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Use of Fluorescence Probes for Detection of Reactive Nitrogen Species: A Review

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The biological and toxicological effects that have been attributed to reactive nitrogen species (RNS) are increasingly stirring the scientific inquisitiveness about the molecular mechanisms involved. However, RNS present some characteristics that complicate their detection, namely their short lifetime and the normal presence of a variety of endogenous compounds capable of reacting with these reactive species, when the studies are performed in biological matrices. The development of methodologies capable of circumvent these difficulties is thus of fundamental importance. Fluorescence probes are particularly important due to their high sensibility and usefulness in temporal and spatial monitoring of RNS, particularly in microanalysis conditions in biological media akin to cells or tissues. In the present review is given an account of the fluorescence probes that have been used for detection of nitric oxide ($^{\cdot}NO$), peroxynitrite anion ($ONOO^-$), as well as of some of its derivatives in biological and nonbiological media.

KEY WORDS: Reactive nitrogen species; nitric oxide; peroxynitrite; fluorescence probes; antioxidant; oxidative stress; scavenging activity; free radicals.

INTRODUCTION

The designation of reactive nitrogen species (RNS) englobes nitric oxide ($^{\cdot}NO$) and its derivatives. RNS represents not only free radicals like $^{\cdot}NO$ and nitrogen dioxide radical ($^{\cdot}NO_2$) but also non-radicals, like peroxynitrite anion ($ONOO^-$), peroxynitrous acid (ONOOH), nitrosoperoxycarbonate anion ($ONOOCO_2^-$), nitronium cation (NO_2^+), and dinitrogen trioxide (N_2O_3) [1,2].

$^{\cdot}NO$ is endogenously produced in a variety of mammalian cells through the conversion of L-arginine to L-citrulline, by nitric oxide synthases (NOS), which occur in three different isoforms [3–5]. The production of $^{\cdot}NO$ at low physiological levels (at the nanomolar range) is mainly involved in homeostatic biochemical and physiological processes such as signal transduction, neurotransmission, smooth muscle relaxation, peristalsis, inhibition of platelet aggregation, blood pressure modulation, immune system control, learning, and memory [6,7]. However, in certain circumstances, the inducible NOS enzymes (iNOS) present in macrophages, activated neutrophils, hepatocytes, smooth muscle cells, chondrocytes, astrocytes, pancreatic β-cells, and several types of tumor cells [8] are activated and may lead to the production of a continuous near-micromolar amounts of $^{\cdot}NO$ for long periods (e.g. up to 72 hr *in vitro*) [5,9]. The sustained $^{\cdot}NO$ generation can cause tissue injury. Indeed, this radical is involved in the pathogenesis of disease states like endotoxin shock and inflammation. iNOS have also been shown to have antimicrobial and tumoricidal activity in the host defense and immune response [10–12].

$ONOO^-$ is formed from the diffusion-controlled reaction between the free radicals $^{\cdot}NO$ and $O_2^{\cdot -}$ ($k \sim 10^{10}$ M^{-1} s^{-1}), which does not require enzymatic catalysis [13–16]. Since $^{\cdot}NO$ is neutral and hydrophobic, capable of traversing membranes, while $O_2^{\cdot -}$ is anionic

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at neutral pH ($pK_a = 4.8$), $ONOO^-$ formation will occur predominantly close to the sites of $O_2^{\cdot -}$ formation [17]. In turn, $ONOO^-$ will traverse membranes by passive diffusion as its conjugated acid form ONOOH ($pK_a = 6.8$) or, through anion channels, in the anionic form [18]. Under physiological conditions $ONOO^-$ is in equilibrium with the protonated form, ONOOH, and has a lifetime of under 1 s [19], yielding oxidizing and nitrating species, namely hydroxyl radical ($HO^{\cdot}$) and $^{\cdot}NO_2$ [20–22]. Ultimately, ONOOH products lead to the formation of nitrate (NO_3^-). $ONOO^-$ can also react with carbon dioxide (CO_2) with formation of an adduct, the $ONOOCO_2^-$ [23] with a rate constant of $(3–5.8)\times10^4$ M^{-1} s^{-1} at 37°C (pH-independent) [23,24]. $ONOOCO_2^-$ decomposition then leads to the formation of CO_2 and NO_3^- (about 70%) and also of the free radicals $^{\cdot}NO_2$ and carbonate radical anion ($CO_3^{\cdot -}$) (about 30%) [25– 26]. Taking into account the high CO_2 concentration in physiological conditions, it is provable that most of $ONOO^-$ effects *in vivo* are in fact mediated by these reactive intermediates [27– 28].

$ONOO^-$ has been shown to oxidize a variety of biomolecules including thiols, lipids, proteins, carbohydrates, DNA, and low-molecule weight anti-oxidants (such as ascorbate, glutathione, NAD(P)H, uric acid, α-tocopherol) via complex and strongly pH-dependent oxidative reaction mechanisms [3,17,21,28–39]. The most studied effect in proteins has been the nitration of tyrosine into 3-nitrotyrosine, although tryptophan and phenylalanine may also suffer nitration. On the other hand, methionine has been shown to be oxidized by $ONOO^-$ into the corresponding sulphoxide [43]. $ONOO^-$ (like HOCl) provokes inactivation of α_1-antiproteinase by this last mechanism [44]. $ONOO^-$ has thus been implicated in an increasing list of diseases, including arteriosclerosis, cardiovascular diseases, inflammation, ischemia-reperfusion, septic shock, cancer, diabetes, asthma, and neurodegenerative disorders such as Alzheimer's or Parkinson's diseases [17,26,27,42,45–47].

RNS present some characteristics that complicate their detection, namely their very short lifetime and the normal presence of a variety of endogenous antioxidants capable of scavenging these reactive species, when the studies are performed in biological matrices. The development of methodologies capable of circumvent these difficulties is thus of fundamental importance. Fluorescence probes are especially important due to their high sensibility and usefulness in temporal and spatial monitoring of RNS, particularly in microanalysis conditions in biological media akin to cells or tissues [48,49]. The objective of the present review is to give an account of the fluorescence probes that have been used for detection of these RNS in biological and nonbiological media.

FLUORESCENCE PROBES USED FOR DETECTION OF NITRIC OXIDE

2,3-Diaminonaphthalene (DAN)

2,3-Diaminonaphthalene (DAN) is an aromatic diamino compound, virtually non-fluorescent, which rapidly reacts with the NO-derived N-nitrosating agent (N_2O_3) generated from the interaction of $^{\cdot}NO$ with O_2 or from the acid-catalyzed formation of nitrous acid from nitrite, leading to the formation of a highly fluorescent product, 2,3-naphthotriazol (NAT) ($\lambda_{excitation} = 375$ nm; $\lambda_{emission} = 415$ nm) (Fig. 1) [50,51].

The fluorescence intensity increases in a concentration-dependent manner on addition of $^{\cdot}NO$ to the DAN solution. Of note, $^{\cdot}NO$, NO_2^-, and NO_3^- cannot directly convert DAN into the corresponding triazole compound, NAT. Therefore, it is possible to detect $^{\cdot}NO$ without the inhibition of its biological functions [50].

The fluorescence intensity for NAT is at least 90- to 100-fold higher than that observed for an equimolar concentration of DAN when the solution is excited at 375 nm and emission is monitored at the emission maximum for NAT at 415 nm. The detection limit for $^{\cdot}NO$ is 10 nmol/L. The N-nitrozation of DAN offers the additional advantages of specificity, sensitivity, and versatility. This assay may be used to quantify $^{\cdot}NO$ generated under biological relevant conditions. The enhanced sensitivity of this assay provides a valuable tool for the determination of small amounts of $^{\cdot}NO$ [50–52]. It is nevertheless necessary to take into account that the production of N_2O_3 follows a slow kinetic profile. Therefore, the use of the oxidant reagent 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO), in the assay system, has been proposed, which accelerates this step-transforming methodology in a fast screening assay (Fig. 1) [53].

Nevertheless, in using DAN as the detection probe for $^{\cdot}NO$ under neutral conditions, some problems have been found, namely cytotoxicity and strong autofluorescente due to the requirement of UV light for excitation, small extinction coefficient, and poor solubility in neutral buffer [53]. Notwithstanding, DAN has been successfully used for detecting not only high $^{\cdot}NO$ concentrations found in activated macrophages, but also low $^{\cdot}NO$ levels released from cultured endothelial cells [54,55] and rat aortic smooth muscle cells [53]. Recently, the use of DAN at physiological pH, was recently improved by previously incorporating DAN in β-cyclodextrin (β-CD) [56]. The inclusion of DAN into the β-cyclodextrin did not prevent the formation of NAT, which remained inside the cyclodextrin, and increasing considerably its fluorescence quantum yield at neutral pH. A plot

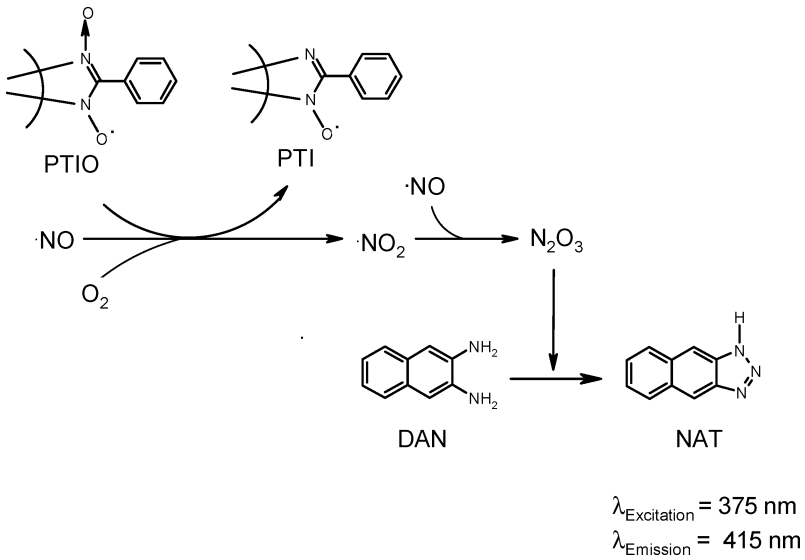

Fig. 1. Mechanism of acceleration by PTIO in the conversion of DAN to NAT (adapted from Ref. 50).

between NO concentration and DAN/β-CD showed a linear relationship and a detection limit of 20 nM.

