Which Are You Watching, an Individual Reactive Oxygen Species or Total Oxidative Stress?

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The fluorometric measurement of an individual reactive oxygen species (ROS) provides useful biological and biochemical information on ROS as important mediators for the pathological conditions of various diseases and yet requires a highly specific probe toward the target ROS. To design such a specific ROS probe, this report proposes a new strategy based on protection–deprotection chemistry between fluoresceins and their derivatives protected with benzenesulfonyl groups. The strategy has allowed developing new fluorescent probes toward extra- and intracellular hydrogen peroxide or superoxide. Herein, I outline the strategy used for the design of these ROS probes and their probe performance with high selectivity toward hydrogen peroxide and superoxide, which could not be achieved until a simple deprotection, namely, a nonredox reaction, was used as a fluorescing process.

Key words: **fluorescent probes; reactive oxygen species; oxidative stress; fluorescent microscopy; probe design**

Introduction

To measure total oxidative stress, or to detect generation of an individual reactive oxygen species (ROS), such as superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•): It is a question. At present, the best answer is believed to be the latter for most researchers working on biological chemistry and biochemistry of ROS.

Existing fluorescent probes, such as dichlorofluorescein (DCFH) and hydroethidine (HE), have played important roles over the last several decades to disclose the relationship between ROS generation and the pathological conditions of various diseases.[1–5] These traditional probes detect ROS sensitively but nonselectively.[3,6–9] This major drawback prevents these fluorescent probes from satisfying those who are anxious to understand the biological and biochemical events, induced or regulated by generation of an individual ROS, as they are. Accordingly, there is a great demand for fluorescent probes with much higher selectivity toward an individual ROS than that of the traditional ones.

Recently, we proposed a new strategy for the design of fluorescent probes based on protection–deprotection chemistry involving fluoresceins (**3**) and their benzenesulfonyl (BES) derivatives (FIG. 1B).[10] Synthetic methods for a wide variety of BES chlorides[11] as well as **3**[12,13] have already been established. Hence, the featured point of our strategy is accessibility to many probe candidates not only through choosing the combination of **3** and BES chlorides from the compound pools but also by selecting protection mode, that is, whether **1** or **2** is used. With this advantage, we developed new fluorescent probes toward H_2O_2 or $O_2^{-\bullet}$ with high specificity.[10,14,15] Herein, I outline our strategy and the full scope of their probe performance.

Strategy

HOO^- behaves as a stronger nucleophile than HO^- (the so-called α-effect), to an extent that depends on the type of electrophile.[16–18] A typical example is perhydrolysis of phenyl acetates. Reaction of phenyl acetates with H_2O_2 proceeds much more rapidly than their hydrolysis, yielding the corresponding phenols and peracids. This fact encouraged us to examine whether acyl resorufins would work as new fluorescent probes for H_2O_2. Resorufin is a water-soluble fluorescent dye with a phenolic hydroxyl group,

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Ann. N.Y. Acad. Sci. 1130: 149–156 (2008).
doi: 10.1196/annals.1430.012

