ROS

RESEARCH ARTICLES

Evaluation of 2′,7′-Dichlorodihydrofluorescein Diacetate and Dihydrorhodamine 123 for the Detection of Reactive Oxygen Species

Jiayuan Zhao and Philip K. Hopke

Center for Air Resource Engineering and Science, Clarkson University, Potsdam, New York 13699, USA

Correspondence: phopke@clarkson.edu (P.K.H.)

Zhao J and Hopke PK. Reactive Oxygen Species 4(10):284–289, 2017; ©2017 Cell Med Press
http://dx.doi.org/10.20455/ros.2017.841
(Received: March 23, 2017; Revised: April 16, 2017; Accepted: April 17, 2017)

ABSTRACT | Exposure to reactive oxygen species (ROS) has been linked to many diseases and is one of the mechanisms suggested for the adverse effects of inhaling airborne particulate matter. The ability to detect and quantify these species collectively could contribute to a better understanding of the mechanisms and the prevention of possible adverse health effects. However, the use of oxidant-sensitive fluorescent probes for ROS detection has been problematic with respect to a reliable, easy-to-use, and related to health outcomes. In the present study, the performance of the commonly used probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for ROS detection in ambient air was compared to a popular probe used in cell culture research, namely dihydrorhodamine 123 (DHR-123). DCFH-DA and DHR-123 were tested with several surrogate compounds from different functional ROS groups (e.g., hydroperoxide and organic peroxide) and a few non-ROS groups. For ROS detection in ambient air, DCFH-DA was often more suitable because it responded to most ROS functional groups. Furthermore, it did not react with many non-ROS. DHR-123, on the other hand, reacted with more species including those that are not oxidizing, and may lead to an overestimation of the ROS present. These results illustrate the species that trigger positive responses from the two probes and could thus be used to understand the mechanisms of these reactions and to decide which probe to choose for ROS detection under different circumstances.

KEYWORDS | 2′,7′-Dichlorodihydrofluorescein; Dihydrorhodamine 123; Particulate matter; Reactive oxygen species

ABBREVIATIONS | BP, benzoyl peroxide; CMHP, cumene hydroperoxide; c-PA, cis-pinonic acid; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHR-123, dihydrorhodamine 123; HMCA, 2,4-hydroxy-3-methoxy-cinnamldehyde; HRP, horseradish peroxidase; PQ, phenantroquinone; ROS, reactive oxygen species; TBHP, tert-butyl hydroperoxide

CONTENTS

©2017 AIMSCI Inc. All Rights Reserved.

1. INTRODUCTION

Oxidative stress has been proven to induce adverse health effects and cause disease. Many oxygen-centered or related reactive species could contribute to increasing the oxidative potential. Therefore, to a certain extent, reactive oxygen species (ROS) concentrations could provide information on the possible hazards of oxidative effects [1, 2].

Oxidant-sensitive fluorescent probes are used to estimate ROS concentrations in ambient air. These reagents can be oxidized by ROS and transform from their reduced (non-fluorescent) forms to oxidized (fluorescent) forms. The intensity of the fluorescent signal therefore indicates the concentration of ROS. Thus, probe effectiveness plays a central role in ROS detection. It will decide the groups of detectable species and the sensitivity of the protocol. The chemical nature of the probe also influences its utility, e.g., if the reagent is light-sensitive.

Based on the target species and the experimental circumstances, several fluorescent probes can be employed. A suitable probe should respond to a maximum number of ROS types and a minimum number of non-oxidizing species. Also, a preferred probe should have a good maneuverability, such that it is insensitive to light or chemically stable over a certain period [3].

