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Recent development in fluorescent probes based on attacking of double bond and masking of functional group

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ABSTRACT

The works on the procedure of fluorescent sensors for the detection of biological analytes are extremely momentous. Among diverse analytical approaches, fluorescence is the most eye-catching due to its high sensitivity, selectivity, rapidity, robustness, ease of measurement and non-destructive approaches. Herein, we show different fluorescent probes synthesized for estimation and detection of biological analytes (H_2S, SO_3^{2-}/HSO_3^-, H_2O_2, HOCl, HNO, $ONOO^-$). These probes were constructed by masking the functional groups (hydroxyl and amino) of fluorophore and formation of active C=C, C=N, C=O and N=N for specific analytes. In this review we concentrate on synthesis of the probe, their photophysical properties and applications to biological studies.

1. Introduction

Fluorescent probes have engrossed immense concentration for systematic sensing and visual illustrating since of their extraordinary compassion and technical simplicity. In common, fluorescent probes reacted to their goal in a reliable method under precise conditions. The connections between goals and probes can hold back fluorescence intensity by reducing ('OFF') or improve it by eliminating reducing effects ('ON'). An OFF-ON control gives a greater signal-to-noise ratio than a reverse ON-OFF control [1]. The planned approaches of fluorescent probes are mostly constructed on relations between probe and the goal moiety, that result in variations in the compounds fluorescence signal [2]. These relations are separated into two categories: covalent and non-covalent connections. The covalent connections based fluorescent probes, are also called as reaction-dependent fluorescent probes, generally have greater compassion and discrimination because they develop precise covalent responses while non-covalent connections include hydrogen bonding, hydrophilic, hydrophobic, electrostatic, donor-acceptor and coordination-based connections, which are extremely dependent on the complementarily between the compounds and analytes [3]. Many methods have been developed to construct compounds for selective detection of analytes. The electron strength of C=C can be shortened by the electron withdrawing group. Therefore, sulfur species, $ONOO^-$ and HOCl can easily break C=C, interrupt the π-conjugation and diverge the electronic configuration, which generally make blue shift phenomenon. The Michael addition dependent fluorescent compounds are categorized into two main groups, uncharged compound and cationic compound, depending on whether electron withdrawing species is charged or not [4]. Similarly, the functional groups such as OH and NH_2 are extremely chemically active. Thus, maskings of these moieties are frequently experiential in multistep synthesis. The masking of these functionalities often brings a noticeable change in electronic properties. This may provide an efficient way for the design of analyte receptive fluorescent probes. To fruitfully utilize this policy, the specific group was added to mask these functionalities which can be removed by specific moieties. In other words, many fluorescent compounds can be constructed for an analyte of interest based on these methods [5].

Although many reviews concerning fluorescent probes have been recently published [6,7], they mainly talk about the overall reaction-based plan for the development of fluorescent probes. Some fluorescent probes based on the masking of functional have been testified recently. Here, we have outlined recent 3-years studies on fluorescent probes based on attacking double bond and masking of functional group. This review is centralized on describing the construction of the probe, photo-physical properties, detection limits, and bioimaging model of the probes. It is our wish that this review may be helpful for readers who are interested in the research field.

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2. The probes based on attacking double bonds

The Michael addition is a nucleophilic addition of a carbanion or another nucleophile to α,β-unsaturated compounds having an electron withdrawing group. It fits to the greater class of conjugate trappings. These methods are useful for the mild formation of C–C bonds. Most of this kind of probes were constructed by formation of active C=C, C=N and N=N bonds. Those double bond could be attacked by active analytes (sulfur species, $ONOO^-$ and HOCl) and in response photophysical properties of the probes were changed.

2.1. Attack of HSO_3^-/HS^- on double bond

The HSO_3^-/HS^- presented key roles in many physiological processes. The deficiency or excess amount of these species may result in serious health problems. Thus, it is necessary to trace these species, for that the fluorescence recognition technique has been broadly employed in biological studies. Under physical environments, H_2S (pK_a ~6.9) disconnects to give HS^- as the main adduct, which is an excellent nucleophile. Similarly sulfur dioxide is hydrophilic which give hydrated bisulfite ion (HSO_3^-), and sulfite ion (SO_3^{2-}) upon closure in water. These species have strong nucleophilic nature. These nucleophiles could attack C=C [8] and N=N [9] and result in spectral properties changed. The electron solidity of C=C can be shortened by the electron withdrawing group. Therefore, HSO_3^-/SO_3^{2-} and HS^- can simply attack C=C or C=N that interrupts the π-conjugation and thus changes the photophysical properties of the probe.

Ahn's group reported a novel benzopyronin based two photon fluorescent probe **1** for selective detection of bisulfite in lysosomal cells [10]. The probe displayed a ratiometric response as C-12 of the benzopyronin-based dyes was attacked by bisulfite ions (Scheme S1 in Supporting information) and NIR emission was blue shifted to green emission. The introduction of morpholine moiety in probe was useful to trace bisulfite ions in lysosomal cells. The probe displayed an excellent selectivity toward HSO_3^- as there was no response to interfering species such as hydrogen sulfide and hypochlorous acid. The probe displayed a blue shift of 192 nm in emission wavelength as emission of the probe was shifted to 512 nm from 704 nm. The probe displayed a very was fast response to bisulfite ions and reaction was completed within 15 s and the detection limit was calculated to be 0.09 μmol/L. The probe itself showed less photo-stability in aqueous buffer, this was considered due to high conjugation in probe but its adduct after the action of bisulfite displayed greater photo-stability. The MIT assesses by a cervical cancer cell (HeLa) revealed that the probe was suit for tracing bisulfite ions in living cells.