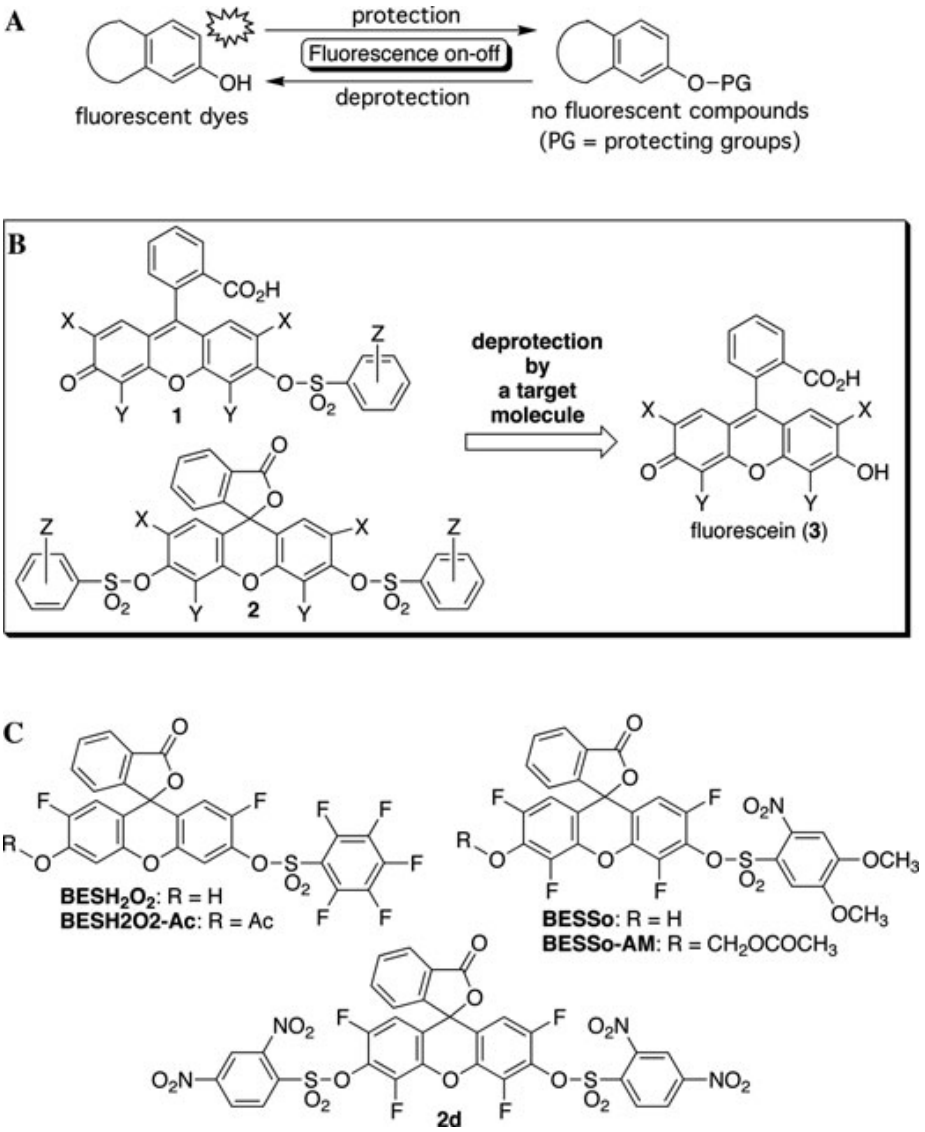

FIGURE 1. Proposed strategy for probe design based on protection–deprotection chemistry. **(A)** General description. **(B)** BES derivatives of fluoresceins as mother compounds. **(C)** ROS probes developed with the strategy.

whereas its acyl derivatives exhibit no fluorescence. As expected, the fluorometry with acyl resorufins was useful for measurements of H_2O_2.[19,20] Unfortunately, acyl resorufins could not find utility as intracellular H_2O_2 probes, because these compounds are hydrolyzed easily by esterase, leading to high background responses within cells. And yet, the work on acyl resorufins enabled us to propose a working hypothesis, that is, a strategy for the design of fluorescent probes based on protection–deprotection chemistry (FIG. 1A).

Protection–deprotection chemistry of **3** has been used for designing some fluorescent probes useful for measuring enzyme activity. When **3** is transformed into artificial enzyme substrates through protection of their phenolic hydroxyl groups, the corresponding protected derivatives work as fluorescent probes toward enzymes, such as esterase, phosphatase, and galactosidase.[21] However, there are no probes that provide fluorescence after deprotection induced not by enzymatic but by chemical reaction.

For a mother compound of our strategy, we have selected **1** and **2** (FIG. 1B), which are prepared by protection of **3** with BES groups, on the basis of the following facts and concepts:

1. Compound **3** exhibits high fluorescence in aqueous medium, whereas **1** and **2** have no and almost no fluorescence, respectively. Thus, deprotection of **1** or **2** to yield **3**, induced by a chemical reaction highly characteristic of a certain

target molecule, would allow the BES derivatives to function as fluorescent probes for the target molecule.

2. Sulfonates are inert to hydrolysis by esterase, to tolerate **1** or **2** finding utility in an intracellular fluorescence-based assay.
3. Compound **3** and BES chlorides with various types of substitutions can be prepared by established methods, and hence **1** and **2** with a wide range of reactivity can be feasibly prepared.
4. Even when the same BES group is used, the reactivity of probe candidates is regulated by choosing protection mode. Compound **1** is much more susceptible to deprotection than **2**.
5. Solubility of probe candidates to aqueous medium is improved by selecting **1** rather than **2**, because the mono-protected compounds are less hydrophobic than the bis-protected ones.