Rao et al. [4] reported that 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (structure shown in **Figure 1A**) reacted with both hydrogen peroxide and nitrogen oxide in human neutrophils. Rothe et al. [5] first introduced dihydrorhodamine-123 (DHR-123) (structure shown in **Figure 1B**) as a structural analog to DCFH-DA, to indicate neutrophil respiratory burst. DHR-123 has amino groups that DCFH-DA does not, but it lacks DCFH-DA's two hydroxyl groups and its diacetate and dichloro substituents [6–8]. Rhodamine 123 can be efficiently trapped within cells because one of the two equivalent amino groups tautomerizes to a charged imino when DHR-123 is oxidized [9]. Royall and Ischiropoulos [9] evaluated DHR-123 and DCFH-DA for the detection of intracellular hydrogen peroxide in cultured endothelial cells. Wrona et al. [10] focused on the reactivity of DCFH-DA and DHR-123 to carbonate, nitrogen dioxide, and hydroxyl radicals. Their study reported that the DHR-123 probe reacted faster than the DCFH-DA probe to carbonate radicals. Both of these probes had lower rate constants with nitrogen dioxide. The rate constants with hydroxyl radicals were close to the diffusion-controlled limit. Qin et al. [11] used DHR-123, DCFH-DA and dihydrorhodamine 6G (DHR 6G) in experiments to detect intracellular hydrogen peroxide levels in tumor cells and they found that DHR-123 was the better choice. Venkatachari and Hopke [12] compared the responses of DCFH-DA, POPHAA (p-hydroxyphenylacetic acid), and DTT (dithiothreitol) to surrogate ROS compounds in ambient air. They found that DCFH-DA was superior to the other two because it reacted with many ROS surrogates and provided linear, stable results with hydrogen peroxide that could be used as standards.

Fairfull-Smith and Bottle [13] reported a kind of novel, polyaromatic, profluorescent, isoindoline nitroxide synthesized probe which they described as a good choice for ROS detection. However, this kind of probe does not dissolve well in water, making it difficult to use to measure water soluble ROS. Moreover, it mostly reacts with radicals and oxidizes transition metals (e.g., Cu^{+} and Fe^{2+}), which, because it may miss some compounds, may lead to an underestimation of ROS. In general, many factors, such as the concentration of a solution, different incubation or sonication times, and different environments, may influence the reaction between a probe and ROS.

Although conditions vary, DCFH-DA has been the probe of choice in many experiments and has provided reproducible results [3, 12, 14–16]. However, this kind of fluorescent probe still has its own specific shortcomings, such as its sensitivity to light and its relatively quick decomposition over time leading to high blank values. Although DHR-123 was widely used in the past as an indicator of reactive peroxide species in cells [8, 9, 11, 17, 18], few studies used it as a fluorescent probe for the measurement of ambient ROS and thus, it was investigated to determine its potential viability as an ROS probe.

©2017 AIMSCI Inc. All Rights Reserved.

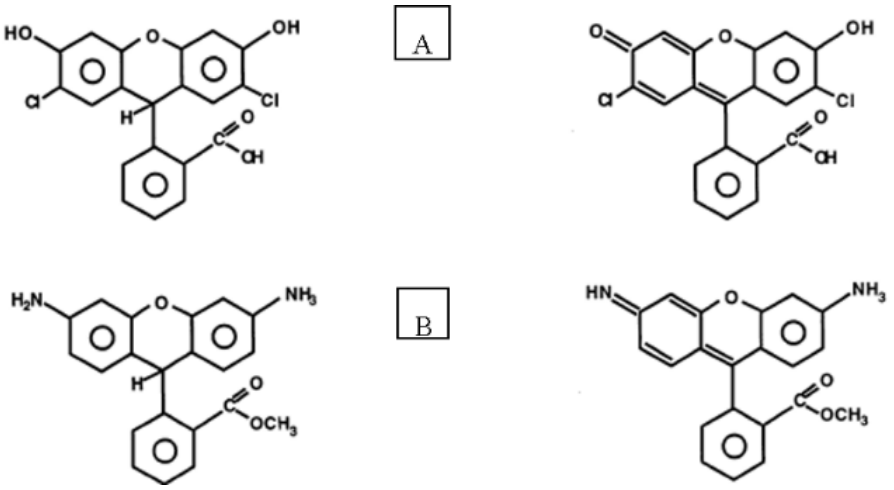

FIGURE 1. **Structures of oxidant-sensitive fluorescent probes.** (A) 2′,7′-Dichlorodihydrofluorescein diacetate. (B) Dihydrorhodamine 123 [18]. The non-fluorescent forms are on the left. The fluorescent forms are on the right.

