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Towards selective detection of reactive oxygen and nitrogen species with the use of fluorogenic probes – limitations, progress, and perspectives

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Abstract

Over the last forty years, there has been tremendous progress in understanding the biological reactions of reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is widely accepted that the generation of ROS and RNS is involved in physiological and pathophysiological processes. To understand the role of ROS and RNS in a variety of pathologies, the specific detection of ROS and RNS is fundamental. Unfortunately, the intracellular detection and quantitation of ROS and RNS remains a challenge. In this short review, we have focused on the mechanistic and quantitative aspects of their detection with the use of selected fluorogenic probes. The challenges, limitations and perspectives of these methods are discussed.

Keywords

Superoxide radical anion; Hydrogen peroxide; Peroxynitrite; Protein hydroperoxides; Fluorogenic probes

1. Introduction

It has been more than forty years since McCord and Fridovich discovered that erythrocuprein, known as superoxide dismutase (SOD), catalyzes the dismutation of the superoxide radical anion ($O_2^{\bullet -}$) [1]. This discovery attracted the attention of the scientific community to the role of reactive molecular oxygen metabolites in biological processes. The group of reactive oxygen species (ROS) and reactive nitrogen species (RNS) consists of

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hydrogen peroxide (H_2O_2), protein and lipid peroxides (ROOH), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$) and radical oxidants, including $O_2^{\bullet-}$, hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$) and thiyl radicals ($RS^{\bullet}$), nitrogen dioxide ($^{\bullet}NO_2$) and carbonate radical anion ($CO_3^{\bullet-}$).

Since the discovery of SOD, tremendous progress in understanding the biological reactions of ROS and RNS and their physiological significance has been made, yet their intracellular detection and quantitation remain a challenge [2–4]. Several ROS-sensitive probes producing easily detectable and relatively stable products have been developed. In addition to spin trapping techniques [5,6], luminescent probes have also become widely used tools in the studies on oxidative stress.

A variety of small molecule fluorescent probes are available for detecting ROS and RNS in cells. Their rational use requires a deep understanding of the mechanism of their action. In most cases, the probe is oxidized to the corresponding fluorescent product. Determination of the reactivity pattern of the primary products of this process (i.e., probe-derived radicals) is of great importance for understanding of reaction mechanisms and proper interpretation of experimental data [2].

In this article, we discuss the mechanisms of oxidative transformation of selected fluorogenic probes: hydroethidine (detection of $O_2^{\bullet-}$), Amplex® Red (detection of H_2O_2), and boronate-based fluorogenic probes (detection of peroxynitrite and hydroperoxides).

Hydroethidine and the detection of superoxide radical anion

Superoxide radical anion is formed by the process of one-electron reduction of molecular oxygen. It can be produced *in vivo* by a number of oxidases (NADPH oxidases family [7] and xanthine oxidase [8]), by the mitochondrial electron transport chain [9], or by redox cycling agents (e.g., paraquat and menadione) in the presence of electron donors [10,11]. In a protic environment, $O_2^{\bullet-}$ undergoes spontaneous ($k = 2 \times 10^5\ M^{-1}s^{-1}$) [12] or SOD-catalyzed dismutation ($k = 1.6 \times 10^9\ M^{-1}s^{-1}$) [13] to form O_2 and H_2O_2. Superoxide radical anion is the primary ROS produced *in vivo* and a precursor of H_2O_2.

Two major groups of probes are used for the detection of $O_2^{\bullet-}$. The first group comprises chemiluminescent probes (e.g., lucigenin, luminol and its derivative, L-012, Fig. 1A) that react with ROS to form a product in the excited state, which relaxes to ground state with the emission of photons (Fig. 1B and C). Chemiluminescent probes have been widely utilized due to their high sensitivity. The mechanism of probes oxidation involves several intermediates, including probes-derived radicals. The major limitation of their use is that the radical intermediates react with O_2 leading to the formation of $O_2^{\bullet-}$ (Fig. 1B and C) [2,14–16]. This reactivity should be considered when interpreting chemiluminescence data. Moreover, those probes do not react directly, or react slowly with superoxide, while other biologically relevant oxidants (or reductants) can react with them, producing radical intermediates, which further complicate the system. The second major group of probes consists of fluorogenic probes: dichlorodihydrofluorescein (DCFH), dihydrorhodamine (DHR) (Fig. 2A), hydrocyanines (Fig. 2B), and hydroethidine (HE) along with its analogs hydropropidine (HPr) and MitoSOX™ Red (Fig. 2C). In the case of

dichlorodihydrofluorescein and dihydrorhodamine, one has to keep in mind that the radical intermediates formed upon one-electron oxidation react rapidly with oxygen to generate $O_2^{\bullet-}$ (Fig. 3A and B) [17,18].

Hydrocyanines, proposed recently as a tool for the imaging of ROS and $O_2^{\bullet-}$ production [19], still await better characterization of their specificity and chemical reactivity towards biologically relevant oxidants.

Hydroethidine is a cell-permeable probe that reacts with superoxide to form a unique marker product, 2-hydroxyethidium (2-OH-E^+), whereas another red fluorescent product, ethidium (E^+), is formed in the reaction with other cellular oxidants (Fig. 4) [20]. HE has been used for the detection of $O_2^{\bullet-}$ in a variety of biological systems, ranging from intracellular organelles to whole organs in live animals [21,22]. The reaction of HE and superoxide involves a radical mechanism. In the first step, hydroethidine reacts with hydroperoxyl radical $HO_2^{\bullet}$ or other one-electron oxidants to form a HE radical cation. Because the oxidation of HE by superoxide at pH 7.4 is rather slow ($k = 6.2 \pm 0.8 \times 10^3\ M^{-1}s^{-1}$) [23], the first step of HE oxidation in cells seems more likely to be achieved by other oxidants. Importantly, the radical intermediate does not react with O_2, thus the generation of superoxide by probe-derived radicals is avoided. HE radical cation combines rapidly with superoxide ($k = 2 \times 10^9\ M^{-1}s^{-1}$) to generate a specific oxidation product, 2-hydroxyethidium [24]. Although superoxide-specific (2-OH-E^+) and non-specific (E^+) oxidation products have slightly different fluorescent spectra, the distinction between them is difficult using currently available fluorescence techniques [25]. The formation of 2-OH-E^+ has to be confirmed and quantitated with the use of other analytical techniques (e.g., HPLC with fluorescence detection or LC-MS) [26,27]. The HE–derived radical can also produce an HE-HE dimer, which can be further oxidized to the HE-E^+ and E^+-E^+. The measurement of E^+ and the dimers (HE-HE, HE-E^+ and E^+-E^+) provides useful information about the cellular oxidation of HE [28]. It has been suggested that 2-OH-E^+ formation should be considered only as a qualitative indicator of intracellular superoxide production [29–31].