Ge *et al*. reported a ratiometric fluorescent probe **2** for HSO_3^- detection in mitochondria [11]. The probe was synthesized by using hemicyanine and benzimidazole derived fluorophore. It works on FRET process. The hemicyanine moiety of the probe was useful as it serves as source of attacking site (acceptor) as well as quencher of the fluorescence of benzimidazole derivative (donor) group. It displayed high selectivity and good solubility (pure water). The probe serve as naked eyes sensor for the detection of HSO_3^- as color changed from colorless to yellow in PBS solution. The probe displayed two different emission intensity due to benzimidazole derivative (470 nm) and hemicyanine (578 nm) unit. After the addition of HSO_3^- the emission of hemicyanine was quenched due to breaking of π-conjugation (Scheme S2 in Supporting information) and the FRET process was inhibited. Kinetically reaction was completed within 2 min. The probe works in a widespread range of pH 5.0-10.0. The limit of detection was determined as 26.7 nmol/L. The Glioma cell line cells were used to check toxicity of the probe in living cells.

Mitochondria are a fundamental organelle of eukaryotic cell which displayed necessary functions in many physiological processes. SO_2 and H_2O_2 are two varieties of energetic signal molecule and biomarker that can be endogenously produced in mitochondria of living cells. Yin group reported a mitochondria-targeted NIR probe **3** for selective detection of reversible H_2O_2/SO_3^{2-} in living cells [12]. The probe was constructed by D-π-A-π-D system in which C=C double bond of benzopyrylium moiety was an active site for SO_2 (Scheme S3 in Supporting information) and after the reaction, the NIR emission of the probe was disappear which was regain by the addition of H_2O_2. The positive charge of benzopyrylium moiety was useful to trace analytes in mitochondria. The quantum yield of the probe was decreased to 0.006 from 0.026 after the reaction of SO_3^{2-}. The probe works at a wide range of pH (6-10) and a fast response was found (5 min).

Huo *et al.* reported dual-sites fluorescent probe **4** for H_2S detection. The probe was fashioned by naphthenylimide derivatives because it has good photochemical stability, high quantum yield, cell transmissibility and visible emission wavelength. The Azide group was added as responsive site for H_2S and two naphthenylimide rings was connected via linker ethylenediamine [13]. The probe has weak fluorescence in absence of H_2S, but when treated with H_2S, azide group was converted to amino group (Scheme S4 in Supporting information) which turn on ICT process and as a result the fluorescence of the probe was enhanced. The probe displayed good water solubility thus spectral properties were recorded e in DMSO and PBS (pH 7.4) (v/v, 8:2) buffer. After the addition of H_2S 20 folds enhancement in intensity along with change in color was observed. The probe shows very fast reaction and fluorescence intensity reaches to maximum within 150 s. The detection limit was measured as 0.02 μmol/L. The probe was significantly stable in the range of pH 2–10. The fluorescence images of H_2S in HepG-2 cells were recorded.

2.2. Attack of HOCl/ClO^- on double bond

Hypochlorite anion (ClO^-) has been accepted as multitude guard destructing incursive bacteria and pathogens, a signal molecule inducing occurrence of apoptosis and a noxious agent when it is produced in excess. The hypochlorous acid/hypochlorite (HOCl/ClO^-), is a one of the important reactive oxygen species (ROS), which takes part in a variability of physiological and pathological processes. The extreme gathering of HOCl may result in tissue damage and lead to many diseases. Many procedures have been reported for the detection of HOCl/ClO^-, but these techniques have many disadvantages. Recently fluorescent probes gained much attention due to its high selectivity and fast response. The fluorescent probes for detection of HOCl/ClO^- were constructed by different method. One of them is forming active C=C and C=N.

Li *et al.* reported probe **5** for selective detection of HOCl in SH-SY5Y neuroblastoma cells and zebrafish larvae with low cytotoxicity, good cell penetrability and biocompatibility [14]. Meldrum's acid was used as reactive site for ClO^-. The probe was a naked eyes sensor for detection of ClO^- as color of solution change from rose red to purple. There was fast response (<1 s) of the probe to ClO^- with detection limit calculated as 78 nm. A large stokes shift was observed (>100 nm) and probe can work at a wide range of pH. The sensing mechanism displayed that addition-ring-opening-elimination reaction was took place (Scheme S5 in Supporting information).