TABLE 1. Excitation and emission wavelengths for DHR-123 methods used in previous research

Source	Excitation Wavelength (nm)	Emission Wavelength (nm)
Royall and Ischiropoulos [9]	500	536
Hempel et al. [18]	488	521
Glebska and Koppenol [19]	500	530
Wrona et al. [10]	497	525
Bukowska et al. [20]	500	538
Qin et al. [11]	488	543

2. MATERIALS AND METHODS

2.1. Solvent Preparation

To prepare 1 mM stock solution of DCFH-DA (Calbiochem, CA, USA), the chemical powder was dissolved into ethyl alcohol (ACS [American Chemical Society] grade, Pharmo, CT, USA). This solution was stored in a refrigerator at 4°C in a sealed brown bottle to protect it from light. This stock solution can be stored for approximately one-and-a-half months, after which it is discarded and fresh solution prepared. To prepare the working solution, a 10 ml stock solution was mixed with 40 ml of 0.01 M sodium hydroxide (NaOH) in a flask covered with aluminum foil to avoid light exposure. The solution was left in a dark environment at room temperature for 30 minutes to hydrolyze. A phosphate buffer was produced by mixing sodium phosphate dibasic (Na_2HPO_4, Sigma-Aldrich, MO, USA) with sodium dihydrogen phosphate anhydrous (NaH_2PO_4, Fluka, Germany) to achieve a pH of 7.2. The phosphate buffer was then added to the DCFH solution. Next, horseradish peroxidase (HRP, Sigma-Aldrich) at a concentration of 0.5 units/ml was added to the mixture as a catalyst. The rest of the buffer solution was then added to the DCFH-HRP solution and mixed well. The final DCFH concentration of this working solution was 5 μM. This can be stored for about 1 week, but was always utilized within 3–4 days so as to ensure freshness and avoid high backgrounds.

The stock solution of 1 mM DHR-123 was prepared by dissolving DHR-123 powder (Invitrogen, OR, USA) in ethanol. This solution was also protected in a

©2017 AIMSCI Inc. All Rights Reserved.

brown bottle and stored in the refrigerator. Normally, this solution could only be kept for 20–30 days before the background florescence increased dramatically and the color of the solution changed to red. Prior to analysis, the 5 μM working solution was freshly made by further dilution with Milli-Q water (resistivity >18.2 MΩ) every day.

Several functional groups were tested by detecting the change in fluorescent intensity from the reactions between fluorescent probes and the selected surrogate compounds in each group. These included: (1) hydrogen peroxide (30%, H_2O_2, ACS grade, Sigma-Aldrich) and tert-butyl hydroperoxide (TBHP, Sigma-Aldrich) in the hydroperoxide group; (2) benzoyl peroxide (BP, Fluka) and cumene hydroperoxide (CMHP, Sigma-Aldrich) in the organic peroxide group; (3) 2,2′-azonbis (2-methylpropion-amidine) dihydro chloride (Sigma-Aldrich) in the alkyl peroxy radicals group; (4) phenanthrenequinone (PQ, Sigma-Aldrich) in the redox-active organics group; (5) sodium hypochlorite (NaOCl, Ricca, TX, USA) in the hypochlorite group; (6) cis-pinonic acid (c-PA, Sigma-Aldrich) in the acids group; and (7) 4-hydroxy-3-methoxy cinnamaldehyde (HMCA, Sigma-Aldrich) in the alcohols group and in the aldehydes group. The concentration of the different chemical species ranged between 0.1 and 10 μM, which is similar to normal pollution concentrations in atmosphere.

2.2. Wavelength Determination

Initially, a spectrofluorophotometer (Model RF-5301PC, Shimadzu Scientific Instruments, Columbia, MD, USA) was used to determine the most appropriate wavelengths for testing DHR-123: these were an excitation wavelength of 485 nm, producing an emission wavelength of 525 nm. For DCFH-DA we used a Turner Quantech Digital Filter Fluorometer (Model FM109535, Barnstead Thermolyne, IA, USA) with an excitation wavelength filter at 490 nm and an emission wavelength filter at 515 nm. Because this 490 nm filter has a 10 nm bandpass, the 485 nm wavelength also passes through it. The sharp cut emission filter starts at 515 nm, but also allows longer wavelengths to be measured. Thus, we could use the same Turner Quantech Digital Filter fluorometer for both DCFH and DHR-123 with the same excitation and emission wavelength filters. The excitation and emission wavelengths for DHR-123-based methods used in previous research are outlined in **Table 1**.

