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Small-molecule luminescent probes for the detection of cellular oxidizing and nitrating species

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Abstract

Reactive oxygen species (ROS) have been implicated in both pathogenic cellular damage events and physiological cellular redox signaling and regulation. To unravel the biological role of ROS, it is very important to be able to detect and identify the species involved. In this review, we introduce the reader to the methods of detection of ROS using luminescent (fluorescent, chemiluminescent, and bioluminescent) probes and discuss typical limitations of those probes. We review the most widely used probes, state-of-the-art assays, and the new, promising approaches for rigorous detection and identification of superoxide radical anion, hydrogen peroxide, and peroxynitrite. The combination of real-time monitoring of the dynamics of ROS in cells and the identification of the specific products formed from the probes will reveal the role of specific types of ROS in cellular function and dysfunction. Understanding the molecular mechanisms involving ROS may help with the development of new therapeutics for several diseases involving dysregulated cellular redox status.

Keywords

reactive oxygen species; fluorescent probes; chemiluminescent probes; bioluminescent probes; superoxide; hydrogen peroxide; peroxynitrite

1. Introduction

Experimental research in the field of redox biology requires the use of probes and rigorous assays for cellular oxidants [1–4]. A wide range of chemical probes has been designed and

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applied for the detection of the oxidants, with new probes constantly being developed and reported [5–18]. However, some of those reports provide contradictory statements, *e.g.*, related to selectivity of the probes and their limitations, or regarding their applicability to biochemical and biological systems (cell-free systems, *in vitro* cell cultures, *in vivo* animal models) [19–23]. This may be confusing not only to beginners, but also to experts in the redox biology field who desperately need new, rigorous assays for the detection and quantitative analyses of cellular oxidants. This review, based on the authors' lectures at the Society for Redox Biology and Medicine (SfRBM, formerly Society for Free Radical Biology and Medicine) over the last decade, is aimed at (i) providing the fundamentals of the luminescent (fluorescent, chemiluminescent, and bioluminescent) detection of cellular oxidants, (ii) describing the major advantages and limitations of the most widely used assays, and (iii) recommending state-of-the-art rigorous experimental approaches for oxidant detection using luminescent probes. The use of genetically encoded probes for ROS measurements is out of the scope of the present review and is the subject of a separate paper in this Special Issue.

ROS and redox biology

Aerobic organisms use oxygen as an electron acceptor to oxidize energy substrates and generate ATP. In this process oxygen is reduced to water by accepting four electrons and two protons. When the reduction of oxygen is not complete, *i.e.*, oxygen receives only one or two electrons, primary reactive oxygen species (ROS), superoxide radical anion and hydrogen peroxide, are formed. These species can be subsequently converted to other reactive oxidizing, nitrosating, nitrating, halogenating, or alkylating agents, as shown in Figure 1 [2, 4]. It is important to emphasize that the term “ROS” does not relate to any particular species but rather is an “umbrella” term covering various species of different chemical identity and reactivity, as shown in red in Figure 1.

Although initially it was assumed that such oxidants are formed as byproducts of aerobic respiration and are exclusively “evil” players, leading to the damage of cellular components and cell death, it has been shown that organisms use such species to fight infection, and the loss of the ability to produce ROS is linked to chronic granulomatous disease [24]. More recently, with the discovery of several members of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family of enzymes and an increasing understanding of the intracellular fate and targets of hydrogen peroxide (H₂O₂), it became apparent that oxidants may serve as signaling messenger molecules [1–4, 25–30].

2. Principles of ROS detection

2.1. Why do we need the probes?

To establish a role of cellular oxidants in physiological and pathological processes, it is imperative to be able to detect and characterize the species involved and monitor its levels in the biological system of interest. Although the rigorous detection and characterization of the cellular oxidant(s) involved in the process of interest may be not a trivial task and may require a significant investment of time and resources, it is the only way to unequivocally demonstrate the involvement of the particular species in the investigated processes. Rigorous

characterization of the oxidant(s) involved may provide the basis for an understanding of the investigated processes at the molecular level and validate the conclusions drawn based on pharmacological and/or genetic approaches. To understand why it is not a trivial task, one has to consider the chemical and spectroscopic properties of the major cellular ROS. For the purposes of this review, we will limit our discussion to superoxide radical anion ($O_2^{\bullet -}$), H_2O_2 , and peroxynitrite anion ($ONOO^-$).

Superoxide radical anion— $O_2^{\bullet -}$ is a one-electron reduction product of molecular oxygen and may act as a reducing agent as well as an oxidant upon protonation to hydroperoxyl radical ($HO_2^{\bullet}$, $pK_a = 4.8$) [31–33]. Therefore, in pure water, it decomposes rapidly via a dismutation reaction

to produce molecular oxygen and H_2O_2 (HO_2^- is the deprotonated form of H_2O_2 , $pK_a = 11.7$). The rate constant of this reaction is pH dependent and second order, which means that, with the increasing the steady-state concentration of $O_2^{\bullet -}$, its half-life time decreases. For example, at the concentration of 1 μM and pH of 7.4, the half-life time of $O_2^{\bullet -}$ in water is 2 s ($k_1 = 2.4 \times 10^5 M^{-1}s^{-1}$ at pH = 7.4). Therefore, even in the absence of scavengers and biological targets, $O_2^{\bullet -}$ is not stable and cannot accumulate significantly over time of incubation. In biological systems, due to the presence of superoxide dismutases (SOD enzymes), which accelerate the dismutation reaction of $O_2^{\bullet -}$, as well the presence of other targets of $O_2^{\bullet -}$, the rate of superoxide decay is significantly higher due to the high rate constant of the reaction of $O_2^{\bullet -}$ with SOD ($k = 2 \times 10^9 M^{-1}s^{-1}$) [34]. Regarding the spectroscopic properties, $O_2^{\bullet -}$ is characterized by a weak absorption band in the ultraviolet (UV) range ($\lambda_{max} = 245$ nm, $\epsilon = 2.4 \times 10^3 M^{-1}cm^{-1}$ [31]), which overlaps with absorption spectra of most cellular biomolecules.

Hydrogen peroxide— H_2O_2 is a product of a two-electron reduction of molecular oxygen, either in a direct reaction with two-electron donors, catalyzed by several oxidases, or via two steps of one-electron reduction of O_2 , with the involvement of $O_2^{\bullet -}$, as described above. In pure water, H_2O_2 is stable and can accumulate over time of incubation with an H_2O_2 -generating system. Organisms, however, evolved efficient enzymatic systems, involving peroxiredoxins (Prxs), glutathione peroxidases (GPxs), and catalase, which rapidly consume H_2O_2 [35, 36]. With regard to spectroscopic properties, H_2O_2 absorbs weakly in the UV range, with no clear absorption maximum above 200 nm. Therefore, spectrophotometry can be used to detect H_2O_2 in the absence of the compounds with overlapping absorption spectra (using extinction coefficient of 39.4 [37] or $43.6 M^{-1}cm^{-1}$ [38] at 240 nm), when present in millimolar concentrations. At such concentrations, H_2O_2 is cytotoxic, so those assays are limited to cell-free systems.

Peroxynitrite— $ONOO^-$ is a product of a diffusion-controlled reaction between nitric oxide ($^{\bullet}NO$) and $O_2^{\bullet -}$ [39–42]:

In pure water, ONOO^- is not stable and decomposes, mainly via protonation to peroxyntitrous acid, with subsequent isomerization to nitrate anion (NO_3^-) and fragmentation to hydroxyl radical ($\bullet\text{OH}$) and nitrogen dioxide radical ($\bullet\text{NO}_2$) [43–46].

This reaction follows a first-order kinetics and limits the lifetime of ONOO^- at pH 7.4 to several seconds [45, 47]. At higher concentrations, ONOO^- may also decompose via a second-order process [48].

This reaction, which produces peroxyntitric acid (O_2NOOH), additionally shortens the lifetime of ONOO^- in pure aqueous solutions [49]. In biological settings the, the lifetime of ONOO^- is further shortened via scavenging by carbon dioxide; rapid reaction with thiol peroxidases, including Prxs; and heme proteins [50, 51]. Thus, similar to the case of $\text{O}_2^{\bullet -}$, one cannot expect significant accumulation of ONOO^- over time of incubation with ONOO^- -generating system, either in biological system or pure aqueous solutions. ONOO^- weakly absorbs UV light ($\lambda_{\text{max}} = 302 \text{ nm}$, $\epsilon = 1.7 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ [52, 53]). This property is used for the determination of ONOO^- in its stock solutions under alkaline conditions.