Ni *et al*. reported a probe **6** based on coumarin fluorophore for the detection of HOCl in HeLa cells [15]. The probe was constructed by the condensation of coumarin moiety and a malononitrile derivative. The Malononitrile has strong electron withdrawing power and diethyl amino group of coumarin ring has elec-

tron donating nature, thus a strong ICT process was operated through coumarin to malononitrile derivative, and the probe displayed NIR emission. When the probe was treated with hypochlorous acid (Scheme S6 in Supporting information), 217 nm large hypsochromic shift was observed. The high π-conjugate systems of the probe was disturbed and result in hypsochromic shift. The color of probe solution changed from brilliant pink to blue light meant that probe was useful as a naked eyes sensor for HOCl detection. The HOCl/ClO^- under mild conditions attack the C=C and the π bond of C=C in the probe is broken and a chloro alcohol derivative is formed. The probe can work in pH range of 5-10 and fast response was observed with low detection limit 65 nmol/L.

Due to their bioactivities and photo physical properties morpholine and its derivatives were widely used in biological medicine and biochemistry. Shen *et al*. reported a simple mitochondria-target morpholine-based probe **7** for selective and sensitive detection of OCl^- with naked eyes [16]. The probe consists of morpholine as a fluorophore and indole cation as mitochondrial targeting group. The C=C bond was an active site for ClO^- (Scheme S7 in Supporting information). The probe exhibited NIR emission due to strong ICT process. When the probe was treated with ClO^-, the π-conjugate system was broken and red solution transformed into a colorless. The probe displayed high sensitivity, low toxicity, fast response (4 s) and detection limit was noted as 0.05 μmol/L.

Guo *et al*. reported a probe **8** for HOCl and the probe is proficient to image HOCl in living HepG2 cells and zebrafish [17]. The probe was based on rhodol dye and to target in mitochondria by triphenylphosphine cation. 2-Pyridylhydrazine was used to synthesis 2-pyridylhydrazone of rhodol dye that is also an active site for HOCl (Scheme S8 in Supporting information). HOCl-promoted triazolopyridine formation and no side product are formed except water. The photophysical properties of the probe were changed after the addition of HOCl. Due to excellent water solubility, the spectral analysis was done in pure water. The probe can work at pH range of 6-9. The color of the solution altered from purple to pink (naked eyes sensor). The quantum yield also moved from 0.03 to 0.12 and there was an enhancement in fluorescence (up to 21-fold). Kinetically reaction was very fast (4 s) and limit of detection was calculated to be 2.2 nmol/L.

Ye group reported a probe **9** for HOCl detection [18]. The fluorescence of the probe was reduced by adding 2-benzothiazole hydrazine. The probes exhibited outstanding selectivity toward HOCl with low detection limit (17.5 nmol/L), fast response (2 min). The probe exhibited "turn-on" response on treatment with HOCl with variations in color from orange to bright yellow. The quantum yield also increased from 0.02 to 0.65. The C=N was oxidized by HOCl to produce carbonyl compound (Scheme S9 in Supporting information). The fluorescence imaging in Hela cells showed that probe was useful to trace HOCl in living cells.

2.3. Attack of $ONOO^-$ on double bond

Peroxynitrite ($ONOO^-$) is one of the endogenous reactive oxygen species (ROS). The $ONOO^-$ was not thought to be a ROS previously, till a study showed by Beckman in 1990. Now, it is documented as a highly reactive species. Owing to its high oxidizing and nitrating capacities, excess $ONOO^-$ can damage a wide variety of bio macromolecules, including DNA and proteins. Numerous approaches have been developed for sensing $ONOO^-$, but they have some drawback. Development of new fluorescent approaches for $ONOO^-$ has expected cumulative courtesy due to their high sensitivities, noninvasive nature, excellent spatial and temporal resolution. To trace $ONOO^-$, some probes were constructed by forming hydrazide (C=N) [19] and C=C [20] bonds. The reaction between C=N and $ONOO^-$ is started by nucleophilic addition of the hydrazide nitrogen to the $ONOO^-$, while in case of C=C bond, it resulted in formation of aldehydic product.

Li *et al*. reported a hemicyanine fluorophore based probe **10** for $ONOO^-$ in living cells [21]. The probe displayed selectivity for $ONOO^-$ duo to its strong oxidation power. The probe was constructed by connecting hemicyanine fluorophore with xanthenes derivative *via* C=C. Due to this connection, the probe displayed high conjugation and emission in NIR region. When the probe was treated with $ONOO^-$, oxidation of this C=C bond take places. As a result, small conjugation was observed thus blue shift in emission band was observed. The probe displayed large emission shift (248 nm), ratiometric response and color of solution changed from blue to colorless. In presence of $ONOO^-$, the probe displayed 1728-fold enhancement in emission intensity. The LOD was found as 33 nmol/L. The C=C bond undergoes a nucleophilic addition (Scheme S10 in Supporting information), followed by an oxidation process to form the epoxy intermediate, then a hydrolysis reaction takes placed resulting formation of cleavage products. The HepG2 cells and RAW264.7 cells were used for cell imaging.

Han *et al*. also reported a probe **11** for $ONOO^-$ [22]. The probe was based on coumarin fluorophore and hydrazone of coumarin derivative was formed which shows high selectivity for $ONOO^-$ based on oxidation mechanism (Scheme S11 in Supporting information). The probe displayed excellent selectivity for $ONOO^-$ over other species. The probe showed no fluorescence in absence of $ONOO^-$ but when treated with it, a strong emission was emerged and 76 folds enhancement was observed with low detection limit 35 nmol/L and fast response time (1 min). The probe was stable to pH as fluorescence intensity enhanced at pH range of 5–9. The RAW264.7 and H1299 cells were employed to visualize $ONOO^-$ in living cells.

