH was obtained as yellow powder with 84 % yield. The compound was characterized by the ESI-MS and ^1H NMR studies.

Cell Culture and Imaging

Breast cancer cell line (MCF-7) was cultured on a cover slip with RPMI 1640 with 5 % fetal bovine serum at 37° C in a

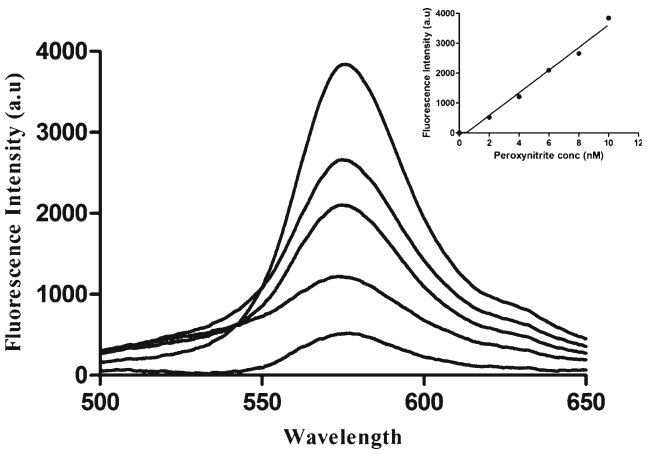


Fig. 1 Fluorescence emission spectra of 1 mL of RBPH (1 mM) in presence of different volumes of peroxynitrite (0–10 nM) (Inset showing the calibration plot of peroxynitrite concentration from 0 to 10 nM)

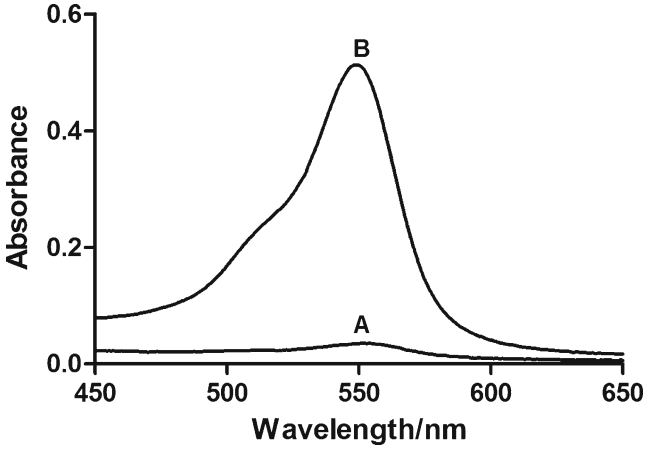


Fig. 2 Absorption spectra of RBPH 1 mL (10 mM) A. In absence of peroxynitrite (10 mM) B. In presence of peroxynitrite (10 nM)

humidified atmosphere with 5 % CO_2. Then 48 h grown culture was incubated with 10 μM RBPH for 2 h and washed five times with phosphate buffer saline (PBS) in order to remove the unabsorbed probe to overcome the background fluorescence and then incubated with 0.5 μM peroxynitrite at room temperature for 1 h and finally washed five times with PBS to avoid fluorescence noise. These cells were subjected for fluorescence imaging. Excitation of cells was carried out at 560 nm and the emission was measured at 570–670 nm.

Recommended Procedure

One mL each of phosphate buffer solution (pH8) and RBPH (1.0 mM) and different concentrations of peroxynitrite (2–20 nM) were added into a series of 10 mL volumetric flasks and diluted to the mark with distilled water and kept at room temperature for 10 min. The solutions were excited at 560 nm and the emitted intensities were measured at 580 nm.

Results and Discussion

The absorption and fluorescence emission spectral characteristics of RBPH were studied in 10 % acetonitrile-water mixture in the pH range 3–12. The RBPH is colorless and non fluorescent similar to that of other xanthene derivatives due to its spiro closed structure. The addition of peroxynitrite to the above solution in presence of phosphate buffer of pH 8 turns the solution into pink colored and highly fluorescent due to the generation of rhodamine B from RBPH by peroxynitrite through spirolactam ring opening process (Figs. 1 and 2). The transformation of RBPH to rhodamine B by peroxynitrite was observed in physiological pH conditions. Hence studies have been carried out to determine the presence of peroxynitrite in biological systems.