In fact, **1** and **2** work well as the mother compounds, and our strategy has been applied in the design of new fluorescent probes for H_2O_2,[14] $O_2^{-\bullet}$,[10,15] thiols,[22] and selenols[23] merely by selecting the appropriate combination between **3** and BES chlorides from the pools of compounds.

H_2O_2-Specific Fluorescent Probe

Among **1** and **2** examined, $BESH_2O_2$ (FIG. 1C) proved to be the best substrate for perhydrolysis, although the reason is not well understood.[14] $BESH_2O_2$ is almost nonfluorescent and reacts with H_2O_2, effectively affording a highly fluorescent compound, difluorofluorescein. A 96-well microtiter plate assay of H_2O_2 (10 μL) was performed with $BESH_2O_2$ (5 μM, 150 μL) through incubating in pH 7.4 HEPES buffer at 37°C for 60 min. The detection limit was 4.6 pmol/well, and a linear calibration curve was obtained from the detection limit up to 92.3 nmol/well. This deprotection is specific for H_2O_2, and hence $BESH_2O_2$ provides fluorescent responses at negligible levels toward $O_2^{-\bullet}$, *tert*-BuOOH (*t*-BuOOH), HO•, NO•, and $ONOO^-$. By exploiting perhydrolysis rather than oxidation as a fluorescing reaction, $BESH_2O_2$ can function as a fluorescent probe for H_2O_2, showing relatively high specificity and sensitivity.

The rate constant of the perhydrolysis was estimated to be 0.25 M^{-1} s^{-1}, which is comparable to or faster than the alkaline hydrolysis of ethyl benzoates.[24] The effect of the pH value on the reaction of $BESH_2O_2$ with H_2O_2 was also examined. The rate of perhydrolysis of $BESH_2O_2$ decreased strikingly below pH 6.6. However, $BESH_2O_2$ still functioned well as a fluorescent probe at pH 6.6, although the fluorescent intensities produced were about 20% of those observed at pH 7.4.

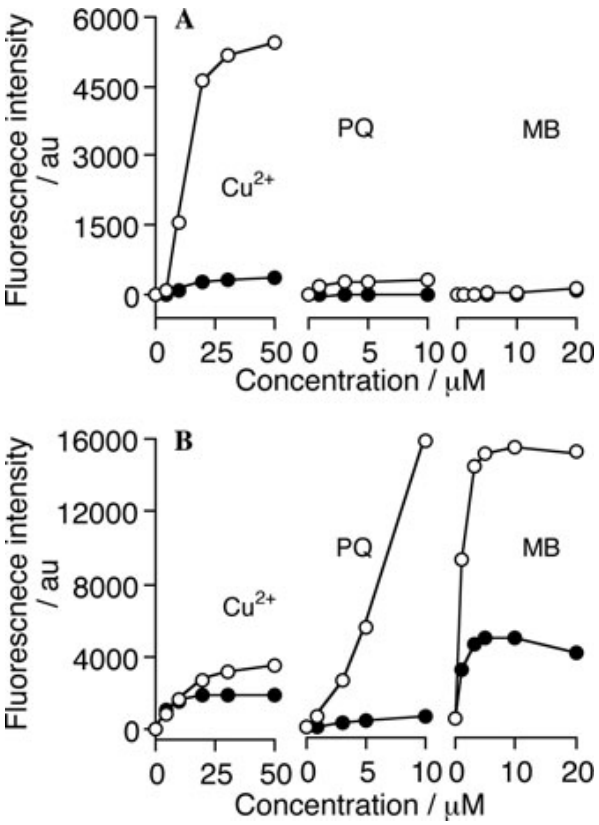

FIGURE 2. Fluorescence intensities measured for *Chlamydomonas reinhardtii* loaded with **(A)** $BESH_2O_2$-Ac or **(B)** DCFH-DA after incubation in the presence of Cu^{2+}, paraquat (PQ), or methylene blue (MB) in the light (open circles) or the dark (closed circles) at 25°C for 60 min.