In summary, a low steady-state concentration of cellular oxidants due to their short lifetime, in combination with unfavorable spectroscopic properties, makes their direct detection practically impossible. Therefore, probes allow for sensitive detection of those species in biological systems by converting ROS into easily detectable and more stable products, which typically accumulate over time of incubation to cross the detection limit. Luminescent probes are examples of compounds that react with cellular oxidants to form the products, which can be detected via chemiluminescence, coupled to a bioluminescent assay or using fluorescence-based techniques.

2.2. Properties of the ideal probes

The first and most important requirement for the luminescent probe to be used for ROS detection is that the amount of the detected product is proportional to the amount of the oxidant produced in the system investigated. All other requirements listed below are, at least in some respects, derivatives of this requirement.

Among the requirements the probes have to fulfill, arguably the least appreciated is that the probe should not affect the system being investigated. This could be compared to the golden rule of journalism, which states that “reporters should make every effort to remain in the

audience, to be the stagehand rather than the star, to report the news, not to make the news” [54].

This means that the probes not only should be nontoxic at the concentrations used, but also should lack effects on cellular function, for example cell metabolism. In the case of probes for ROS, it is important that they do not induce ROS formation or change the redox status of the cell.

However, the concentration of the probe should not be too low; not only should it be present in amounts higher than the total amount of the oxidant to be trapped by the probe, it should also be high enough to compete with other oxidant decay pathways. Thus, a sufficient amount of the product is formed for luminescent detection and for the detected signal to be proportional to the amount of oxidant or the rate of its formation.

Ideally, the uptake/availability of the probe for the oxidant should be the same between the biological variables studied. In case of differences in probe availability/cellular levels, there should be a simple way to correct the results, so that they accurately reflect the oxidant levels rather than the probe availability.

Ideally, the probe should be selective toward a single oxidant and resistant to ROS-independent consumption. In case of a lack of selectivity, different oxidants should form different, specific products, easily distinguishable by the detection technique used.

The product of the reaction between the probe and the oxidant should be stable enough to allow quantitative analyses of the amount formed. For chemiluminescent probes, that means the intermediates formed during the reaction should not be scavenged by other cell components, and for fluorescent probes, the product should be able to accumulate over the time of the experiment (except for quasi-reversible probes, as discussed in a subsequent section).

It is preferable that the amount of the product formed, rather than the extent of probe consumption, is measured. For the extent of probe consumption to be measured accurately, it must account for at least three times the standard deviation (SD) of the measurement of probe level. Thus, even if the relative SD (or coefficient of variation) is 10%, probe depletion of 30% would be required, which—taking into account low levels of ROS in cells—would require very low concentrations of the probe and, in turn, would limit the ability of the probe to compete with other targets to trap the oxidant.

2.3. Luminescence-based approaches for ROS detection

Of many the modes of detection, luminescence-based techniques garnered the most interest and were widely applied in biological systems. This is due to the high sensitivity of luminescence detection as well as the ability of nondestructive, real-time monitoring of the formation of luminescent products and of subcellular localization of the products formed. A range of biocompatible fluorophores, differing in their brightness and fluorescing in various spectral regions, including *in vivo*-compatible near infrared (NIR), is available and still expanding [55–60].

In the simple case of the fluorogenic probe, the probe by itself is not fluorescent, but upon reaction with oxidants, it forms a fluorescent product, with characteristic excitation and emission wavelengths (Fig. 2A). In such a case, it is difficult to determine the level of the probe. This is the case for most small-molecule fluorogenic probes for ROS currently in use. In some cases, the probe is also fluorescent, but upon reaction with oxidants, the fluorescent product exhibits fluorescence in a different spectral region (Fig. 2B). These probes, called ratiometric, enable monitoring of the levels of the probe and the oxidation product, which helps in interpretation of the results [61].

In case of chemiluminescent probes, the reaction of the probe with ROS leads to the formation of the intermediate in the excited state, which relaxes to the ground state with the emission of light, detected as chemiluminescence (Fig. 3). The examples of chemiluminescent probes include luminol (LH₂), L-012, and *Cypridina* luciferin methoxy-analogue (MCLA).

The bioluminescent probes react with oxidants to form a product, which is a substrate in the subsequent bioluminescent reaction. The example is a peroxy-caged luciferin (PCL-1) probe, which upon oxidation releases luciferin (Luc). In the presence of luciferase and its co-factors (ATP, O₂), Luc is oxidized to oxyluciferin with the emission of light.

2.4. The lack of reversibility of the reaction of the probes for ROS

Most fluorescent probes for the real-time measurement of stable analytes in cells (*e.g.*, calcium cation, transition metal ions) are based on the formation of a reversible complexes with the analytes (Fig. 5A) [62–66]. The fluorescent properties of the probe are affected by the binding of the analyte and from the changes in fluorescence intensities and the known binding constant, one can determine the absolute concentration of the analyte. The differences in the binding constants for various analytes will determine the selectivity of the probe. Also, the decrease in the concentration of the analyte will reverse the changes in fluorescence intensity. This enables relatively straightforward monitoring of the dynamics of the changes of analyte concentration over time. The probes for ROS, however, are based on the irreversible reaction of the probe; thus, the product formed cannot yield the probe and the oxidant in the reverse reaction (Fig. 5B). In this regard, the ROS probes resemble the probes used to monitor enzymatic activity in intact cells, and often are based on similar fluorophores using a different chemical sensing moiety [63, 66–69].

This implicates that the fluorescent products accumulate over time of incubation and inhibition of oxidant formation or scavenging of the oxidant would not result in a decrease in the amount of the product formed but rather would slow down the rate of product formation. Therefore, any decrease in fluorescence intensity during the experiment should be interpreted as indicative of additional factors affecting the signal intensity, in addition to ROS level/rate of formation.

Over the last decade, new probes have been proposed, in which the product formed upon probe oxidation, can be reduced back to the probe by cellular reducing systems (Fig. 6) [70].

These probes are based on the chemistry of biologically compatible redox couples, including quinones, nitroxides, and selenium or tellurium-containing compounds, in which the redox state of the sensing moiety affects the fluorescence of the probe. Because the reduction reaction (probe recovery) is not a reverse of the reaction of the probe with the oxidant, but a separate reaction with cellular reductant(s), these should be called *quasi-reversible* probes. In many respects, they behave as true reversible probes, but the extent of their oxidation (or the redox status of the probe) is a function of both the level of oxidants (forward reaction) and the activity of the reducing system (reverse reaction). Due to the nature of quasi-reversible probes and the mechanism employed, these probes may act as catalytic antioxidants (by analogy to the biological activity of ebselen), and thus should be used at low concentration, to not perturb the redox status of the cell.

2.5. Rate of ROS formation vs. total amount trapped

Depending of the type of probe, the luminescence signal intensity may report a different parameter of cellular ROS production. The most widely used fluorogenic probes are nonreversible, and their products accumulate over time of experiment/incubation. Thus, the fluorescence signal intensity will reflect the total amount of the oxidant, which reacted with the probe. Theoretically, even if the amount of oxidant is very low, by extending the time of incubation, one can accumulate a sufficient amount of the product to cross the threshold of detection limit. In practice, however, this may be limited by the rate of autooxidation and/or nonspecific oxidation of the probe under the conditions used.

In case of quasi-reversible probes, the signal intensity reflects the redox status of the probe, which reflects the rates of two opposite processes, the oxidation of the probe and the reduction of the product, as mentioned above.

In case of chemiluminescent and bioluminescent probes, there are two possibilities: (i) the rate-limiting step is the reaction of the probe with the oxidant, and (ii) the rate-limiting step is the reaction of the intermediate product leading to the emission of light. In the first case, there is no accumulation of the reaction intermediates, and the luminescence intensity reflects the rate of probe oxidation. In the second case, the signal intensity may reflect the extent of accumulation of the reaction intermediate, similar to irreversible fluorescent probes.

2.6. Selectivity of the probes

One of the goals in studying the role of oxidants in redox biology is to identify the species involved in the process. Therefore, high selectivity of the probe toward a specific oxidant has been the “holy grail” in the development of new probes for ROS. In reality, however, no probe has a complete selectivity for a single biological oxidant, and numerous selectivity claims that appeared in the literature have been dismissed by follow-up studies.