Park and Kostkra [57] developed a DAN-based methodology for detection of biological *S*-nitrosothiols based on quantitative conversion of nitrous acid released from *S*-nitrosothiols to NAT in a reaction with acidified DAN. Disruption of the S–NO bond was achieved by treatment of samples with an excess of mercuric chloride ($HgCl_2$) (182 mM). Under such conditions, $HgCl_2$-mediated decomposition of *S*-nitrosothiols is sufficiently rapid to ensure that the decomposition rate is not a limiting factor in the formation of NAT [57].

Of note, the nitroxyl (NO^-/HNO) donor Angeli's salt (disodium diazen-1-ium-1,2,2-triolate) is highly effective in nitrosating DAN [58]. Thus, formation of NAT must not necessarily result from autoxidation of ·NO.

Diaminofluoresceins (DAFs)

Diaminofluoresceins (DAFs) (Fig. 2) are fluorescein derivatives widely used as ·NO probes [59,60]. The fluorescent chemical transformation of DAFs is based on the reactivity of the two aromatic vicinal amines, attached to fluorescein, with ·NO in the presence of O_2, via triazole ring formation, yielding a highly green-fluorescent compound [59,60].

DAFs do not react directly with ·NO, but rather with the oxidized form of ·NO. In fact, it has been proposed that the reaction mechanism of DAFs with ·NO involves N_2O_3 (Fig. 3) [66]. The lack of direct reactivity of DAFs with ·NO is important because the measurement should not interfere with signal transduction mediated by ·NO.

Kojima *et al.* [60] showed that DAFs do not react in neutral solution with other oxidized forms of ·NO, such as NO_2^- or NO_3^-, neither with other reactive species like $O_2^{\cdot-}$, H_2O_2, and $ONOO^-$, to yield any fluorescent product. Also, under physiological conditions, triazolofluorescein is not formed in the absence of ·NO [60].

Conversion of DAFs to the corresponding triazole forms causes little change in the absorbance maxima, but greatly increases the quantum efficiency [60]. DAF-2 and DAF-4 possess a slightly higher sensitivity for ·NO than the other DAF derivatives. The detection limit for ·NO by DAF-2 is 5 nmol/L [50,60].

In order to efficiently load the cells with the probe, the DAF diacetate derivative (DAF-2 DA) was prepared and tested in cellular systems. DAF-2 DA was shown to permeate readily into the cells, where it is hydrolyzed by intracellular esterases existing in many cell types, to generate DAF-2 (Fig. 4) [50,60]. Since the reactive DAF-2 is only generated within the cells, DAF-2 DA cannot be used to monitor extracellular NO and, thus, is not suitable for spatial NO-imaging.

The fluorescence intensity of the fluorescein structure is almost completely quenched when the phenolic OH group is protonated. The protonation of the phenolic OH significantly reduces the fluorescence intensity because of the blue shift of the absorption wavelength due to hindrance of electron delocalization, whereas deprotonation

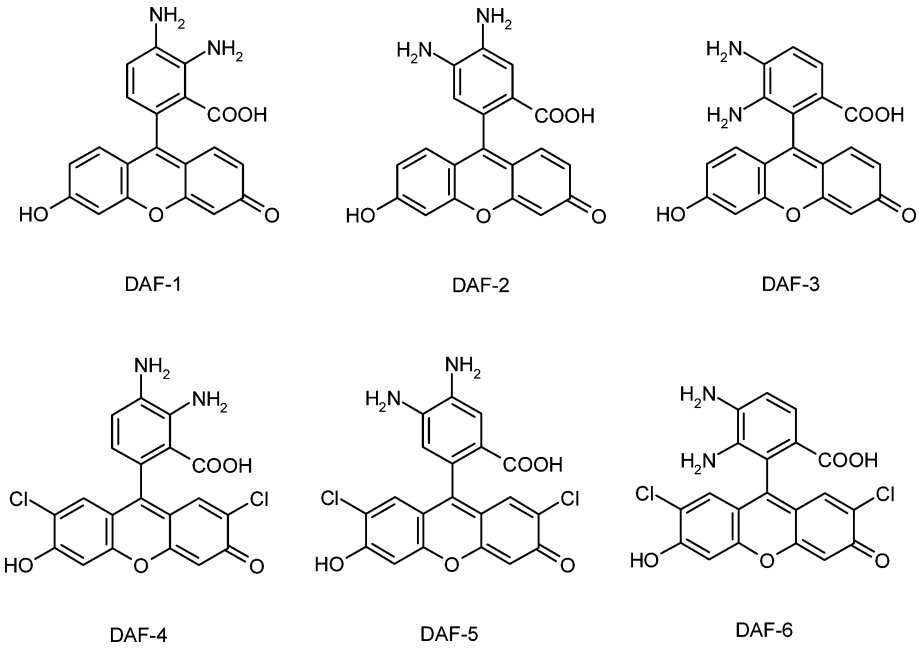

Fig. 2. Chemical structures of DAFs, numbered 1–6 (adapted from Ref. 53).

of the triazole ring slightly decreases the intensity because of the small electron donation to the conjugated double bonds. DAFs exhibit similar properties. Thus, the fluorescence of DAF-2T decreases significantly below pH 7 due to the protonation of the phenolic OH. For the same reason, DAF-1, DAF-2, and DAF-3 are only useful in media above pH 7. DAF-4, DAF-5, and DAF-6 with the electron-withdrawing chlorine substituent adjacent to the phenolic OH were synthesized in order to lower the pK_a value, with the aim of obtaining a stable fluorescence intensity at physiological pH. The resulting excitation and emission wavelengths of these probes were shifted to longer wavelengths comparatively to those of DAF-1, DAF-2, and DAF-3. However, the lower pK_a results in fluorescence instability at pH values from 7 to 9. Indeed, the chlorine substitution, lowering the pK_a value of phenolic OH, results in unstable fluorescence intensity around physiological pH [50,60]. Since the instability may arise from the triazole proton, Nagano *et al*. [53] introduced a methyl group in the molecule, leading to the formation of DAF-4 M1, DAF-4 M2, DAF-5 M1, and DAF-5 M2 (Fig. 5). DAF-4 M2 was subsequently shown to be the most sensitive to ·NO. The fluorescence intensity of the triazole form of DAF-4 M2 is stable at pH > 5.8.

Taking into account that fluorinated fluorescein derivatives are more resistant to photobleaching than fluorescein, Nagano *et al*. [50] also replaced the chlorine atom by a fluorine atom, and this new probe was named DAF-FM (Fig. 5). Examination of photobleaching in sunlight showed that the triazole form of DAF-FM (DAF-FM T) is more stable than DAF-2T and DAF-4T [50]. The sensitivity of DAF-FM is 1.4 times higher than that of DAF-2, which may be due to the electron-donating effect of the methyl group. Its detection limit for ·NO is 3 nmol/L [50].

DAFs possess favorable properties for cellular imaging applications. Indeed, DAFs have an excitation wavelength at visible light (495 nm), which is less damaging to cells than the UV light needed for other probes, and is not subject to interference from the autofluorescence of biological samples [60]. It was also shown that the reactivity of DAFs is independent of Ca^{2+} or Mg^{2+} at physiological concentrations [62]. Other advantage of these compounds is that the wavelength associated with fluorescein can be used, making equipment currently used for other bioassays as well as cell and tissue imaging, already using fluorescein, easily adapted for detection of ·NO *in vitro* and *in vivo* [51]. Fluorescein has been used in many applications because of its pH sensitivity and high fluorescence quantum yield [62].

Several studies have shown the usefulness of DAFs for the detection of ·NO in biological tissues. DAFs can readily detect either the high concentration of ·NO produced by activated macrophages or the low concentration of ·NO in endothelial cells in a concentration-dependent manner. In both types of cells, the use of NOS inhibitors prevents the increase in fluorescence intensity [50,61].

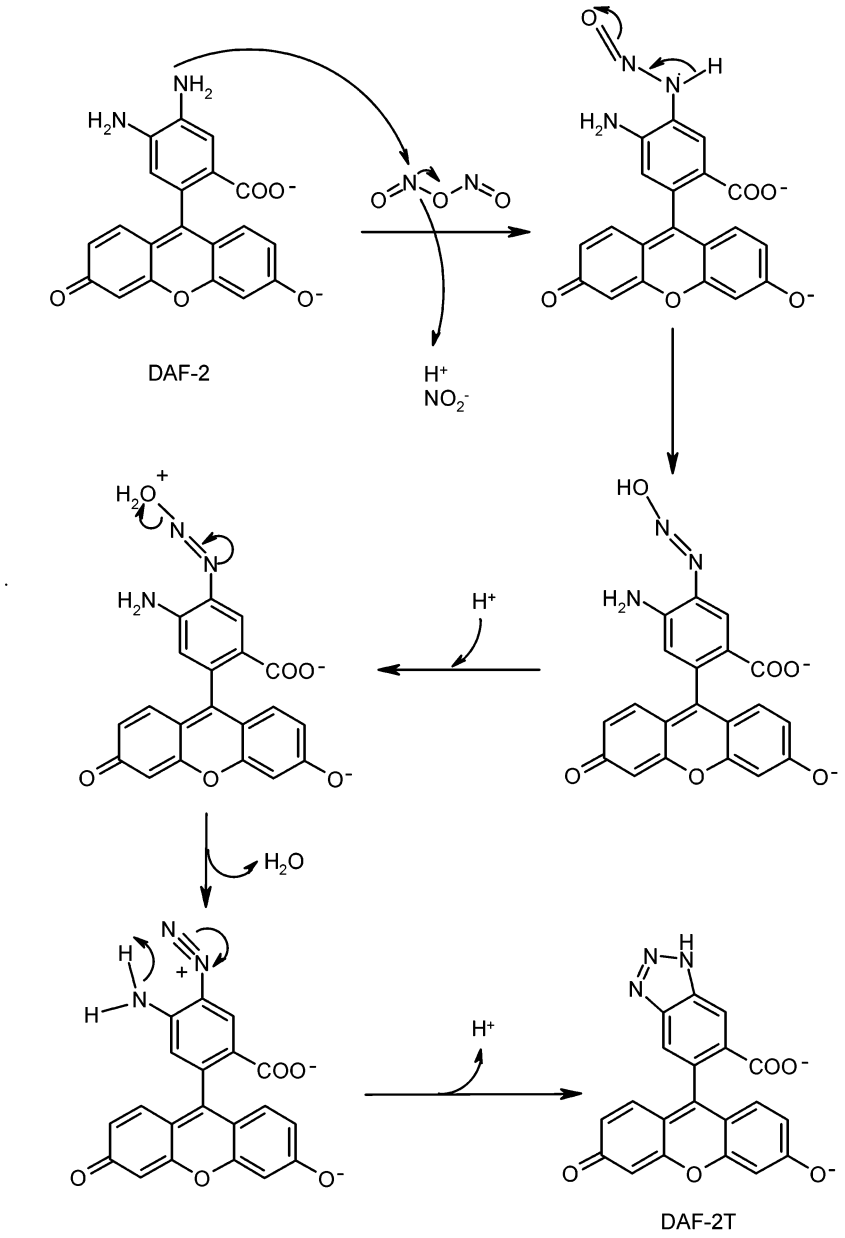

Fig. 3. Proposed mechanism in the reaction of DAF-2 with ·NO in presence of O_2 (adapted from Ref. 61).

