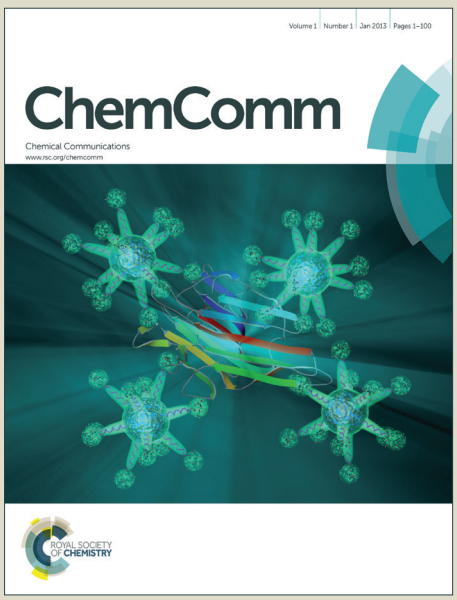
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

A highly selective fluorescent probe for hypochlorite and its endogenous imaging in living cells

Shahi Imam Reja[†], Vandana Bhalla[†], Anuradha Sharma[‡], Gurcharan Kaur[‡] and Manoj Kumar[†]*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

An oxime based fluorescent probe has been designed and synthesized which detects free as well as enzymatically generated hypochlorite with low detection limit and high sensitivity. In addition, the probe was successfully utilized for the monitoring of endogenously produced hypochlorite in LPS stimulated cell lines, C6 glioma and BV2 microglial.

Recently there has been lot of interest in the development of chemosensors for reactive oxygen species (ROS) due to vital role played by these ROS in many biological processes.[1] Hypochlorite (ClO^-) is one of those important reactive oxygen species (ROS) which plays a crucial role in the immune defence against microorganisms and also in inflammation.[2] In living organisms, hypochlorite (ClO^-) is the major oxidant generated by the hydrogen peroxide and chloride (Cl^-) ions in a chemical reaction catalysed by the heme protein myeloperoxidase (MPO) and which then can react with $O_2^{\cdot}$ to produce $OH^{\cdot}$.[3] Although hypochlorite has strong antibacterial properties but excess and abnormal production of hypochlorite in living systems has been linked to a variety of diseases, including cystic fibrosis, kidney disease, and certain types of cancer and can also have harmful effect on host tissues.[4] Thus, the development of effective chemosensors for selective detection of hypochlorite in the presence of H_2O_2 and other ROS to understand the role of hypochlorite in cellular physiology has immense potential.[5] Recently, a few fluorescent probes for hypochlorite detection have been reported in literature, [6] but these probes fail to fulfil the appropriate criteria for imaging of endogenous hypochlorite. The fluorescent probe for biological chemosensing should be permeable through cell membrane, act fast under physiological conditions, be chemically stable, low interference from other species and should have low molecular weight.

Recently from our laboratory we reported a charge transfer assisted fluorescent probe for selective sensing of hydrogen peroxide among the different reactive oxygen species tested.[7] In continuation of this work we have now designed and synthesised a dimethylaminocinnamaldehyde linked oxime based probe **1** which undergoes fluorescent enhancement in the presence of hypochlorite. In addition probe **1** can detect endogenously produced ClO^- in living cells. We believe that this is one of the best reaction based chemosensors for ClO^- among various chemosensors reported in literature[8] (S17 ESI†).

Thus, condensation of *N,N*-dimethylaminocinnamaldehyde with hydroxylamine in ethanol furnished compound **1** in 78% yield (**Scheme 1**). The structure of compound **1** was confirmed from its spectroscopic data (S12-13 ESI†).