One of the ways to overcome the limitation of the lack of selectivity is to inhibit the sources or to selectively scavenge a specific ROS (Fig. 7).

By inhibiting specific pathways of probe oxidation, or efficient scavenging of the oxidant, one can estimate the contribution of different oxidants to the total rate of probe oxidation.

For example, in the case of boronate-based probes, inhibition of ONOO^- formation by inhibiting NO production or scavenging $\text{O}_2^{\bullet-}$ will block ONOO^- -dependent pathways, while scavenging H_2O_2 by catalase will prevent the H_2O_2 -mediated probe oxidation.

2.7. Formation of specific products

Another possibility to monitor a single oxidizing species is to follow a specific product, which is formed by only one oxidant. If the probe forms a specific product for a single oxidant, even if the probe is not completely selective, it can still be used to detect this oxidant, assuming there is a way to selectively monitor such a product (Fig. 8).

There are several examples of the formation of oxidizing species-specific products, including the oxidation of hydroethidine by $\text{O}_2^{\bullet-}$ to 2-hydroxyethidium or the formation of the minor products from boronate probes by ONOO^- . In most cases, however, the detection of those specific products requires a combination of the luminescence measurements with chromatographic or other techniques, which allows selective detection of the oxidant-specific products.

2.8. Kinetics, bioorthogonal approach

The amount of the detectable product formed is controlled by the rate of the reaction between the probe and the oxidizing species. At any time during incubation, this rate (V) is directly proportional to the concentrations of the oxidant (c_{oxidant}) and the probe (c_{probe}):

$$V = k \times c_{\text{oxidant}} \times c_{\text{probe}}.$$

As discussed above, the cellular oxidants undergo self-decay and/or consumption by cellular antioxidant defense or other cellular targets and do not significantly accumulate in cells. The added probe has to compete with those processes to be able to detect the oxidant (Fig. 9). Therefore, for efficient scavenging of the oxidant, the rate constant (k) of its reaction with the probe and the probe concentration should be sufficiently high. In reality, while it is possible to completely scavenge extracellular oxidants for their quantitative analyses, intracellularly, typically only a portion of the oxidant pool reacts with the probe.

Therefore, the quantification of the product formed may be not sufficient to determine the rate of oxidant formation, unless the probe-independent pathways of ROS decay are outcompeted by the probe, or the relative contribution of the probe to the total oxidant consumption can be quantitatively determined. The complete scavenging of the oxidant by the probe can be experimentally verified by testing the effect of probe concentration on the yield of the product. If doubling the probe concentration does not significantly affect the product yield, one can assume that the probe is used at a sufficiently high concentration to outcompete other pathway(s) of oxidant decay.

The opposite situation, when the probe reacts with only a small fraction of the oxidant, is more typical for intracellular detection of ROS. In such case, only a small amount of the oxidant is intercepted by the probe and a small amount of the detectable product is formed, requiring highly sensitive detection. This can be achieved, for example, by using the

fluorogenic probe, which yields the product characterized by high brightness (the product of the extinction coefficient and the fluorescence quantum yield). Scavenging only a small fraction of the oxidant by the probe has its own advantages, as the probe would not significantly perturb any oxidant-mediated processes, and thus would work in a *bioorthogonal* mode (Fig. 10).

Another aspect of this case is that the extent of product formation should be directly proportional to the intracellular probe concentration. Therefore, the amount of the product could be “normalized” to probe availability by dividing the product concentration by the probe concentration. This approach, however, requires validation in the system tested, and one has to be able to determine probe concentration/availability. Determination of the probe uptake can be done using fluorometric measurements of the probe concentration in case of ratiometric ROS probes [61] or by high-performance liquid chromatography (HPLC)-based analyses.

Finally, the differences between homogenous chemical systems and a highly heterogeneous cellular environment should be taken into account when considering the rates of cellular processes, including trapping the oxidants by ROS probes. In addition to subcellular compartmentalization, the phenomenon known as cellular or macromolecular crowding alters the properties of the intracellular compounds due to a limited solvent volume available for the dissolved molecules [71]. This may significantly affect the reaction kinetics, including decreased solute diffusion, as demonstrated experimentally for enzymatic reactions [72, 73]. However, the practical effects of cellular crowding on the efficiency of small-molecule probes in trapping various oxidants remain to be experimentally established.

2.9. Subcellular localization of the probe and the product

One of the advantages of fluorescence-based detection is the ability to localize the signals at the subcellular level using microscopy techniques. To be able to determine the site of oxidant production, the probe should be distributed over all subcellular components to a similar extent. In such a case, the distribution of the signal intensity may reflect the compartmentalization of the ROS production (Fig. 11).

It should be noted, however, that the differences in signal intensities may also be due to the changes in the photophysical properties, including the fluorescence yield of the product, due to differences in the microenvironment (different pH, dielectric constant, binding to DNA, etc.) [74]. Finally, the timing of the experiment should be shorter than the time needed for redistribution of the product between subcellular compartments as well as the influx/efflux of the product into the extracellular space [75–78]. This can be exemplified by the case of hydroethidine probe oxidation, where upon initial oxidation the product(s) may redistribute to the nucleus, due to its DNA binding affinity. The subcellular distribution of the fluorescent products (*e.g.*, rhodamines, cyanines, ethidium analogs) and fluorescence intensity may be also affected by the changes in mitochondrial membrane potential, a confounding factor in the measurement of localization and rates of ROS production [75, 76, 79]. Clearly, not always the subcellular localization of the fluorescence can be equated to the site of oxidant production and the subcellular distribution of the probe and the cellular

retention and affinity of the fluorescent product(s) to subcellular organelles should be determined.

The ability of the probe to accumulate in a specific subcellular compartment, however, may be used for site-specific detection of oxidants. Thus, several strategies have been made to modify the chemical structure of the probes to increase their cellular uptake/retention and target them to various organelles [80–83]. As shown in Fig. 12, the probe may be linked to the triphenylphosphonium cation to selectively accumulate it in cell mitochondria [84]. Because mitochondria are characterized by the most negative membrane potential among cellular organelles, cell-permeable cationic compounds accumulate in mitochondria to a significantly higher extent than in other organelles, as according to the Nernst equation [84, 85].

It should be emphasized that the use of a targeted probe does not allow for determination of the major source of the oxidant in the cells; rather, it enables selective monitoring of oxidants in a specific compartment.

2.10. Major limitations in the use of luminescent probes

Understanding the limitations of the probes is a prerequisite for their successful use and accurate interpretation of the experimental data. Some of the most common limitations of the luminescent probes are discussed in the following sections.

The lack of efficiency in trapping the oxidant—As discussed above, the reaction of a probe with the oxidant should be fast enough to compete with other pathways of oxidant decay (Fig. 9) to produce a sufficient amount of the product, enabling its detection by the technique of choice. The efficiency of trapping the oxidant depends on the relative rates of its reaction with the probe and of the other pathways of oxidant consumption, including scavenging by the intracellular low molecule antioxidants and antioxidant enzymatic systems. One way to improve the trapping efficiency is to increase the concentration of the probe. Low trapping efficiency may be explored for bioorthogonal detection, without affecting cellular function and/or ROS signaling. However, if the probe cannot compete with other cellular targets of the oxidant, the amount of the product formed may be too low for its detection.

As an example, detection of the hydroxyl radical in biological systems is extremely difficult, as it is rapidly scavenged by most cellular components. Thus, to scavenge even 1% of the radical formed, the probe would need to be present at >10 mM concentration. Additionally, the solvent used for preparing a stock solution of the probe should not be reactive toward the oxidant of interest. For example, when using a 10 mM probe in a dimethyl sulfoxide (DMSO) solvent, DMSO is present at >1000-fold molar excess over the probe, and may outcompete the probe for the hydroxyl radical or hypochlorous acid.

The lack of selectivity—Despite numerous claims of the probes' selectivity, the “inconvenient truth” is that there are no fully selective probes for the oxidants. Many claims regarding the probes' selectivity have been based on a limited number of oxidants tested and/or less than optimal reaction conditions (*e.g.*, use of an oxidant-reactive solvent,

consumption of the luminescent product by the excess of oxidant used). The lack of selectivity may prevent identification of the oxidant detected, unless specific inhibitors and/or scavengers are used, as discussed above (Fig. 7). Also, in some instances, the lack of selectivity may be overcome by the specificity of the products formed.