Evidence for the Reaction Between RBPH and Peroxynitrite

The peroxynitrite quantitatively reacts with RBPH in the pH range 7–8 to generate rhodamine B by cleaving the spirolactam ring. It has been proved by characterizing the generated molecule through various spectroscopic tools including UV-Vis spectroscopy, fluorescence spectroscopy,

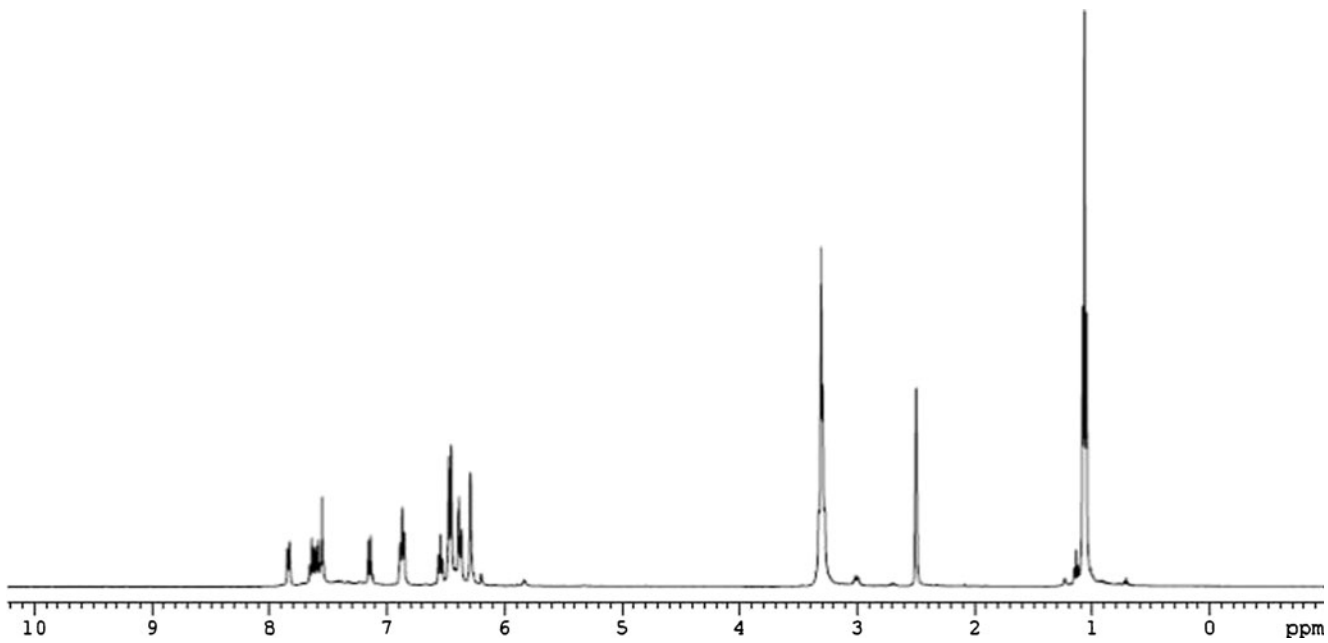


Fig. 3 ^{1}H nmr spectrum of RBPH

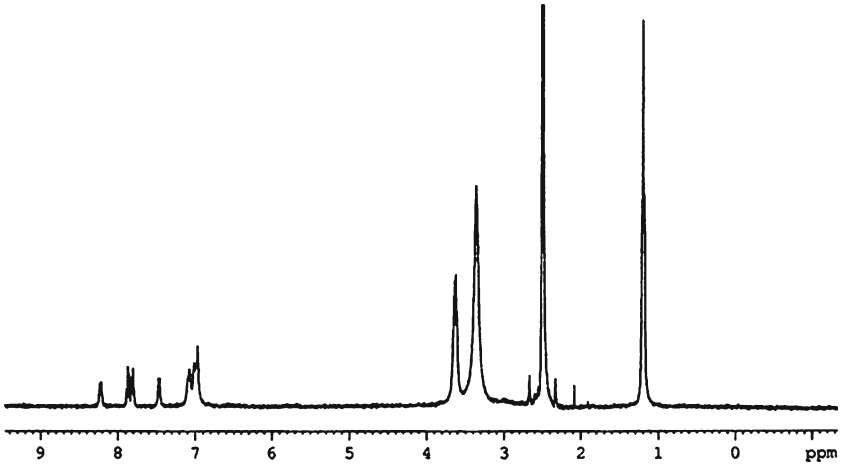


Fig. 4 ^{1}H nmr spectrum of reaction product obtained in the reaction RBPH and peroxynitrite

proton NMR and mass spectrometry. The UV-Vis absorption spectra of the RBPH in acetonitrile-water (1:10 v/v) with phosphate buffer (pH-8) showed very little absorption whereas upon addition of peroxynitrite to the above solution showed a new band at 560 nm which corresponds to the rhodamine B generated in the reaction.