The molecular recognition behaviour of probe **1** toward different reactive oxygen species (ROS) was studied by UV-vis and fluorescence spectroscopy. The absorption spectrum of probe **1** (5.0 μM) in HEPES, 50 mM, buffer solution pH 7.4 and 0.5% acetonitrile as cosolvent (Fig. S11 ESI†), shows an absorption band at 391 nm ($\varepsilon = 6.44 \times 10^4\ M^{-1}\ cm^{-1}$) (**Fig. 1a**) due to the transition from S_0 to S_1 states.[9] This absorption band at 391 nm is due to π-π* transitions. Upon addition of ClO^- (0-25 μM) to the solution of receptor **1**, the absorption band at 391 nm apparently decreases ($\varepsilon = 4.64 \times 10^4\ M^{-1}\ cm^{-1}$) and a new red shifted band appears at 450 nm ($\varepsilon = 2.52 \times 10^4\ M^{-1}\ cm^{-1}$) with an isosbestic point at 418 nm ($\varepsilon = 4.3 \times 10^4\ M^{-1}\ cm^{-1}$) (**Fig. 1a**). The colour of the solution changes from colourless to dark yellow, allowing colorimetric detection of hypochlorite in aqueous medium by naked eye (**Inset, Fig. 1a**). No such colour change was observed in the presence of other ROS's (H_2O_2, *tert*-butyl hydroperoxide (TBHP), HO•, ^{t}BuO•, OONO•) (Fig. S8 ESI†) tested.

The fluorescence spectrum of receptor **1** (1.0 μM) does not exhibit any emission when excited at 450 nm. This non-emissive (Φ_F=0.008) nature of receptor **1** in solution is due to non radiative deactivation through rapid isomerization of the C=N-OH bond.[10] On addition of ClO^- to the solution of receptor **1** an emission band at 556 nm (Φ_F = 0.51) is observed (**Fig. 1b**), the fluorescence intensity of which increases with increase in concentration of ClO^- (0-25 μM) with a linear regression coefficient of 0.993. The formation of an emission band at 556 nm is due to the conversion of oxime into nitrile oxide.[11] The nitrile oxide has a positive charge on nitrogen atom as result of which the intramolecular charge transfer (ICT) from nitrogen atom of the dimethylamino group to nitrogen atom of nitrile oxide takes place which gives an emission band at 556 nm when

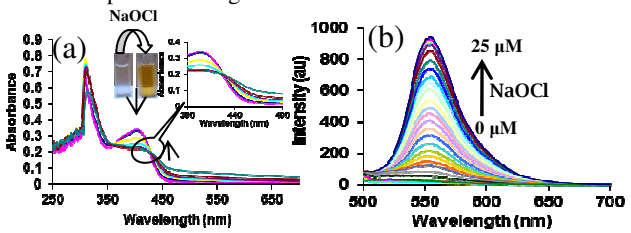
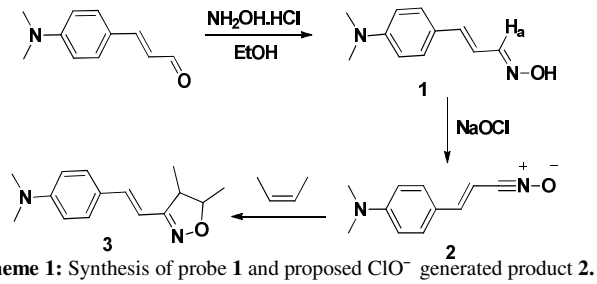


Fig. 1: (a) Absorption spectra of probe **1** (5.0 μM) upon addition of increasing concentration of hypochlorite (0-25 μM) in H_2O:CH_3CN (99.5:0.5, v/v) buffered with HEPES (50 mM), pH = 7.4. **(b)** Emission spectra of probe **1** (1.0 μM) upon the addition of increasing concentration of hypochlorite (0-25 μM) in H_2O/CH_3CN (99.5:0.5, v/v) buffered with HEPES (50 mM), pH = 7.4; (λ_{ex} = 450 nm).



Scheme 1: Synthesis of probe **1** and proposed ClO^- generated product **2.**

excited at 450 nm. To confirm the formation of nitrile oxide we performed a separate reaction of compound **1** with the hypochlorite and recorded the spectroscopic data of the product. In the ^1H NMR spectrum of the isolated product **2** the signal corresponding to the imino proton (H_a) of the compound **1** disappears. Further the ^{13}C NMR spectrum shows a characteristic peak at 99.90 ppm corresponding to the carbon atom of nitrile oxide. In the mass spectrum a peak corresponding to the nitrile oxide **2** appears at m/z-189.103. These spectroscopic data corroborates the structure **2** for this compound (S14-15 ESI†). Further we also carried out the reaction of *in-situ* generated nitrile oxide with 2-butene which gave isoxazoline **3**.[12] The formation of isoxazoline **3** was confirmed from its mass spectrum which shows a peak at m/z 267.13 corresponding to the cycloaddition product **3**. (S16 ESI†).