Probes may affect the cellular function—When used at high concentrations, some probes become toxic to cells, for example, due to the probe or its oxidation product binding to DNA or accumulating in mitochondria and diminishing the mitochondrial membrane potential [86, 87]. Even if the probe is nontoxic, it may still affect cellular function, which should be reflected in altered metabolism and cell growth rate. On the other hand, a high concentration of the probe may be needed for efficient trapping of the oxidant. Therefore, it is recommended that the “safe” concentration of the probe be determined experimentally, by testing its effect on the cell growth rate, cellular respiration, etc.

Photosensitivity of the probe—Many luminescent probes are photosensitive and may undergo photooxidation upon exposure to visible and/or UV light (Fig. 13). The product could be the same as that formed upon reaction of the probe with the oxidant of interest. In addition, molecular oxygen may serve as the electron acceptor during the photooxidation process, leading to the formation of $O_2^{\bullet-}$ and H_2O_2 , which can further drive the oxidation of the probe. Thus, the samples, as well as stock solutions of the probes, should be shielded from light to minimize the photooxidation reaction. If the excitation light used in fluorescence measurements can be absorbed by the probe, it may lead to probe photooxidation during the detection event.

In such case, the photooxidation cannot be avoided, and an effort should be made to minimize the time of sample exposure to the excitation light, and to make sure that all samples to be compared receive the same light exposure.

Oftentimes, the absorption spectra of the probes and products differ sufficiently, so that the excitation light cannot be directly absorbed by the probe and the direct photooxidation is negligible. It is still possible, however, that the fluorescent products formed upon probe oxidation would act as photosensitizers for the probes (Fig. 14). In this case, light is absorbed by the fluorescent product, generating the product in the electronically excited state. The product in the excited state may relax to the ground state, emitting light (fluorescence). If the lifetime of the excited state of the product is long enough, it may also react with the probe to oxidize it. Then, the fluorescent product would sensitize the probe to excitation light-induced oxidation. Also, the higher the amount of oxidation products, the larger extent photooxidation of the probe is possible.

Regardless of the mechanism, photo-induced oxidation is a common limitation of many fluorogenic probes, contributing to the common observation that the longer the fluorescence is measured, the stronger the signal detected.

Redox cycling and generation of ROS by probes and products—The nature of many reactions of luminescent probes with ROS is the involvement of many steps leading to the detectable product(s), with the formation of transient products of radical character. If the

radical formed from the probe can reduce oxygen, $O_2^{\bullet-}$ and H_2O_2 may be generated in this process. In case the initial oxidant was $O_2^{\bullet-}$ or H_2O_2 , this may lead to a chain reaction, amplifying the signal. However, if the initial one-electron oxidant was different, for example a heme protein, this may lead to a shift in the identity of the oxidant responsible for the formation of the detectable product, confusing data interpretation (Fig. 15).

An example of such a case is the measurement of oxidants during cell apoptosis using a dichlorodihydrofluorescein (DCFH) probe. When cell undergoes apoptosis, cytochrome *c* is mobilized and released from mitochondria into cytosol. DCFH probe reacts with cytochrome *c*, to produce a DCFH-derived radical, which reacts with oxygen to produce $O_2^{\bullet-}$, which then dismutates into H_2O_2 . In turn, H_2O_2 will drive further, cytochrome *c*-catalyzed oxidation of DCFH. Thus, while the initial event in probe oxidation was the reaction with cytochrome *c* (ROS-independent oxidation), further reaction of the probe radical with oxygen will switch the mechanism into H_2O_2 -dependent oxidation, inhibitable by H_2O_2 -depleting agents. Without any knowledge of the reactivity of the DCFH probe toward cytochrome *c* or the reactivity of the DCFH-derived radical toward oxygen, one would make a conclusion that apoptosis is driven by, or results in, ROS generation, although in such a case, the probe just reports the mobilization of cytochrome *c*.

Sensitivity to pH changes—An additional factor, which may affect the signal intensity from ROS probes, is the pH of the probe/product microenvironment. Although changes in pH are typically considered as a confounding factor in the measurement of oxidants using genetically encoded probes (e.g., roGFP and Hyper probes) [88, 89], the possibility that pH may affect the results of measurements using small-molecule probes for oxidants is typically neglected. The changes in pH may, however, affect the kinetics of the reaction of the oxidant with the probe, modulate the reactivity of probe-derived intermediate(s), and modify the fluorescent properties of the product(s). The kinetics may affect the portion of the oxidant scavenged by the probe (see Fig. 9), and the changes in pH may affect the kinetics of the reaction due to a change in the protonation state of the probe and/or the oxidant. For example, $O_2^{\bullet-}$ may undergo protonation to form a much stronger oxidant, $HO_2^{\bullet}$, while deprotonation of H_2O_2 will increase its reaction rate with boronate- or carbonyl-based probes.

Changes in pH may also affect the luminescent properties of the products formed, especially if the pK_a of the ground and/or excited state of the product is not far from the physiological pH (between 5 and 10). Changes in the protonation state of the luminescent product in its ground state typically result in the changes in the absorption spectra, resulting in a shift in maximum excitation wavelength. Changes in the protonation state of the product in its excited state will affect the fluorescence emission properties, including the maximum emission wavelength and the quantum yield. Although such properties can be used to measure intracellular pH [90], they may result in misinterpretation of the experimental data, when monitoring cellular oxidants using luminescence-based techniques. Typically, fluorophores containing hydroxyl groups possess pK_a sufficiently close to physiological pH, to exhibit fluorescence intensity changes with changes in cellular pH (pK_a of umbelliferone, fluorescein, and resorufin are 7.8, 6.4, and 5.8, respectively) [57, 58]. On the other hand, the fluorophores containing amine groups (e.g., 7-aminocoumarin, rhodamines) are much less

sensitive to pH changes in the physiologically relevant range. The pH sensitivity may be modulated by introducing electron donating or by withdrawing substituents into the fluorophore structure. As an example, the halogenation of phenolic fluorophores stabilizes their deprotonated, anionic form, leading to a lower pK_a and decreasing pH sensitivity in the physiologically relevant pH range. The confounding effects of the pH sensitivity of the products can also be overcome by performing the detection under controlled pH conditions, as exemplified by HPLC- or microfluidic device-based measurements.

3. Probes used for cellular oxidants

3.1. Reduced fluorophores

2', 7'-dichlorodihydrofluorescein—The DCFH probe is a non-fluorescent product of a chemical reduction of the fluorescent dye, 2',7'-dichlorofluorescein (DCF). For improved probe stability and cellular uptake, the probe is produced and stored in the acetylated forms (diacetate ester, DCFH-DA). In the alkaline solution or in the presence of esterases, DCFH-DA undergoes hydrolysis to DCFH, the form reactive toward oxidants. Upon reaction with oxidants, DCFH undergoes reoxidation via two consecutive one-electron oxidation steps to DCF with a significant increase in the fluorescence signal (Fig. 16).

Although it has been widely used to measure intracellular H_2O_2 , it must be emphasized that the reaction requires a catalyst, typically heme proteins or transition metal ions [77, 91, 92]. In fact, the initial report on the use of DCFH for H_2O_2 measurement included horseradish peroxidase as a catalyst [93, 94]. Heme proteins alone have been shown to induce DCFH oxidation [77]. Various oxidants, including ONOO⁻-derived radicals, peroxy and thiyl radicals are capable of oxidizing DCFH [22, 95]. Therefore, the probe is currently used to measure “general ROS” without any selectivity toward a specific species. Due to the requirement of a catalyst for the reaction with hydroperoxides, it has been postulated that DCFH reports changes in intracellular iron concentration and cytochrome *c* mobilization rather than in H_2O_2 production [96, 97]. This property seems to be important for the application of the probe to monitor ferroptosis, an iron-dependent programmed cell death [98–101].

However, the lack of selectivity is not the only limitation of the probe. The ability of the probe to generate ROS upon reaction with a non-ROS oxidant (*e.g.*, heme protein) is one of the major problems. As discussed above (Fig. 16), one-electron oxidation of DCFH by the oxidants leads to the formation of the DCFH radical (DCFH^{•-}). Further oxidation of the DCFH-derived radical produces the fluorescent product, DCF. Molecular oxygen is one of the species capable of oxidizing DCFH derived radical to DCF (Fig. 16) [102]. This reaction is relatively fast ($k = 5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4), and leads to the formation of $O_2^{\bullet-}$. Dismutation of $O_2^{\bullet-}$ produces H_2O_2 , which may drive further iron- or heme-catalyzed probe oxidation, as exemplified in Fig. 15. Additional confounding factors are the ability of the product, DCF, to “leak” to the extracellular medium and the photochemical behavior of DCF, possibly generating additional $O_2^{\bullet-}/H_2O_2$ during the exposure to the excitation light.

