This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
A Dual Colorimetric-Ratiometric Fluorescent Probe NAP-3 for Selective Detection and Imaging of Endogenous Labile Iron (III) Pools in C. elegans

Atul Goel,*a Shahida Umar,a Pankaj Nag,a Ashutosh Sharma,a Lalit Kumar,b Shamsuzzama,b Zakir Hossain,c Jiaur R. Gayen,c Aamir Nazirb

The discovery of an iron (III)-selective ratiometric fluorescent probe to detect and visualize endogenous labile iron pools in living organisms at molecular-level has been long awaited. Herein we report the first dual colorimetric and ratiometric fluorescent probe Naphtho[2,1-b][1,10]phenanthroline NAP-3 for selective and ‘direct’ visualization of labile iron (III) pools in a multicellular organism Caenorhabditis elegans.

Iron is an essential redox-active element in all living organisms controlling indispensable physiological and pathophysiological functions.1 The bulk of the iron present in biological systems is tightly bound to various proteins.2 Only a fraction of iron is free and exists as cytosolic labile iron pool (LIP) consisting of both the ionic forms (Fe2+ and Fe3+) in dynamic equilibrium.3 If the cellular homeostasis of labile iron gets disturbed, it can trigger uncontrolled Fenton reaction4 leading to peroxidative tissue damage and thus causing various diseases, such as Alzheimer’s disease (AD), Parkinson’s disease (PD), and different types of cancers.5 The complexity of redox chemistry of labile iron and its role in biological processes remain a challenge for chemists and biologists due to the dearth of iron-selective, sensitive and cell-permeable ratiometric fluorescent probes.6 Although fluorogenic iron probes like calcein, fluorescein-deferoxamine (FL-DFO), Phen green SK and Phen green FL have been explored for cell imaging applications, they provide information through fluorescence quenching readouts, and exhibit poor selectivity between the two spin states of iron and nonspecific turn-off response with other interfering transition metal ions.6,7 Few examples of Fe (II)8 and Fe (III)9 selective fluorescent sensors and chemodosimeters are reported in the literature with or without cell imaging studies. Remarkably, Tang et al. in 2011 reported the first ratiometric fluorescent probe for the selective detection of both exogenous and endogenous labile Fe (II) in live human liver HL7702 cells.10 Recently, Guo et al in 2012 reported rhodamine based turn on fluorescent probe ‘RPE’ for imaging labile Fe (III) pools in live neuronal cells.11 To the best of our knowledge, none has reported Fe (III)-selective ratiometric probe for in vivo visualization of LIP in a multicellular organism. From the perspective of accurate and quantitative analysis, ratiometric sensors and reporters have distinct advantage over single emission intensity-based probes because of their self-calibration/built-in environmental correction via two selected emission wavelengths.12

Commercially available Phen green probes possess [1,10]-phenanthroline (PHT) scaffold, which is an entropically balanced iron-metal chelating ligand but exhibits weak fluorescence due to low lying π–π* and n–π* singlet excited states.13 Though enormous efforts have been made in the past to improve its emission intensity by introducing π-conjugated systems mainly at the 2,9-, 3,8- and 4,7-positions, researchers still rely on fluorescent tags. Henceforth PHT-based commercial iron probes Phen green SK/FL or siderophores (deferoxamine) are appended with fluorescein label, yet they suffer with nagging problems of quenching readouts and/or poor selectivity.1,16 Addressing these issues and considering the necessity for selective ratiometric fluorescent probes for iron, we conceptually designed a novel14 naphtho[2,1-b][1,10]-phenanthroline (NAP) framework (surprisingly yet unknown in the literature) equipped with well-conjugated donor-acceptor (D–A) and chromophoric π-groups to induce effective charge transfer during preferential binding of metal to the phenanthroline receptor (Fig. 1).