We also studied the effect of pH on recognition behaviour of probe **1** towards hypochlorite and it was found that there was fluorescence enhancement until pH 5 and pH 9 during the addition of ClO^- to the solution of **1** (**Fig. 2b**). However when ClO^- was added to the solution of probe **1** at pH <5 there was very weak fluorescence enhancement. This is due to the fact that at pH <5 ClO^- is present as HOCl which is less reactive than ClO^-. Moreover at pH <5 the probe **1** which contains imino moiety is likely to undergo hydrolysis. Both these factors contribute to poor responses of probe **1** at pH <5. Similarly when ClO^- is added to the solution of probe **1** at pH >9 the fluorescence response is poor. This is again due to the hydrolysis of probe **1** under basic conditions,[13] although in basic condition ClO^- will be present exclusively and therefore the maximum enhancement should be observed. Based on these studies we may propose that probe **1** can detect ClO^- in broad pH range (pH 5-pH 9). We also carried out time dependent fluorescence studies and it was found that there was no fluorescence enhancement after 35 minutes which indicate that reaction is complete during this time. The detection limit of the probe **1** for ClO^- was found to be 1.63×10^{-7} M (Fig. S9 ESI†), which is sufficiently low for monitoring

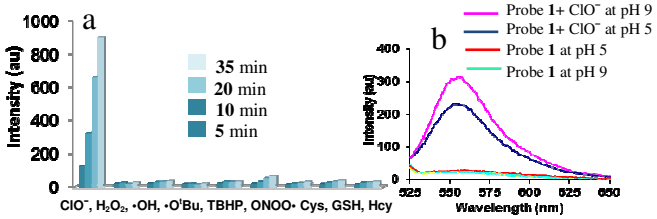


Fig 2. (a) Fluorescence response of **1** (1.0 μM) in H_2O:CH_3CN (99.5: 0.5, v/v) with HEPES buffer at pH = 7.4 (λ_{ex}= 450 nm) with various analyte (25 μM each). Bars represent selectivity (fluorescence intensity at 556 nm after the addition of analyte) of **1** upon addition of different analytes. Data were collected after incubation with the appropriate analyte after 5, 10, 20 and 35 min; **(b)** Fluorescence response of receptor **1** (1.0 μM) in H_2O:CH_3CN (99.5:0.5, v/v) with HEPES buffer in pH 5 and 9 (λ_{ex}= 450 nm) to addition of Sodium hypochlorite (NaOCl) upto 25 μM and spectra recorded after 35 mins.

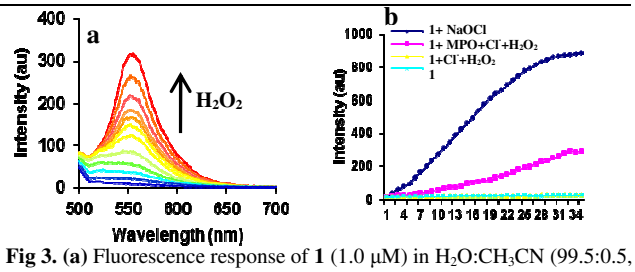


Fig 3. (a) Fluorescence response of **1** (1.0 μM) in H_2O:CH_3CN (99.5:0.5, v/v) on addition of H_2O_2 on the receptor **1** + myeloperoxidase + Cl^- ions in PBS buffer, pH 7.4 (λ_{ex} = 450 nm) at 37 °C. **(b)** Fluorescence response at 556 nm of **1, 1+Cl⁻ +H_2O_2, 1+Cl⁻ +H_2O_2+MPO, 1+NaOCl** within 35 mins time scale (X-axis) at 37 °C (λ_{ex} = 450 nm).

the endogenously produced hypochlorite.