Hydroethidine analogs (e.g., hydropropidine and MitoSOX™ Red, Fig. 2C) can be used for the detection of superoxide with the same limitations as assigned to HE [28]. Hydropropidine, a water-soluble analog of HE, possesses a highly localized positive charge that prevents its cellular uptake, making this probe a convenient tool for measurements of extracellular superoxide. It seems possible that in the presence of horseradish peroxidase, (HRP) the sensitivity of detection for extracellular superoxide with hydropropidine can be significantly increased. Recently, we have shown that the addition of HRP to HE and xanthine/xanthine oxidase system dramatically enhances 2-OH-E^+ formation [31].

The requirement of HPLC separation for 2-OH-E^+ from other HE oxidation products is the major limitation of superoxide detection in cells. The rational design and synthesis of new hydroethidine analogs is needed to make real-time monitoring and/or imaging of superoxide in biological systems possible [32].

Amplex Red and the detection of hydrogen peroxide

Hydrogen peroxide can be generated *in vivo*, directly by an enzymatic two-electron reduction of O_2 or from dismutation of $O_2^{\bullet-}$ [13,33]. H_2O_2 can be produced by several enzymes, such as L-amino acid oxidase, urate oxidase, glycolate oxidase and monoamine oxidase [34,35]. H_2O_2 is assumed to be an important signaling molecule regulating enzymes such as protein kinases and phosphatases [36–38].

Hydrogen peroxide is a neutral molecule that can diffuse through lipid membranes. It can slowly react with thiols, which can lead to the formation of sulfenic acids or sulfoxides [39]. High H_2O_2 toxicity is ascribed to $^{\bullet}OH$ formed through the Fenton reaction. To protect cells from H_2O_2 toxicity, the iron pool in cells is tightly controlled, and cells possess endogenous enzymatic H_2O_2 scavengers: peroxiredoxins, glutathione peroxidases, and catalase [40–42].

Currently, there are very few methods that yield reliable quantitative data on H_2O_2 production in cells. One method is the Amplex® Red (AR, 10-acetyl-3 7-dihydroxyphenoxazine) assay, which is based on the enzymatic oxidation of a resorufin derivative [43]. Amplex® Red is a colorless and non-fluorescent compound that upon oxidation by H_2O_2, in the presence of HRP, is transformed into a highly fluorescent resorufin [43]. The mechanism of its oxidation involves a multi-step reaction (Fig. 5). In the first step Amplex® Red undergoes one-electron oxidation to form an AR-derived radical, which subsequently can be oxidized by H_2O_2/HRP. AR-derived radicals can also undergo dismutation to form the secondary transient product. Further de-*N*-acetylation of that product leads to the formation of resorufin [44]. The Amplex® Red assay is assumed to be highly selective toward H_2O_2. However, our results indicate that AR can also be oxidized by other oxidants [45]. Moreover, the traces of resorufin present in the sample are able to photosensitize Amplex® Red when exposed to visible light (e.g., excitation light during monitoring of Amplex® Red oxidation). The rate of photooxidation is increased when HRP is present [46,47]. Therefore, real-time, fluorescence-based monitoring of Amplex® Red oxidation should not be used for quantitative analysis of H_2O_2 formation. Additionally, NADH and reduced glutathione in the presence of HRP can interfere with the measurement [4,48]. Our results indicate that the addition of peroxynitrite can lead to the oxidation of Amplex® Red [44]. The mechanism of this reaction involves the formation of oxidizing radicals (i.e., $CO_3^{\bullet-}$ and $^{\bullet}NO_2$) from the decomposition of peroxynitrite. Pulse radiolysis experiments have indicated that those radicals are able to oxidize Amplex® Red. HRP can significantly increase the yield of resorufin from Amplex® Red oxidation by peroxynitrite [44].

Boronate probes and the detection of peroxynitrite and hydroperoxides

Boronate-based probes have recently emerged as a versatile tool for detecting ROS and RNS. It has been proposed that boronate-derivatives of fluorescent dyes can be used as convenient and selective probes for H_2O_2. The first reported fluorogenic boronate probe for H_2O_2 was *p*-dihydroxyborylbenzyloxycarbonyl derivative of 7-aminocoumarin. The mechanism of detection was based on the oxidative deprotection of the fluorescent aminocoumarin reporter (Fig. 6A) [49]. In 2004, Chang *et al.* described the synthesis of a diboronate derivative of fluorescein PF1 (Fig. 6B) [50]. During the next decade many

boronate-based fluorogenic probes were designed and synthesized. Generally, the mechanism of action of boronate probes is based on oxidative transformation of non- or weakly fluorescent boronates into strongly fluorescent products [49–51].

Reactivity towards oxidants—The oxidation of arylboronates in alkaline solutions of H_2O_2 was reported for the first time over 80 years ago [52]. However, at physiological pH, the reaction between boronate and H_2O_2 is slow ($k \sim 1–2\ M^{-1}s^{-1}$) [53]. Thus, it has been postulated that in biological systems oxidation of boronate probes by H_2O_2 seems rather unlikely [54].

Several other oxidants can also convert boronates into corresponding phenols. That reaction is typical for hypohalous ions and nucleophilic peroxy oxidants (Fig. 7) [53,55,56]. A few years ago, we demonstrated that boronates react rapidly with hypochlorite and peroxynitrite anions [51,53] (Fig. 7). We also showed that boronates can be oxidized by selected organic hydroperoxides [57,58].

