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AN OVERVIEW ON RECENT DEVELOPMENT OF FLUOROGENIC PROBES FOR NITRIC OXIDE IMAGING IN LIVE CELLS

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ABSTRACT: Nitric oxide (NO), considered as an important signaling molecules, involves in numerous pathological and physiological processes, such as cardiovascular, immune, and nervous systems. The effects of NO depend on its concentration and spatiotemporal constraints of the cell-environment. The abnormal level of NO production is associated with a large number of pathological processes such as endothelial dysfunction, cancer and neurodegenerative diseases. That is why, huge efforts have been put to the development of sensitive and selective methods to analyse NO generation and distribution in living cells. Thus the selective monitoring and detection of low levels of NO remains challenging for researchers. Fluorescence detection technique is highly attractive because of its high sensitivity and real-time approach almost in a non-destructive way. This review article encompasses the design strategies, mechanistic outlook of the fluorogenic probes towards NO and their application in living cells imaging fluorescence microscopy.

KEYWORDS: Fluorescence, probe, nitric oxide, detection

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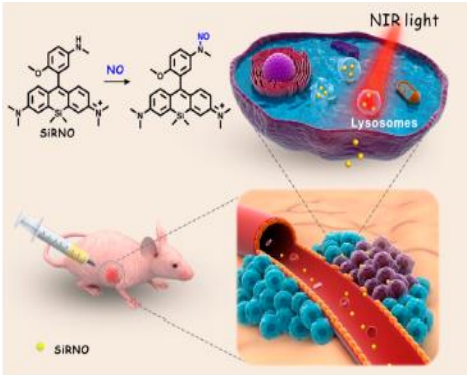
1.INTRODUCTION

Nitric oxide is a colorless, heteronuclear diatomic, paramagnetic and highly reactive gaseous molecule which plays various physiological as well as pathological roles through rapid reaction with other radicals or metalloproteins in the organisms. In mammals, nitric oxide acts as a signaling molecule in many physiological and also pathological processes. The Nobel Prize of 1998 in Physiology Medicine was awarded for discovering the roles of nitric oxide as a cardiovascular signalling molecule [1]. NOx (NO and NO_2) are toxic gases and environmental pollutants. The major sources of NOx are the exhausts from the industrial combustion of fossil fuels and automotive engines. The increasing number of vehicles still increases NOx emissions. As a matter of fact, NOx is responsible for photochemical smog in urban areas and contributes to acid rain. Nitric oxide damage the human respiratory organs and nerves system. It is considered as an endogenously generated bioactive gas messenger molecule. It has been implicated in a number of diverse physiological process, including smooth muscle relaxation, plays critical roles in a wide range of physiological and pathophysiological processes, including platelet inhibition, neurotransmission, immune regulation, vasodilation, respiration, immune response and apoptosis [2-3]. It is one of the most toxic, poisonous and abundant air pollutants and its toxicity depends on concentrations and duration of NO exposure [4]. It can play an crucial role in cancer biology [4]. Moreover, frequent exposure of the higher concentrations of NO may also responsible for acute respiratory illness in children. The abnormal level of NO production is related with a large number of pathological processes such as endothelial dysfunction, cancer and neurodegenerative diseases. That is why, huge efforts have been put to the development of sensitive and selective methods to analyse NO generation and distribution in living cells. Molecular sensors for the detection of NO gas are developed based on graphene because of its high chemical and thermal stability, high reliability, remarkably high carrier mobility, quick recovery and also low cost [5-7]. The NO sensors and their derivatives have been performed excellently both at high and low temperature [8-13]. Unfortunately, most of these devices are operated at higher temperatures. In recent years owing to the advantages of fluorescence microscopy with the assistance of fluorescent probes, noninvasive visualization of biological molecules with high temporal-spatial resolution [14,15] of NO release and or detection can be realized within one molecule [16,17]. NO detection is usually conducted by using different platform, such as chemiluminescence [18], fluorescence NO probe [19], electrochemical sensors [20], *o*-phenylenediamine (OPD) moiety [21], metal-ligand complex [22] etc. Fluorescence quencher has been harnessed in a variety of fluorescent probes for NO imaging both in cells and tissues triggering permits an exquisite control of location and timing of NO delivery [23].

