Oxidative chemistry of fluorescent dyes: implications in the detection of reactive oxygen and nitrogen species

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Abstract
HE (hydroethidine), a widely used fluorescent dye for detecting intracellular superoxide, undergoes specific oxidation and hydroxylation reactions. The reaction between HE and O$_2$•− (superoxide radical) yields a diagnostic marker product, 2-hydroxyethidium. This is contrary to the popular notion that O$_2$•− oxidizes HE to form ethidium. HE, however, undergoes a non-specific oxidation to form ethidium in the presence of other oxidants (hydroxyl radical, peroxynitrite and perferryl iron) and other dimeric products. The mitochondria-targeted HE analogue Mito-SOX$^\text{®}$ undergoes the same type of oxidative chemistry to form products similar to those formed from HE. On the basis of the oxidative chemical mechanism of HE and Mito-SOX$^\text{®}$, we conclude that fluorescence microscopy or related techniques are not sufficient to measure the superoxide-specific hydroxylated products. HPLC methodologies are required to separate and identify these products. Peroxynitrite reacts rapidly and stoichiometrically with boronates to form specific products. Assays using fluorescent-based boronate probes will be more reliable for peroxynitrite determination than those using either dichlorodihydrofluorescein or dihydrorhodamine.

Oxidative chemistry of HE (hydroethidine)
Nearly 10 years ago, we discovered that the cell-permeant redox probe HE is selectively oxidized and hydroxylated by superoxide to a specific product, 2-OH-E+ (2-hydroxyethidium) (see Figure 1 for structures) [1,2]. This finding disproved the long-held belief that O$_2$•− (superoxide radical) reacts with HE to form E+ (ethidium) as a major product [3]. Like E+, whose fluorescence quantum yield is enhanced by binding to DNA, the fluorescence yield of 2-OH-E+ is also enhanced by DNA [1]. Other oxidants, including hydroxyl radical (OH•), perferryl iron, peroxynitrite (ONOO•−), or peroxidase and hydrogen peroxide (H$_2$O$_2$) react with HE, but do not form 2-OH-E+ as a specific product [1,3]. The structure of 2-OH-E+ was characterized further by MS and NMR techniques [2]. To our knowledge, O$_2$•− is the only ROS (reactive oxygen species) that forms 2-OH-E+ upon reaction with HE. We also showed that both 2-OH-E+ and E+ can be separated and quantified by HPLC techniques [1–3]. Investigators have since used HE and HPLC to detect and quantify O$_2$•− formed in cells and tissues [4,5].

Previously, it was suggested that several oxidants including O$_2$•− oxidize HE via a one-electron oxidation mechanism to the corresponding aminyl radical (HE•), which then rapidly reacts with O$_2$•− to form 2-OH-E+ [6]. The aminyl radical HE• does not react with molecular oxygen [6]. However, in the absence of O$_2$•−, the HE• radical undergoes a disproportionation and dimerization reaction forming E+ and HE–HE, HE–E+ or E+–E+ dimeric products. The dimeric product E+–E+ is non-fluorescent, although the fluorescence characteristics of HE–HE and HE–E+ have not yet been established [7]. Dimeric oxidation products were identified during oxidation of HE by cytochrome c, peroxidase and H$_2$O$_2$, OH• formed from the Fenton reaction (Fe$^{2+}$ and H$_2$O$_2$), co-generated nitric oxide (NO•) and O$_2$•−, and pre-formed ONOO•−. It is conceivable that HE can undergo a series of oxidations, generating a variety of oxidation and hydroxylated products depending upon the redox status of the cell. Figure 2 shows the different products formed from intracellular oxidation of HE in the presence of ROS and RNS (reactive nitrogen species) and iron/cytochrome c.

Oxidative chemistry of Mito-HE (mitochondria-targeted HE)/Mito-SOX$^\text{®}$
We showed that O$_2$•− reacts with Mito-SOX$^\text{®}$ (also known as Mito-HE) in the same manner as it does with HE [7], although the rate constant for the Mito-SOX/ O$_2$•− reaction is 2-fold higher than the rate constant between O$_2$•− and HE [8]. Mito-SOX reacts with O$_2$•− to form the hydroxylated product 2-OH-Mito-E+ (2-hydroxy-Mito-ethidium) [7]. This is different from the two-electron oxidation product, Mito-E+ [mitochondria-targeted E+,
which is TPP⁺ (tetraphenylphosphonium) conjugated to E⁺ via an alkyl side chain. As with HE, Mito-SOX-derived aminyl radical disproportionates and dimerizes to form Mito-E⁺ and the products Mito-HE–Mito-HE, Mito-HE–Mito-E⁺ and Mito-E⁺–Mito-E⁺ [7]. Various oxidants (haem protein and H₂O₂, iron and H₂O₂, and ONOO⁻) are able to oxidize Mito-HE to form the corresponding oxidation products, but not 2-OH-Mito-E⁺, the superoxide-specific marker product. Figure 2 shows the different oxidation and hydroxylation products of Mito-SOX or Mito-HE that can be generated during redox activation in cells.