Its acetyl derivative, $BESH_2O_2$-Ac (FIG. 1C), is useful for measuring intracellular H_2O_2.[14] Oxidative stress can be induced in green algae by incubating with suitable reagents in the presence of light. Stimulation with Cu^{2+} ions causes intracellular formation of various ROS, such as $O_2^{-\bullet}$, H_2O_2, and HO•.[25] Cells also undergo oxidative stress upon generation of $O_2^{-\bullet}$ or HO• through specific activation by paraquat (PQ) or methylene blue (MB), respectively.[26] Thus, experimental models using *Chlamydomonas reinhardtii*, a freshwater green alga, were informative for evaluating the applicability of $BESH_2O_2$-Ac as a cell-permeable H_2O_2 probe. FIGURE 2 summarizes the results obtained when cells, loaded with $BESH_2O_2$-Ac or diacetyl DCFH (DCFH-DA) for 30 min at 25°C in the dark, were incubated in a 96-well microtiter plate for 60 min in the light or dark in the presence of Cu^{2+} ions, PQ, or MB. When $BESH_2O_2$-Ac was used (FIG. 2A), fluorescence responses were produced only in cells incubated

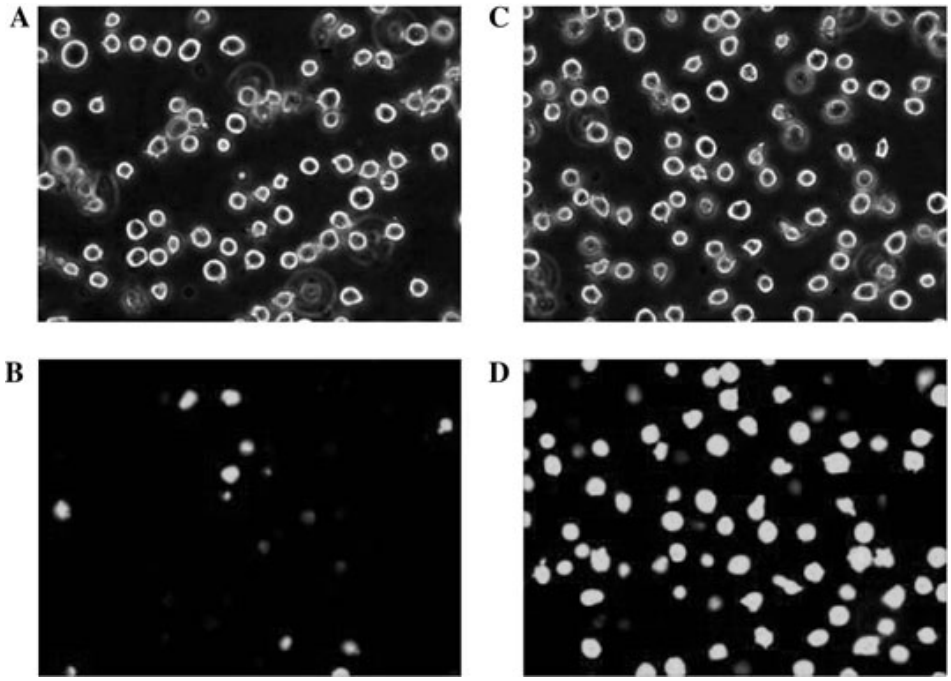

FIGURE 3. Phase contrast **(A, C)** and fluorescence images **(B, D)** obtained after incubating human Jurkat T cells, loaded with $BESH_2O_2$-Ac, at 37°C for 1 h in the absence **(A, B)** or the presence **(C, D)** of 5 mM butyric acid.

with Cu^{2+} ions in the light, the extent of which depended on the Cu^{2+} concentration. When one considers the high H_2O_2 selectivity of $BESH_2O_2$-Ac, these results demonstrate that $BESH_2O_2$-Ac permeates the cells and is transformed into $BESH_2O_2$, which then detects the oxidative stress arising from intracellular formation—not of $O_2^{-\bullet}$ and $^1O_2$ but of H_2O_2 on stimulation by Cu^{2+} ions in the light. In contrast to the action of $BESH_2O_2$-Ac, DCFH-DA detected the oxidative stress caused by PQ and MB, as well as Cu^{2+} ions (FIG. 2B). These results are consistent with the usefulness of DCFH as a probe for providing an index for total oxidants, thereby confirming that $BESH_2O_2$-Ac can serve as a probe for cell systems without loss of selectivity.