3. The probes based on masking functional groups

Masking groups are used to temporarily mask the specific chemistry of a functional group because it affects other reaction. A good protecting group should be easy to put on, easy to remove and in high yielding reactions, and inert to the conditions of the reaction required. In this review article, we are mentioning the probe based on masking of hydroxyl and amino group to trace analytes.

3.1. Hydroxyl group masking

Hydroxyl group can be masked by specific substituent to trace specific analytes. The electron donating ability to fluorophore is prohibited which mostly result in fluorescence turn off and by removing this protection fluorescence of fluorophore is regained.

3.1.1. Masking for HS^-

Fluorescent probes can be constructed by masking the hydroxyl group. Specifically for sulfur species, hydroxyl can be masked by 2-formyl benzoic acid [23], thiophene-2-carboxylic acid [24], acryloyl chloride, cyanoxy group [25], di-pyridyl disulfide [26], 2,4-dinitrobenzenesulfonyl group [27], 2,4-dinitrofluorobenzene [28] and nitrobenzofurazan (NBD) [29]. All these groups have electron withdrawing effect and quench the fluorescence of fluorophores. Thus, upon removal of these groups, fluorescence of fluorophores are regained.

Gore group reported a novel green fluorescent protein (GFP) chromophore-based probe **12** for hydrogen sulfide [30]. The probe showed extraordinary selectivity and sensitivity, fast response (15 min), lower detection limit (15.85 ppb), large stokes shift and high fluorescence stability for H_2S in complete aqueous medium. The acryloyl ester was formed by protecting the hydroxyl group of

GFP fluorophore thus spectral changes were found. This ester linkage was broken by the addition of H_2S and Cys (Scheme S12 in Supporting information). The probe displayed selectivity for Cys and H_2S. The color of solution changed from colorless to yellow, thus the probe was useful to trace analytes by naked eyes. There was 290-folds enhancement in emission intensity after the addition of H_2S. The 1, 2 nucleophilic attacks of HS^- on acryloyl ester free the phenolate ions and thus enhanced fluorescent properties of the probe. The probe was used to trace H_2S in human colon cancer cells (HCT116) and normal human dermal fibroblasts (HDF).

Inspired by the work of Erman group, Yang group reported a NIR probe **13** based on isophoronitriles scaffold [31]. The cyanoxy group was added to protect the hydroxyl group, which causes changes in spectral properties. The probe exhibited great sensitivity, high selectivity (towards H_2S over Cys, GSH and HSO_3^-), good linear relationship, low detection limit (50 nmol/L), high quantum yield (0.54) after the action of H_2S, good fluorescence enhancement (21-folds) and pH stability (6-10). The ICT process of the probe was weakened by protecting the hydroxyl group of the probe. When the probe was treated with Na_2S, a nucleophilic reaction occurred between H_2S and cyano bond, which was converted to thiocarbamates and subsequently was hydrolyzed (Scheme S13 in Supporting information). The cell membrane penetrability and low cytotoxicity of the probe was useful to trace H_2S in MDA-MB-231 and Kunming mice cells.

Currently, main issue for researcher is selectivity of the probe for detection of H_2S because biothiols interfere with it and other thing are auto-fluorescence background, light scattering, and destruction to the tissues caused by the ultraviolet to the visible light emission and excitation. Part issues were solved by using DCM-OH chromophore because it is a red emission dye having many advantages such as large Stoke's shift *via* ultrafast ICT, manageable NIR fluorescence emission by alteration electron donor aptitude, and excellent photostability. Han *et al.* reported a thienopyridinone derivative (KF)-based probe **14** for H_2S in serum [32]. The probe displayed high selectivity to H_2S over biothiols. The hydroxyl group of the probe was masked with 2,4-dinitrobenzene sulfonyl (DNBS) group which was highly selective for H_2S. The fluorescence of KF was associated with specific binding to human serum albumin (HSA). H_2S causes thiolysis of DNBS (Scheme S14 in Supporting information) and releasing the fluorophore (KF) which bind with HSA and then exhibited strong emission. HSA and KF has strong interaction which was confirmed by the bonding constant based on the Benesi-Hildebrand plot ($K_a = 1.9 \times 10^4$ L/mol). The probe displayed fast response (20 min), large Stoke's shift, low detection limit (3.2 μmol/L) and wide range of pH stability (5-9). The probe was used for quantitative analysis of spiked H_2S in human serum.

Previously most probes shown short wavelength that have limitation such as deep tissue penetration and auto emission. To control these effects, Li *et al.* reported a novel NIR probe **15** based on DCM-xanthenes fluorophore which has strong emission in NIR zone and NBD ether as a reaction site for H_2S [33]. The NBD based probes were not selective unexpectedly this probe shows great selectivity for H_2S. The masking of hydroxyl group made the probe as non-fluorescent due to strong electron withdrawing nature of NBD group. When the probe was treated with H_2S the thiolysis of ether linkage takes place which released fluorophore (Scheme S15 in Supporting information). The probe has many advantages such as extraordinary selectivity for H_2S over various other analytes including biothiols, fast response (within 3 min), a 40-fold increase in fluorescence intensity, large Stoke's shift (166 nm), low cytotoxicity and high sensitivity (detection limit 26 nmol/L). The confocal imaging of exogenous H_2S in MCF-7 cells with the probe was also recorded.