Fluorogenic probe for nitric oxide detection

Zhihong Liu and coworkers developed Si-rhodamine scaffold fluorophore based first “near-infrared”

two photon fluorescent probe (**P1**) of high sensitivity, specificity and fast responsive nature towards nitric oxide (NO) detection by a photoinduced electron transfer (PeT) principle [24]. Also **P1** was able to track the various intracellular NO content in live cells. In the design strategy, Si-rhodamine derivate was used as the two-photon excitable platform and 4-methoxy-N-methylaniline moiety was selected as an NO recognition site based on the N-nitrosation reaction under aerobic condition. The two moieties were covalently linked by a short carbon−carbon single bond (**Scheme 1**), which facilitated the fluorescence quenching of the probe. Upon the N-nitrosation of the methylamino group by NO, the fluorescence of the probe, **P1** was lightened up due to the suppression of the PeT process. Fluorescence intensity was enhanced 81-fold within 90s which reveals the quick responsive nature of **P1**. The value of the limit of detection (LOD) was calculated as low as 14 nM which resembles with the high sensitivity among the reported small-molecule probes for NO. Probe (**P1**) is suitable for the detection of NO in the physiological pH range. As This probe is localized in lysosomes in the cell (**Figure 1**), it would be useful as lysosome tracker in the cell. The probe exhibits a low cytotoxicity to HeLa cells (cell viability >90%) at a concentration up to 15 μM and thus it could be utilize to detect intracellular NO.



Scheme 1. Design of a silicon-rhodamine based NIR two-photon fluorescent probe, **P1** for the detection of NO in cells and mouse. Reprinted with permission from ref. 24, Copyright 2017 ACS.

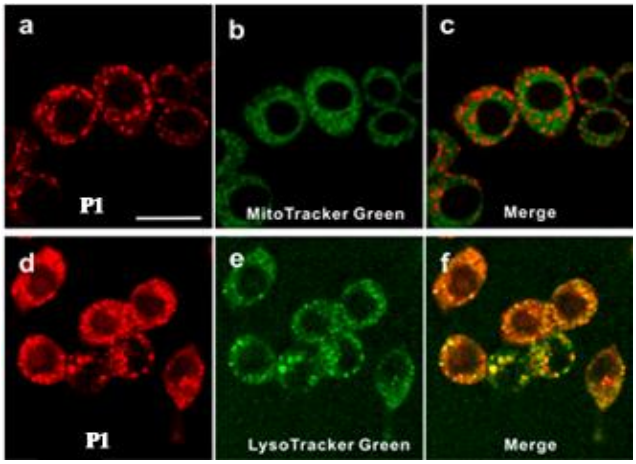
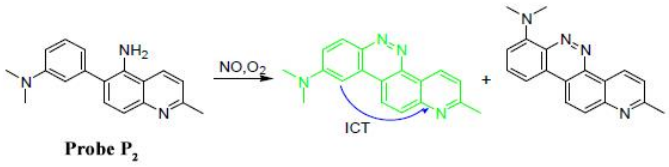


Figure 1. Colocalization images of prob (**P1**) with MitoTracker Green (a−c) and LysoTracker Green (d−f) in RAW 264.7 cells. Reprinted with permission from ref. 24, Copyright 2017 ACS.

H.-P. Zhouc, Q.-H. Song and their co-workers developed a two-photon and water-soluble fluorescent probe, **P_2**, for selective detection of NO [25]. In the designing of the probe molecule, quinoline was chosen as the fluorophore due to its excellent photostability and the pyridine moiety can act as an electron acceptor. Here, *o*-amino-3'-dimethylaminopheny (AAP) moiety was linked to quinoline at 6-position such that the constructed molecule could react with NO to form a diazo ring donor-acceptor (D-A) derivative (**Scheme 2**). Limit of detection (LOD) of **P_2** is calculated to be 15 nM. **P_2** can sense NO selectively and rapidly in a wide pH range (6-11), and achieve a real-time detection of NO in aqueous solutions. This probe was used to visualize both exogenous and endogenous NO in living cells (**Figure 2**). Sodium nitroprusside (SNP), a NO releaser, was employed as exogenous NO in living cells.



Scheme 2. The proposed mechanism for detection of NO using the probe **P_2**. Reprinted with permission from ref. 25, Copyright 2017 ACS.