Thus, since their development in 1998 by Kojima *et al.* [59], DAFs have been widely applied for ·NO detection and imaging in biological systems, being employed in more that 100 scientific reports in the last two years. This type of probes can also be extremely useful for the screening of ·NO scavenging activities [64–66].

Concerning to the limitations of DAFs as indicators of ·NO, it has recently been shown that NO^-/HNO reacts with DAF-2 giving even higher yields of triazole than ·NO [67]. In light of the potential presence of NO^-/HNO in biological systems, it could be interesting to entertain the possibility that some of the ·NO detected by DAF-2 is in fact NO^-/HNO. One cautionary note is that using powerful light sources such as lasers can result in photochemistry that can lead to false positives [51]. Taking into account that, like DAN, DAFs do not react directly with ·NO, but react with the oxidized form of ·NO (probably N_2O_3), there is a slow step during the process that

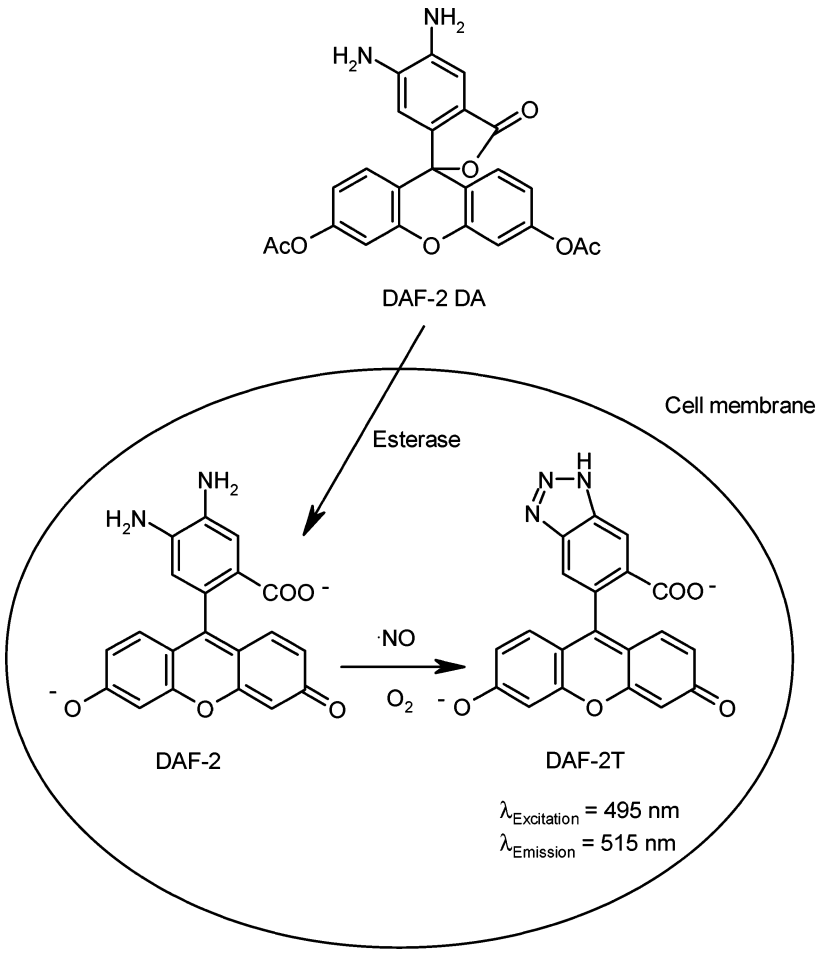

Fig. 4. Schematic representation of DAF-2 DA loading mechanism in the cell and formation of DAF-2T from DAF-2 by $^{\cdot}NO$ in the presence of O_2 (adapted from Ref. 61).

generates this species, which is the formation of $^{\cdot}NO_2$ from $^{\cdot}NO$. Thus, the rate at which $^{\cdot}NO$ is oxidized imposes an intrinsic limitation on detection by this method [60]. DAF-2 cross-reacts with dehydroascorbic acid (DHA) to produce fluorescent compounds, termed DAF-2-DHAs, while ascorbic acid (AA) and other reducing agents like glutathione, $O_2^{\cdot -}$, catecholamines, dithiothreitol, and 2-mercaptoethanol, attenuate or abolish the formation of DAF-2T [68,69]. One possible mechanism for the inhibition of the fluorescence reaction is the direct interaction of the above-mentioned reducing compounds with DAF-2, by protonation of its phenolic OH groups. An alternative mechanism could be considered with respect to the interaction of $^{\cdot}NO$ with those compounds, which would reduce the effective concentration of $^{\cdot}NO$ for the reaction with DAF-2 [69]. Since several of these compounds are found in mM levels in the same cell types that are often involved in $^{\cdot}NO$ signaling, this is a particularly confounding cross-reaction. Although the use of NOS inhibitors and $^{\cdot}NO$ scavengers along with DAF-2 allows the detection of $^{\cdot}NO$ production, quantification using DAF-2 is hampered by the complexity of biological systems and the cross-reactivity of DAF-2. Nevertheless, it was shown that incubation of tissues with DAF-2 DA produces intracellular concentrations of DAF-2 that approach the mM range [70]. Such a high concentration is beneficial, inasmuch as it enables the probe to compete with scavenging by cellular antioxidants, but detrimental as its autofluorescence contributes significantly to the overall background fluorescence from tissue samples. The resulting loss in sensitivity (approaching three orders of magnitude compared to those in buffer solutions) makes detection of sources and targets of NO in tissues by fluorometry difficult under basal conditions [70]. A new method of assaying $^{\cdot}NO$ using DAF-2 that eliminates these interferences was recently reported [71]: when frozen on dry ice, the $^{\cdot}NO$ in the

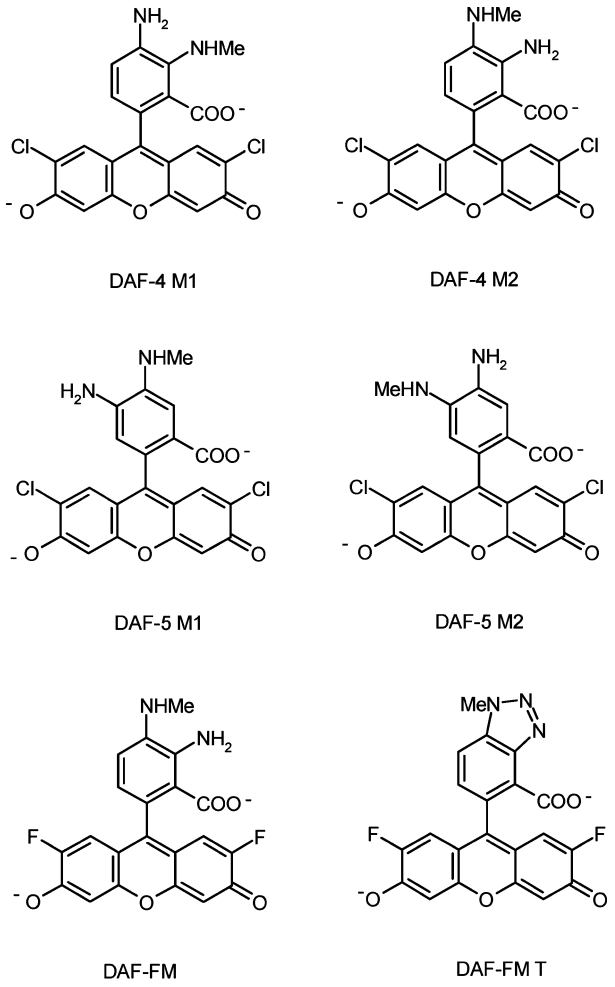

Fig. 5. Chemical structures of DAF-4, DAF-5, and DAF-FM derivatives (adapted from Ref. 50).

original solution still diffuses and can react with an adjacent frozen block of DAF-2, but the confounding compounds such as DHA do not. Thus, placing the microliter-volume frozen blocks of solutions containing ·NO and the solutions of DAF-2 adjacent to each other for 30 min results in the concentration-dependent formation of fluorescent product (DAF-2T) from the reaction of ·NO with DAF-2.

Jour'heuil [72] verified that incubation of DAF-2 with $ONOO^-$ or with peroxidase and H_2O_2, increases the production of DAF-2T in the presence of a ·NO generator. This is due to the formation of a relative stable intermediate of DAF-2 that directly combines with ·NO to bypass the ·NO/O_2 reaction (slow step in DAF-2T production) [72]. This finding suggests that results from DAF-2 fluorimetric assays are quantitatively difficult to interpret in cells and in solutions when oxidants and ·NO are co-generated. The intracellular oxidation of DAF-2 may result in increased ·NO-dependent fluorescence that may be mistakenly taken as an increase in ·NO production [72].

Diaminorhodamine (DAR-4M AM)

In 2001, Kojima *et al.* [73] reported the development of a new membrane-permeable fluorescent indicator for ·NO. This indicator is based on the rhodamine chromophore and, in contrast to DAF-2, shows no pH-dependent fluorescence and is more photo-stable [72]. This indicator was named DAR-4M AM (acetoxymethyl ester of DAR-4M) (Fig. 6). The DAR-4M reacts irreversibly with ·NO in the presence of O_2 to produce the triazole form of DAR-4M, DAR-4M T ($\lambda_{excitation} = 560$ nm; $\lambda_{emission} = 575$ nm) (Fig. 6). The fluorescence quantum yield of the product after reaction with ·NO is 840 times higher than that of DAR-4M. The detection limit for ·NO was 7 nM, and the fluorescence showed no pH dependency above pH 4 [73].