Our research group is engaged in target-based design and synthesis of new fluorescent dyes for their application in material15 and biological sciences.16 We have previously shown that controlled tuning of π-conjugation and appropriate positioning of D–A moieties dramatically modulate the optical properties of aromatic and heterocyclic compounds.15 The synthetic methodology adopted for the synthesis of designed D–A NAPs is outlined in Scheme 1. The key intermediates 2H-pyran-2-ones 3a-d were prepared from easily accessible precursor α-oxo-ketene-S,S-acetal (I) and substituted acetophenones (2a-d).13 This was followed by the substitution of the methylsulfanyl group in
3a-d by a piperidine donor group to furnish D-A appended 6-aryl-2-oxo-4-piperidin-1-yl-2H-pyrano-3-carbonitriles (4a-d) in good yields.\textsuperscript{15} The chelating precursor 10,11-dihydronaphtho[2,1-b][1,10]phenanthroline-8(9H)-one (8) was prepared via three-component condensation of 8-aminoquinoline with formaldehyde and 1,3-cyclohexane-dione in good yield (Scheme 1). Further, Michael addition of the conjugate base of 8 to the lactones 4a-d at position 6 followed by intramolecular cyclization afforded 11-aryl-9-(piperidin-1-yl)-12,13-dihydronaphtho[2,1-b][1,10]-phenanthroline-8-carbonitriles 9a-d. To extend π-conjugation, the compounds 9a-d were aromatized with DDQ in dioxane at reflux conditions to get the desired NAPs 10a-d.

The absorption and emission characteristics of the designed NAPs 9a-d and 10a-d were investigated in aqueous medium (10\textsuperscript{-5} M, triple distilled water TDW/DMSO, 9:1, v/v) at pH 7.0-7.4 (Figs. S1 and S2, Table S1, ESI\textsuperscript{†}). The iron-sensing studies of NAPs in the presence of perchlorate salts of Fe\textsuperscript{3+} and Fe\textsuperscript{2+} revealed that only Fe\textsuperscript{3+} exhibited a visible color change (colorimetric response, Fig. 2a, Figs. S3-S6, ESI\textsuperscript{†}) and a ratiometric fluorescence response in 10a-d (Figs. S3-S5, ESI\textsuperscript{†}) that allowed the quantification of Fe\textsuperscript{3+} by a ratiometric fluorescence method\textsuperscript{13}. Further Job’s plot\textsuperscript{18} established a 2:1 binding stoichiometry of NAP-3 with Fe\textsuperscript{3+} (Fig. S10, ESI\textsuperscript{†}), which was supported by mass spectrometry (Fig. S11, ESI\textsuperscript{†}). The binding constant for NAP-3 was determined as 3.96 × 10\textsuperscript{15} M\textsuperscript{-2} by non-linear regression analysis\textsuperscript{19} (Fig. S12, ESI\textsuperscript{†}). The fluorescence property of the probe NAP-3 at different pHs (1.8–10.7) revealed no change in fluorescence intensity within pH 5.5–8.3 but a gradual decrease in the intensity was observed beyond this range (Fig S13, ESI\textsuperscript{†}).