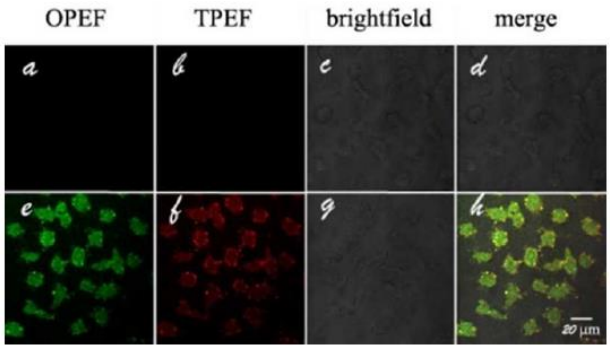
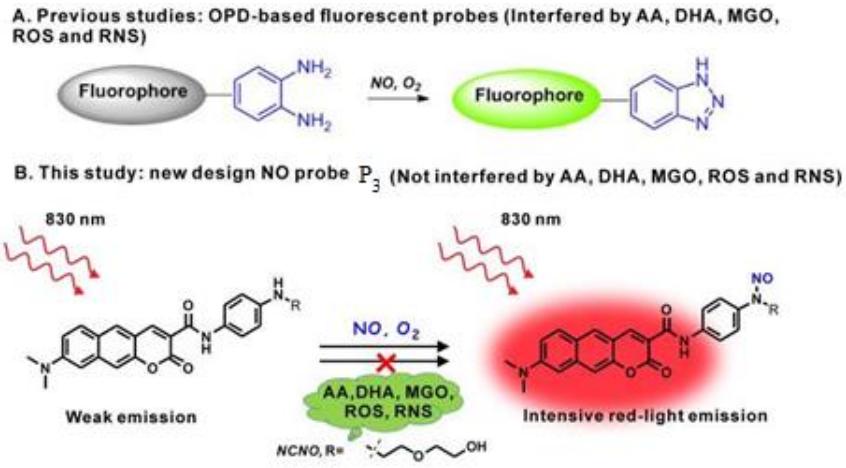


Figure 2. Confocal fluorescence images: RAW 264.7 cells stained with **P_2** for 1 h and then incubated with 100 μM SNP for 1 h (e–g). (a, e) one-photon fluorescence image; (b, f) pseudo-colour two-photon fluorescence image; (c, g) brightfield image; (d and h) overlay of (a, e), (b, f) and (c, g). The images were collected at 500−650 nm; excitation at 405 nm (one-photon excitation) and 760 nm (two-photon excitation). Reprinted with permission from ref. 25, Copyright 2017 ACS.

Zhihong Liu and coworkers was developed a red-emissive two-photon (TP) fluorescent probe (**P_3**) containing benzo[g]coumarin derivate (8-(dimethylamino)-2-oxo-2H-benzo[g]chromene-3-carboxylic acid) as red-emissive TP fluorophore, and *p*-phenylenediamine derivate as NO recognition group [26]. This probe (**P_3**) overcomes the drawbacks of *o*-phenylenediamine(OPD)-based NO probe. It detect the NO accurately without any interference (**Scheme 3**) from AA, RCS (DHA, MGO) and ROS, RCS, RNS in complicated biosystems [27]. The probe was able to detect

both exogenous and endogenous NO in living cells with a detect limit of 37 nM. According to cytotoxicity test it was revealed that the probe shows a low cytotoxicity so for this it was suitable for use in living cells. The probe (**P3**) can readily enter and localizes in cytoplasm into the living cells which was shown by red fluorescence in cytoplasm (**Figure 3**) and capable of imaging NO in deep tissues taking the advantages of two photon excitation and red-light emission. With the help of two-photon microscopy the group established the Probe (**P3**) could be a competent probe for NO detection in live tissues.



Scheme 3. Design of the N-nitrosation reactivity- based two-photon fluorescent probe **P3** for NO.