**Intracellular red fluorescence from HE or Mito-SOX: does it measure intracellular O₂⁻• ?**

Clearly, the HPLC-based methods can unequivocally determine a wide range of products formed from HE and Mito-SOX; however, because of the cumbersome processing of cell samples, investigators have, for the most part, used the fluorescence methods (i.e. fluorescence microscopy) to assess intracellular O₂⁻•. The red fluorescence detected from cells incorporated with HE or Mito-SOX is often equated to intracellular O₂⁻•. We reported that, because of the overlapping fluorescence spectra from 2-OH-E⁺ (diagnostic marker product of the O₂⁻•/HE reaction) and E⁺ (non-specific oxidation product of HE), the red fluorescence derived from HE cannot be used to quantify intracellular O₂⁻• [2]. The overlapping fluorescence spectra obtained from 2-OH-E⁺ and E⁺ are shown in Figure 3. Although we initially felt that by using appropriate optical filters, the individual contribution of 2-OH-E⁺ and E⁺ to the overall fluorescence can be assessed, there are many unknowns that make fluorescence microscopy data interpretation highly problematic [9]. For example, the levels of E⁺ detected in cellular systems which are nearly 10-fold higher than those of 2-OH-E⁺ and E⁺ will significantly contribute to O₂⁻•-induced red fluorescence.
**Figure 3** Optimal experimental conditions for detection of 2-OH-E⁺–DNA and E⁺–DNA complexes using fluorescence microscopy

Fluorescence spectra of 2-OH-E⁺ (shown in yellow) or E⁺ (shown in red) (10 μM) in the presence of DNA using the excitation wavelengths (EX) of 490 (A), 494 (B) and 510 (C) nm. Fl, fluorescence intensity. Reproduced from [2] with permission.

**HPLC measurements of 2-OH-E⁺, the marker product of the HE and O₂⁺⁻ reaction**

We have shown previously that the HPLC profiles of 2-OH-E⁺ and E⁺ are well-resolved and clearly separated [1,2]. The HPLC peak height of 2-OH-E⁺ was measured to be nearly 3-fold greater than the peak height of E⁺ over the same range of concentrations (0–20 μM) [2]. Using this technique, ceramide-induced intracellular O₂⁺⁻ in endothelial cells was measured [2]. Ceramide treatment was reported to synergistically enhance quinone-mediated oxidative stress in neuronal cells. HPLC analysis using HE as the detection probe indicated that ceramide and menadione enhanced the intracellular formation of both O₂⁺⁻ (increased 2-OH-E⁺) and iron-induced oxidant formation (increased E⁺). Ceramide-induced intracellular oxidant formation was attributed to TfR (transferrin receptor)-dependent iron signalling, NO⁺ signalling and mitochondrial H₂O₂ formation [10].

**Peroxynitrite reaction with DHR (dihydrorhodamine) and boronates**

DHR is one of the most frequently used probes to measure ONOO⁻ [11,12]. This assay is based on the formation of a fluorescent product rhodamine formed from one-electron oxidation of DHR by OH⁺, nitrogen dioxide (NO₂⁺), [or carbonate anion radical (CO₃⁻•) in the presence of bicarbonate] derived from ONOO⁻ decomposition. Other oxidants (OH⁺ formed from the Fenton reaction or compounds I and II formed from H₂O₂ and peroxidase or cytochrome c) will also oxidize DHR to rhodamine [11,13].