The mechanism of peroxynitrite-derived oxidation—Peroxynitrite ($ONOO^-$) is a primary product of the reaction between nitric oxide ($^{\bullet}NO$) and $O_2^{\bullet-}$ ($k \sim 10^{10}\ M^{-1}s^{-1}$) [59]. We have recently demonstrated that peroxynitrite is also produced in the reaction of O_2 with HNO ($k = 1.8 \times 10^4\ M^{-1}s^{-1}$) [60]. Although peroxynitrite possesses a relatively short half-life in biological systems, it is able to diffuse through biological membranes, and it can diffuse up to two cell diameters distance [61]. $ONOO^-$ is a relatively strong non-radical oxidant. Under physiological conditions, it exists in acid-base equilibrium with its protonated form, peroxynitrous acid (ONOOH, $pK_a = 6.8$). Peroxynitrite can directly oxidize low molecular weight thiols, as well as sulfhydryls groups in proteins. This compound can also react with CO_2 (high concentration *in vivo*, $k = 4.6 \times 10^4\ M^{-1}s^{-1}$) [62]. The product of this reaction is unstable and decomposes up to 35% into $^{\bullet}NO_2$ (nitrating radical) and $CO_3^{\bullet-}$ (strong one-electron oxidant). In the absence of scavengers, homolysis of ONOOH, considered as a minor pathway in biology, yields $^{\bullet}OH$ and $^{\bullet}NO_2$ radicals [63]. Formation of such radicals leads to the oxidation and nitration of relevant targets, such as amino acids (tyrosine, phenylalanine and histidine), sugars (deoxyribose) and lipids [59,64].

Boronates react rapidly, directly and stoichiometrically with $ONOO^-$ ($k \sim 10^6\ M^{-1}s^{-1}$) to form corresponding phenols (80–90% yield) [53]. We showed that the remaining 10–20% can be ascribed to minor products derived from radical intermediates [53,57,60,65].

The reaction mechanism for peroxynitrite-derived oxidation of boronates has been studied and described in detail (Fig. 8) [57,60,65]. The formation of anionic adduct of peroxynitrite to the boronate moiety is the first reaction step. There are two pathways of subsequent transformation of the adduct. The first pathway (80–90%) proceeds *via* heterolytic cleavage of the O-O bond in the anionic adduct and results in the formation of the major phenolic product. The second, minor pathway involves homolytic cleavage (~ 10–20%) of the peroxide bond and results in the formation of a $RB(OH)_2O^{\bullet-}$ radical anion, which undergoes further spontaneous fragmentation with the formation of phenyl radicals [57,60,65]. As the minor free-radical pathway is specific for the reaction of boronates with peroxynitrite, the formation of phenyl radical-derived minor products may be used to support its identification

(Fig. 8) [60]. During the last few years, the formation of such peroxynitrite specific products was shown for simple arylboronate compounds [57], mitochondria targeted isomeric arylboronates [65] as well as for fluorogenic boronate probe coumarin 7-boronic acid (CBA) [69]. We have demonstrated that such products are of diagnostic value and can be used as a "peroxynitrite fingerprint" [60,66].

Detection of peroxynitrite in cell culture studies—The usefulness of boronate probes for monitoring peroxynitrite generation in cellular systems was first shown in a study performed on activated macrophages, RAW 264.7 [67]. Recently [54], a boronate-based bioluminescent probe Peroxy Caged Luciferin-1 (PCL-1, Fig. 9), previously reported as a selective probe for hydrogen peroxide [68], was shown to detect peroxynitrite in macrophage cells [54]. After reaction of PCL-1 with an oxidant, luciferin is released, and in the presence of ATP and luciferase, luciferin is oxidized producing a bioluminescence signal.

Detection of protein hydroperoxides with the use of boronate probes—Boronate probes can also be used to detect other oxidants in a complex biological system. Recently, we have shown that CBA is oxidized to the highly fluorescent 7-hydroxycoumarin by amino acid and protein hydroperoxides [58]. Based on this observation, we have developed a fluorometric real-time assay to detect protein hydroperoxides (Fig. 10) generated in cells. The use of CBA probe is a convenient way to detect and determine protein hydroperoxides, superior to other assays i.e., FOX or iodometric assays (both FOX and iodometric assays possess several limitations, as discussed elsewhere [69]).

Global profiling of oxidants

Recently, we proposed a "global profiling approach" [67] with the use of a set of selected fluorogenic probes for simultaneous real-time monitoring of oxidants formation in living cells. Such an approach seems to be an ideal tool for complete characterization of cellular ROS and RNS generation under oxidative stress conditions. Hydroethidine or hydropropidine [23] can be used to monitor superoxide and/or other oxidants formation. Boronate probes can be used to detect peroxynitrite, and the Amplex Red/HRP assay can be used to detect both H_2O_2 and peroxynitrite [67,70]. As it has been emphasized, the proper identification of the oxidant is not possible without simultaneous HPLC-based measurements verifying the identities of the monitored fluorescent species [67,70].

With a better understanding of the redox chemistry of the probes and the recent developments in rapid HPLC analyses of 2-hydroxyethidium and hydroxycoumarin [67], rigorous high throughput profiling of various reactive oxygen and nitrogen species in biological systems seems closer than ever to being a reality.