DAR-4M should offer a good signal-to-noise ratio in the examination of biological samples due to the low background fluorescence with the longer wavelength excitation, even though the quantum yield of DAR-4M T is lower than that of fluorescein dyes.

DAR-4M AM was applied to the bioimaging of ·NO in bovine aortic endothelial cells, for which a high sensitivity to ·NO is required [73] and also to the measurement of ·NO production in the postnatal mouse neocortex [73]. Nevertheless, further control experiments are required to confirm that this diamino-rhodamine-derivative reacts specifically with ·NO and not with other reactive oxygen species (ROS) and RNS.

1,2-Diaminoanthraquinone (DAQ or DAA)

1,2-Diaminoanthraquinone (DAQ or DAA) (Fig. 7) has been shown to be a ·NO-indicator with high sensitivity, specificity, and accuracy. DAQ itself is dark-violet and non-fluorescent [75]. The reaction of DAQ with ·NO in the presence of O_2 leads to the formation of water-insoluble and red fluorescent 1H-anthra [1,2d] [1,2,3] triazole-6,11-dione (ATD) (Fig. 7). ATD can be visualized using a filter currently used for rhodamine ($\lambda > 580$ nm) under a fluorescence microscope. The change in the fluorescence behavior is attributed to the reaction of ·NO, in the presence of O_2, with the aromatic amino groups at neutral pH and formation of a condensed triazole ring by subsequent reaction with the vicinal amino group [75].

Since the product of the reaction of DAQ and ·NO (ATD) is water insoluble, it precipitates and, therefore, is not able to diffuse passively in the tissue. This feature

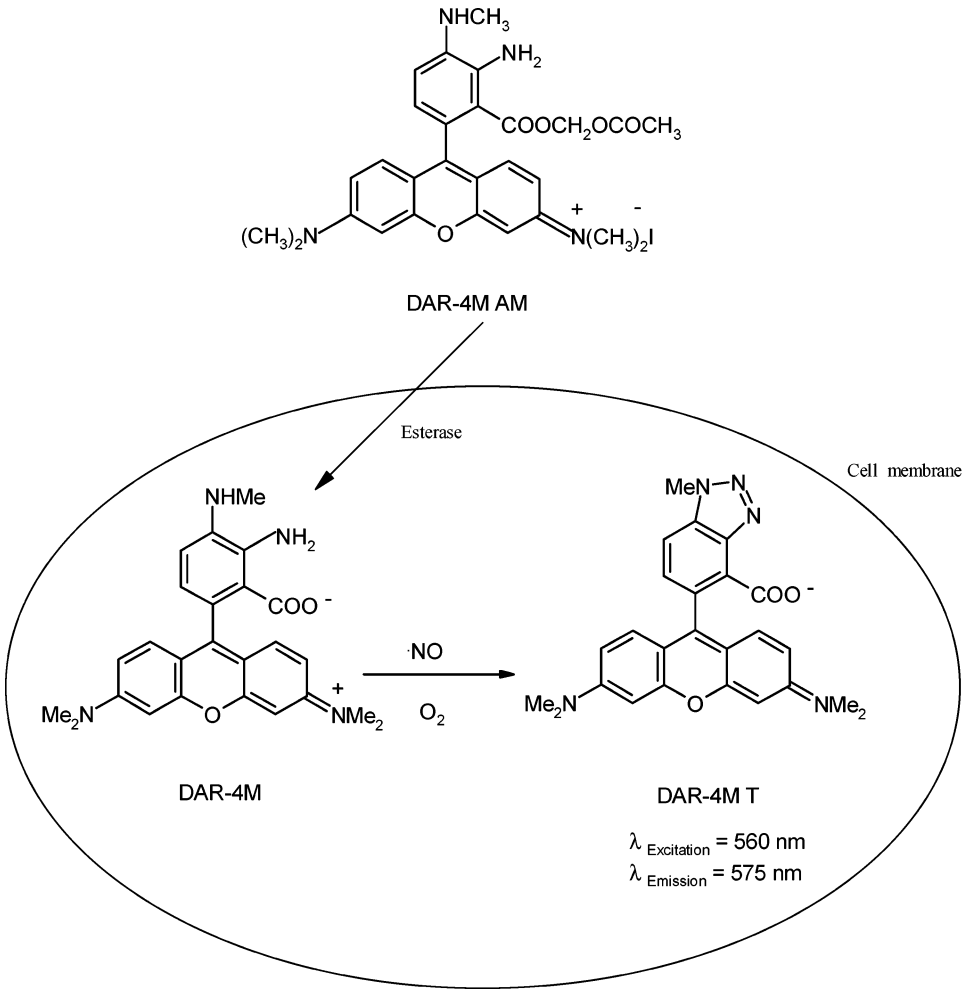

Fig. 6. Formation of DAR-4MT from DAR-4M induced by ·NO in the presence of O_2 (adapted from Ref. 73).

allows the examination of the fluorescence pattern even in fixed tissues. In addition, taking into account that DAQ does not display neurotoxic properties [76], it allows real-time imaging of ·NO production in cultured hippocampal neurons [77] and in brain slices [76,78].

Importantly, the increase in fluorescence can be inhibited by ·NO-scavengers or NOS-inhibitors [4,75], and no reaction of DAQ was observed with H_2O_2, a potential source of oxygen radicals [75,77].

Fluorescent Nitric Oxide Cheletropic Traps (FNOCT)

Fluorescent nitric oxide cheletropic traps (FNOCT) are fluorescent molecules that specifically trap ·NO by means of a formal cheletropic reaction (NOCT: nitric oxide cheletropic trap). This methodology is different to most of the other known fluorescence assays for ·NO in the sense that the fluorophore is produced directly by the reaction of the reagent with ·NO, and also leads to a permanent incorporation of the latter in the fluorescent product. Thus, ·NO is trapped without its prior transformation to other nitrogen oxides, which assures its specificity for ·NO [79,80].

The fluorescence methods proposed for biological applications so far produce fluorescence only indirectly and require prior transformation of ·NO into other more reactive RNS, for example, NO^+, $\cdot NO_2$, or N_2O_3. This affects the specificity of such methods, always leaving some ambiguities concerning the truly detected species [79,80].

The principle of methodologies using FNOCT is based on the fact that the reaction of *o*-quinodimethane derivatives (A) (Fig. 8) with ·NO generates nitroxide radical adducts with an aromatic phenanthrene unit as the active fluorophore (B) (Fig. 8). The parent compounds

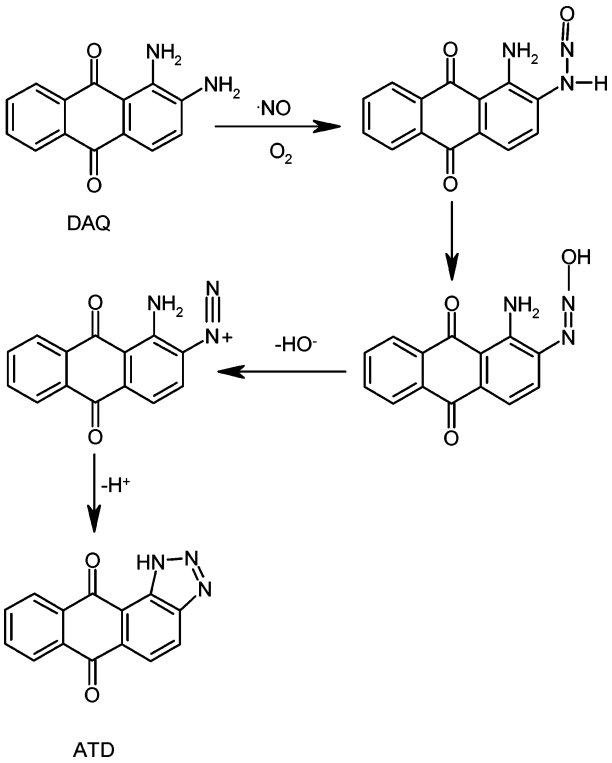

Fig. 7. Postulated reaction of DAQ with ·NO, in the presence of O_2, to form the fluorescent triazole derivative (ATD) (adapted from Ref. 75).

(A) should be either non-fluorescent or should show fluorescence at different excitation and emission wavelengths relatively to the products (B). Taking into account that nitroxide radicals are fluorescence quenchers, the fluorescence intensity increases dramatically when the nitroxide radicals (B) are reduced to the corresponding hydroxylamines (C) (Fig. 8) [79].

The FNOCT methodology can be advantageously applied in cell biological studies (i) to specifically and quantitatively determine overall ·NO production in the nanomolar concentration range by a small number of cells, and (ii) by using fluorescence microscopy to monitor ·NO production by single cells with high temporal and spatial resolution [80].

The specificity of FNOCTS for the trapping of ·NO is demonstrated by the fact that the increase in fluorescence intensity in the presence of H_2O_2, H_2O_2/Fe(II), or $O_2^{\cdot-}$ is very similar to the increase in pure buffer solution, indicating insensitivity toward these ROS [79]. However, $ONOO^-$ reacts with FNOCT-4 as it may be noticed by an increase of fluorescence and the disappearance of the color of FNOCT-4 after addition of a small excess of $ONOO^-$. Thus, a large excess of $ONOO^-$ leads to a decrease of the fluorescence [79]. Nevertheless, this methodology has been applied successfully in cell cultures [80–82].

One of the most studied FNOCT compounds, FNOTC-5 (Fig. 8), is a red fluorescent substance ($\lambda_{excitation} = 460$ nm; $\lambda_{emission} = 600$ nm), which is loaded into the cells. Within the cell, FNOCT-5, an acetoxymethylester of a dicarboxylic acid of an *o*-quinodimethane derivative, is enzymatically cleaved (Fig. 8). This product remains in the cells because of its negative charge. The product then reacts within the cell with ·NO and the formed product displays a blue fluorescence ($\lambda_{excitation} = 380$ nm; $\lambda_{emission} = 460$ nm) [4,80].

Metal-Based NO Sensing by Selective Ligand Dissociation

Franz *et al*. [83] prepared a ligand containing a fluorophore that is quenched at the metal center, in this case Co(II) (Fig. 9). In the absence of ·NO this ligand only gives little residual fluorescence signal. In the presence of ·NO, however, the formation of a metal–nitrosyl adduct selectively displaces the fluorescent ligand, thereby removing it from the quenching environment and turning the fluorescence "on."

The complex [Co(DATI-4)] (Fig. 9) is stable in both the solid state and solution, and reacts readily with ·NO. The ·NO detection limit of [Co(DATI-4)] was estimated as 50–100 μM [83].