It is interesting to note that donor-acceptor functionalized naphtho[2,1-b][1,10]phenanthroline NAP-3 selectively binds to Fe\textsuperscript{3+} but not to Fe\textsuperscript{2+} (Figs. S6-S7, ESI\textsuperscript{†}). This is contrary to the nature of 1,10-phenanthroline, which selectively binds to Fe\textsuperscript{2+} (Figs. S14-S15, ESI\textsuperscript{†}). To understand the electronic behaviour of NAP-3, the geometries were optimized at DFT/B3LYP level using a 6-31G(d,p) basis set and time-dependent density functional theory (TDDFT) calculations were performed using a B3LYP/6-311++G(d,p) method.\textsuperscript{20} The energy of the HOMO and LUMO levels, HOMO-LUMO gap, main orbital transition, and oscillator strength \( f \) are listed in Table S2 (ESI\textsuperscript{†}). Fig. 3a shows the theoretically computed molecular orbitals in the ground states for the probe NAP-3. TDDFT results revealed two prominent electronic transitions observed at 305 nm (HOMO-2 to LUMO+1) and 383 nm (HOMO to LUMO). The electron density of the HOMO is delocalized over the piperidine donor moiety and the neighbouring naphthophenanthroline ring, while the LUMO is delocalized on the naphthophenanthroline moiety and the nitrile acceptor group (Fig. 3a). The electronic transition at higher wavelength (HOMO to LUMO) corresponds to an intramolecular charge-transfer (ICT) band. The presence of conjugated donor-acceptor moieties enhanced the charge density over phenanthroline ring of NAP-3 facilitating the formation of ligand to metal charge transfer (LMCT) complex with Fe\textsuperscript{3+}. In contrast, 1,10-phenanthroline forms metal to ligand charge transfer (MLCT) complex with Fe\textsuperscript{2+}.\textsuperscript{21}
The selectivity studies of NAP-3 in the presence of high concentration (20 equiv.) of perchlorate salts of Li⁺, Na⁺, K⁺, Cs⁺, Mg²⁺, Ca²⁺, Ba²⁺, Sr²⁺, Co²⁺, Mn²⁺, Fe²⁺, Fe³⁺, Cu²⁺, Al³⁺, Zn²⁺, Pb²⁺, Cd²⁺, Hg²⁺, Ni²⁺ revealed that only Fe³⁺ triggered a dramatic increase in the emission ratio I_{505/550}, while other metal ions were either silent or induced minor ratiometric response (Fig. 3b, Fig. S17, ESI†). In order to confirm the selectivity of NAP-3 for Fe³⁺ over Fe²⁺, we conducted experiments under reducing (Fe²⁺ and hydroxylamine) and oxidizing (Fe²⁺ and hydrogen peroxide) environments (Figs. S18 and S19, ESI†). To our delight, NAP-3 successfully discriminated Fe³⁺ over Fe²⁺. We further conducted competitive experiments of NAP-3 with a known strong Fe³⁺ chelator deferoxamine (DFO). We observed that in the presence of DFO, NAP-3 could not bind to Fe³⁺; while in the presence of NAP-3, DFO failed to remove Fe²⁺ from the complex (NAP-3,Fe²⁺) possibly due to steric hindrance (Fig. S20, ESI†).

Promising selectivity of NAP-3 for Fe³⁺ in both reducing and oxidizing environments and aqueous compatibility encouraged us to detect and visualize labile Fe³⁺ in living systems. Since small molecules like cysteine and glutathione present in cellular environment may interact with the probe, we examined the fluorescence response of NAP-3 in the presence of these molecules (Fig. S21, ESI†). Gratifyingly, no change in the fluorescence spectra of NAP-3 probe was observed. Thereafter confocal fluorescence imaging experiments were carried out with HepG2 cells considering liver as a major iron storage organ of the body.⁸ We observed that treatment of cells with different concentrations (nanomolar to micromolar) of NAP-3 exhibited a concentration-dependent staining pattern with 3 µM showing the best results (Fig. 4a, S22, ESI†). The cells incubated with NAP-3 (3 µM) for 24 h exhibited intense intracellular fluorescence in the green channel (505–550 nm) but no fluorescence in the red channel (Long pass 575 nm) was observed (Fig. 4a). However, HepG2 cellspretreated with ferric citrate (5 × 10⁻⁸ M, 24 h) followed by incubation with NAP-3 (3 µM, 24 h) showed significant increase in intracellular fluorescence in the red channel due to the formation of the complex of NAP-3 in situ with exogenously supplemented Fe³⁺ (Fig. 4b). Furthermore, NAP-3 exhibited significant intracellular ratiometric fluorescence response (Fig. 4c). NAP-3 showed no cytotoxicity under the conditions used for cell imaging (Fig. S23, ESI†). Therefore, the in vitro cell studies demonstrated that NAP-3 is a cell-permeable fluorescent probe to detect elevated levels of Fe³⁺ via exogenous supplementation and may be used for in vivo imaging of labile iron.