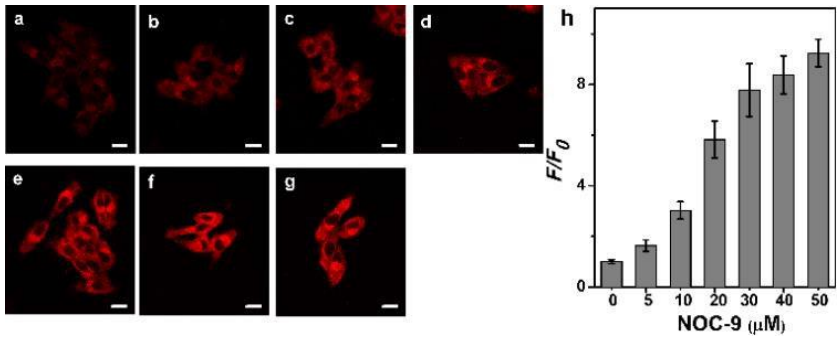


Figure 3. (a-g) TP images of 5.0 μM Probe (**P3**) loaded HepG2 cells incubated with various amounts of NOC-9 (0, 5, 10, 20, 30, 40, 50 μM) for 30 min. (h) Relative TP fluorescence intensity in (a)-(g). To overcome some drawbacks, such as reduced long-term stability in solution and deactivation by some bio-analytes, S. J. Lippard and coworkers designed and characterized two new water-soluble transition metal Cu(II)-based probes (**P4** and **P5**) for NO detection [28]. The structure of probes consist of CuFL sensing motif, hydroxycoumarin and fluorescein chromophores. The structure employed the Forster resonance energy transfer (FRET) mechanism between the appended 7-hydroxycoumarin and fluorescein chromophores, compatible donor acceptor fluorophore pair, separated by a polyproline helix (in case of **P4**) and piperazine unit (in case of **P5**) as spacers (**Figure 4**). Both the probes showed high sensitive, selective and ratiometric response to NO by direct

sensing method at physiological pH medium. Due to the membrane permeability nature of the piperazine-based probe (**P5**), it was employed to detect NO in live cells (HeLa and A_{549}) at incubation concentrations as low as 2 μM (**Figure 5**).

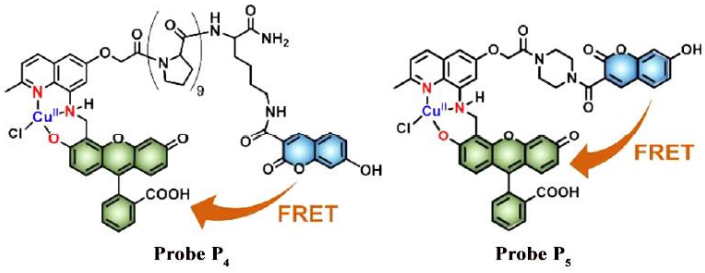


Figure 4. Probes (**P4** and **P5**) having NO sensor obeying FRET mechanism between the pairs of fluorophores and the ligating atoms of the metal-binding sites. Reprinted with permission from ref. 28, Copyright 2017 RSC.

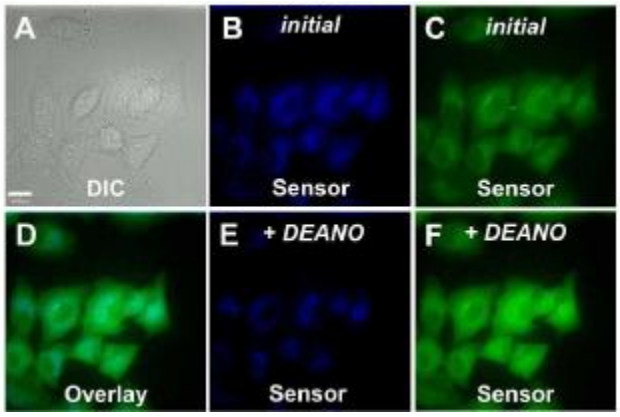
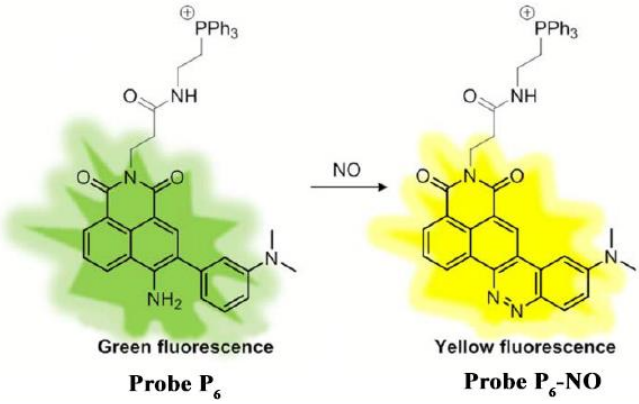


Figure 5. Fluorescence microscopy images of live A549 cells incubated with 2 μM of Probe (**P5**) at 37 °C for 30 min in DMEM. (A) Differential interference contrast (DIC) image. (B) Signal in the blue channel. (C) Signal in the green channel. (D) Overlay of (B) and (C). (E, F) Signal in the blue and green channels, respectively. Reprinted with permission from ref. 28, Copyright 2017 RSC.