Acridine-TEMPO-DTCS-Fe(II)

Acridine-TEMPO-DTCS-Fe(II) is a complex composed of 2,2,6,6-tetramethyl-piperidine-*N*-oxyl (TEMPO) labeled with acridine and *N*-dithiocarboxysarcosine (DTCS)-Fe(II) (Fig. 10) [48,84]. The use of this fluorescent probe is based on a novel "spin exchange" concept that was developed for monitoring ·NO production. This spin exchange concept was inspired in the activation mechanism of guanylate cyclase by ·NO.

Guanylate cyclase is one of the major intracellular targets of ·NO. This enzyme has a heme moiety in the regulation domain to which the imidazole group in the histidine residue coordinates. ·NO binds strongly to the heme iron to dissociate the imidazole group through a strong electron-withdrawing effect. This event effects a conformational change of the protein to promote the enzymatic activity. This reaction is highly selective for ·NO, because ·NO binds strongly to Fe(II) to create an extremely stable nitrosyl–Fe complex.

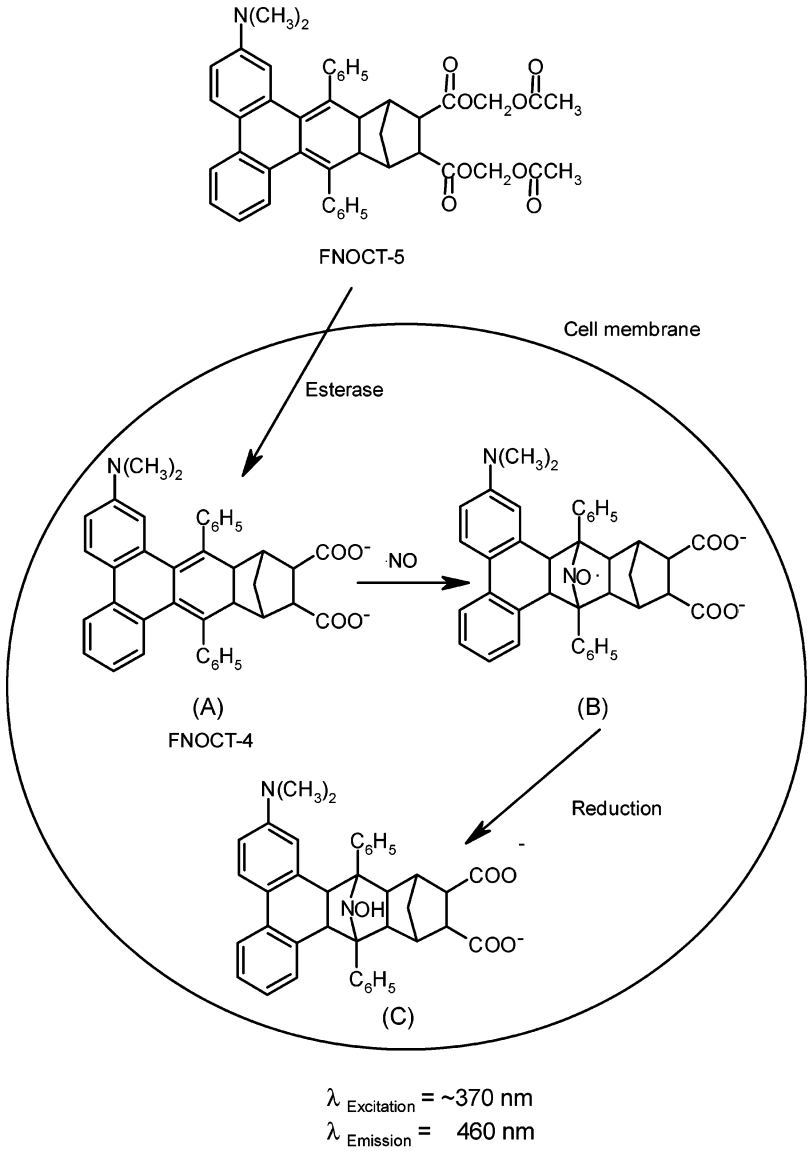

Fig. 8. Schematic representation of FNOCT-5 loading mechanism in the cell (adapted from Ref. 80).

In the "spin exchange" methodology, the DTCS–Fe(II) complex and the TEMPO derivatives are used instead of the heme and the imidazol group, respectively. ·NO binds to the iron in the DTCS–Fe(II) complex to liberate the TEMPO due to the extremely high stability of NO–DTCS–Fe(II). Thus, the unstable ·NO radical is exchanged for the stable TEMPO radical, which can be detected in high sensitivity by the EPR technique [48,84]. To utilize this mechanism in the fluorescent detection of ·NO, the acridine–TEMPO may be used.

When the non-fluorescent acridine–TEMPO is incubated with DTCS–Fe(II) complex in buffer solution, the nitroxide radical in the acridine–TEMPO interacts with the Fe(II) through a redox interaction (Fig. 10). This interaction recovers the fluorescence based on the acridine moiety. The presence of ·NO causes the displacement of acridine–TEMPO from the DTCS–Fe(II) complex, due to the irreversible binging of ·NO to the Fe(II). This effect leads to a fluorescent decrease of the probe in a ·NO concentration-dependent manner.

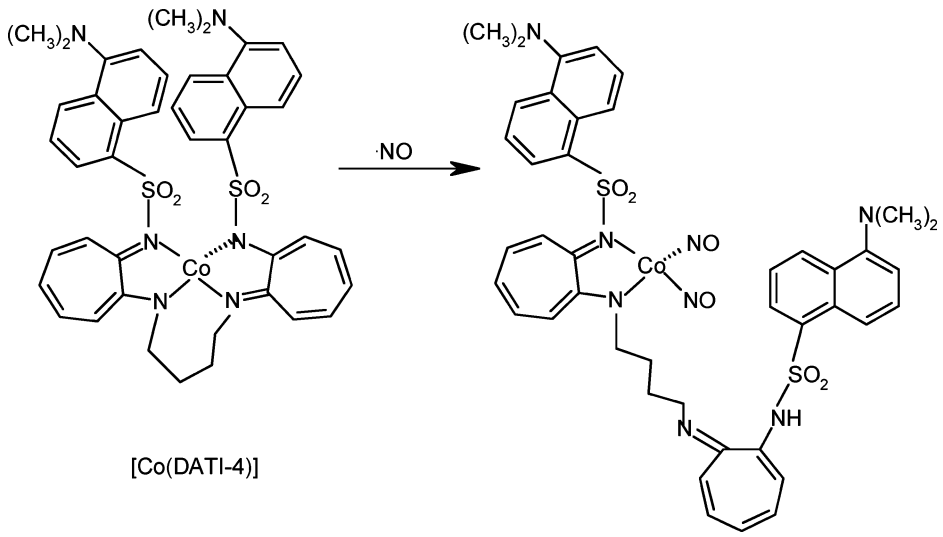

Fig. 9. Formation of a cobalt–dinitrosyl complex results in ligand dissociation and fluorescence enhancement (adapted from Ref. 83).

Using this probe, less than 100 nM of ·NO can be detected [48]. A problem to be considered is the effect of O_2, which tends to compete with the acridine–TEMPO for the binding to DTCS–Fe(II) complex. Although the binding ability of O_2 is weaker than that of acridine–TEMPO, a small inhibition of the fluorescent recovery of acridine can be seen [48].

8-(3,4-Diaminophenyl)-2,6-bis(2-carboxyethyl)-4,4-difluoro-1,3,5,7-tetra-methyl-4-bora-3a,4a-diaza-s-indacene (DAMBO-P^H)

8-(3,4-Diaminophenyl)-2,6-bis(2-carboxyethyl)-4,4-difluoro-1,3,5,7-tetra-methyl-4-bora-3a,4a-diaza-s-indacene (DAMBO-P^H) (Fig. 11) was recently developed by Gabe *et al.* [85] based on the boron dipyrromethene

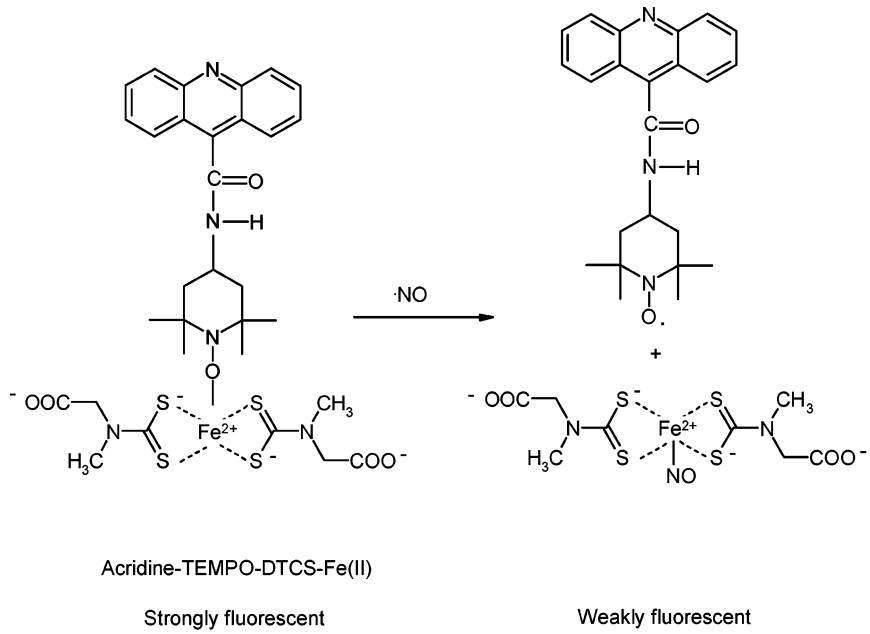

Fig. 10. A fluorescent probe for ·NO using the spin-exchange concept and its detection mechanism of ·NO (adapted from Ref. 48).