Dihydrorhodamine—Dihydrorhodamine (DHR) is a two-electron reduction product of the red fluorescent cationic dye, rhodamine 123 (Rh123). Similar to the case of DCFH,

H₂O₂ does not react directly with DHR but can oxidize it in the presence of a catalyst (*e.g.*, peroxidase, cytochrome *c*) [78]. Also, it has been shown that peroxynitrite-derived radicals can oxidize the probe to the fluorescent product [22, 95]. DHR oxidation by one-electron oxidants proceeds via the intermediacy of the DHR-derived radical (Fig. 17).

Similar to the DCFH-derived radical, the DHR radical reacts with oxygen to form the fluorescent product (Rh123) and O₂ ($k = 5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4) [103]. Thus, oxygen concentration may affect the rate of formation the fluorescent product and lead to production of O₂^{•−}/H₂O₂. In contrast with DCFH, the product of oxidation does not “leak” to the medium, but is retained in cells [78] and due to its positive charge may accumulate in mitochondria.

Hydroethidine, MitoSOX Red, Hydropropidine—Hydroethidine (HE; or dihydroethidium, DHE) is a product of the reduction of another fluorescent dye, ethidium (E⁺), widely used for DNA staining [21]. Upon oxidation via two one-electron oxidation steps, with the involvement of the HE-derived radical, E⁺ may be formed (Fig. 18).

In contrast with DCFH and DHR, HE can be directly oxidized by O₂^{•−} (or its protonated form, HO₂[•]). The product of the reaction of HE with superoxide is 2-hydroxyethidium (2-OH-E⁺) and not E⁺ [104–106]. 2-OH-E⁺ has been shown to be a specific product for O₂^{•−}, as there is no other biologically relevant oxidant that can convert HE into 2-OH-E⁺ unless O₂^{•−} is involved. Thus, the conversion of HE into 2-OH-E⁺ has been used as a basis for specific detection for O₂^{•−}. Similar to DCFH and DHR probes, HE does not react directly with H₂O₂, but in the presence of a catalyst (iron, heme proteins), H₂O₂ will drive the oxidation of HE to E⁺, as well as dimeric products (*e.g.*, E⁺-E⁺) [107]. Once formed, E⁺ may translocate to the nucleus and bind DNA, which is accompanied by an increase in fluorescence intensity [76].

Because both E⁺ and 2-OH-E⁺ are formed in cells, with typically much higher levels of the former, the selective detection of 2-OH-E⁺ is required for O₂^{•−} detection. With typical fluorescence parameters used for E⁺ detection, it is nearly impossible to distinguish 2-OH-E⁺ and E⁺ in the presence of excess of E⁺. Typically, HPLC (or liquid chromatography-mass spectrometry [LC-MS])-based separation and detection of 2-OH-E⁺ is used [108–110]. Due to the differences in the excitation spectra of 2-OH-E⁺ and E⁺ in the 350–450 nm region, it was proposed that using this property may allow for selective monitoring of O₂^{•−} using fluorescence-based assays [111, 112]. In fact, several reports show data obtained with excitation at 405 nm, for more selective O₂^{•−} detection [113, 114]. This, however, has been not validated against the HPLC-based method in cellular systems, so the applicability and robustness of such an approach remains to be experimentally established.

E⁺ and 2-OH-E⁺ are not the only products of HE oxidation (Fig. 19). As already mentioned, one-electron oxidants also form diethidium (E⁺-E⁺), among other dimeric products. It has been also demonstrated that, in the presence of hypochlorous acid (HOCl), HE is converted to 2-chloroethidium (2-Cl-E⁺), which can be used as a marker of the activity of myeloperoxidase *in vitro* and *in vivo* [115, 116]. As these products (E⁺-E⁺ and 2-Cl-E⁺) are non- to low-fluorescent, one has to use the chromatographic techniques, HPLC or LC-MS

for their detection and quantification. The additional benefit of those techniques is the ability to monitor the intracellular level of the probe (HE). This may help answer the question of whether the changes in product amount are due to changes in the level of oxidant or in the level (availability) of the probe.

In addition to HE, which is expected to be distributed over most cellular organelles due to its lack of charge, two probes have been reported—MitoSOX Red (or Mito-HE [111]) and hydropropidine (HPr⁺ [117])—for more selective subcellular localization. MitoSOX Red (or Mito-HE) is an HE derivative, where the probe has been linked to the triphenylphosphonium cation to target the probe to mitochondria. HPr⁺ is an HE analog formed by the reduction of propidium iodide, a dye used to stain dead cells, as live cells do not accumulate it. HPr⁺ is a cell-impermeable analog of HE and can be used to monitor extracellular generation of O₂^{•-}, for example, produced by neutrophils (NADPH oxidase-2).

Recently, a new analog of MitoSOX Red, MitoNeoD, has been reported [118]. The chemical modifications used resulted in a loss in the DNA-binding ability of the oxidation products and slowed down two-electron oxidation of the probe, resulting in improved probe stability. The probe has been used to detect O₂^{•-} formation *in vivo* using the LC-MS approach. While the chemical reactivity of the probe toward O₂^{•-} has been well-characterized, the reactivity toward other biologically relevant small-molecule oxidants remains to be determined but may be expected to be similar to that of the HE probe, except for a decreased rate of radical dimerization due to the steric hindrance and a decreased rate of hydride transfer (two-electron oxidation) due to the presence of deuterium. Improved stability also has been previously reported for a deuterated isotopomer of the HE probe [119].

While much effort has been made to improve the probe and the detection method selectivity toward O₂^{•-}, all the HE analogs synthesized thus far share the same or a similar chemical reactivity to HE and are subject to similar limitations, including the requirement of HPLC-based detection of the specific products.

Hydrocyanines—Hydrocyanines (HCy) are a class of compounds formed upon reduction of Cy dyes [120, 121]. Several structurally related compounds, of different lipophilicities, numbers of methylene groups between the indole moieties, and fluorescence properties, belong to this class of probes. In the presence of oxidants, HCy may be converted to Cy, a fluorescent cationic dye (Fig. 21). Several probes based on a similar approach have been reported [122, 123].

The major advantage of HCy probes is that the product formed, Cy, fluoresces in red or in the near-infrared (NIR) region, compatible with *in vivo* fluorescence imaging. Although it has been proposed that HCy probes are selective for one-electron radical oxidants, the rate constants for specific oxidants remain to be determined. Also, no data are available on the ability of peroxidases to catalyze the reaction of HCy probes with H₂O₂. The possibility of the formation of different oxidation products and the fate of the probe-derived radical in the presence and absence of oxygen is currently unknown. Clearly, more mechanistic studies on the chemical reactivity of HCy probes and reaction mechanisms are needed for the application of this promising class of compounds for rigorous detection of ROS in biological

systems. Similar to HE, deuterated analogs of hydrocyanines have been synthesized and are reported to exhibit improved stability [119]. As with many other positively charged fluorophores, the fluorescence properties of Cy dyes are affected by the mitochondrial membrane potential, which may confound HCy-based fluorescence measurements of the oxidants [79].

3.2. Chemiluminescent probes

Luminol and analogs—LH₂, isoluminol, and L-012 are structurally similar chemiluminescent probes that have been used to detect oxidants in biological systems, specifically O₂^{•−} [124–128]. The probes also have been used to measure H₂O₂ in the presence of peroxidases [129, 130]. Luminol-derived chemiluminescence induced by peroxidatic activity is widely used in bioassays [131]. The first step of the reaction is oxidation of the probe to its radical. Reaction of luminol radical with O₂^{•−} leads to the formation of aminophthalic acid in the excited state, which relaxes to its ground state with the emission of blue light (Fig. 22) [132].