H. Zhang and coworkers synthesized a mitochondria-target two-photon fluorescence Probe (**P6**) for selective NO detection. The structure of the probe was based on 1,8-naphthalimide system having mitrocondrial targetable moiety and NO sensing unit [29]. A robust turn-on fluorescence response (from green fluorescence to yellow fluorescence) of probe (**P6**) to NO was obtained (**Scheme 4**). This fluorescence enhancement was very much selective over a variety of reactive nitrogen, oxygen, and sulfur species. The detection limit was calculated as low as 21 μM and these result noticeably indicated that probe **P6** could be utilized for selective and qualitative detection of NO. The co-localization experiments were studied by co-staining Raw 264.7 macrophages with Mitochondrial-tracker Red CMXRos and probe **P6** (**Figure 6**). After the incubation for 30 min at 37 °C in the presence of exogenous NO (20 μM), yellow fluorescence was detected which merged well with the image from incubated with Mitochondrial-tracker. These results suggest that **P6** would be a potential

and mitochondrion-targetable probe.



Scheme 4. Reaction mechanism between probe (**P6**) and NO. Reprinted with permission from ref. 29, Copyright 2017 RSC.

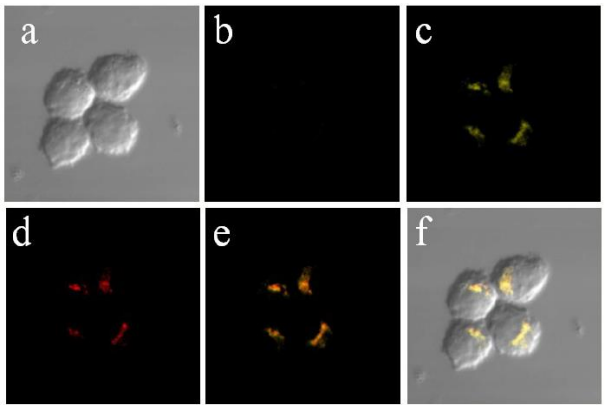
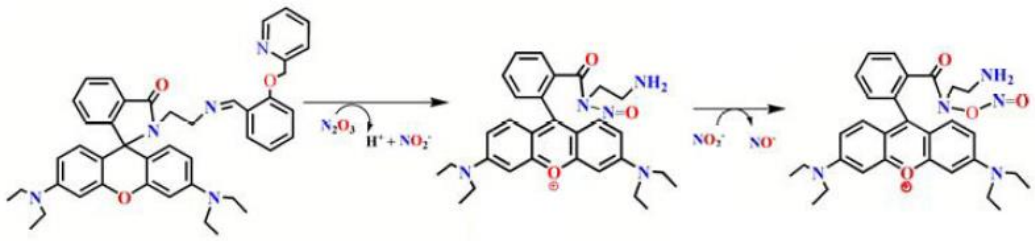


Figure 6. (a) and (b) Brightfield image and fluorescence images of Raw 264.7 macrophages without any treatment respectively. (c) Raw 264.7 incubated with 20 μM DEA·NONOate for 20 min, further incubated with 20 μM **P6** for 30 min. (d) Incubated with 1.0 μM Mitochondrial-tracker Red CMXRos for 30 min. (e) Overlay of b, c and d. (f) Overlay of a, c and d (λ_{em} = 575-625 nm). Reprinted with permission from ref. 29, Copyright 2017 RSC.

Mahammad Ali and coworkers synthesized a novel Rhodamine B based dual channel Probe (**P7**) (**Scheme 5**), which is highly selective and sensitive to NO in pure aqueous medium [30]. The probe, **P7** selectively recognized NO with a high formation constant value, without any interference of 5 equivalent metal ions, anions, amino acids and various biological species. The fluorescence enhancement was obtained more than 11-fold, leading to the opening of spirolactam ring in the biological pH range. The probe **P7** was reported as a cell permeable molecule and thus it is suitable for both *in vitro* and *in vivo* NO sensing species. The LOD was calculated as 83.4 nM suitable for *in□vivo* monitoring of NO mostly in the cytoplasmic compartment. The *in vivo* compatibility of the sensor was also checked on live Zebra fish.



Scheme 5. Proposed reaction mechanisms of ring opening of the probe **P**7 with NO. Reprinted with permission from ref. 30, Copyright 2018 RSC.

2. CONCLUSION

The approaches discussed above in this review provide examples of the designing and mechanistic aspects of recently reported fluorogenic probes for the detection of NO in a selective manner. Most of the reported probes are based on PeT, FRET, ICT inducible systems with various types of NO responsive moiety. Also probes discussed above exhibited very low detection limit (LOD) value towards NO and very good selectivity over a variety of interfering species. Though, many of them still have limitations e.g. less selectivity, long response time and high back ground emission. Although, it is still strongly desirable to develop novel fluorescent probes with high sensitivity, and quick response for real-time detection of NO for practical applications.

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