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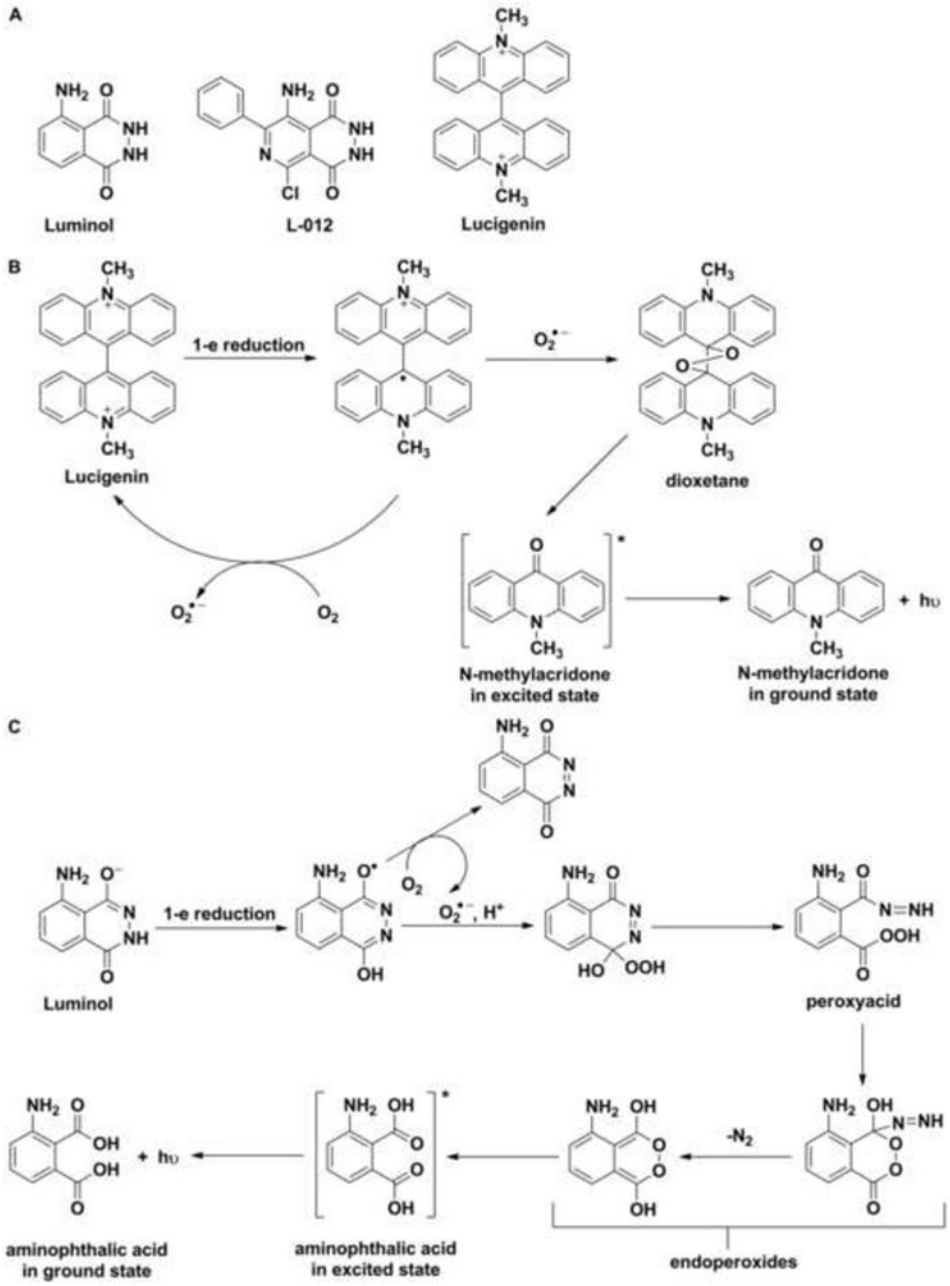

Figure 1.

(A) Chemical structures of chemiluminescent probes for the detection of superoxide radical anion, (B) The mechanism of superoxide-dependent oxidative transformation of lucigenin, (C) The mechanism of oxidative transformation of luminol.

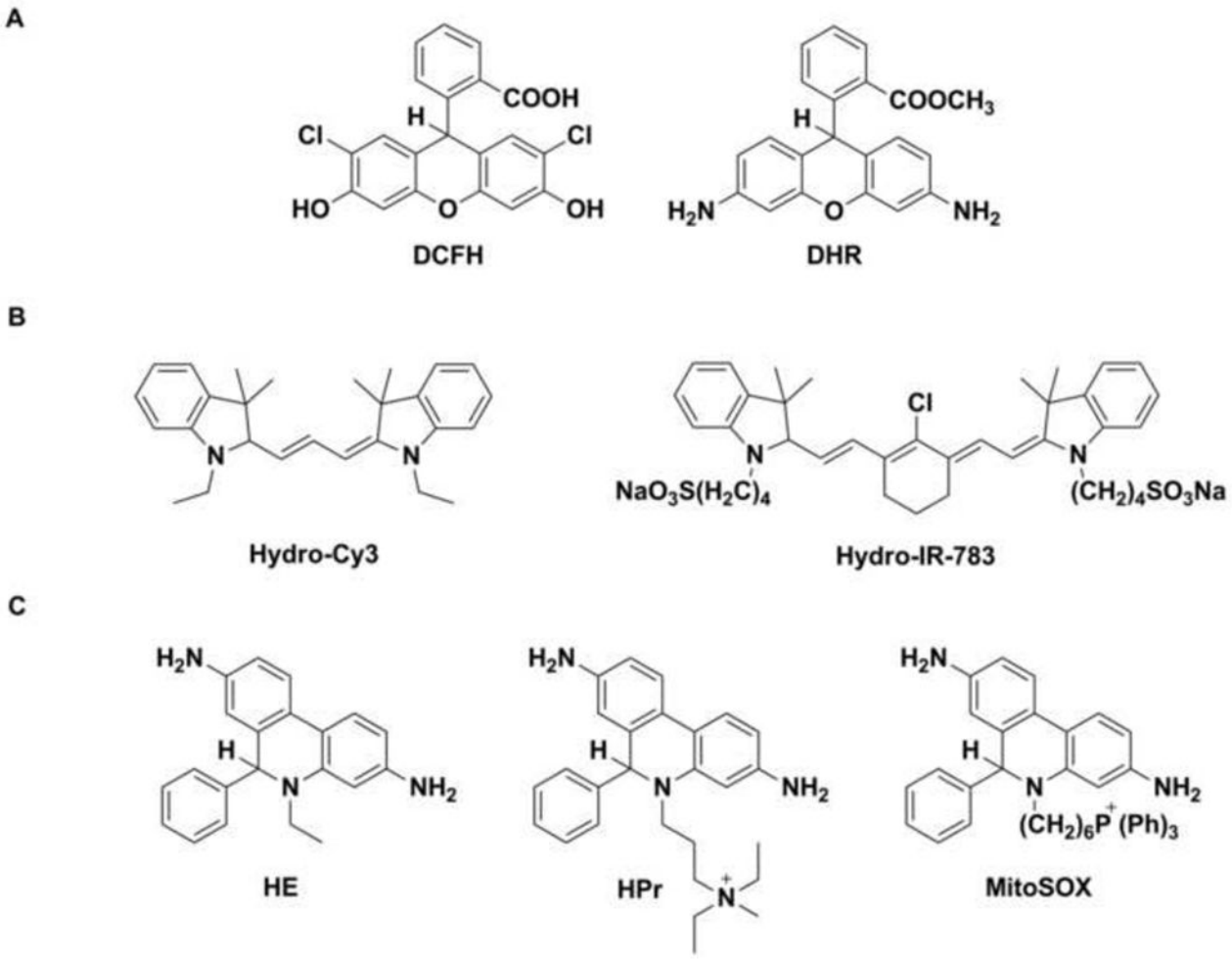

Figure 2.