Although the probes have been used to detect O₂^{•−}, it was shown that they do not react directly with O₂^{•−}, but need activation to their radicals (one-electron oxidation) for the reaction to occur [133]. Thus, the presence of a stronger oxidant will affect the yield of chemiluminescence. Furthermore, it has been shown that, in the presence of molecular oxygen, oxidation of L-012 to its radical is sufficient to generate chemiluminescence, which is inhibitable by the scavenging of O₂^{•−} by SOD [129, 133]. Clearly the L-012-derived radical can react with oxygen to produce O₂^{•−}, a feature it shares with LH₂ (Fig. 22) [19, 133]. Therefore, while LH₂ and L-012 probes may report one-electron oxidizing species, the probes should not be used for selective detection of O₂^{•−}, and the availability of oxygen may be one of the parameters controlling the luminescence intensity.

Lucigenin—Lucigenin (Luc²⁺) is a dicationic chemiluminescent probe that has been used for the detection of O₂^{•−} (*e.g.*, in cell-free systems and tissue homogenates) [134]. It has been widely used in luminescent assays of NADPH oxidase in cell and tissue homogenates [135]. In contrast with the probes described above, the mechanism of producing luminescence is based on reduction of the probe to its radical (Luc^{•+}) (Fig. 23). The Luc²⁺-derived radical of reacts with O₂^{•−} to produce 10-methylacridone in the excited state. Upon relaxation to its ground state, 10-methylacridone produces blue luminescence.

Similar to the LH₂-derived radical, Luc^{•+} can react with O₂ to produce O₂^{•−} [136–138]. In fact, the reaction of Luc^{•+} with O₂ is thermodynamically preferred over the reaction of Luc²⁺ with O₂^{•−}, as the one-electron reduction potential of O₂, E(O₂(1 M)/O₂^{•−}) = −0.16 V, is more positive than of Luc²⁺, E(Luc²⁺/Luc^{•+}) = −0.28 V [139]. This is reflected in the reaction kinetics, with the rate constant of the reaction of Luc²⁺ with O₂^{•−} (*k* ~ 6 × 10⁴ M^{−1}s^{−1}) lower than of the reduction of O₂ by Luc^{•+} (*k* = 3 × 10⁶ M^{−1}s^{−1}) [139]. It has been proposed that cellular flavoproteins are responsible for the one-electron reduction of Luc²⁺ to form Luc^{•+} [20]. Because in the presence of oxygen Luc^{•+} can produce O₂^{•−}, the occurrence of one-electron reduction of Luc²⁺ is sufficient to generate superoxide-dependent luminescence, inhibitable by SOD. This conclusion may explain why NADPH-dependent

Luc²⁺-derived luminescence can be observed in tissue homogenates obtained from NADPH oxidase-1, -2 and -4 triple knockout animals [140].

Coelenterazine and analogs—Coelenterazine and its structural analogs, including *Cypridina* luciferin analog (CLA) and MCLA, are another class of chemiluminescent probes used to detect O₂^{•−} [141–143]. Similar to LH₂, these probes require oxidation to the probe radical, followed by the reaction of the radical with O₂^{•−}, to produce luminescence (Fig. 24) [144]. It was proposed that HO₂[•] is responsible for the observed reaction of CLA with superoxide and that the deprotonated form of CLA (pK_a = 7.6) is the reactive form of the probe [145].

While the reactivity of the radical formed has not been studied in detail, the major reported limitation of those probes has been a high rate of autoxidation (the reaction of the probe with oxygen to produce O₂^{•−}, Fig. 24), leading to high background signal. Also, the chemiluminescence signal can be generated in the presence of nitric oxide or peroxynitrite, and it was reported that peroxidase can produce the chemiluminescence from MCLA in the presence of H₂O₂ [146]. Although new analogs of coelenterazine have been reported, producing a stronger chemiluminescence signal than MCLA, their reactivity toward other oxidants has not been studied in detail.

3.3. Probes for the peroxidase-based detection of extracellular H₂O₂

Simple phenolic probes—Although H₂O₂ is not a highly reactive oxidant, it can oxidize many compounds in the presence of a catalyst. Horseradish peroxidase is widely used to catalyze oxidation of phenolic compounds by H₂O₂. Several phenolic compounds upon oxidation to their radicals dimerize to produce a diphenolic product, exhibiting blue fluorescence (Fig. 25). This property has been used for selective detection of H₂O₂, using e.g., homovanillic acid (HVA) and p-hydroxyphenylacetate probes in the presence of HRP [147–150]. Due to the requirement of HRP, which is not cell membrane permeable, the assay is limited to H₂O₂ released extracellularly. The HPLC-based separation and detection of the product may improve assay sensitivity and detection selectivity.

Amplex Red—In some regards, Amplex Red is similar to the class of phenolic products described above, as it requires HRP and is used to detect extracellular H₂O₂ [151–153]. However, upon oxidation of Amplex Red to its radical, it does not dimerize, but instead undergoes further oxidation and deacetylation to produce a highly fluorescent product, resorufin (Fig. 26) [154, 155], with a 1:1 stoichiometry in a pure chemical system [151, 156].

Because of the good stability of Amplex Red and high brightness of resorufin, Amplex Red is one of the most preferred probes for H₂O₂ produced in cell-free systems or permeabilized cells or for H₂O₂ released to extracellular medium [157–161]. One of the major limitations of the assay is photosensitivity of the probe and the product [162–164]. It has been shown that resorufin may photosensitize oxidation of Amplex Red by oxygen, leading to overestimation of the amount of H₂O₂. Also, due to the requirement of HRP and the involvement of Amplex Red-derived radical, reducing agents, including antioxidants and peroxidase substrates may interfere with the assay [165–167]. This includes NADH and

NADPH, significantly limiting the applicability of the assay for cell-free assays of NADPH oxidases. Although one may assume that the requirement of HRP ensures the selectivity of the assay toward H_2O_2 , it has been demonstrated that ONOO^- can also serve as an oxidant in HRP-catalyzed probe oxidation [154]. In addition, carboxylesterase has been reported to convert Amplex Red to resorufin, in the absence of H_2O_2 or HRP [168]. Clearly, the Amplex Red-based detection of H_2O_2 should be always based on the determination of catalase-inhibitable fluorescence signal.

3.4. Probes based on deprotection of “caged” fluorophores

Phenylether derivatives of fluorophores—HPF (hydroxyphenylfluorescein) and APF (aminophenylfluorescein) probes have been developed for the detection of one-electron oxidants in biological systems [169]. In both cases, one-electron oxidation leads to the formation of the probe derived radical, which upon further reaction(s), releases fluorescein to produce green fluorescence. While the exact chemical mechanism of the reactions of various oxidants with APF and HPF has not been fully studied, it has been proposed that the radical formed requires the reaction with a second oxidizing/radical species, followed by hydrolysis, for the release of fluorescein fluorophore (Fig. 27) [170].

The derivatives of other fluorophores, including phenylamine derivatives, using a similar approach have also been reported [80, 171–177] as has a probe for bioluminescent ROS imaging [178]. Because of the difference in the chemical reactivity of phenols (present in HPF) and anilines (present in APF), the probes differ in terms of the selectivity and kinetics of the reaction with various biologically-relevant oxidants. Interestingly, APF, but not HPF, was shown to efficiently release fluorescein upon reaction with HOCl and hypobromous acid (HOBr), while other strong oxidants were reactive toward both probes. This may be due to a different mechanism of the reaction of APF with hypohalogenous acids (HOCl , HOBr) not involving probe-derived radical. Like other probes discussed above, APF and HPF do not react directly with H_2O_2 , but may be oxidized by H_2O_2 to release fluorescein in the presence of a catalyst, including iron or peroxidases. The probe-derived radical was proposed to be not reactive toward oxygen, as the yield of the product of the reaction of HPF with ferricyanide was shown to be independent of the presence of oxygen.

Boronate-based fluorogenic probes—Boronate-based probes were proposed for selective detection of H_2O_2 more than a decade ago [179, 180], and since then, tens of boronate-based probes have been developed that use the same chemical principle [181–185], including coumarin-7-boronic acid (CBA; Fig. 28) [42, 186]. Boronate-based probes have been designed to accumulate in various cellular organelles, including nuclei [187, 188], lysosomes [189, 190], and mitochondria [191–194], for site-specific H_2O_2 detection. It has been demonstrated that boronates react significantly faster with ONOO^- ($k \sim 1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) and HOCl ($k \sim 1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) than with H_2O_2 ($k \sim 1 \text{ M}^{-1}\text{s}^{-1}$) [42, 183, 195].