To investigate whether the probe **1** can selectively detect hypochlorite under physiological conditions, the fluorescence response of the probe was also studied with other competing ROS's and biothiols like cysteine, glutathione and homocysteine whose concentration is generally high in biological systems (Fig. S10A & B ESI†). As shown in the selectivity bar diagram (**Fig. 2a**), only ClO^- shows dramatic fluorescence enhancement. Other ROS like H_2O_2, *tert*-butyl hydroperoxide (TBHP), HO•, ^{t}BuO•, OONO• and biothiols like cysteine, glutathione and homocysteine did not show any significant fluorescence enhancement. Further, since the probe **1** contains an imino nitrogen atom and hydroxyl groups which are known to interact with different metal ions and anions respectively, we studied the fluorescence response of probe **1** toward different metal ions (Ag^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}) (Fig. S7A & D ESI†) and anions (Br^-, CH_3COO^-, CN^-, F^-, SO_4^{2-}, $H_2PO_4^-$, I^-, SCN^-, HSO_3^-) (Fig. S7B ESI†), but no noticeable change in fluorescence intensity was observed in the presence of these cations and anions which indicate that receptor **1** is selective for ClO^- only. Having done all these studies, we were then interested to check whether the probe **1** can be used for the detection of endogenous hypochlorite generated in living cells by oxidation of chloride ions in presence hydrogen peroxide catalysed by heme protein myeloperoxidase (MPO).[14] We studied the feasibility of probe **1** for monitoring the generation of ClO^- in an MPO/H_2O_2/Cl^- enzymatic reaction. For this 1 μL of MPO solution was added to 3 mL probe solution followed by addition of H_2O_2 and then recorded the fluorescence spectra. The fluorescence intensity of probe **1** increased but the increase in intensity was low (30%) as compared to direct addition of hypochlorite to the solution of probe **1** (**Fig. 3b**). This is probably due to the fact that the *in-situ* generation of hypochlorite is slow and it takes time. On the other hand in the absence of myeloperoxidase no change in emission was observed at 556 nm. Thus, the above results indicate that ClO^- generated in the enzymatic reaction can be easily detected by probe **1**.

The potential biological application of probe **1** was evaluated by *in-vitro* detection of hypochlorite (ClO^-) using a cell based model system of LPS stimulated murine BV2 microglial cell line (brain resident macrophages) and C6 glial cell line. Both the cell types are known to be activated by lipopolysaccharide (LPS), which is secreted by bacterial cell wall during neuroinfections and generate ROS.[15] The experiments were performed on four groups of cells (I) control group, (II) unstimulated cells exposed to probe (5.0 μM) alone, (III) unstimulated cells exposed to exogenous ClO^- ions (1.0 μM) and probe (5.0 μM), (IV) LPS stimulated

cells exposed to probe (5.0 μM) for the detection of endogenous ClO^- ions. The images of the cells were taken using Confocal Microscope in both fluorescence and DIC modes (**Fig. 4**). No fluorescence was detected in control cells (**Fig. 4** A-Ia and B-Ia). The cells in group II were incubated with probe **1** (5.0 μM) for 30 min at 37 °C and washed with PBS (pH 7.4) to remove excess of receptor **1**. Microscopic observation did not exhibit any intracellular fluorescence in red channel, which indicates the presence of oxime group in the receptor **1** (**Fig. 4** A-IIa and B-IIa). In the third group, the cells loaded with probe **1** (5.0 μM) were then exposed to exogenous ClO^- ions (1.0 μM) for 30 min at 37 °C and washed with PBS buffer. The cells showed fluorescence in red channel which suggests that the probe **1** is permeable through cell membrane and is an effective hypochlorite imaging agent (**Fig. 4**A-IIIa and B-IIIa). To further