2.3. Fluorescence Measurement

Surrogate compounds were incubated with the probes in cuvettes at 37°C for 15 min. Rhodamine 123 and 2′,7′-dichlorofluorescein formation were monitored by measuring their fluorescence using the fluorometer and wavelengths described above.

3. RESULTS AND DISCUSSION

Table 2 summarizes the responses obtained. DHR-123 is not significantly oxidized by hydroperoxide. To some extent, this property makes this probe inappropriate for ROS detection of atmospheric species because the hydroperoxide functional group plays a key role in ambient air pollution. Failure to respond to the species in this group could lead to a serious underestimation of the ROS concentration. DCFH does react with hydroperoxide and provides a stable and linear response. Both the DHR-123 and DCFH probes reacted with a number of ROS species (e.g., R-OO-R′, $ROO^{\bullet}$, and OCl^{-}). These two probes also reacted with non-ROS species such as HMCA (2,4-hydroxy-3-methoxy-cinnamldehyde), and this may lead to overestimation of the ROS concentration. Compared with DCFH, DHR-123 responds to more non-ROS compounds, such as acids. This behavior increases the risk of overestimation. Based on previous research, both probes are good indicators of peroxynitrite anion [8].

DHR-123 stock solution can only be stored in a refrigerator for 20–30 days. After this period, the solution turns red and does not provide stable results. We tested DHR-123's blank working solution at different times during the same day, and found that the background florescence kept rising relatively rapidly. A fresh working solution had to be prepared at least every day. To maintain a relatively low background florescence, we shortened this time period to ~4–8 h. The fact that the DHR-123 solution has such a short useful shelf-life was a considerable disadvantage for this probe.

In general, DHR-123 is reactive to many species. However, since some of them are not ROS, using this probe may lead to overestimations. Moreover, the fact that DHR-123 did not provide a stable and linear response to the species in the hydroperoxide group further harms its usefulness. DCFH appears to be more suitable for the detection of ROS in the ambient atmosphere.

©2017 AIMSCI Inc. All Rights Reserved.

TABLE 2. Responses of different probes with the surrogate compounds

Functional Group	Surrogate Compounds	DHR-123		DCFH	
		Response?	If Yes, Linear?	Response?	If Yes, Linear?
Hydroperoxyl (HOO-R)	Hydrogen peroxide (HP)	Yes (very little)	No	Yes	Yes, $r^2 = 0.999$
	tert-Butyl hydroperoxide (TBHP)	Yes (little)	No	Yes	Yes, $r^2 = 0.708$
Organic Peroxide (R-OO-R')	Benzoyl peroxide (BP)	Yes	Yes, $r^2 = 0.993$	Yes	No
	Cumene hydroperoxide (CMHP)	Yes	Yes, $r^2 = 0.905$	Yes	No
Peroxyl Radicals (ROO•)	2,2′-Azobis (2-aminopropane) dihydrochloride	Yes	Yes, $r^2 = 0.925$	Yes	No
Redox Active Organics	Phenantroquinone (PQ)	Yes	Yes, $r^2 = 0.850$	No	N/A
Hypochlorite (OCl^-)	Sodium hypochlorite (NaOCl)	Yes	Yes, $r^2 = 0.998$	Yes	No
Acids (R-COOH)	cis-Pinonic acid (c-PA)	Yes	No	No	N/A
Alcohols (ROH)	2,4-Hydroxy-3-methoxy-cinnamldehyde (HMCA)	Yes	No	Yes	No
Aldehydes (RCHO)	2,4-Hydroxy-3-methoxy-cinnamldehyde (HMCA)	Yes	No	Yes	No

4. CONCLUSION

This study explored ROS detection methods by evaluating the performances of different fluorescent probes. We conclude that the commonly used DCFH-DA probe was superior to the DHR-123 probe in detecting the existing ROS in the atmospheric aerosol. This conclusion is based on the responses to ROS functional groups and the feasibility of applying the two probes. We also explored the most suitable situations for using DHR-123. We conclude that this would be when the aim is to detect both ROS and non-ROS groups, and hypochlorite could be chosen as the positive control. In order to achieve reliable data and to decide upon the most suitable probe for each situation, the specific objectives of the experiment should govern the choice of indicator species.