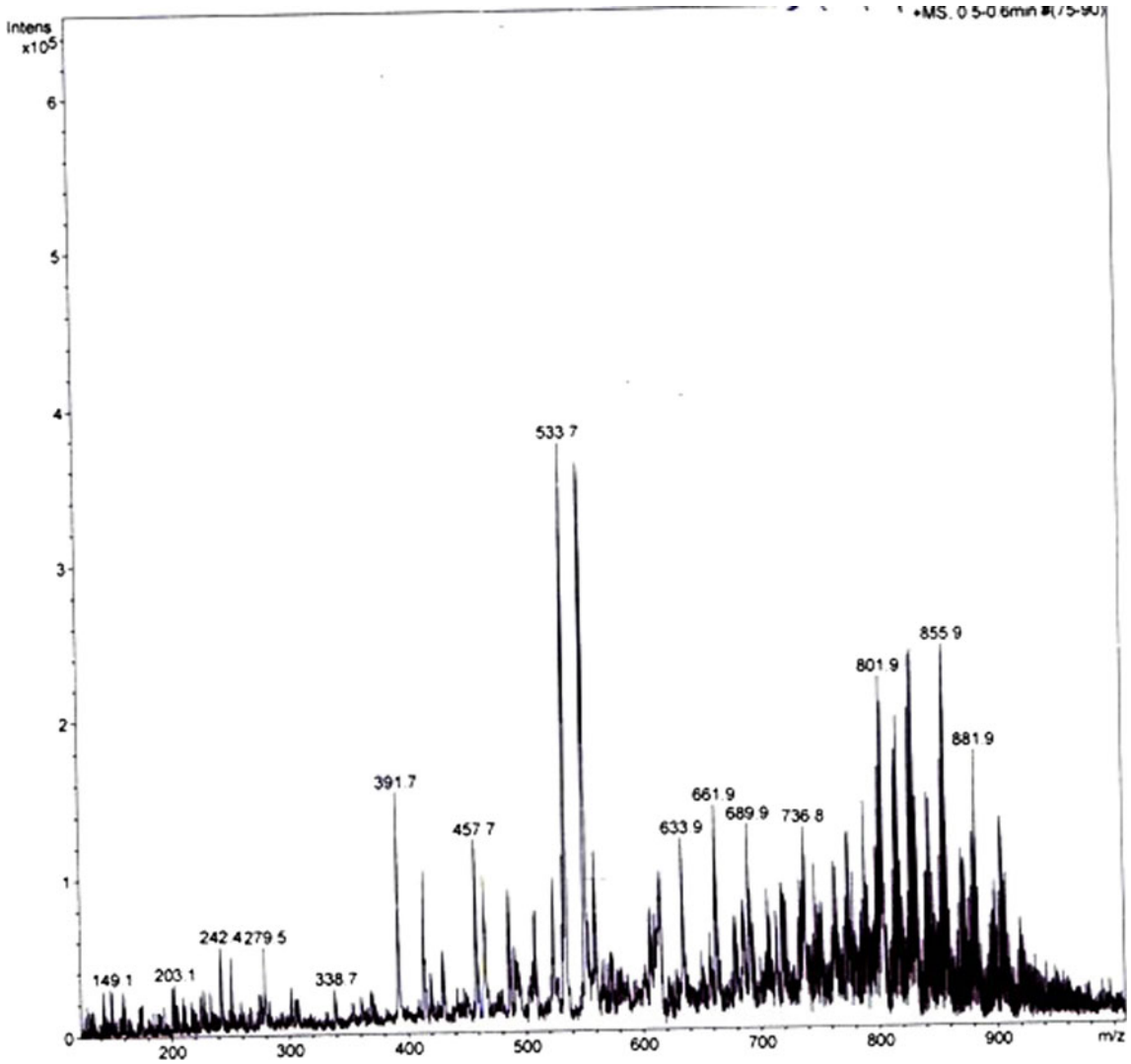


Fig. 5 ESI MS spectra of RBPH

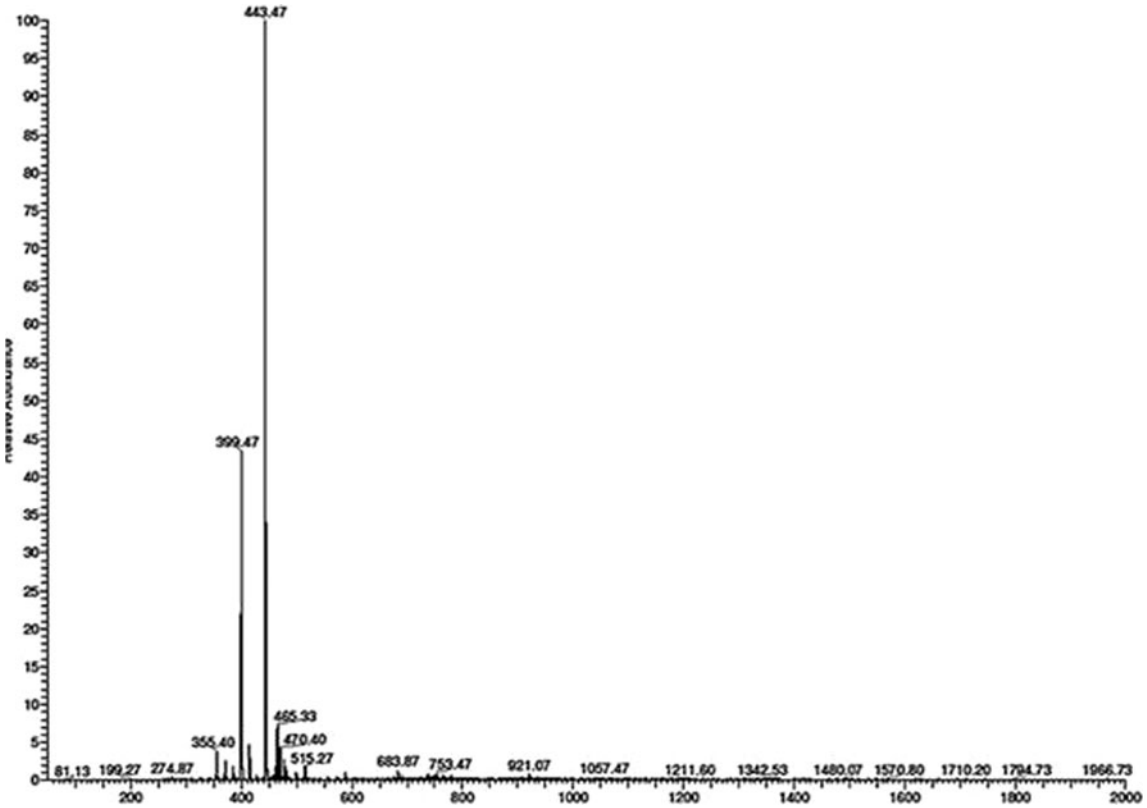
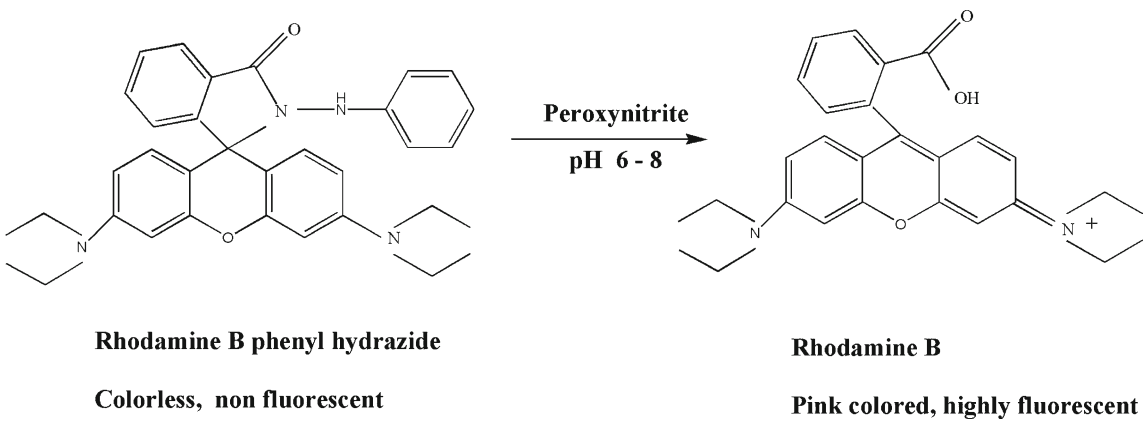


Fig. 6 ESI-MS spectrum of reaction product obtained in the reaction between RBPH and peroxynitrite

Similarly the fluorescence spectrum comparison of the excitation and emission fluorescence spectra of the reaction system with that of authentic rhodamine B showed identical spectra, both having excitation maximum at 560 nm and emission maximum at 580 nm (not shown), indicating that the fluorescent product generated in the reaction mixture is rhodamine B. In order to carry out spectral analysis, the reaction between RBPH and peroxynitrite was carried out in bulk quantities at pH8. The pink colored product obtained was extracted using dichloromethane and the residue was isolated by evaporation method. The solid substance was recrystallised using methanol and the red powder obtained was used for NMR and mass spectral study. The ^{1}H NMR spectra of RBPH contains the NHC_6H_5 signal at 5.9 ppm where as the product obtained in the reaction lacks this peak instead its proton NMR spectra well matched with that of ^{1}H NMR spectra of authentic rhodamine B (Figs. 3 and 4).