Human Jurkat T cells undergo apoptosis upon treatment with butyric acid, which induces the production of ROS as well as ceramide formation in the cytosol.[27] The oxidative burst in the mammalian cells was visualized with $BESH_2O_2$-Ac also. FIGURE 3 compares the fluorescent images obtained from the cells loaded with the cell-permeable H_2O_2 probe 30 min after incubation in the absence and presence of butyric acid. The results indicate that stimulation of Jurkat T cells with butyric acid induces the oxidative burst because of intracellular generation of H_2O_2.

Tang *et al.* also applied our strategy to designing a red fluorescent probe for H_2O_2, namely, bis (4-methylBES) naphthofluorescein.[28] As an alternative nonoxidative fluorescing mechanism to perhydrolysis of BES groups, deprotective hydroxylation of boronates is useful for designing H_2O_2 fluorescent probes.[29,30]

$O_2^{-\bullet}$-Specific Fluorescent Probe

Compound **2d** (FIG. 1), prepared from tetrafluorofluorescein and 2,4-dinitroBES chloride, worked as an $O_2^{-\bullet}$ fluorescent probe.[15] The bis-protected derivative provides sensitive and specific responses toward $O_2^{-\bullet}$. When $O_2^{-\bullet}$ was generated in a 96-well microtiter plate by the enzymatic reaction of hypoxanthine (HPX) and xanthine oxidase at 37°C for 10 min, $O_2^{-\bullet}$ was detected with **2d** over the concentration range between 1 and 2000 pmol of HPX. Under essentially the same conditions, reaction of **2d** with H_2O_2, *t*-BuOOH, NaOCl, HO•, $^1O_2$, NO•, or $ONOO^-$ brought about almost no fluorescence. Esterase induced no fluorescent responses from **2d** either. The applicability of **2d** as a probe for a fluorescence-based assay of cell-derived $O_2^{-\bullet}$ was demonstrated by experiments using neutrophils stimulated with phorbol myristate acetate (PMA). A cell suspension (10^6 cells/mL, 100 μL) was incubated for 90 min at 37°C with **2d** (25 μM, 50 μL) in the presence or absence of

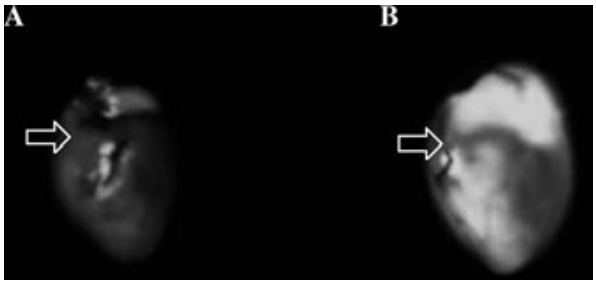

FIGURE 4. Fluorescence images obtained for a coronary artery occlusion–reperfusion model of myocardial infarction. Arrow indicates where the left anterior descending coronary artery of each mouse heart was ligated. Reperfusion was sustained for **(A)** 5 or **(B)** 7 min.

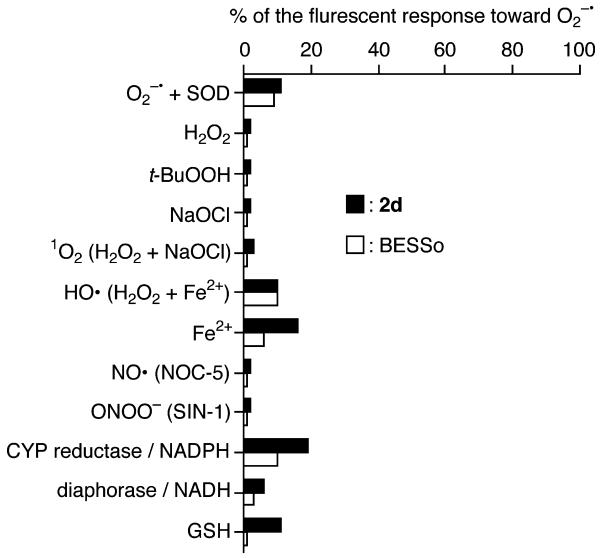

FIGURE 5. Comparison of fluorescence augmentations observed on reaction of BESSo or **2d** with ROS, reductases, and GSH. All data are shown as percentages of the fluorescence intensity produced by BESSo or **2d** in the presence of $O_2^{-\bullet}$.