3.1.2. Masking for HOCl/ClO⁻

Hypochlorous acid (HOCl) is a kind of ROS which is formed by hydrogen peroxide (H_2O_2) and chloride ion (Cl^-) from myeloperoxidase. It is vital to develop suitable and selective approaches for the detection of HOCl. Many methods have been developed such as colorimetric, electrochemical, and chemiluminescence. These methods hurt the cells and are also not extensively used in sensing of HOCl in living system. But fluorescence approaches have been extensively used in recent years and delivered excellent membrane permeability, high selectivity, good sensitivity and even real-time detection. A batch of fluorescent probes in which OH of fluorophore is protected with sulfide moiety have been reported [34–37]. Interestingly, HOCl can oxidize sulfide-type amino acids and this trend of HOCl is different from other ROS.

Gong group recently reported a coumarin fluorophore based probe **16** for selective detection of HOCl in living cells [38]. The dimethylthiocarbamates (DMTC) was used to protect hydroxyl group of the probe which was easily hydrolyzed by HOCl and in response fluorophore is released. It was a pH dual activatable fluorescent probe. The specific organelle targeting (Morpholine was added as a lysosomal targeting group) group was added. When the probe was treated with HOCl in neutral solution, a weak fluorescence of the probe was observed due to oxidation of DMTC group (Scheme S16 in Supporting information). But strong emission was emerged in acidic environment due to the protonation of phenolic anion and morpholine group. The probe showed excellent sensitivity, high specificity, fast response, low detection limit (24.3 nmol/L), and low cytotoxicity (85% cell survive) under the acidic conditions. The Bright field and fluorescent images of HeLa cells were captured to confirm the HOCl detection in living cells.

Wang *et al.* reported dual functional probe **17** for selective detection of ClO^- and H_2O_2 in living cells [39]. Many probes have been reported for ClO^- and H_2O_2 separately but this probe was useful as it detects both species collectively. The FRET process was operated because 7-hydroxycoumarin and 4-hydroxy-1,8-naphthalimide were used as donor system. The electron donating ability of donors was diminished by adding specific sites of action for ClO^- and H_2O_2. The ethylenediamine was used as linker because of its suitable length. Borate and dimethylthiocarbamate were used as reaction site for H_2O_2 and HOCl. Both these sites are highly selective for respective analytes. When the probe was treated with HOCl the emission in blue channel (due to deprotection of hydroxyl group of coumarin fluorophore) was observed (Scheme S17 in Supporting information). When treated by H_2O_2 the emission of the probe in green region was observed (due to deprotection of hydroxyl group of naphthalimide fluorophore). But when treated with both H_2O_2 and HOCl, emissions in green channel and FRET process could be observed. By the action of HOCl 213-folds enhancement in fluorescence intensity were found and the detection limit was calculated to be 28.2 nmol/L. In case of H_2O_2 260-folds enhancement in fluorescence intensity was found and the detection limit was calculated as 64.6 nmol/L. The probe displayed a very fast response in case of HOCl (180 s), while in case of H_2O_2 fluorescence reaches to saturation point within 70 min. The probe displayed low toxicity for both analytes and can be used for imaging in mouse liver tissues.

Lin *et al.* reported anendoplasmic reticulum targeting probe **18** based on naphthalimide fluorophore for selective detection of HOCl [40]. The *N,N*-dimethylthiocarbamates was used as the recognition site for HOCl. The naphthalimide derivative was used because of its good photostability, deep tissue penetration and high quantum yield. The hydroxy group of naphthalimide was masked with the help of *N,N*-dimethylthiocarbamates. As compared to *N,N*-dimethylthiocarbamates, hydroxyl group has strong ICT process. When the probe was treated with HOCl, the fluorescence of the probe enhanced. The chloride ion (Cl^+) coming from the HOCl will

help in thiolysis of *N,N*-dimethylthiocarbamates and in result released fluorophore which displayed strong emission (Scheme S18 in Supporting information). The probe showed change such as from colorless to yellow, 60-fold enhancement in fluorescence intensity, low detection limit (0.13 μmol/L), fast response time (2 min) and low cytotoxicity which were useful to trace HOCl in living cells (HepG2 cells and zebrafishes).

3.1.3. Masking for H_2O_2

Hydrogen peroxide (H_2O_2), is another ROS, associated with numerous physiological and pathological developments in biological systems. Several probes for its detection have been quantified in recent years. To construct probes for H_2O_2, one of good method is a boronate oxidation reactions which could be divided into different categories: (a) The probes based on direct linkage strategy [41,42], (b) the probes based on ether linkage strategy [43], (c) the probes based on ester linkage strategy [44], and (d) the probes based on pyridinium linkage strategy [45,46]. Some probes were constructed by adding α-ketoamide group as an active site for H_2O_2. These groups usually involved the masking of hydroxyl and amino group of the probe.