The ESI-MS spectra of RBPH has $[M+H]^+$ around 533.21 where as the product obtained in the reaction showed $[M+H]^+$ 443.47 well matched with the $[M+H]^+$



Scheme 1 Schematic representation of reaction between Rhodamine B Phenyl Hydrazide and Peroxynitrite

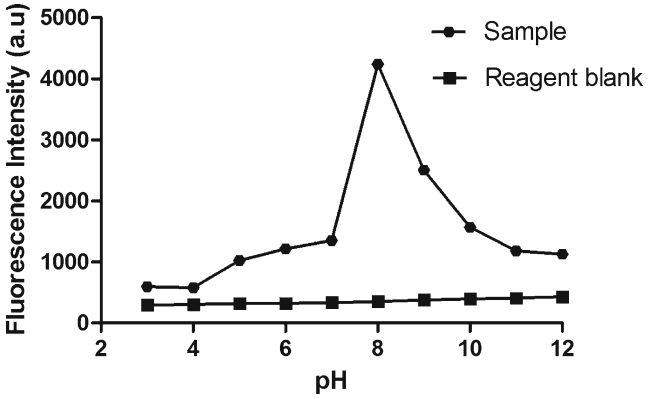


Fig. 7 Effect of pH

443.47 of authentic rhodamine B (Figs. 5 and 6). Based on these spectroscopic experimental evidences, we can conclude that the reaction between RBPH and peroxynitrite undergoes quantitatively to generate rhodamine B compound. The plausible mechanism of the reaction between the reactants has been proposed (Scheme 1).

Optimization Study

Effect of pH

The effect of pH on the reaction between RBPH and peroxynitrite was studied by keeping all other parameters constant. In a set of 10 mL volumetric flasks, 1 mL of 10 nM of peroxynitrite, 1 mL of 1 mM RBPH and 1 mL of phophate buffer solutions of pH in the range of 3–12 were added into 10 mL volumetric flask and diluted upto the mark and allowed for 10 min. at room temperature. The fluorescence intensity of the solutions were measured ($\lambda_{ex/em}$=560/580 nm). It is clear that fluorescence intensity of the solution has increased with pH up to 8 and decreased beyond 9 (Fig. 7). Hence pH 8 of phosphate buffer was chosen as an optimum pH for the quantitative reaction between RBPH and peroxynitrite. Therefore the proposed probe can be applied for bioimaging of peroxynitrite from cells in the physiological pH condition.

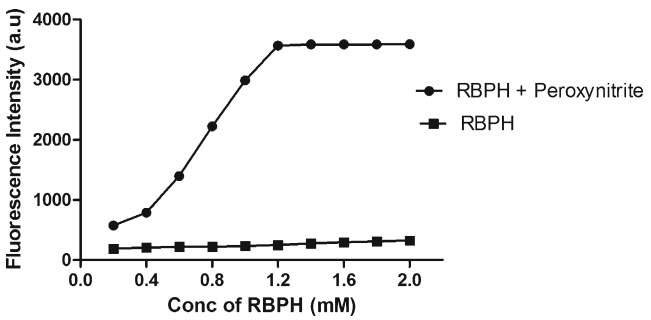


Fig. 8 Effect of RBPH concentration

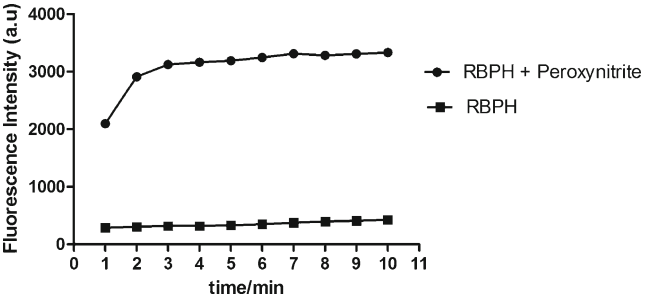


Fig. 9 Effect of Reaction time

Effect of Concentration of RBPH

The optimium concentration of RBPH required for the reaction was studied. Into a series of 10 mL volumetric flasks containing 1 mL of phosphate buffer solution (pH8) 1 mL of 10 nM of peroxynitrite were added and different volumes of 1 mM RBPH were added and diluted up to the mark. It is clear from the figure (Fig. 8) that the fluorescence emission intensity increases with increasing concentration of RBPH and remains constant when the overall concentration of RBPH beyond 1 mM. Hence in all further studies 1 mL of 1 mM RBPH was used as an optimum concentration.