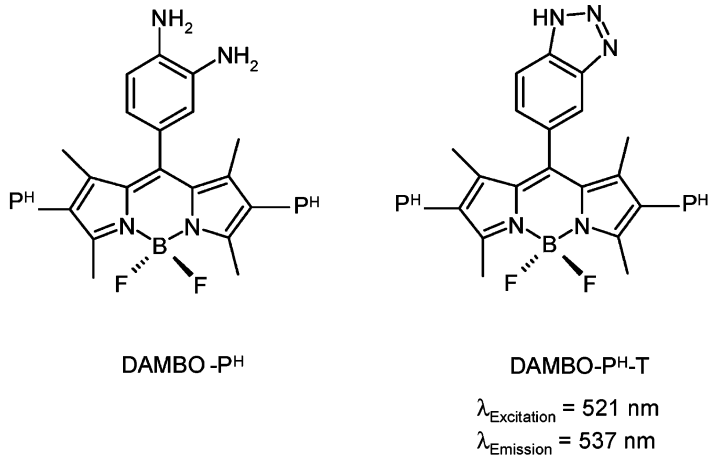

Fig. 11. Chemical structures of DAMBO-P^H and DAMBO-P^H-T (P^H: $CH_2CH_2CO_2H$) (adapted from Ref. 85).

BODIPY chromophore, as a pH-independent and highly sensitive fluorescence probe for ·NO. BODIPYs seem to be a promising platform for sensitive fluorescence probes, since these compounds generally have high extinction coefficients and high quantum efficiencies in water as well as in organic solvents. BODIPY-based fluorescence probes are easy to design and synthesize with an electron-rich reactive site for ·NO. In addition, BODIPY derivatives can emit fluorescence over a wide range from 500 to 700 nm [85].

DAMBO-P^H, which is almost non-fluorescent, in the presence of ·NO is converted into the respective highly fluorescent triazole form DAMBO-P^H-T ($\lambda_{excitation} = 521$ nm; $\lambda_{emission} = 537$ nm) (Fig. 11). However, all *o*-phenylenediamine derivatives, e.g. the DAF and DAR family, DAN and DAQ, are sensitive to intermediates that exhibit a formal NO^+ activity, like N_2O_3 [89]. Thus, DAMBO-P^H reacts not with ·NO radical but with NO^+ species to yield triazole forms.

DAMBO-P^H can be used as a probe in a wide range of pH values (3–13) [85], while most fluorescein-based probes lose fluorescence owing to lactonization reaction under acidic conditions. DAMBO-P^H can also be used in buffer solution, so it should be potentially useful for detection of ·NO in biological applications [85].

When compared with DAF-2, DAMBO-P^H showed to be a much more sensitive probe for ·NO [85]. *o*-Phenylenediamine derivatives with high electron density are essential for improvement of the ·NO probe's sensitivity. DAF-2 possesses a serious disadvantage in this regard, as the *o*-phenylenediamine moiety of DAF-2 has an electron-withdrawing carboxyl functional group. DAMBO-P^H does not have any electron-withdrawing functional group on *o*-phenylenediamine. Though DAMBO-P^H had higher reactivity, its selectivity for ·NO over various ROS remained high. Even $HO^·$, the most potent oxidant among ROS, did not generate fluorescence [85].

Pyrene-nitronyl

Pyrene-nitronyl is nitronyl nitroxide radical covalently linked to an organic fluorophore, pyrene (Fig. 12), that can be used to detect ·NO [87].

This approach is based on the phenomenon of the intramolecular fluorescence quenching of the fluorophore fragment by the nitroxide [88]. The stable radical pyrene-nitronyl reacts with ·NO to yield a pyrene-imino nitroxide radical and eliminate $·NO_2$ (Fig. 13). Transformation of the EPR spectrum of the nitronyl nitroxide to the EPR spectrum of imino nitroxide is accompanied by a drastic (32-fold) increase of the fluorescence intensity because

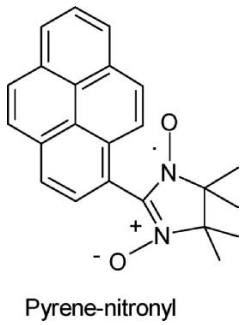

Fig. 12. Chemical structure of pyrene-nitronyl (adapted from Ref. 87).

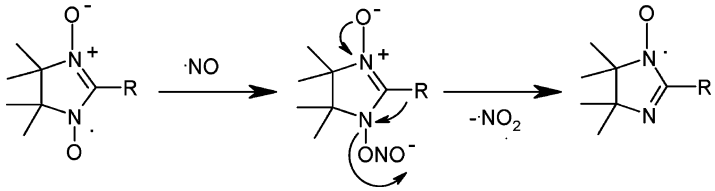

Fig. 13. Postulated mechanism for production of imino nitroxide from the reaction between ·NO and nitronyl nitroxide (adapted from Ref. 88).

the imino nitroxide is a weaker quencher of the fluorescence [87]. The fluorescence measurements enable detection of nanomolar concentrations of ·NO compared to a sensitivity threshold of only several micromolar for the EPR technique [87].

A possible problem with the detection of ·NO by nitronyl nitroxide is the fact that nitronyl nitroxides are prone to fast reduction in living systems, mainly by $O_2^{\cdot -}$ and ascorbate. Indeed, in living systems, especially in blood, which contains a relatively high concentration of ascorbate, the nitronyl nitroxides are reduced to the corresponding hydroxylamines, leading to a decrease of the EPR signal intensity and increase in fluorescence intensity [89,90]. This problem may be solved by the use of Tempol. A high concentration of Tempol (e.g. 0.1 mM) ensures that the greater part of the reducing agents present in the solution are oxidized before the added nitronyl nitroxide [87].

The reaction of the nitronyl nitroxide with ·NO was shown to be fast, with a rate constant of about 10^4 M^{-1} s^{-1} [88]. However, ·NO reacts also with O_2, which requires that the analysis should be performed under anaerobic conditions. Since the reaction is fast (rate constant of 76.4 M^{-1} s^{-1}) and essentially irreversible, the time of incubation of the tissues in absence of O_2 is up to 5 min. On the other hand, the reactions of nitronyl nitroxides with ·NO are practically irreversible processes; therefore, after completion of the reaction, the complex (nitronyl nitroxide-NO) is not affected by the presence of $\cdot NO_2$, NO_2^-, or O_2 [88].

The ability to measure ·NO or *S*-nitroso compounds released from living cells at the basal and the excited states by pyrene-nitronyl was demonstrated on mucociliary epithelial tissue from pig trachea [87]. The measured basal flux of *S*-nitroso compounds obtained from the tissues was about 1.2 nmol/g×min, and NO-synthase stimulated by extracellular adenosine 5′-triphosphate produced ·NO flux of 0.9 nmol/g×min. The universality of the application of the proposed technique to systems other than mucociliary epithelial tissue from pig trachea needs to be further evaluated.

FLUORESCENCE PROBES USED FOR DETECTION OF PEROXYNITRITE

Dihydrorhodamine 123 (DHR)

Dihydrorhodamine 123 (DHR) is a non-fluorescent molecule that, by oxidation, yields rhodamine 123 (RH), a fluorescent cationic and lipophilic probe ($\lambda_{excitation} = 505$ nm; $\lambda_{emission} = 529$ nm) (Fig. 14) [91]. The lipophilicity of DHR facilitates its diffusion across cell membranes. Upon oxidation of DHR to the fluorescent RH, one of the two equivalent amino groups tautomerizes into an imino, effectively trapping RH within cells [92].

The oxidation of DHR by $ONOO^-$ has a linear pattern over a large concentration range (0–1000 nM) [91]. The oxidation keeps constant from pH 4.2 to 8.3, and significantly decreases at pH values greater than 8.3 [91]. Kooy *et al.* [91] suggested that formation of rhodamine 123 from DHR is the result of a direct reaction with $ONOO^-$ and does not require $HO^{\cdot}$ or NO_2^+ intermediates. The use of free radical scavengers and specific enzyme inhibitors allowed Kooy *et al.* [91] to distinguish between oxidation induced by $ONOO^-$ versus other oxidant species. In that study, $HO^{\cdot}$ scavengers mannitol and DMSO had minimal effect (<10%) while SOD slightly enhanced RH production. L-Cysteine was an efficient inhibitor of DHR oxidation through competitive oxidation of free sulfhydryls. Uric acid was also an efficient inhibitor, possibly by reduction of an intermediate DHR radical and recycling of DHR. $ONOO^-$-mediated formation of RH was not dependent on metal ion catalyzed reactions because studies were performed in metal ion-free buffer and RH formation was not enhanced in the presence of Fe(III)/EDTA. Under anaerobic conditions, ·NO did not oxidize DHR and inhibited spontaneous oxidation of DHR. In the presence of O_2, ·NO induced a relatively slow oxidation of DHR due to the formation of $\cdot NO_2$ [91]. Figure 15 represents the mechanism proposed by Kooy *et al.* [91] for this reaction. DHR may undergo a single electron transfer to $ONOO^-$

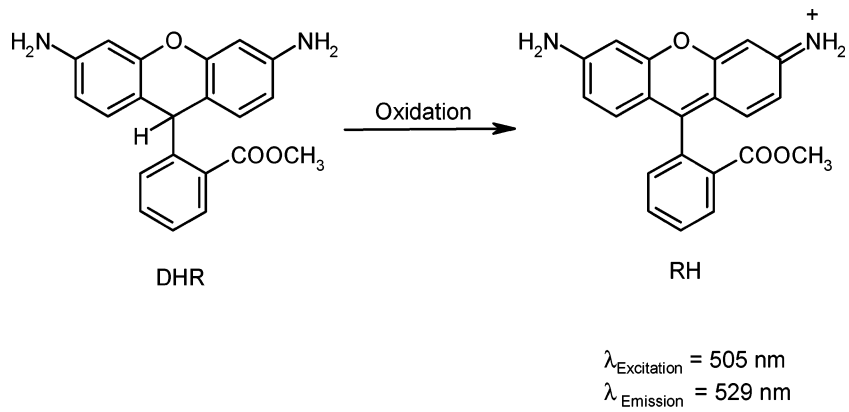

Fig. 14. $ONOO^-$ oxidation of dihydrorhodamine 123 to rhodamine 123 (adapted from Ref. 92).

to form a DHR radical, which can then dismutate to form RH and DHR. The DHR radical may reversibly reduce O_2 to form $O_2^{\cdot -}$ and RH in an aerobic environment. This reaction is very slow comparatively to the dismutation of DHR radicals and therefore its contribution to the total fluorescent product in the absence of SOD is small [91].