Thus the DHR assay for determining oxidants including ONOO⁻ is very non-specific. The oxidative free radical chemistry of DHR to rhodamine is very similar to that of DCFH (dichlorodihydrofluorescein) to the DCF (2′,7′-dichlorofluorescein) radical (DCF⁺). Like the DCF radical, the DHR radical also rapidly reacts with oxygen to form additional O₂⁺⁻ and H₂O₂ that can participate in reactions leading to artefactual amplification product, 2-OH-Mito-HE. However, extraction of Mito-SOX- or Mito-HE-derived products from cell lysates is complicated by its binding to plastic vials. On the basis of the similarities in reaction chemistry between HE and Mito-SOX, all of the limitations attributed to the HE assay are applicable to Mito-SOX as well and thus the mitochondrial red fluorescence observed from Mito-SOX cannot be equated to mitochondrial O₂⁺⁻. Further characterization of mitochondrial red fluorescence using additional confocal imaging technique and/or separation and identification of Mito-SOX hydroxylation and oxidation products responsible for red fluorescence is clearly needed. Failure to recognize these limitations often leads to erroneous interpretations with regard to O₂⁺⁻ formation and diverts the researcher’s focus away from other oxidative signalling mechanisms.
of rhodamine fluorescence. The limitations and caveats postulated for the DCF assay apply to the DHR assay as well. Thus DHR can only be used as a non-specific indicator of 

\[ \text{ONOO}^- \] or other one-electron oxidant formation.
The role of NO\(^*\), O\(_2\)\(^*\) or other iron-derived oxidants in intracellular oxidation of DHR to rhodamine should be independently confirmed with appropriate enzymatic inhibitors [1. NAME (N\(^\text{O}^-\)-nitro-l-arginine methyl ester) or SOD (superoxide dismutase)], iron chelators (defereroxamine) or the anti-TIR antibody.

We have recently shown that aromatic boronates can be rapidly and directly oxidized by ONOO\(^-\) to form a corresponding phenol as a major product (− 85% yield) [14]. Boronates containing fluorophores (e.g. coumarin boronate) will react in a similar fashion with ONOO\(^-\) to form the corresponding fluorescent product 7-hydroxycoumarin [15]. Other boronate-based fluorescent-containing compounds also can be effectively oxidized by ONOO\(^-\) to the corresponding fluorescent product [16]. There is remarkably high activity in the research area of syntheses of novel cell-permeant and mitochondria-targeted boronate probes [17]. These probes have been developed for intracellular measurement of H\(_2\)O\(_2\). Peroxynitrite reacts with most boronate-containing probes nearly a million times faster than H\(_2\)O\(_2\) [14]. Thus boronate-based fluorescent probes that are cell-permeant will probably be the ‘next generation’ probes for measuring intracellular ONOO\(^-\).

**Detection of intracellular oxidant formation using DCFH-DA (dichlorodihydrofluorescein diacetate) and its analogues**

DCFH-DA is one of the most frequently used probes for measuring intracellular H\(_2\)O\(_2\) and oxidative stress [18,19]. However, it is becoming increasingly clear that this probe cannot be used to measure H\(_2\)O\(_2\) as this probe does not react with H\(_2\)O\(_2\) at any significant rate to form the fluorescent product [20]. Published reports, however, suggest that there is a role for redox-active iron or haem in the oxidation of DCFH to DCF [21]. In previous studies, it was shown that H\(_2\)O\(_2\)- and lipid hydroperoxide-induced intracellular DCFH oxidation is mediated by TIR-dependent uptake of iron in response to oxidative signalling initiated by a cellular iron-sensing mechanism [22]. A role for iron, endothelial nitric oxide synthase activity and mitochondrial complex II activity in oxidized low-density-lipoprotein-dependent DCF fluorescence was proposed [23]. There are other examples in the literature that support the role of oxidant-induced iron signalling in intracellular DCF fluorescence [24]. More recently, investigators have used the probe CM-H\(_2\)DCFDA [5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate, acetyl ester], an analogue of DCFH-DA, to measure intracellular H\(_2\)O\(_2\) in response to Toll-like receptor signalling in macrophages activated with lipopolysaccharide and other Toll-like receptor agonists [25]. These results probably implicate some other oxidant signalling mediated by iron or cytochrome c and possibly H\(_2\)O\(_2\) as part of innate immune signalling. DCFH-DA may be used as a redox indicator probe for detecting intracellular oxidant formation caused by changes in iron or haem signalling or ONOO\(^-\) formation. However, DCF fluorescence cannot be equated with intracellular H\(_2\)O\(_2\) levels because of the artefactual amplification of the ‘green fluorescence’ intensity due to DCF radical-mediated redox chemistry in the presence of oxygen [26].

In the present review, I have addressed some aspects of the oxidation/reduction chemistry of commonly used fluorescent dyes. Understanding the mechanism of oxidation of fluorescent dyes with ROS/RNS, the limitations and artefacts will enable one to use these dyes with more confidence and conviction in oxidative signalling.

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