Zhang group reported a probe **19** based on DCM fluorophore for selective detection of H_2O_2 in living cells and organism [43]. The boronate ester group added to mask the hydroxyl group of the probe which resulted inhibition of ICT process. When the probe was treated with different oxygen reactive specie, change in spectral properties was observed with H_2O_2. This change was attributed due to breaking of ether linkage by cascade reaction of H_2O_2 to boronate ester and released the fluorophore which has strong ICT process (Scheme S19 in Supporting information). This probe displayed large stoke shift (131 nm), a wide range (pH 4-12), short response time (0.5 h), good photostability and the limit of detection was determined as 0.07 μmol/L. The probe was also useful to trace choline and glucose.

Tetrahydroquinoxaline iminocoumarin has strong emission in NIR region, high quantum yield and large Stoke's shift. Due to these advantages, they are used as a fluorophore to construct the probes for the detection of analytes. On the basis of these properties Song *et al*. reported a novel NIR probe **20** to trace H_2O_2 in living cells (HeLa cell) [47]. The aromatic boronic ester functionality was added to mask the hydroxyl group of the probe which made it as fluorescence off. When the probe was treated with H_2O_2, the boronic ester group was broken and under goes a cyclization reaction to give iminocoumarin derivative (Scheme S20 in Supporting information). The probe could be used as naked eyes sensor to trace H_2O_2 as color change from red to orange. The probe displayed high selectivity toward H_2O_2, 22-folds fluorescence enhancement, low detection limit (6.0×10^{-8} mol/L), large Stoke's shift (148 nm), fast response (40 min), pH stability (6–11 with H_2O_2) and low toxicity (cell survival rate was above 95%).

3.1.4. Masking for HNO

The nitroxyl (HNO) is formally the protonated product of nitric oxide. However, owing to its exclusive chemical possessions, it manifests definite biological properties. Several diverse plans have been projected for the detection of HNO in chemical, enzymatic and biological systems. There were four classes of HNO reactive probes: (1) Triphenylphosphine-based probes [48,49], (2) copper(II) complexes based probes [50], (3) nitroxide-based fluorescent probes [51,52] and (4) esters of fluorescent dyes (2-mercapto-2-methylpropionic acid) [53].

Ye's group reported dual-channel fluorescent probe **21** to trace HNO in living HepG-2 cells [54]. The probe was constructed by coumarinsemi-rhodamine mix fluorophore and phosphine moiety was introduced to protect hydroxyl group of the probe which was also a reactive site for HNO. The probe displayed outstanding sensing performance at long wavelength in terms of high specificity, brilliant sensitivity, large Stock's shift (273 nm), low limit detection (111.6 nmol/L), and rapid response rate (< 5 min). The probe displayed two channels, one in green region and the other one in red region. This was the first probe that works dually for detection of HNO. The competitive experiment shows that SO_3^{2-}, HSO_3^- and GSH cause the interference with HNO. The GSH is a good scavenger of HNO and other two sulfur species attack on C=C double bond of aromatic ring thus no change in fluorescence enhancement was observed in these cases. The phosphine moiety was attacked by HNO and the release the fluorophores (Scheme S21 in Supporting information).

Zhu *et al*. reported probe **22** for visualization of HNO in endoplasmic reticulum [55]. Two-photon imaging knowledge has the sole rewards of lower photo toxicity, profounder penetrability, and longer comment time. To construct two photons probe they used 1,8-naphthalimide fluorophore for its two-photon properties and methyl sulphonamide was applied as an ER targeting group. The phosphine moiety was used to protect the hydroxyl group of fluorophores which stop the free ICT process (Scheme S22 in Supporting information). The probe displayed excellent selectivity, 10-folds enhancement in fluorescence intensity with large Stoke's shift (105 nm), stability over a period of time, low detection limit (32 nmol/L), pH range of 7−10 and low cytotoxicity (cells survival rate 80%). One-photon and two-photon fluorescence images of HNO in MCF-7 cells were captured successfully.

3.1.5. Masking for $ONOO^-$

The peroxynitirite ($ONOO^-$) is an important ROS. Beckman firstly confirmed the role of $ONOO^-$ as a reactive oxygen species in 1990 [56]. Now a day it was known as a highly reactive species generated endogenously by diffusion-controlled reaction of nitric oxide (NO) with $O_2^{\bullet-}$ ions. It has short biological half-life (<10 ms) and more stable in basic form as compared to acidic form. The $ONOO^-$ plays fundamental roles in living cells such as signal transduction and also takes part in antibacterial and antimicrobial activities. The fluorescent probes constructions method is important and easily handling method to trace this ROS in living cells. $ONOO^-$ probes involved different construction method such as oxidation of chalcogenide [57,58], oxidation of boronic acids [59,60] or protection of hydroxyl group of fluorophore [61–63], oxidation of hydrazides [64,65], cleavage of the C=C double bonds [66,67] and the oxidative *N*-dearylation or protecting amino group [68,69].