Also, amino acid- and protein-based hydroperoxides have been shown to oxidize the boronate-based probes, as exemplified in Figure 28 by tyrosine hydroperoxide (TyrOOH), with the rate constant one order of magnitude higher than determined for H_2O_2 [196]. The major advantage of boronate-based probes for the detection of H_2O_2 is that the reaction is

direct, without the requirement of the presence of a catalyst. Another advantage for the detection of H_2O_2 by boronates is the lack of radical intermediate during the conversion of the probe into the fluorescent product. This significantly limits the possibility of interference with the reaction by other compounds present in cells or extracellular medium, and it avoids the frequent problem of the generation of ROS by the probe-derived radicals, as discussed above for many widely used probes.

As mentioned, ONOO^- is much faster oxidant of boronates than H_2O_2 . To visualize the differences, Figure 29 shows the fluorescence of vials containing the same amounts of the boronate probe, CBA, with increasing the amounts of the added oxidants [42]. Blue fluorescence due to the oxidation of CBA to umbelliferone (COH [7-hydroxycoumarin]) develops within the mixing time (< 1 s) in the case of ONOO^- , with an intensity similar to the standard for COH. In the case of H_2O_2 , after 5 min, very little fluorescence is observed, and even after 30 min, the fluorescence intensity is much lower than in the case of added ONOO^- , or authentic standards for COH.

Boronates are currently the probes of choice and superior to any other class of probes for ONOO^- reported so far. This is due to the high rate constant of the reaction, enabling direct detection of ONOO^- in biological systems [156, 197–202]. It should be noted that several other probes used for ONOO^- detection react with ONOO^- much slower than boronates, or are based on the detection of ONOO^- -derived radicals ($\cdot\text{OH}$, $\cdot\text{NO}_2$ and $\text{CO}_3^{\cdot-}$). Although the reaction of boronates with ONOO^- is stoichiometric (1:1), the phenolic product formed typically accounts for only 85–90% of the amount of ONOO^- [203–205]. This is due to a minor pathway of the reaction of boronates with ONOO^- , including a phenyl radical-type intermediate, to form ONOO^- -specific product(s) (Fig. 30) [203, 206]. Thus, detection of those minor products has been proposed as a way to identify the oxidant detected [203–205, 207], as exemplified by identification of ONOO^- as the product of the reaction of HNO with molecular oxygen [206].

While most of boronate-based probes have been designed for fluorescence-based detection, the chemical principle involved allows for development of probes with a wide range of detection modalities, including chemiluminescence [208], bioluminescence (as described below) [209] and PET [210] for *in vivo* ROS detection.

Boronate-based bioluminescent probes—Boronate-based bioluminescent probes have been designed to release Luc upon oxidation by H_2O_2 . The first such a probe, PCL-1, was shown to react with H_2O_2 , with the formation of the phenolic product, which spontaneously decomposes to yield Luc (Fig. 31) [209]. More probes based on the same principle have been subsequently reported [211, 212]. In the presence of luciferase enzyme and additional cofactors, luciferin is oxidized to form oxyluciferin and emit light.

As with other boronates, it was demonstrated that PCL-1 reacts with ONOO^- nearly million times faster than with H_2O_2 [198]. Analysis of the products formed with different oxidants indicates that, although Luc is the only final product of oxidation by H_2O_2 , ONOO^- forms a minor but specific nitrated product (Luc-Bz- NO_2), while HOCl oxidizes PCL-1 to Luc,

which may be subsequently chlorinated by HOCl to 2-chloroluciferin (Luc-Cl, Fig. 32) [214].

Thus, combination of the bioluminescence measurements with structural characterization of the products by HPLC or LC-MS has been proposed for detection and identification of the oxidants in the *in vivo* systems. In addition to PCL-1, other probes based on the same principle, but with modified structures, have been reported and proposed for *in vivo* detection of H₂O₂. One of the limitations of the use of the PCL-1 probe and analogs is the requirement of luciferase enzyme, as well as ATP and O₂. Thus, the cells or animals need to be engineered to express luciferase. Fortunately, many luciferase-expressing cancer cells as well as transgenic mice are commercially available. The dynamics of the bioluminescence signal from PCL-1 or other bioluminescent probe should be compared with that from Luc to account for ROS-independent factors affecting bioluminescence intensity.

3.5. Other probes

Because the need for reliable and convenient-to-use probes has been widely realized, many laboratories remain focused on the development and validation of new probes and assays for cellular oxidants. Below are some examples of the reported probes, of which some may not yet be commercially available. However, with further characterization of their chemical reactivity, reaction mechanism and kinetics, and behavior in biological settings, these probes may have the potential to become useful tools to unravel the chemical biology of cellular small-molecule oxidizing species.

Benzenesulfonate-based probes—Several derivatives of fluorescein and other fluorescent dyes, with the hydroxyl or amine groups substituted with benzyisulfonate groups, have been synthesized and reported for detection of reduced thiols [215, 216], H₂S, O₂^{•−} [217, 218], and H₂O₂ [219–221], depending on the substituents present on the benzene ring [222]. The advantage of those probes is that reaction with the analytes is direct and is based on the nucleophilic displacement of the fluorescein moiety from the probe. Also, in case of H₂O₂ detection, the reaction proceeds via a nonradical mechanism. In case of the superoxide probe, the fate of the benzenesulfonate peroxy radical may need to be considered for accurate interpretation of the experimental observations.

Probes based on nucleophilic addition to a carbonyl group—Another promising approach for a catalyst-free detection of H₂O₂ is based on the reaction with α-keto carbonyl compounds, including α-ketoacids [223, 224] and α-ketoamides [225]. The reaction is direct and without involvement of radical intermediates [226]. Although selective detection of H₂O₂ has been claimed, there remains a possibility that ONOO[−] may react with such probes faster than H₂O₂, as has been shown for several carbonyl compounds, including pyruvate [227], methylglyoxal [228], and others [229].

Addition to a carbonyl group is also the basis for a series of probes designed for selective detection of ONOO[−] [230–232]. These probes (HKGreen-1–3) contain the ONOO[−]-reactive trifluoromethylketone moiety, and are based on the formation of fluorescent, ONOO[−]-specific products. The advantage of such probes is that the reaction with ONOO[−] is direct, in contrast to DHR or DCFH probes, which react with ONOO[−]-derived secondary oxidants.

The limitation of this approach is that the yield of the specific product is not well defined, as the adduct formed may decompose via several pathways [233, 234]. Thus, a more detailed analysis of the reaction mechanism and kinetics is needed for this class of probes.

Probes based on nitrative potential of ONOO⁻—Similar to nitration of protein tyrosine residues, nitration of fluorogenic probes can be used to monitor cellular “nitrative stress” involving generation of the nitrogen dioxide radical ($\cdot\text{NO}_2$). Several BODIPY-based probes (NISPY1–3) have been developed based on the principle of increased fluorescence intensity upon probe nitration [235]. The reaction typically includes oxidation of the probe to the radical, which recombines with $\cdot\text{NO}_2$ to form a fluorescent product. The identity of the product formed can be further confirmed by HPLC or LC-MS to corroborate the fluorescence data. It should be noted that ONOO⁻ is not the only biological source of nitration and peroxidase-mediated nitration by a nitrite in the presence of H₂O₂ should be also considered.

Quasi-reversible probes—Most of the probes described above react with oxidants to produce products that cannot be reduced back to the probes by cellular reductants, and the products accumulate during incubation. In contrast, quasi-reversible probes, while reacting with the oxidants irreversibly, produce products that can be reduced back to probes by cellular thiols or other nonenzymatic and/or enzymatic reductants [70, 82, 236, 237]. Many redox couples have been used for the design of such probes, including quinone-hydroquinone [238, 239], flavin-dihydroflavin [240], sulfhydryl-disulfide [241, 242], nitroxide-hydroxylamine [243, 244], selenium, and tellurium-based redox systems [245–248]. The fluorescent properties of such probes are affected by the redox state of the redox couple, which is controlled by both the rates of oxidation of the probe and of the reduction of the product. For optimal use, it is preferable that both probe and the oxidation product exhibit fluorescence, but in different spectral regions, enabling the ratiometric analyses to account for changes in probe concentration. Due to the nature of the redox couples, typically such probes lack selectivity toward a specific oxidant. Understanding the nature of oxidants and the processes responsible for probe recovery (product reduction) is important for accurate interpretation of the changes in the redox state of the probes. This class of probes may provide invaluable information on the dynamics of redox changes in biological systems and become a complementary tool to the irreversible probes described above, in the analyses of cellular redox signaling.