Effect of Reaction Time

The optimum time required for the reaction between RBPH and peroxynitrite was studied by keeping all other parameters constant. The solutions were prepared as above in 10 mL volumetric flasks and allowed for different time intervals. Then the fluorescence intensity of the solutions were recorded as a function of reaction time ($\lambda_{ex/em}$=560/580 nm). It was observed that the fluorescence intensity of the solutions increased up to 3 min and remained constant even after 1 h, but the fluorescence background of the solution in the absence of

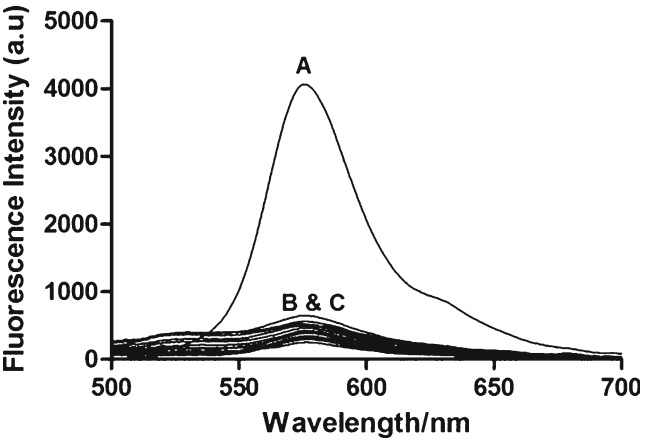


Fig. 10 Fluorescence emission spectra of 1 ml of pH-8 phosphate buffer with **a** 1 ml of 1 mM RBPH and 10 nM Peroxynitrite **b** 1 ml of 1 mM RBPH and **c** 1 ml of 1 mM RBPH with other oxidizing species

Table 1 Effect of foreign ions

Interference	Tolerance limit (μM)
Glucose, Urea, Methionine, Threonine	800
Ascorbic acid, Glutamic acid, Cystein, H_2O_2	500
SO_4^{2-}, NO_2^-, NO_3^-	2,500
Na^+, K^+, Fe^{3+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Ca^{2+}, Zn^{2+}	1,000

peroxynitrite remained unchanged (Fig. 9). Hence 10 min. reaction time was allowed in all further experimental studies.

Interference Study

In order to check the selectivity of RBPH towards the peroxynitrite signaling, the effect of various interfering species normally oxidising agents and other biological species present in the cells along with analyte has been studied. Under the optimised conditions only the analyte facilitates the transformation of RBPH to rhodamine B compound. The spectral behavior of RBPH in presence of various interfering species at pH 8 of phosphate buffer is shown in Fig. 10. The metal ions like Cu^{2+} and Hg^{2+} ions did not induce any fluorescence under the physiological pH condition used in the present investigation. Various other metal ions, anions and oxidizing species generally present in the biological systems like H_2O_2 does not trigger any fluorescence changes of the probe in the pH range 7–9. The tolerance limits of the various species studied are given in the Table 1.

Analytical Merits

The proposed method obeyed Beer's law in the concentration range 2–20 nM with a regression coefficient r^2=0.9991. The limit of detection (LOD) and relative standard deviations were found to be 1.4×10^{-9} and±4.1 % respectively.

Fluorescence Imaging of Living Cells

The biological application of the probe RBPH has been demonstrated through fluorescence imaging experiments. These studies were carried out in MCF-7 breast cancer cells. In view of its good water solubility, favorable spectroscopic properties and the instantaneous interaction with peroxynitrite, RBPH should be well-suited for fluorescence imaging in living cells. The cells were incubated with RBPH (10 μM) for 2 h at 37 ° C, then followed by the addition of peroxynitrite (0.5 μM) and incubation for another 1 h. The cells were washed with PBS solution and their fluorescence images were recorded before and after addition of peroxynitrite (Fig. 11).