Nevertheless, the exact mechanism of the oxidation of DHR by $ONOO^-$ is still a question of debate. Indeed, Jourd'heuil *et al*. [93] proposed that the oxidation of DHR by $ONOO^-$ occurs via free radical intermediates ($HO^{\cdot}$ and $^{\cdot}NO_2$) formed during the spontaneous decomposition of ONOO− and excluded a direct reaction with $ONOO^-$. These findings were later corroborated by Glebska and Koppenol [94]. In their opinion, the reaction of DHR with $ONOO^-$ is zero-order; thus, it can hardly be a direct reaction between $ONOO^-$ and DHR. Similarly, if oxidation of DHR was caused by reaction of intermediates formed during the decay of HONOO, one would expect a higher yield of oxidation at lower pH, rather than at higher pH, as it was observed by Glebska and Koppenol [94]. On the basis of these findings, Glebska and Koppenol [94] proposed possible reaction pathways depicted in Fig. 16. These pathways were also proposed to be applicable to other fluorescence probes as is the case of 2,7-dichlorodihydrofluorescein.

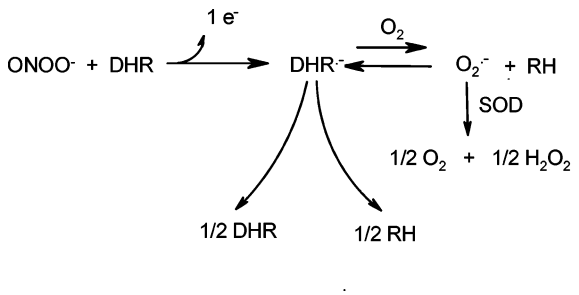

Fig. 15. Proposed mechanism for $ONOO^-$-mediated oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123 (RH) (adapted from Ref. 91).

Crow [92] corroborated the ability of DHR to react efficiently with $ONOO^-$ even at very low concentrations, which makes it useful for detecting $ONOO^-$ formation both *in vitro* and in cells. This probe has also been used in several *in vitro* studies to evaluate $ONOO^-$ scavenging activity of different compounds [65,66,95–97].

As mentioned before, at physiological levels of carbon dioxide the dominant pathway of reaction involves interaction between ONOO− and CO_2 and leads to rapid production of $^{\cdot}NO_2$ and $CO_3^{\cdot -}$ with a yield of about 30%. It has been shown that these species were capable of oxidizing DHR [98]. Of note, RH also interacts with $CO_3^{\cdot -}$, although much more slowly than DHR, and does not interact with $^{\cdot}NO_2$ [98].

DHR can also be oxidized by Fe(II), Fe(III)/ascorbate, Fe(III)/EDTA, cytochrome *c*, HOCl, or H_2O_2 in the presence of peroxidases [95,102–104]. DHR is not oxidizable by H_2O_2 alone, by $O_2^{\cdot -}$, and by xanthine/xanthine oxidase [91,92,99,100].

2,7-Dichlorodihydrofluorescein (DCFH)

2,7-Dichlorodihydrofluorescein (DCFH) oxidation yields 2,7-dichlorofluorescein (DCF), a fluorescent compound ($\lambda_{excitation} = 498$ nm; $\lambda_{emission} = 522$ nm) (Fig. 17). DCFH was initially referred as an indicator for H_2O_2 [105]. However, it was already avowed that DCFH can also be oxidized by other ROS like $HO^{\cdot}$ and $ROO^{\cdot}$ as well as RNS like $^{\cdot}NO$ and $ONOO^-$ [92,94,103,104]. Yet, the suitability of DCFH for $^{\cdot}NO$ detection is still controversial. Although some authors refer DCF formation in the presence of $^{\cdot}NO$, in cells and cell-free systems [105,106], others suggest that DCFH hardly reacts with $^{\cdot}NO$ while

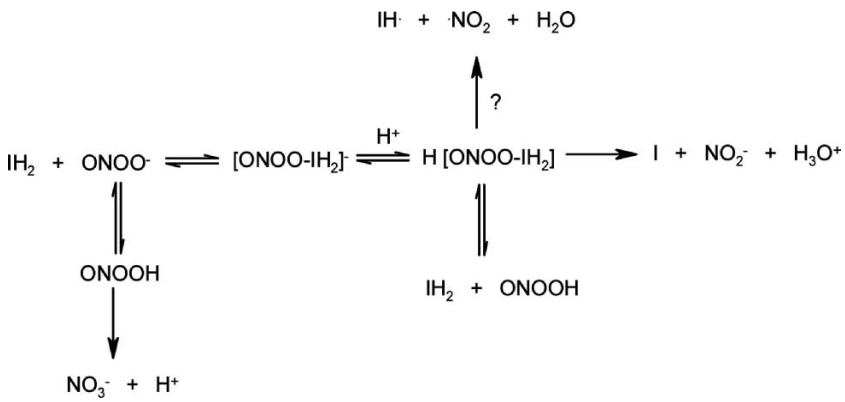

Fig. 16. Proposed mechanism for $ONOO^-$-mediated oxidation of dihydrorhodamine 123 (IH_2) and 2,7-dichlorodihydrofluorescein (IH_2) (adapted from Ref. 94).

$ONOO^-$ strongly oxidizes the probe [92,103,107]. In the sequence of these experiments, Myhre *et al*. [108] investigated, in cell-free systems, the DCF formation in the presence of *S*-nitroso-*N*-acetylpenicillamine (SNAP), a ·NO-generator, and 3-morpholinosydnonimine hydrochloride (SIN-1), that generates both ·NO and $O_2^{\cdot -}$ resulting in the formation of $ONOO^-$. While, in the presence of SNAP, the fluorescence increase did not occur to a significant extent, the addition of SIN-1 induced a robust and fast increase of DCF formation. An identical experiment had already been performed by Wang and Joseph [104]. These authors used sodium nitroprusside (SNP) instead of SNAP as a ·NO generator. While SNP did not lead to a significant fluorescence increase, SIN-1 showed to be a potent DCFH pro-oxidant [104]. These results support the idea that $ONOO^-$ is more potent than ·NO in oxidizing DCFH. Thus, it may be considered that those who suggest that ·NO is responsible for DCF formation may not have taken into account the formation of $ONOO^-$. On the other hand, Crow [92] suggests that, in those cases where ·NO

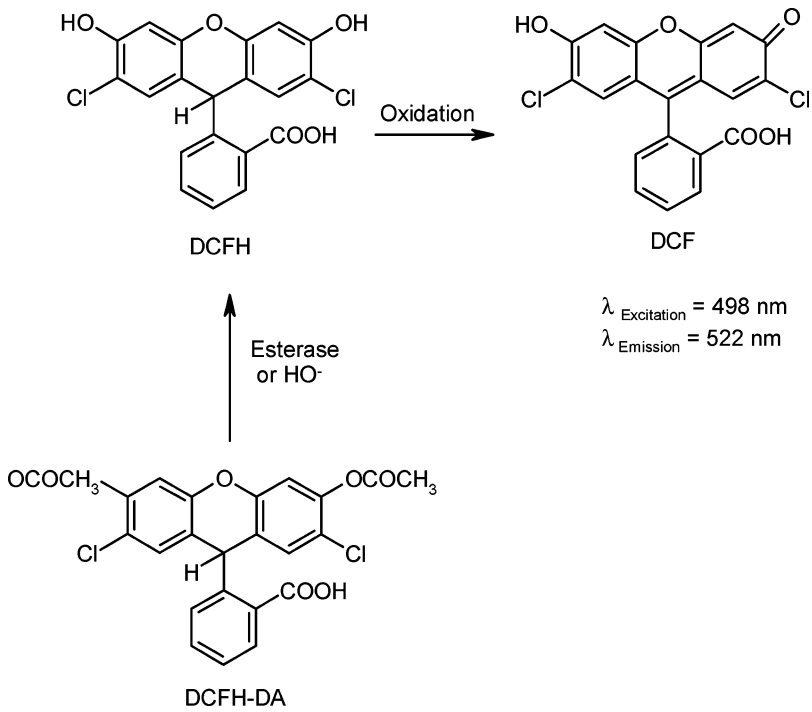

Fig. 17. Mechanism of DCFH-DA de-esterification to DCFH, which is oxidized to fluorescent DCF by ROS and RNS (adapted from Ref. 92).

was reported to oxidize DCFH, $^{\cdot}NO_2$ might have been the responsible species. However, in this situation, the $^{\cdot}NO$ concentrations needed to oxidize DCFH would be 10- to 100-fold greater than those that would be encountered *in vivo*, even under pathologic conditions [92].

This probe can be applied in cellular studies by using the derivative 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Fig. 17), which crosses the cell membranes and is enzymatically hydrolyzed by intracellular esterases to DCFH [101,102,109].

Exogenously applied $ONOO^-$ to DCFH-DA loaded cells resulted in a steady increase in DCF fluorescence. This effect strongly suggests that $ONOO^-$ is capable of crossing intact cell membranes [107]. In contrast to the commonly used $ONOO^-$ probe DHR, when DCFH is oxidized it is not translocated to subcellular structures as it happens with RH, the oxidation product of DHR, which accumulates within intact mitochondria. This feature of DHR limits its application especially in experimental designs that alter the mitochondrial state such as ischemia, hypoxia, and cell death [107].

In the presence of $ONOO^-$, DCFH shows a greater increase in the fluorescence intensity when compared with DHR [101,107], although the rate of $ONOO^-$-dependent oxidation is similar for both compounds or even greater for DHR [92,107]. Moreover, Possel *et al*. [107] have also shown that DCFH is more sensitive for $ONOO^-$ than it is to other reactive species like $^{\cdot}NO$ or H_2O_2. According to this study, low $ONOO^-$ concentrations (1–5 μM) result in a rapid and efficient increase of DCF fluorescence. Hempel *et al*. [101] managed to detect DCF fluorescence with even lower $ONOO^-$ concentrations (400 nM). Yet, DCFH is also oxidizable by Fe(II), Fe(III)/ascorbate, cytochrome *c*, and by the enzyme xanthine oxidase [101].

As in the case of DHR, it has been shown that $^{\cdot}NO_2$ and $CO_3^{\cdot -}$ were capable of oxidizing DCFH. However, the oxidized form of this probe also interacts efficiently with these reactive species, resulting in fluorescence quenching, which makes results difficult to interpret [98].

Dihydrofluorescein (HFLUOR)

Dihydrofluorescein (HFLUOR) oxidation by $ONOO^-$ yields fluorescein, a fluorescent compound ($\lambda_{excitation} = 488$ nm; $\lambda_{emission} = 512$ nm) (Fig. 18) [101].

HFLUOR is not oxidizable by H_2O_2 alone, Fe(II), H_2O_2/Fe(II), Fe(III)/H_2O_2, $^{\cdot}NO$, and cytochrome *c*. On the other hand, HFLUOR was shown to be oxidizable by H_2O_2 in the presence of horseradish peroxidase, Fe(III)/ascorbate, Fe(III)/EDTA/H_2O_2, and by KO_2 in the presence of Cu/Zn-SOD and/or catalase [101].