Our group has reported a novel NIR probe **23** for selective detection of peroxynitrite in EC1 cells [70]. The probe was based on quinoline-malononitrile fluorophore and hydroxyl group of the probe was protected with arylboronates (Scheme S23 in Supporting information). The quinoline malononitrile fluorophore has many advantages such as obvious photostability, the red emission, good biocompatibility and high brightness. The probe displayed high selectivity for $ONOO^-$ as compared to other reactive oxygen species especially H_2O_2 which are very reactive to arylboronates. The probe displayed high sensitivity (27.52 nmol/L) for $ONOO^-$, large Stoke's shift (∼190 nm) and work at a wide range of pH values 7.4–12.0. The probe displayed fast response (4 min) to $ONOO^-$ and low cytotoxicity was measured by MIT assay (More than 85.3% of cells survived.).

Zeng's group reported a novel probe **24** for selective detection and bioimaging of $ONOO^-$ in living cells and zebrafish [71]. The probe was synthesized by coumarin-fused coumarin dye and borates as the specific recognition group for $ONOO^-$. Two coumarin rings were fused to form fluorophore which has excellent optical properties, including large Stoke's shift, high fluorescence quantum yield. The probe showed quite few noticeable concerts such as high specificity, excellent sensitivity (4 nmol/L), and fast response (5 s),

large stokes shift (100 nm) and it could promptly respond toward $ONOO^-$ (Scheme S24 in Supporting information). Due to low cytotoxicity the probe can be used in biosystems.

3.2. Amino group masking

Similar to hydroxyl group masking, amino group are also masked to construct probes. Nitrogen atom has free lone pair of electrons which could be donated to the fluorophore. As a result, the probe displayed fluorescence which was diminished by the addition of electron withdrawing group. The electron withdrawing group pulls lone pair of electrons toward them and fluorescence of the probe turn off. When this protection was removed, it again displayed photophysical properties.

3.2.1. Masking for HS^-

These probes were constructed by masking amino group of fluorophores. Many probes for HS^- were constructed based on masking amino group with 7-nitro-1,2,3-benzoxadiazole (NBD) [72–74] and 2,4-dinitrobenzenesulfonyl chloride (DNBS) [75]. These groups stop the free transfer of lone electrons pair to fluorophore and make the probe fluorescence off. When treated with H_2S, these groups were removed and fluorescence properties of the probe were regained.

Feng *et al.* reported a NIR probe **25** for selective detection of H_2S in living cells [76]. Iminocoumarin based dyes displayed short wavelength. To overcome this shortcoming, Feng group designed tetrahydroquinoxaline (imino) coumarin which displayed NIR emission. The 2,4-dinitrobenzenesulfonamide (DNBS) group was used as reactive site for sulfur. Amino group of the probe was protected with DNBS which has strong electron withdrawing ability. When the probe was treated with H_2S, thiolysis of NDS take place and a strong emission was emerged (Scheme S25 in Supporting information). A large Stoke's shift (128 nm), fast response (2 min), and about 35-fold enhancement were found. The high viability (>84%) of cells illustrated it was useful to trace H_2S in living RAW264.7 cells.

Han *et al.* reported a novel probe **26** for H_2S and Cys in living MCF-7 cells [77]. The probe was synthesized by using 1,8-naphthalimide and 7-chlorobenzofurazan-4-sulfonyl chloride (CBD) linked by a rigid piperazine group. Addition of triethylene glycol moiety to fluorophore makes it having good water soluble. The probe can also be used to differentiate Cys and H_2S. Both analytes showed different mode of action to active site CBD. H_2S causes thiolysis of sulfonamide bond between fluorophore and benzofurazan moiety, and result in properties of fluorophore turn on (Scheme S26 in Supporting information). The reaction of the probe with H_2S was fast (10 min) as compared to Cys (40 min). The detection limit was calculated as 11.5 nmol/L and 16.7 nmol/L for H_2S and Cys respectively.

3.2.2. Masking for H_2O_2

Hydrogen peroxide (H_2O_2) in existing systems is getting growing concentration due to its diverse assistance as an indicator for oxidative stress and scratch events connected with aging and disease. The fluorescent probes act as precious gear to sense the amount, chronological and spatial division of H_2O_2 in living cells. The design of such fluorescence probes is mainly based on the sole chemical properties of H_2O_2. Numerous fluorescent probes have been developed based on various chemical reactions (section 2.1.3). One of them is by protecting amino group of fluorophore which include protection by ester linkage [44], by quaternarized pyridine unit [45,46] and by α-ketoamide moiety [78].

Many probes have been reported for detection of H_2O_2 in living cells. These probes have some shortcoming, such as the probes which based on sulfonate hydrolysis may response to mercaptans and other reactive oxygen, and probes based on boronate esters also response to nitric oxide (NO) species. Another shortcoming is that they showed short emission wavelength. Zhou *et al.* reported a probe **27** that was based on DCM fluorophore which has strong emission in NIR region, and α-ketoamide was used as the recognition group which displayed high selectivity toward H_2O_2 [79]. The probe gives specific colorimetric (yellow to pink) and fluorescence response to H_2O_2 as compared to other biothiols, other reactive oxygen species and ions. The probe displayed 22-folds enhancement in intensity after treatment with H_2O_2, low detection limit (5.3 μmol/L) and fast response (15 min). After the α-ketoamide cleavage by H_2O_2, the free fluorophore released (Scheme S27 in Supporting information) and changes in photophysical properties could be observed. Thus, the probe is proficient of detecting H_2O_2 in aqueous media with good sensitivity. The cell feasibility of probe was judged adjacent to a variety of cancer cells, counting A549, SMMC-7721, HeLa, MCF-7, 4T1 and normal HL-7702 cell lines.