In absence of peroxynitrite, free RBPH showed no detectable fluorescence signal in living cells. After incubation with peroxynitrite, a bright fluorescence was observed in cells. These results suggest that the probe RBPH can penetrate the cell membrane and can be applied for in vitro imaging of peroxynitrite in living cells and could be used potentially in vivo too.

The proposed fluorescent probe has unique features in comparison with the existing probes which suites for imaging studies also in live cells *viz*. (1) Its specificity towards the particular analyte in the physiological pH conditions in presence of other potent oxidants. (2) Its long emission wavelengths (probes with short emission wavelengths are cytotoxic because cell have to irradiate with higher energies which may result in cell damage) and (3) water solubility of the probe which is essential during imaging studies.

Conclusion

We have proposed a novel fluorogenic probe for peroxynitrite recognition in biological cells. The probe exhibits excellent selectivity, sensitivity and it is very simple for the determination of peroxynitrite. The fluorogenic probe is also suitable for bioimaging of peroxynitrite because the probe specifically reacts with peroxynitrite in the physiological pH range. Hence peroxynitrite in biological samples can be easily detected at physiological conditions. The response time of the proposed probe for peroxynitrite is instantaneous with a detection limit of 1.4 nM. Other biological oxidants which are commonly present in the human physiology do not induce the fluorescence with the

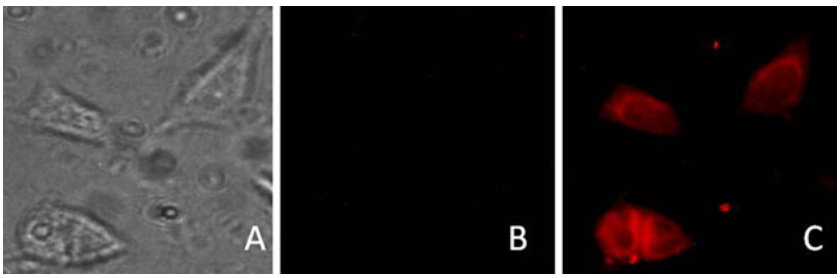


Fig. 11 Fluorescence images of Peroxynitrite in MCF-7 cells with RBPH. **a** Bright-field transmission image of MCF-7 cells incubated with RBPH (10 μM) for 2 h. **b** Fluorescence image of MCF-7 cells incubated with RBPH (10 μM) for 2 h. **c** Fluorescence image of MCF-7 cells incubated with RBPH (10 μM) for 2 h, washed five times with PBS, and then further incubated with 0.5 μM Peroxynitrite for 30 min

proposed probe i.e RBPH. Hence the intensity of the emitted radiant energy is directly proportional to the amount of peroxynitrite present in a particular cell or tissue.