The soil nematode Caenorhabditis elegans is a model of choice for studying regulatory mechanisms associated with iron metabolism due to homology with human gene sequences.³² Electron paramagnetic resonance (EPR) spectroscopy has been used as a tool to ‘indirectly’ measure free Fe³⁺ levels in vivo in C. elegans.²⁷ In an attempt to visualize LIP ‘directly’ in an organism, we examined in vivo iron binding specificity of NAP-3 in wild type (N2) C. elegans by confocal fluorescence imaging experiments. We observed that treatment of worms with 3 µM of NAP-3 for 24 h exhibited staining in the green channel, while staining was weak in the red channel (Fig. 5a, see zoomed images). The staining in the red channel implicated that NAP-3 has access to Fe³⁺ present in LIP of wild type C. elegans. In order to confirm that the fluorescence in the red channel is due to the formation of Fe³⁺ complex and not by NAP-3 alone, we measured the fluorescence of NAP-3 at 561 nm excitation and no fluorescence in red channel was observed (Fig. S25, ESI†). These results implicated the detection of Fe³⁺ present in the LIP by the probe NAP-3.

On exogenous Fe³⁺ supplementation, there was no increase in the fluorescence intensity in the red channel as compared with only NAP-3 treated groups (data not shown). These results indicated that to maintain iron homeostasis, worms might have triggered iron regulatory mechanisms associated with ferritins (FTN-1 and FTN-2), which are transcriptionally regulated by iron.²⁴ Thereby we repeated the experiments with ftn-1 silenced C. elegans (RNAi induced silencing of gene). Interestingly at this time we observed significantly enhanced signals in the red channel (Fig. 5b) as compared to wild type strain (Fig. 5a). These results supported the previous findings²⁹ suggesting that reduced level of ferritins causes elevation of free iron content in the cytosol. Furthermore, the ratio of the intensities (I_{505/550}) was increased by 3 fold (p < 0.001) (Fig. 5c). The confocal imaging results showed a marked staining pattern in the worm body, posterior to pharyngeal region particularly concentrated around the gut region. It has been reported that ftf-1 is up-regulated in the presence of exogenous iron supplementation, and down-regulated on treatment with an iron chelator.²⁸ Therefore to cross-examine whether NAP-3 detected Fe³⁺ present in endogenous iron pool, we carried out quantitative real time PCR (qPCR) assay to quantify the levels of ftf-1 mRNA in worms. We observed 2.8 fold (p < 0.01) down-regulation and 3.7 fold (p < 0.01) up-regulation of ftf-1 mRNA levels in worms from NAP-3 treated and Fe³⁺-treated groups respectively (Fig. 5d). Upon treatment with both NAP-3 and Fe³⁺, a 1.5 fold reduction (p < 0.05) in levels of ftf-1 mRNA was observed as compared to iron-treated group (Fig. 5d). These results unambiguously confirmed that ratiometric probe NAP-3 is capable of detecting labile iron pool in C. elegans. This is the first report to visualize LIP in an organism with remarkably high selectivity and in analytically advantageous ratiometric manner.

In summary, we have designed and synthesized a novel Fe³⁺ selective dual colorimetric and ratiometric fluorescent probe NAP-3 for detection and visualization of exogenous Fe³⁺ in in vitro (HepG2 cell line) and for the first time in in vivo (Wild type and ftn-1 silenced C. elegans) models. The potential of NAP-3 for binding to labile iron pool in wild type C. elegans was further established by monitoring the
mRNA levels of iron responsive gene *fnr-1* via qPCR analysis. The high selectivity of NAP-3 towards Fe$^{3+}$ and its biocompatibility open new avenues to unravel the mysteries of the cellular dynamics of iron homeostasis and the role of labile iron pools associated with oxidative stress produced during Fenton chemistry. Further studies in these directions are underway.

The work is supported by the Council of Scientific and Industrial Research, New Delhi (BCS0102 and BCS0114). Authors thank K. Mitra and K. Singh for confocal fluorescence imaging of the samples. The CDRI communication number is 8873.

### Notes and References

*Medical and Process Chemistry Division, †Department of Toxicology ‡Department of Pharmacokinetics and Metabolism, CSIR-Central Drug Research Institute, Lucknow 226031, India; fax: +91-522-2771941; Tel.: +91-522-2772450; e-mail: atul.goel@cdri.res.in*

†Electronic Supplementary Information (ESI) available: [Spectroscopic Characterization data, 1H, 13C NMR spectra and absorption, and fluorescence spectra of all the compounds 9a-d and 10a-d. Cytotoxicity data, materials and methods]. See DOI: 10.1039/b600000x/