REFERENCES

1. Hopke PK. Reactive Particles as a source of human health effects. *Atmospheric Environ* 2008; 42:3192–4.
2. Hopke PK. Reactive ambient particles. In: *Air Pollution and Health Effects: Molecular and Integrative Toxicology* (SS Nadadur and JW Hollingsworth) . Springer-Verlag, London, UK. 2015, pp. 1–24.
3. Zhao J, Hopke PK. Concentration of reactive oxygen species (ROS) in mainstream and sidestream cigarette Smoke. *Aerosol Sci Technol* 2012; 46:191–7.
4. Rao KM, Padmanabhan J, Kilby DL, Cohen HJ, Currie MS, Weinberg JB. Flow cytometric analysis of nitric oxide production in human neutrophils using dichlorofluorescein diacetate in the presence of a calmodulin inhibitor. *J Leukoc Biol* 1992; 51(5):496–500.
5. Rothe G, Oser A, Valet G. Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Naturwissenschaften* 1988; 75(7):354–5.
6. Black MJ, Brandt RB. Spectrofluorometric analysis of hydrogen peroxide. *Anal Biochem* 1974; 58(1):246–54.
7. Banati RB, Rothe G, Valet G, Kreutzberg GW.

©2017 AIMSCI Inc. All Rights Reserved.

Respiratory burst activity in brain macrophages: a flow cytometric study on cultured rat microglia. *Neuropathol Appl Neurobiol* 1991; 17(3):223–30.

8. Crow JP. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide* 1997; 1(2):145–57. doi: 10.1006/niox.1996.0113.
9. Royall JA, Ischiropoulos H. Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch Biochem Biophys* 1993; 302(2):348–55. doi: 10.1006/abbi.1993.1222.
10. Wrona M, Patel K, Wardman P. Reactivity of 2',7'-dichlorodihydrofluorescein and dihydrorhodamine 123 and their oxidized forms toward carbonate, nitrogen dioxide, and hydroxyl radicals. *Free Radic Biol Med* 2005; 38(2):262–70. doi: 10.1016/j.freeradbiomed.2004.10.022.
11. Qin Y, Lu M, Gong X. Dihydrorhodamine 123 is superior to 2,7-dichlorodihydrofluorescein diacetate and dihydrorhodamine 6G in detecting intracellular hydrogen peroxide in tumor cells. *Cell Biol Int* 2008; 32(2):224–8. doi: 10.1016/j.cellbi.2007.08.028.
12. Venkatachari P, Hopke PK. Development and laboratory testing of an automated monitor for the measurement of atmospheric particle-bound reactive oxygen species (ROS). *Aerosol Sci Tech* 2008; 42:629–35.
13. Fairfull-Smith KE, Bottle SE. The synthesis and physical properties of novel polyaromatic profluorescent isoindoline nitroxide probes. *Eur J Org Chem* 2008; 32:5391–400.
14. Hung HF, Wang CS. Experimental determination of reactive oxygen species in Taipei aerosols. *J Aerosol Sci* 2001; 32:1201–11.
15. Huang MF, Lin WL, Ma YC. A study of reactive oxygen species in mainstream of cigarette. *Indoor Air* 2005; 15(2):135–40. doi: 10.1111/j.1600-0668.2005.00330.x.
16. See SW, Wang YH, Balasubramanian R. Contrasting reactive oxygen species and transition metal concentrations in combustion aerosols. *Environ Res* 2007; 103(3):317–24. doi: 10.1016/j.envres.2006.08.012.
17. Szabo C, Salzman AL, Ischiropoulos H. Peroxynitrite-mediated oxidation of dihydrorhodamine 123 occurs in early stages of endotoxic and hemorrhagic shock and ischemia-reperfusion injury. *FEBS Lett* 1995; 372(2–3):229–32.
18. Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM. Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. *Free Radic Biol Med* 1999; 27(1–2):146–59.
19. Glebska J, Koppenol WH. 20. Bukowska B, Rychlik B, Krokosz A, Michalowicz J. Phenoxyherbicides induce production of free radicals in human erythrocytes: oxidation of dichlorodihydrofluorescine and dihydrorhodamine 123 by 2,4-D-Na and MCPA-Na. *Food Chem Toxicol* 2008; 46(1):359–67. doi: 10.1016/j.fct.2007.08.011.

©2017 AIMSCI Inc. All Rights Reserved.