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References

1. Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82(1):47–95
2. Ahmad R, Rasheed Z, Kaushal E, Singh D, Ahsan H (2008) Biochemical evaluation of human DNA-lysine photoadduct treated with peroxynitrite. Toxicol Mech Methods 18(7):589–595
3. Denicola A, Souza JM, Radi R (1998) Diffusion of peroxynitrite across erythrocyte membranes. Proc Natl Acad Sci U S A 95(7):3566–3571
4. Niles JC, Wishnok JS, Tannenbaum SR (2006) Peroxynitrite-induced oxidation and nitration products of guanine and 8-oxoguanine: structures and mechanisms of product formation. Nitric Oxide 14(2):109–121
5. Yang H, Zhang Y, Poschl U (2010) Quantification of nitrotyrosine in nitrated proteins. Anal Bioanal Chem 397(2):879–886
6. Khan MA, Dixit K, Jabeen S, Moinuddin, Alam K (2009) Impact of peroxynitrite modification on structure and immunogenicity of H2A histone. Scand J Immunol 69(2):99–109
7. Good PF, Hsu A, Werner P, Perl DP, Olanow CW (1998) Protein nitration in Parkinson's disease. J Neuropathol Exp Neurol 57(4):338–342
8. Reyes JF, Reynolds MR, Horowitz PM, Fu Y, Guillozet-Bongaarts AL, Berry R, Binder LI (2008) A possible link between astrocyte activation and tau nitration in Alzheimer's disease. Neurobiol Dis 31(2):198–208
9. Browne SE, Ferrante RJ, Beal MF (1999) Oxidative stress in Huntington's disease. Brain Pathol 9(1):147–163
10. Parastatidis I, Thomson L, Fries DM, Moore RE, Tohyama J, Fu X, Hazen SL, Heijnen HF, Dennehy MK, Liebler DC, Rader DJ, Ischiropoulos H (2007) Increased protein nitration burden in the atherosclerotic lesions and plasma of apolipoprotein A-I deficient mice. Circ Res 101(4):368–376
11. Xu J, Wang S, Wu Y, Song P, Zou MH (2009) Tyrosine nitration of PA700 activates the 26S proteasome to induce endothelial dysfunction in mice with angiotensin II-induced hypertension. Hypertension 54(3):625–632
12. Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87(1):315–424
13. Yang XF, Guo XQ, Zhao YB (2002) Development of a novel rhodamine-type fluorescent probe to determine peroxynitrite. Talanta 57(5):883–890
14. Possel H, Noack H, Augustin W, Keilhoff G, Wolf G (1997) 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. FEBS Lett 416(2):175–178
15. Digerness SB, Harris KD, Kirklin JW, Urthaler F, Viera L, Beckman JS, Darley-Usmar V (1999) Peroxynitrite irreversibly decreases diastolic and systolic function in cardiac muscle. Free Radic Biol Med 27(11–12):1386–1392
16. Thomson L, Trujillo M, Telleri R, Radi R (1995) Kinetics of cytochrome c2+ oxidation by peroxynitrite: implications for superoxide measurements in nitric oxide-producing biological systems. Arch Biochem Biophys 319(2):491–497
17. Radi R, Cosgrove TP, Beckman JS, Freeman BA (1993) Peroxynitrite-induced luminol chemiluminescence. Biochem J 290(Pt 1):51–57
18. Kooy NW, Royall JA (1994) Agonist-induced peroxynitrite production from endothelial cells. Arch Biochem Biophys 310(2):352–359
19. Xue J, Ying X, Chen J, Xian Y, Jin L (2000) Amperometric ultramicrosensors for peroxynitrite detection and its application toward single myocardial cells. Anal Chem 72(21):5313–5321
20. Liang J, Liu Z-H, Cai R-X (2005) A novel method for determination of peroxynitrite based on hemoglobin catalyzed reaction. Analytica Chimica Acta 530(2):317–324
21. Wei F, Lu Y, He S, Zhao L, Zeng X (2012) Highly sensitive fluorescent chemosensor for hypochlorite anion based on a novel irreversible ring-opening strategy. Analytical Methods 4(3):616–618
22. Zheng H, Shang GQ, Yang SY, Gao X, Xu JG (2008) Fluorogenic and chromogenic rhodamine spirolactam based probe for nitric oxide by spiro ring opening reaction. Org Lett 10(12):2357–2360
23. Rathel TR, Leikert JJ, Vollmar AM, Dirsch VM (2003) Application of 4,5-diaminofluorescein to reliably measure nitric oxide released from endothelial cells in vitro. Biol Proced Online 5:136–142
24. Yang YK, Cho HJ, Lee J, Shin I, Tae J (2009) A rhodamine-hydroxamic acid-based fluorescent probe for hypochlorous acid and its applications to biological imagings. Org Lett 11(4):859–861
25. Kim HN, Lee MH, Kim HJ, Kim JS, Yoon J (2008) A new trend in rhodamine-based chemosensors: application of spirolactam ring-opening to sensing ions. Chem Soc Rev 37(8):1465–1472
26. de Silva AP, Gunaratne HQ, Gunnlaugsson T, Huxley AJ, McCoy CP, Rademacher JT, Rice TE (1997) Signaling recognition events with fluorescent sensors and switches. Chem Rev 97(5):1515–1566
27. Dujols V, Ford F, Czarnik AW (1997) A long-wavelength fluorescent chemodosimeter selective for Cu(II) Ion in water. Journal of the American Chemical Society 119(31):7386–7387
28. Pandurangappa M, Kumar KS (2011) Micellar mediated trace level mercury quantification through the rhodamine B hydrazide spirolactam ring opening process. Journal Cover:Anal Methods 3:715–723, Analytical Methods 3 (3):715–723
29. Yang XF, Li Y, Bai Q (2007) A highly selective and sensitive fluorescein-based chemodosimeter for Hg2+ ions in aqueous media. Anal Chim Acta 584(1):95–100
30. Kooy NW, Royall JA, Ischiropoulos H, Beckman JS (1994) Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic Biol Med 16(2):149–156
31. Kooy NW, Royall JA, Ischiropoulos H (1997) Oxidation of 2′,7′-dichlorofluorescin by peroxynitrite. Free Radic Res 27(3):245–254
32. Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM (1999) Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic Biol Med 27(1–2):146–159
33. Uppu RM, Pryor WA (1996) Synthesis of peroxynitrite in a two-phase system using isoamyl nitrite and hydrogen peroxide. Anal Biochem 236(2):242–249