Chemical structures of fluorogenic probes for the detection of superoxide radical anion: (A) dichlorodihydrofluorescein (DCFH) and dihydrorhodamine (DHR), (B) hydrocyanines, (C) hydroethidine (HE) and its analogs.

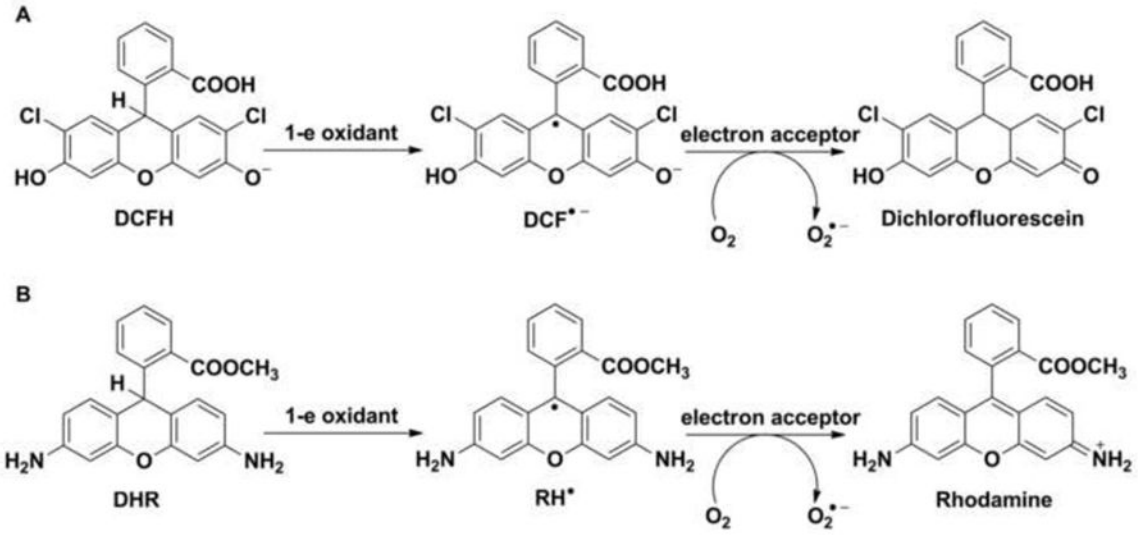

Figure 3.
Mechanisms of one-electron oxidation of (A) dichlorodihydrofluorescein (DCFH) and (B) dihydrorhodamine (DHR).

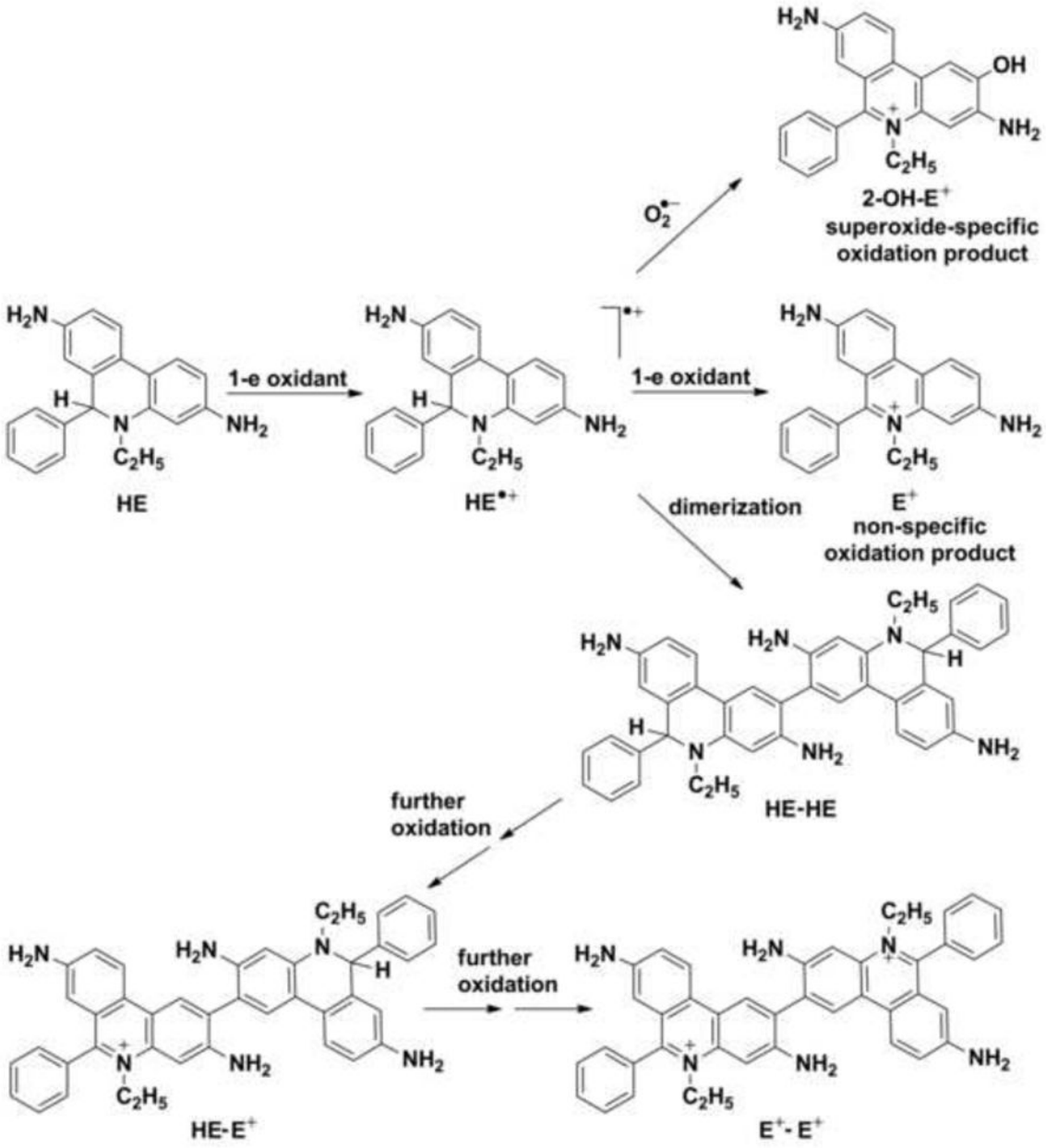

Figure 4.
Proposed radical mechanism of the oxidative transformation of hydroethidine (HE).