Hydrogen peroxide can be formed in mitochondria and shows important part in physiological metabolism. Excess amount of H_2O_2 causes many diseases. Therefore, it is extremely significant to build up an extremely sensitive technique for detecting H_2O_2 together *in vitro* and *in vitro*. Benzil based fluorescence probes are better to those based on boronate ester in conditions of reaction selectivity. Though, NIR probe with biocompatibility has been hardly ever reported for the detection of endogenous hydrogen peroxide. Hemicyanine fluorophore has been established to be successful gallows for NIR fluorescent probes for optical imaging *in vitro*. Thus Hu's group reported a probe **28** based on hemicyanine fluorophore and benzil group was added as reactive site for H_2O_2 [80]. The probe works by PET process because it shows no fluorescence, and shows high fluorescence as treated with H_2O_2. The nucleophilic attack of hydrogen peroxide demolishes the amide bond of the probe, then rearrangement undergoes and the fluorophore frees (Scheme S28 in Supporting information). The probe showed high specificity and sensitivity, and has good water solubility and short response time (within 10 min) for the detection of hydrogen peroxide *in vitro* and *in vitro*. The detection limit was determined as 65 nmol/L.

3.2.3. Masking for $ONOO^-$

The probe for peroxynitrite could also be synthesized by masking amino group of the probe. The masking can be done by using amino methoxyphenol/phenol [69], borate group [81] and benzil group [68,82].

Jia's group synthesized a probe **29** for detecting exogenous and endogenous $ONOO^-$ levels in living cells and inflammation sites of zebrafish [83]. The probe was based on 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran fluorophore which has strong ICT process and displayed emission in NIR region. The boronate/boronic acids were used to mask the amino group of the probe, and ICT process was blocked which resulted in quenching of the fluorescence (Scheme S29 in Supporting information). The probe displayed high selectivity toward $ONOO^-$, fast response time (40 s) and high sensitivity (21 nmol/L). The probe displayed low cytotoxicity as more than 90% cells survived after incubation with 20 μmol/L $ONOO^-$ for 12 h.

4. Conclusion

In disparity to outdated analysis approaches for exact detection, dual-emission ratiometric fluorescence approach contains an essential integral alteration to the inspiration from recognition systems and background signals, presenting a noteworthy probable to increase accuracy and consistency. Particularly, ratiometric fluorescence approach can realize exact, measurable illustrative and

real-time recognition of goals. In current years, there is increasing interest in the dominant research field of ratiometric fluorescence probes for specific analytes recognition. Various types of dual-emission ratiometric fluorescence probes have been extensively working for the chemo/biosensing and bioimaging detection of analytes. The strategy moralities of fluorogenic molecules are well recognized. Upcoming developments in small-molecule fluorogenic combinations will be determined by better synthetic approaches to competently build excellently adjusted derivatives for specific biological experiments. Successful generation of dyes will allow the discovery of additional probes with essential sensitivity and attraction for different cellular environments.

Excellent progress in improvement and application of fluorescent probes has been found over the last decades. This has been an extremely creative and stimulating period. Of course, around remain many dares to report, such as developing probes for the reversible recognition, bigger claim of the probes for eco-friendly detection, enhanced the probes for controlling real-time surgical procedures.

In this review, we have summarized fluorescent probes for imaging and detection of biological analytes. The probes contained of several fluorophores and recognition moieties, providing an effective site for biological analytes detection in living organisms. In specific, the design plans, sensing mechanisms, and deprotection modes of the fluorescent probes were showed. The fluorescent probes were ordered according to Michael addition, protection-deprotection of two types of functional groups: OH and NH_2 groups. While major progress has been made in the development of fluorescent probes centered on the Michael addition, protection-deprotection strategy, challenges remain in the development of these probes for biological applications. The protection-deprotection of functional groups and formation of double bond were initially done in organic solvents. In addition, the same shield group may be removed by different species. Thus, the discrimination for a specific target of interest may be difficult. Although the detection of endogenous analytes *in vitro* is generally accessed, the imaging depth is still limited because of low light penetration ability and stability of the probes. NIR and two-photon probes with low background interference help solve part of this, but not all of it. The significant improvements may be afforded by using NIR-II window (1000−1700 nm)-emissive probes, which is characteristic of good imaging resolution and depth. In some cases, influence of the cellular micro environment on the probe response characteristics cannot be neglected. The preliminary experiment in cell extracts or simulated cell physiological conditions may contribute to the identification of the difference, guiding the observation in living cells or *in vitro*. We believe that with the pains of chemists in different areas, more protection, deprotection methods and formation of double bond will be recognized for the development of fluorescent probes, which are else not available by current methods. The fluorescent probes based on the formation of double bond and protection-deprotection strategy will continue to act as powerful molecular tools for biological and medical studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2021.12.092.

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