4. Recommended approaches for the detection of O₂^{•-}, H₂O₂, and ONOO⁻ using luminescent probes

As discussed above, all assays for cellular oxidizing and nitrating species have limitations. However, the probes used are not all equal and, based on the reaction mechanism, kinetics, and stoichiometry, the assays currently regarded as “state-of-the-art” can be identified and recommended for rigorous detection of ROS/RNS in cell-free and cellular systems. As a general rule, luminescence-based measurements should be used in combination with chromatographic techniques to determine probe levels/uptake and identify the product(s) formed [5, 11–13, 213, 249–251].

Superoxide radical anion

Currently, among the luminescent probes, the HE-based assay remains the most rigorous approach to detect superoxide in biological systems. The availability of HE analogs, targeting mitochondria (MitoSOX) and extracellular space (HPr⁺), allow for site-specific detection of superoxide production (Fig. 20). As discussed above, the detection of superoxide using those probes requires a combination of fluorescence measurements with HPLC or LC-MS-based determination of probe level/availability and identification of the oxidants formed. An additional benefit from the HPLC-based approach is the detection of oxidant-specific nonfluorescent products, including diethidium and 2-chloroethidium (Fig. 19).

Hydrogen peroxide

Although boronates are oxidized slowly by H₂O₂, they are currently the preferred way to measure H₂O₂ in cells, because the reaction does not require a catalyst. The identity of the oxidant should, however, be confirmed using genetic and/or pharmacologic approaches, as other acidic hydroperoxides (especially peroxynitrous acid) may also contribute to the oxidation of boronates.

For extracellular H₂O₂, the Amplex Red-based assay remains the most widely used and produces reliable quantitative data, assuming the catalase-sensitivity of the signal is validated and photochemical oxidation is avoided. Other phenolic probes in combination with peroxidase can be also used, but they may suffer from lower sensitivity compared with the Amplex Red assay.

Peroxynitrite

Due to the direct and fast reaction with peroxynitrite, boronate-based probes are currently the best tools for peroxynitrite detection. The identity of the oxidant typically can be confirmed by monitoring the specific products formed, as well as by pharmacologic treatments. A more detailed discussion of the luminescent approaches and recommended assays for the detection of peroxynitrite is discussed in a separate paper in this Special Issue [252].

5. Outlook

In the detection of cellular oxidizing species, it is of high importance to use a wide selection of probes to correctly determine the source, identity, and the production rate of the species involved in the biochemical/biological process studied. Although currently available probes are subject to numerous limitations, they allow identification and semiquantitative analyses of O₂^{•−}, H₂O₂, and ONOO[−], as discussed and recommended above.

Understanding the applicability and limitations of the recommended probes and assays is primarily based on knowledge of their experimentally determined chemical and biochemical reactivity [9, 13, 250, 253, 254]. Thus, although newly developed probes may provide an opportunity to overcome some of the current limitations, it is important that the chemical structure of the probe is known, a basic chemical reactivity toward various oxidants is

established, and the applicability to biological systems is validated against an established method.

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Highlights

- Luminescent probes for cellular oxidizing species are reviewed
- Luminescence probes can be used for real-time monitoring of cellular oxidants
- Chemical mechanisms of the probe reaction with the oxidants should be established
- HPLC- or LC-MS-based identification of the probe oxidation products is recommended

Fig 1.

A network of reactive oxidizing, nitrosating, and nitrating species. Primary products of oxygen reduction ($O_2^{\bullet-}$ and H_2O_2) can be converted to other species of different chemical reactivity, which can affect cellular redox status and/or lead to the damage of cellular components.

Fig 2.

Principles of the use of the fluorescent probes for the detection of ROS. (A) The probe is nonfluorescent and undergoes oxidation to a fluorescent product. (B) Both probe and products are fluorescent, but they differ in their excitation/emission spectral properties.

Fig 3.
Principle of ROS detection using the chemiluminescent probes.

Fig 4.
Principle of ROS detection using the bioluminescent probes.

Fig 5.
Comparison of probes based on (A) reversible formation of a complex with an analyte (exemplified by a metal cation M^{n+} binding) with (B) irreversible reaction of probes for ROS.

Fig 6.
Principles of ROS detection using quasi-reversible probes.

Fig 7.

Approaches to overcome the lack of selectivity of the probes. Inhibiting the sources of specific oxidants, or using selective scavengers, may help identify the oxidant(s) detected.

Fig 8.
Monitoring the specific product to overcome the lack of probe selectivity.

Fig 9.
Competition between the probe and other pathways of oxidant consumption.

Fig 10.
Principle of the bioorthogonal approach in the detection of cellular oxidants.

Fig 11.

Uniform distribution of the probe over subcellular compartments may allow the location of oxidant production to be determined using fluorescence microscopy.

Fig 12.
Targeting the probe to cell mitochondria by conjugation to a triphenylphosphonium (TPP⁺) moiety.

excitation light

Fig 13.

Direct photooxidation of the probe by the fluorescence excitation light in the presence of oxygen. Probe oxidation leads to formation of $O_2^{\bullet-}$ and H_2O_2 , which may further contribute to probe oxidation.

Fig 14.

Photosensitization of probe oxidation by the fluorescent product formed. The reaction of the probe with the excited state of the product is indicated by the red arrow.

Fig 15.

Example of the switch from heme to H₂O₂-driven oxidation of the probes, due to reaction of the probe-derived radical with oxygen.

Fig 16.
Chemical basis for dichlorodihydrofluorescein diacetate (DCFH-DA)-based detection of cellular oxidants.

Fig 17.
Chemical basis for DHR-based detection of cellular oxidants

Fig 18.
Oxidation of HE to fluorescent products on O₂^{•-}-dependent and independent pathways.

Fig 19.

Oxidant-dependent products identified from the HE probe. (Adapted from [11]. Reprinted by permission from Springer Customer Service Centre GmbH: Springer Nature, Cell Biochemistry and Biophysics, Recent Developments in the Probes and Assays for Measurement of the Activity of NADPH Oxidases, J. Zielonka, M. Hardy, R. Michalski, A. Sikora, M. Zielonka, G. Cheng, O. Ouari, R. Podsiadly, B. Kalyanaraman, © 2017)

Fig 20.

Targeting different cellular locations by HE derivatives, HPr⁺, and MitoSOX Red (Mito-HE).

Fig 21.
Oxidation of HCy to fluorescent product, Cy.

Fig 22.

Oxidation of LH₂ analog, L-012 probe, to produce luminescence, and the effect of the presence of oxygen.

Fig 23.
Reduction of lucigenin probe to produce luminescence, and the effect of the presence of oxygen.

Fig 24.

Oxidation of the coelenterazine analog, the MCLA probe, to produce luminescence, and the effect of the presence of oxygen.

Fig 25.

Oxidation of the HVA phenolic probe to produce a fluorescent dimer.

Fig 26.

Oxidation of the Amplex Red probe to produce fluorescent resorufin.

Fig 27.
Oxidation of the HPF and APF probes to produce fluorescein.

Fig 28.

Oxidation of the CBA probe by various two-electron oxidants to produce fluorescent COH.

The rate constants for various oxidants differ significantly in the following order: $\text{ONOO}^- \gg \text{HOCl} \gg \text{TyrOOH} > \text{H}_2\text{O}_2$.

Fig 29.
Visual comparison of the rates of oxidation of the CBA probe to a fluorescent product by H_2O_2 and ONOO^- .

Fig 30.
Reaction of CBA probe with ONOO^- , with the formation of ONOO^- -specific products, CNO_2 (7-nitrocoumarin) and CH (coumarin).

Fig 31.

Principle of *in vivo* bioluminescence-based detection of cellular oxidants using the PCL-1 boronate probe. (Adapted from [213]. Reprinted by permission from Springer Customer Service Centre GmbH: Springer Nature, Current Pharmacology Reports, Critical Review of Methodologies to Detect Reactive Oxygen and Nitrogen Species Stimulated by NADPH Oxidase Enzymes: Implications in Pesticide Toxicity, B. Kalyanaraman, M. Hardy, J. Zielonka, © 2016)

Fig 32.
Oxidant-specific products formed from the PCL-1 boronate probe.