PMA (0.64 μM, 50 μL). The assay with **2d** provided much larger fluorescence augmentation at each measurement point for PMA-stimulated neutrophils than for unstimulated cells. The responses observed for the stimulated cells were effectively reduced by adding superoxide dismutase (SOD) (1000 μU/mL, 10 μL). This result clearly indicated that the fluorescent responses observed with **2d** on PMA-stimulated neutrophils arise from $O_2^{-\bullet}$ released by the cells.

The fluorometric method with **2d** was also successfully applied to a coronary artery occlusion–reperfusion model of myocardial infarction. The $O_2^{-\bullet}$ probe was administered to mice by intraperitoneal injection of its dimethyl sulfoxide solution (20 mM, 100 μL). After 1 h, the mice were subjected to left thoracotomy, followed by isolation and ligation of the left anterior descending coronary artery. The ligation was sustained for 1 h. After reperfusion for 5 or 7 min, the coronary artery was religated. On the incised heart, fluorometric measurement was performed. Reperfusion for 5 min induced no fluorescence at all, whereas the downstream region of the ligated coronary artery obtained exhibited strong fluorescence when the reperfusion was carried out for 7 min (FIG. 4). The imaging with **2d** demonstrated that the oxidative burst through occlusion–reperfusion on mouse hearts occurs after an appropriate induction period.

From the results obtained with neutrophils and mouse hearts, it was accepted that **2d** works as a useful probe for extracellular $O_2^{-\bullet}$. However, the specificity of this prototype $O_2^{-\bullet}$ probe had yet to be optimized. Fluorescence augmentation from **2d** also occurred through reaction with glutathione (GSH). The extent of the augmentation toward GSH was around 15% of that observed for $O_2^{-\bullet}$. Because GSH is ubiquitous in cells at millimolar levels, the considerable fluorescent response toward GSH is likely to compromise the high specificity of **2d** for $O_2^{-\bullet}$ among the other ROS in intracellular measurement of $O_2^{-\bullet}$ with this probe.[31] Thus, further tuning of the probe performance of **2d** through structural modification of its BES group has been conducted with a view to developing a practical $O_2^{-\bullet}$ probe that shows high specificity over GSH as well as other ROS. From our strategy, various BES derivatives have been designed in a stepwise manner from **2d**, and their performance as $O_2^{-\bullet}$ probes has been examined. Screening of these carefully designed candidates indicated that BESSo (FIG. 1C) works as a more practical $O_2^{-\bullet}$ probe than **2d**.[10]

A 96-well microtiter plate assay with BESSo provided a highly sensitive method for measuring $O_2^{-\bullet}$ generated by a xanthine oxidase–HPX system in pH 7.4 HEPES buffer at 37°C for 10 min. The detection limit corresponded to the amount of $O_2^{-\bullet}$ generated from HPX at 0.1 pmol/well. A linear calibration curve for $O_2^{-\bullet}$ was obtained over the range from 1.0 to 1000 pmol of HPX/well. Although the linear concentration range produced by BESSo was similar to that obtained with **2d**, the sensitivity of BESSo toward $O_2^{-\bullet}$, as expressed by the slope of the calibration curve, was approximately threefold greater than that of **2d**.

FIGURE 5 compares the reactivity of BESSo and **2d** toward $O_2^{-\bullet}$, other ROS, GSH, Fe^{2+}, and reductases, such as cytochrome P450 reductase/NADPH and diaphorase/NADH, in pH 7.4 HEPES buffer. The observed fluorescent responses are shown as percentages of the fluorescence intensity in response to $O_2^{-\bullet}$ produced by BESSo (24,700 au) or **2d** (9000 au), and a