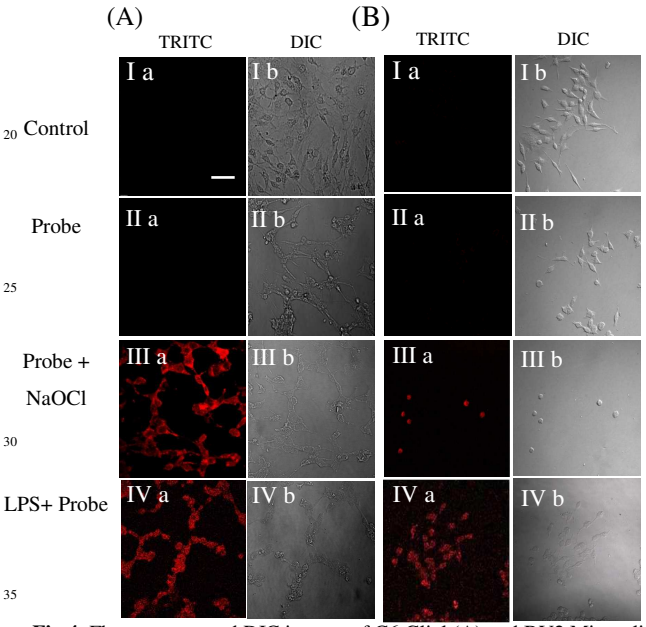


Fig 4. Fluorescence and DIC images of C6 Glial (A) and BV2 Microglial (B) cell lines. (Ia) Control of C6 Glial and BV2 Microglial cell lines respectively. (Ib) DIC images of Ia. (IIa) Images of cell lines (C6 Glial and BV2 Microglial) upon treatment with probe **1** (5.0 μM). (II b). DIC images of IIa. (IIIa) Red fluorescence images of cell lines (C6 Glial and BV2 Microglial) upon treatment with probe **1** (1.0 μM) and then NaOCl (5.0 μM). (IIIb) DIC images of IIIa. (IVa) Red fluorescence images of cell lines (C6 Glial and BV2 Microglial) of probe **1** upon treatment with LPS, 2 μg/ml (C6-Glial cells) and 100 ng/ml (BV2 Microglial cells). (IVb) DIC images of IVa. Scale bar represents length of 50 μm.

test the efficacy of probe to detect endogenous ClO^- ions, C6 cells were stimulated with 2μg/ml of LPS for 24 hr and BV2 cells with 100 ng/ml LPS for 48 hr and then exposed to probe **1** (5.0 μM) for 30 min at 37 °C and washed with PBS buffer. After exposure to probe, the images of the cells were taken and cells showed fluorescence in red channel which indicates that the probe **1** is an effective intracellular hypochlorite sensing agent (**Fig. 4** A-IVa and B-IVa).

BV2 cells exposed to exogenous ClO^- and probe (**Fig. 4B** IIIa) showed rounding and reduction in number of cells as compared to control as well as the corresponding group of C6 cells (**Fig. 4A** IIIa) but LPS activated cells on treatment with probe retained their normal morphology thus indicating the nontoxicity of probe. Although the intensity of fluorescence in both the cell types

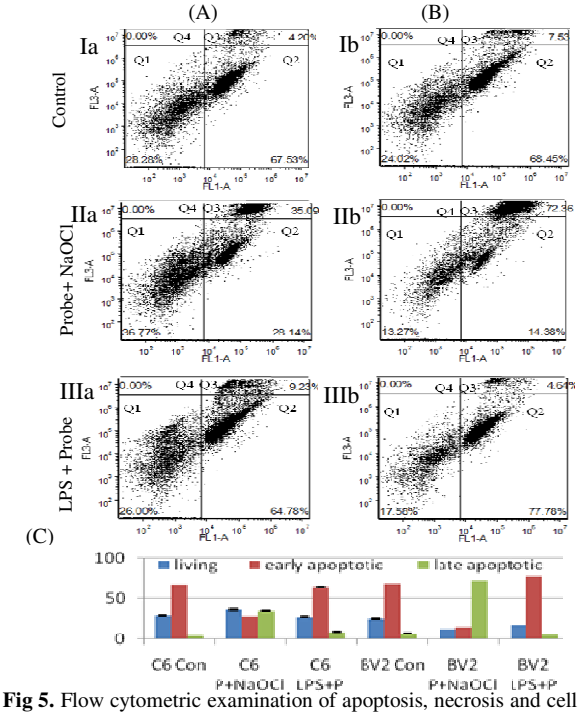


Fig 5. Flow cytometric examination of apoptosis, necrosis and cell viability of C6 Glial (A) and BV2 Microglial (B) cells by Annexin V/FITC assay. Viable (Q1), early apoptotic (Q2), late apoptotic (Q3) and necrotic (Q4) cell populations. (Ia) Control of C6 Glial cells and (Ib) Control of Microglial cells. (IIa) C6 cells treated with probe and exogenous hypochlorite. (IIb) BV2 cells treated with probe and exogenous hypochlorite (IIIa) C6 cells activated with LPS and exposed to probe.. (IIIb) BV2 cells activated with LPS and exposed to probe. (C) Histogram represents percentage distribution of the cells in different quadrants for control cells (Con), probe plus exogenous NaOCl treated cells (P+NaOCl), LPS stimulated and probe treated cells (LPS+P) for C6 and BV2 Cell line.

exposed to exogenous ClO^- with probe was almost similar but in case of group IV of LPS activated cells secreting endogenous HOCl, BV2 cells showed lower fluorescence intensity as compared to C6 (Fig. S18 ESI†). This may be due to less generation of HOCl in the BV2 cells. Further, our probe could detect HOCl in both the cell line tested, thus suggesting that this probe can be used for HOCl detection in different types of cells exposed to oxidative stress.