Dihydrofluorescein diacetate (HFLUOR-DA) is a fluorescence probe suitable for imaging of intracellular structures [101]. Indeed, comparatively to DCFH-DA, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (5&6DH-DA) (see later), and DHR, HFLUOR-DA originated the brightest images in human umbilical vein endothelial cells either in control conditions or when treated with menadione [101]. HFLUOR and 5&6DH reacted with $ONOO^-$ to a similar degree, but were less reactive than DCHF.

5(and 6)-Carboxy-2′,7′-dichlorodihydrofluorescein (5&6DH)

5(and 6)-Carboxy-2′,7′-dichlorodihydrofluorescein (5&6DH) oxidation by $ONOO^-$ yields 5(and 6)-carboxy-2′,7′-dichlorofluorescein, a fluorescent compound ($\lambda_{excitation} = 504$ nm; $\lambda_{emission} = 530$ nm) (Fig. 19) [101].

5&6DH is not oxidizable by H_2O_2 alone, Fe(II), H_2O_2/Fe(II), Fe(III)/H_2O_2, Fe(III)/ascorbate, Fe(III)/EDTA/H_2O_2, cytochrome *c*, $^{\cdot}NO$. However,

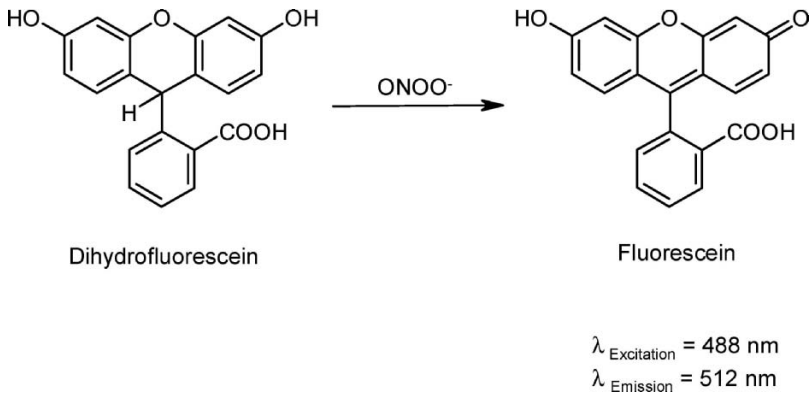

Fig. 18. ONOO− mediated oxidation of dihydrofluorescein to fluorescein (adapted from Ref. 101).

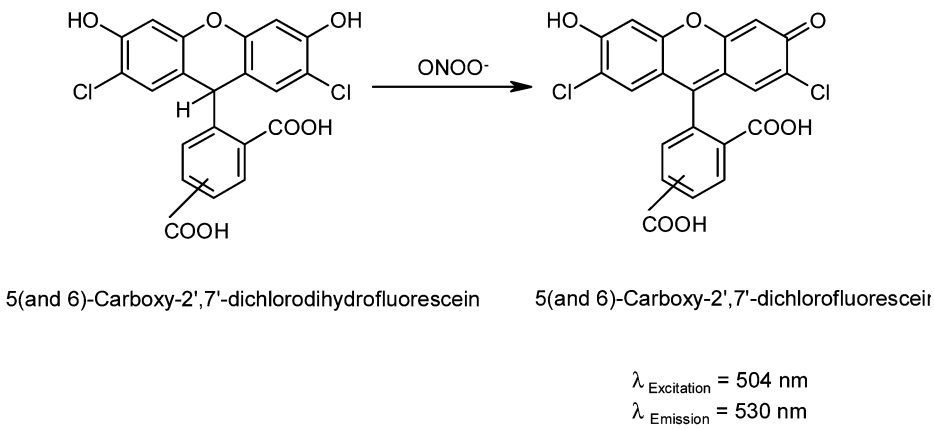

Fig. 19. ONOO− mediated oxidation of 5(and 6) carboxy-2′,7′-dichlorodihydrofluorescein to 5(and 6) carboxy-2′,7′-dichlorofluorescein (adapted from Ref. 101).

5&6DH is oxidizable by H_2O_2 in the presence of horseradish peroxidase and by KO_2 in the presence of Cu/Zn-SOD and/or catalase [101].

5(and 6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (5&6DH-DA) is supposed to be retained in cells better than DCHF, thereby yielding more fluorescence [101].

Rhodamine B Hydrazide

Rhodamine derivatives are prized for their great photostability, pH insensitivity over a broad range (low to neutral pH), high quantum yield in aqueous solution and excitability at long wavelengths [112]. The reaction of $ONOO^-$ with rhodamine B hydrazide (Fig. 20), a colorless, non-fluorescent rhodamine derivative, results in a dramatic increase in fluorescence intensity of the reaction mixture ($\lambda_{excitation} = 556$ nm; $\lambda_{emission} = 574$ nm) [110].

The response time of rhodamine B hydrazide for $ONOO^-$ is less than 30 s, and it has a detection limit of 24 nmol/L for this RNS. Compared with the two fluorescent probes (DCFH and DHR) reported in the literature, rhodamine B hydrazide has some advantages: (i) longer excitation wavelength (DCFH and DHR have the excitation wavelength of 502 and 500 nm respectively [111], while this probe has the longer wavelength of 556 nm); this is desirable for probing $ONOO^-$ formation in biological samples because of the low background fluorescence with the longer wavelength excitation and less cytotoxicity caused by longer UV excitation. (ii) Insensitivity to pH variations in a wider range; because the rhodamine fluorophore does not have phenolic hydroxyl groups, the fluorescence of the rhodamine B hydrazide reaction product with $ONOO^-$ is independent of physiological pH variations and can be applicable in a wider pH range. (iii) Higher stability; rhodamine B hydrazide demonstrates greater photostability than those of DCFH and DHR, which are extremely sensitive to light induced oxidation [110].

2-[6-(4′-Hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) and 2-[6-(4′-Amino) phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF)

2-[6-(4′-Hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) and 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) are non-fluorescent derivatives of fluorescein, designed and

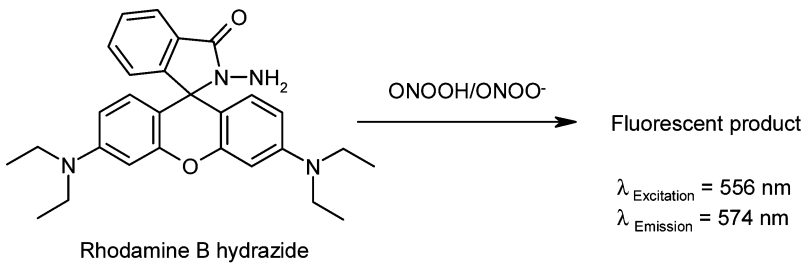

Fig. 20. ONOO− mediated oxidation of rhodamine B hydrazide (adapted from Ref. 11).

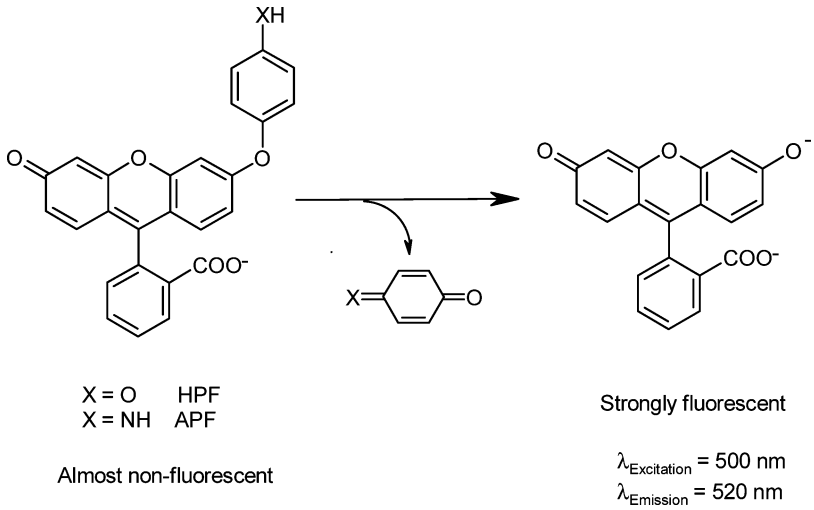

Fig. 21. Scheme of HPF and APF O-dearylation induced by reactive species (adapted from Ref. 112).

synthesized by Setsukinai *et al.* [112], that originate fluorescein by O-dearylation, leading to its characteristic fluorescence ($\lambda_{excitation} = 500$ nm; $\lambda_{emission} = 520$ nm) (Fig. 21).

APF and HPF were classified by Setsukinai *et al.* [112] as appropriate probes for detecting highly reactive ROS and RNS, meaning, $HO^{\cdot}$, $ONOO^{-}$, and HOCl, while the reactive species H_2O_2, $^{\cdot}NO$, $O_2^{\cdot -}$, $^1O_2$, and $ROO^{\cdot}$ do not cause any fluorescence increase in any of the probes. According to Setsukinai *et al.* [112], this constitutes an advantage that these probes have relatively to DCFH in what concerns selectivity, since DCFH was demonstrated to be reactive for all ROS and RNS, in an experiment carried out by the same authors. Besides, APF and HPF present high resistance to auto-oxidation, contrarily to DCFH and can be used in enzymatic and cellular systems. Thus, although the sensibility of DCFH is higher than of APF and of HPF, the use of these last when detecting certain ROS and RNS might be worthwhile.

FINAL NOTES

The use of fluorescence probes for detection of $^{\cdot}NO$ and $ONOO^{-}$ is becoming increasingly important as new sensitive and specific compounds are developed for this type of application. The choice of the most adequate probe should be criteriously made in order to assure the best result in the experimental conditions under study. Several factors should be weighted in order to make the right choice, although it may be possible that all the best qualities may not be found in just one of the above-described probes. Thus, the probe should have high fluorescence quantum yield for the species to be detected, high specificity, and accuracy, but low interference from the pH, solvents, or other biological and nonbiological experimental conditions in the system under study. The probe should be competent to be used to quantify the reactive species generated under biological relevant conditions. Signal transduction hampering due to the scavenging of the reactive species by the probes and the possible toxicity of probes and/or $UV_{excitation}$ light should also be the factors to be taken into account. Hopefully, the present review will help readers to find the probe that better fits their scientific objectives.

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