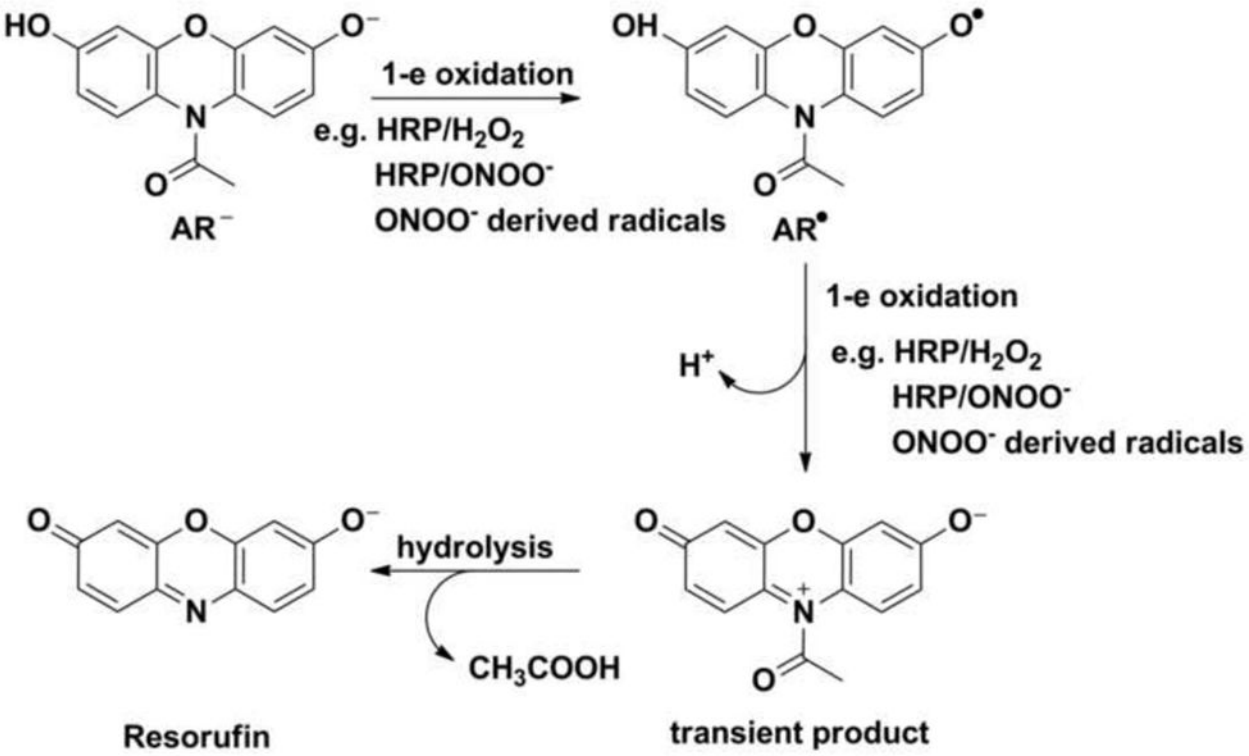

Figure 5.
Mechanism of the oxidative transformation of Amplex® Red (AR) in its deprotonated form under basic conditions.

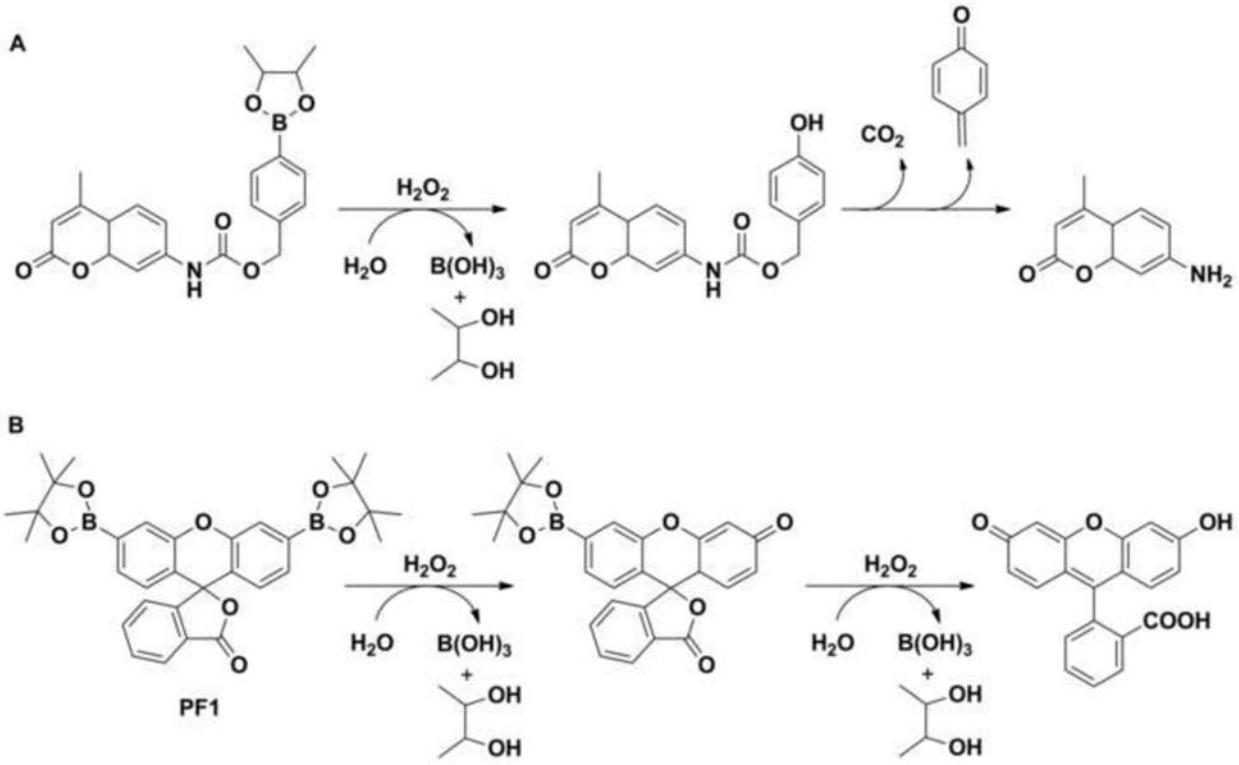

Figure 6.
The reaction of hydrogen peroxide with (A) *p*-dihydroxyborylbenzyloxycarbonyl derivative of 7-aminocoumarin and (B) diboronate derivative of fluorescein (PF1).