The cytotoxicity of probe **1** was further tested using Annexin V/ FITC apoptosis assay. FITC conjugated Annexin V has strong affinity for the exposed phosphotidyl serine which is translocated from inner to outer side of plasma membrane during early appoptosis and is used to estimate the population of early and late apoptotic cells.[16] After treatment cells were trypsinized, washed with PBS, stained with Annexin V/ FITC and PI and then viable, early apoptotic, late apoptotic and necrotic cells were detected and analysed by using BD Accuri C6 flow cytometer. Percentage of late apoptotic cells in control group was 9.23% and 4.64% for C6 and BV2 cells, respectively (**Fig. 5** A-Ia and B-Ib). Cells exposed to exogenous ClO^- (1.0 μM) showed cytotoxicity as is apparent from high percentage of late apoptotic cells (C6 cells 35.06% and BV2 cells 72.36%) in this group (**Fig. 5** A-IIa and B-IIb). The probe was not cytotoxic in LPS stimulated C6 and BV2 cells producing endogenous ClO^- as is evident from the low percentage of late apoptotic cells (4.20% and 7.53% respectively) and the values are similar to control cells (**Fig. 5** A-IIIa and B-IIIb). The percentage changes in living, early apoptotic and late apoptotic cells are shown in **Fig. 5** C for both the cell lines.

Conclusions

In conclusion, we designed and synthesised a turn-on fluorescent probe **1** for selective detection of hypochlorite among the various reactive oxygen species tested. The probe **1** can also be used for the detection of enzymatically generated hypochlorite in cell based model system under physiological conditions. Furthermore, the probe **1** was successfully tested for the detection of endogenous hypochlorite in LPS activated C6 glia and BV2 microglia cells.

Acknowledge

MK is thankful to UGC (F. No. 42-282/2013(SR)) and DST (SR/S1/OC-69/2012) New Delhi for financial support. S.I.R. and A. S. acknowledges UGC/ DST-Inspire for SRF/JRF. We are also thankful to UGC for providing infrastructural facilities under UPE and CPEPA schemes.

Notes and references

[†]*Department of Chemistry, UGC Sponsored Centre for Advanced Studies-1 and* [‡]*Department of Biotechnology, Guru Nanak Dev University, Amritsar, Punjab, India; E-mail: mksharmaa@yahoo.co.in*

† Electronic Supplementary Information (ESI) available: [Experimental details, NMR and mass spectra]. See DOI: 10.1039/b000000x/

1 (*a*) E. A. Podrez, H. M. Abu-Soud and S. L. Hazen, *Free Radical Biol. Med.,* 2000, **28**, 1717; (*b*) X. Chen, X. Tian, I. Shin and J. Yoon, *Chem. Soc. Rev.,* 2011, **40**, 4783.

2 (a) S. Kenmoku, Y. Urano, H. Kojima and T. Nagano, *J. Am. Chem. Soc.*, 2007, **129**, 7313; (b) T. Chen, Y. Hu, Y. Cen, X. Chu, and Y. Lu, *J. Am. Chem. Soc.,* 2013, **135**, 11595.

3 (*a*) N. R. Madamanchi, A. Vendrov and M. S. Runge, *Arterioscler Thromb Vasc Biol.*, 2005, **25**, 29;(*b*) D. I. Pattison and M. J. Davies, *Biochemistry,* 2006, **45**, 8152; (*c*) D. I. Pattison, C. L. Hawkins and M. J. Davies, *Biochemistry,* 2007, **46**, 9853

4 M. J. Davies, *J. Clin. Biochem. Nutr.,*2011, **48**, 8.

5 (a) X. Chen, K.-A. Lee, E.-M. Ha, K. M. Lee, Y. Y. Seo, H. K. Choi, H. N. Kim, M. J. Kim, C.-S. Cho, S. Y. Lee, W.-J. Lee and J. Yoon, *Chem. Commun.,* 2011, **47**, 4373; (b) Q. Xu, K.-A. Lee, S. Lee, K. M. Lee, W.-J. Lee and J. Yoon, *J. Am. Chem. Soc.,* 2013, **135**, 9944; (*c*) G. Chen, F. Song, J. Wang, Z. Yang, S. Sun, J. Fan, X. Qiang, X. Wang, B. Dou and X. Peng, *Chem.Commun.,* 2012, **48**, 2949; (*d*) W. Lin, L. Long, B. Chen and W. Tan, *Chem. Eur. J.,* 2009, **15**, 2305.

6 (*a*) X. Cheng, H. Jia, T. Long, J. Feng, J. Qin and Z. Li, *Chem. Commun.,* 2011, **47**, 11978; (*b*) R. Zhang, Z. Ye, B. Song, Z. Dai, X. An and J. Yuan, *Inorg. Chem.*, 2013, **52**, 10325; (*c*) Y. Xiao, R. Zhang, Z. Ye, Z. Dai, H. An and J. Yuan, *Anal. Chem*., 2012, **84**, 10785.

7 M. Kumar, N. Kumar, V. Bhalla, P. R. Sharma and Y. Qurishi, *Chem. Commun.*, 2012, **48**, 4719.

8 (*a*) S. Goswami, A. Manna, S. Paul, C. K. Quah and H.-K. Fun, *Chem. Commun*., 2013, **49**, 11656; (*b*) L. Yuan, W. Lin, J. Song and Y. Yang, 2011, **47**, 12691; (*c*) S. T. Manjare, J. Kim, Y. Lee, and D. G. Churchill, *Org. Lett.*, 2014, **16**, 520; (*d*) G. Cheng, J. Fan, W. Sun, K. Sui, X. Jin, J. Wang and X. Peng, *Analyst*, 2011, **136**, 2277; (*e*) K. Chen, J. W. Bats and M. Schmittel, *Inorg. Chem.,* 2013, **52**, 12863.

9 A. Chakraborty, S. Kar, N. Guchhait, *Chem. Phys.,* 2006, **324**, 733.

10 J. S. Wu, W. M. Liu, J. C. Ge, H. Y. Zhang and P. F. Wang, *Chem. Soc. Rev.,* 2011, **40**, 3483.

11 M. Emrullahoğlu, M. Üçüncü and E. Karakuş, *Chem. Commun.,* 2013, **49**, 7836.

12 H. Feuer, *Nitrile oxide, nitrone and nitronates in organic synthesis: novel strategies in synthesis,* John Wiley and Sons, 2008.

13 K. Koehler, W. Sandstrom and E. H. Cordes, *J. Am. Chem. Soc.,*1964, **86**, 2413.

14 (*a*) M. J. Davies, *J. Clin. Biochem. Nutr.,* 2011, **48**, 8; (*b*) M. Sun, H. Yu, H. Zhu, F. Ma, S. Zhang, D. Huang and S. Wang, *Anal. Chem.,* 2014, **86**, 671; (*c*) G. Li, D. Zhu, Q. Liu, L. Xue and H. Jiang, *Org. Lett.,* 2013, **13**, 2002; (*d*) Y. Yan, S. Wang, Z. Liu, H. Wang and D. Huang, *Anal. Chem.,* 2010, **82**, 9775.

15 (*a*) A. Haegens, P. Heeringa, R. J. Suylen, C. Steele, Y. Aratani, R. J. J. O'Donoghue, S. E. Mutsaers, B. T. Mossman, E. F. M. Wouters and J. H. J. Vernooy, *J. Immunol*., 2009 ,**182**, 7990; (*b*) C. Y. Chang, M. J. Song, S.-B. Jeon, H. J. Yoon, D. K. Lee, I.-H. Kim, K. Suk, D.-K. Choi and E. J. Park, *Am. J. Pathol.,* 2011, **179**, 964.

16 M. Engeland, L. J. W. Nieland, F. C. S. Ramaekers, B. Schutte and C. P.M. Reutelingsperger, *Cytometry,* 1998, **31**, 1.