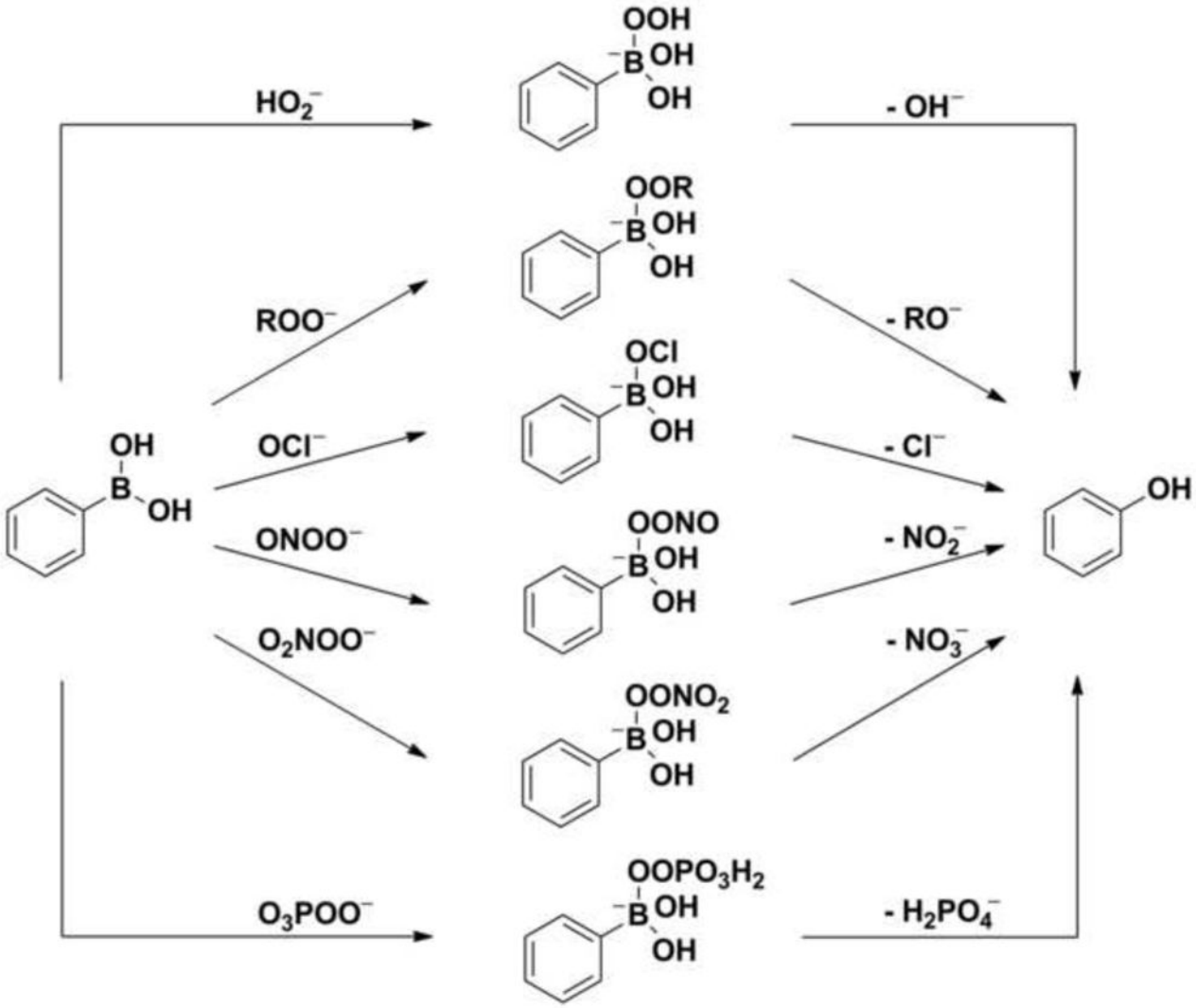

Figure 7.
Proposed mechanisms of oxidation of boronate probes by various oxidants.

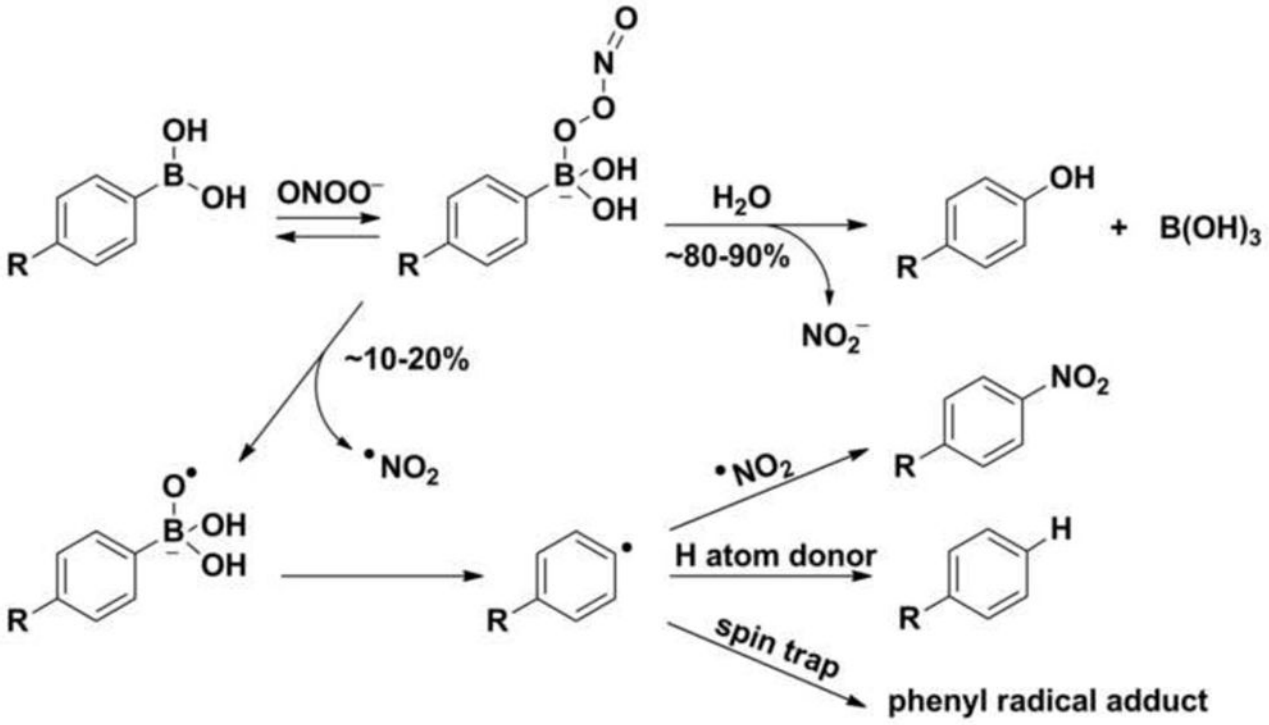

Figure 8.
Mechanism of the reaction between peroxynitrite and boronic probes.

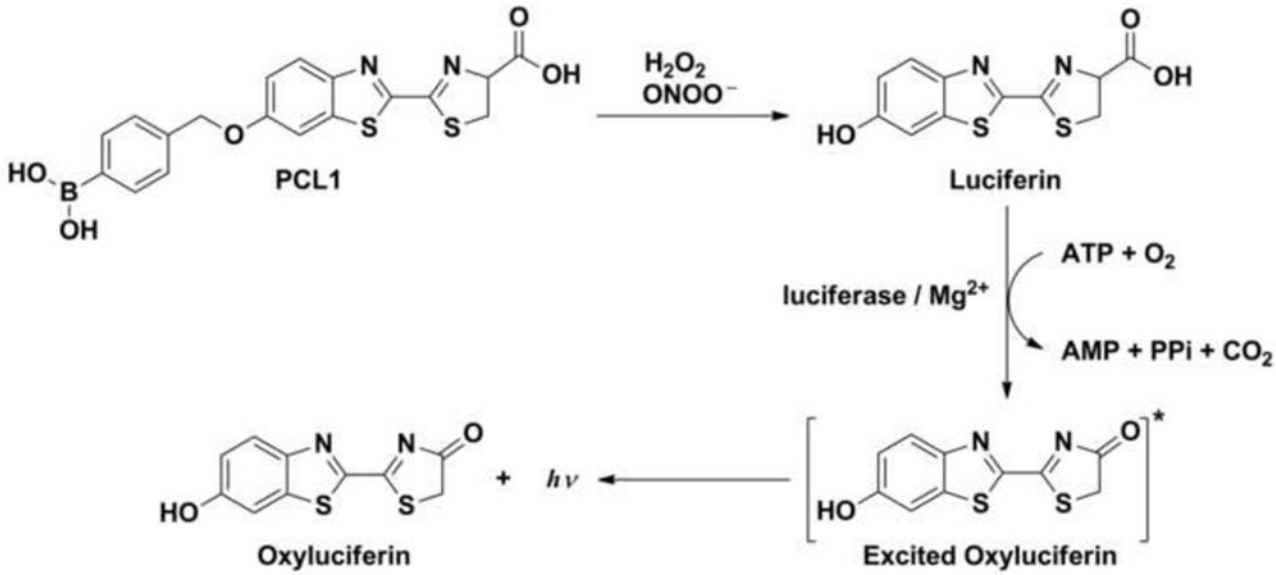

Figure 9.

Mechanism of the detection of hydrogen peroxide or peroxynitrite by Peroxy Caged Luciferin-1 (PCL-1).

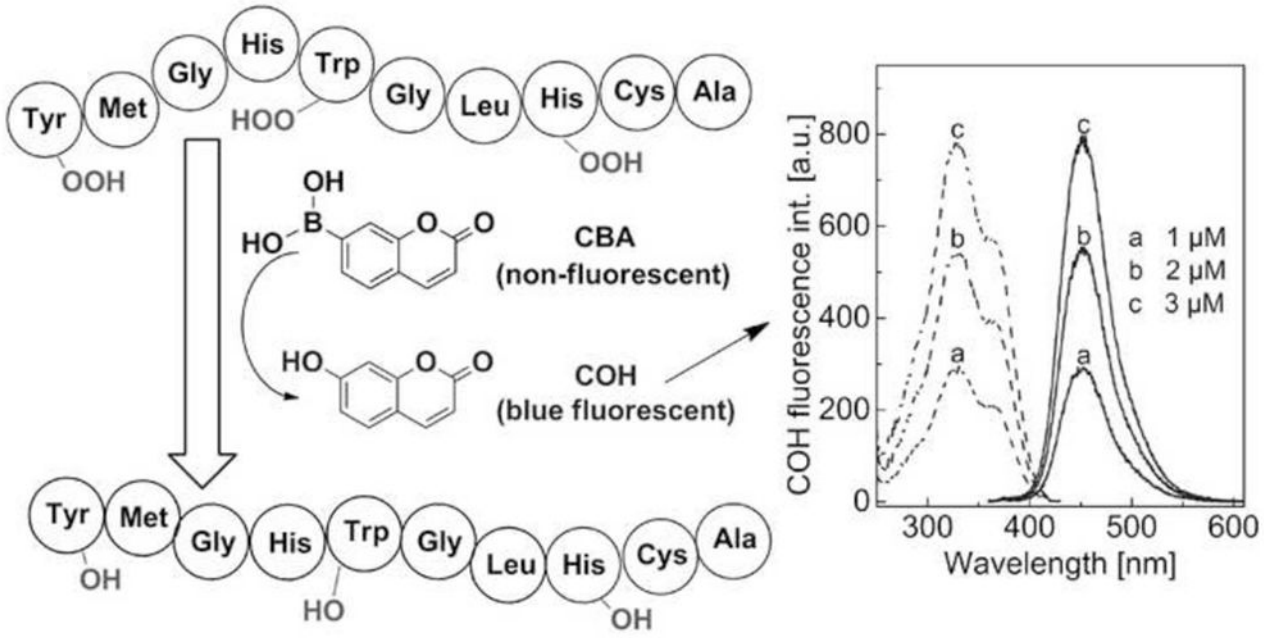

Figure 10.
Fluorometric, real-time assay for protein hydroperoxides based on the CBA probe.