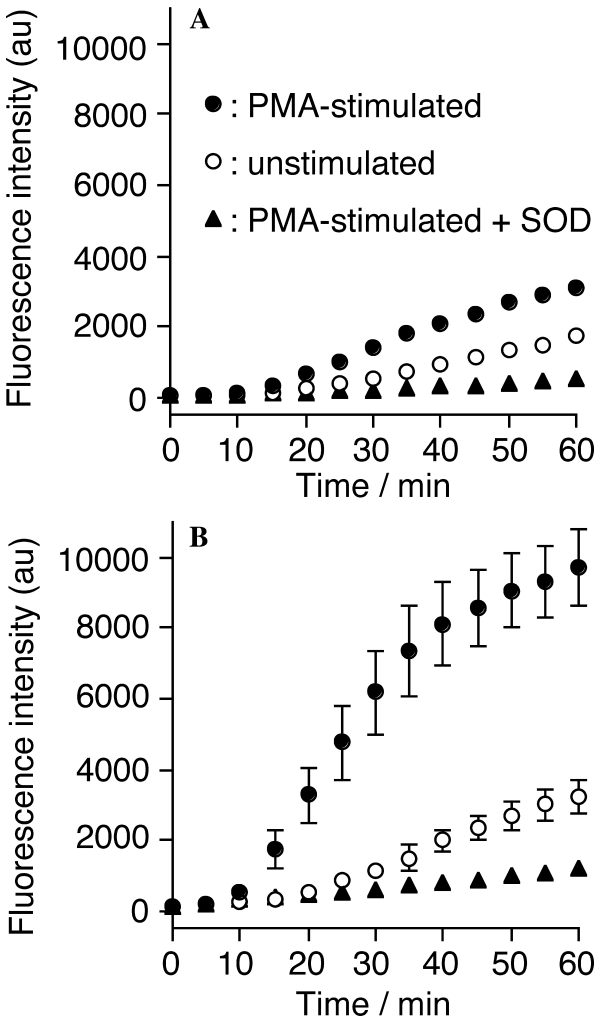

FIGURE 6. Temporal changes in fluorescence intensities observed with **(A)** compound **2d** or **(B)** BESSo for PMA-stimulated or unstimulated human neutrophils (10^5 cells/well). Data are expressed as mean ± standard deviation ($n = 8$).

value of 1% indicates almost no difference in fluorescent intensity from the blank response. BESSo exhibited a highly specific response to $O_2^{-\bullet}$, not only over GSH but also over H_2O_2, *t*-BuOOH, NaOCl, $^1O_2$, NO•, and $ONOO^-$. The specificity of BESSo over these ROS was even better than that of **2d**. These results demonstrate that BESSo is a more practical $O_2^{-\bullet}$ probe than the prototype probe **2d** for specificity and sensitivity.

The rate constant, k_{obsd}, for the conversion of BESSo to tetrafluorofluorescein by $O_2^{-\bullet}$ was estimated by competitive kinetic analyses, as are generally applied to determine rate constants for $O_2^{-\bullet}$ probes or scavengers.[32–34] As a competitor to BESSo, SOD was used. Reactions of BESSo and $O_2^{-\bullet}$ in the presence of SOD at various concentrations were monitored fluorometrically, and k_{obsd} was estimated to be $4.0 \pm 0.2 \times 10^3\ M^{-1}\ s^{-1}$. The rate constant for HE has been reported to be $2.6 \pm 0.6 \times 10^5\ M^{-1}\ s^{-1}$.[33] Although the rate constant for BESSo was thus 65 times smaller than that for HE, the specificity and sensitivity of BESSo as an $O_2^{-\bullet}$ probe are better than those of HE.

The usefulness of BESSo as a probe for fluorescence-based assays of extracellular $O_2^{-\bullet}$ was compared with that of **2d** in experiments using neutrophils stimulated with PMA. A cell suspension (1.0×10^5 cells/well) was incubated at 37°C with BESSo or **2d** in the presence or absence of PMA. An assay with BESSo or **2d** produced greater fluorescence response to PMA-stimulated neutrophils than in response to unstimulated cells 10 min after incubation (FIG. 6). The responses observed to the stimulated cells with both probes were reduced upon adding SOD. These results indicate that the fluorescence responses observed with BESSo or **2d** to PMA-stimulated neutrophils result from $O_2^{-\bullet}$ release. However, the differences between fluorescence augmentations from stimulated and unstimulated cells, and from stimulated cells in the absence and presence of SOD, were larger with BESSo than with **2d**. Thus, BESSo showed improved specificity and sensitivity and hence represents a more practical probe than **2d** for fluorescent assays of extracellular $O_2^{-\bullet}$.

BESSo was also applied to detect intracellular $O_2^{-\bullet}$ generation. For this purpose, we synthesized its acetoxymethyl derivative, BESSo-AM (FIG. 1C). Probe-loaded human Jurkat T cells were incubated in the presence or absence of butyric acid at 37°C for 1 h and were subjected to the fluorescence-based assays. Flow cytometric measurements yielded mean fluorescence intensity values of 793 and 331 au for stimulated and unstimulated cells, respectively. When cells were loaded with 4,5-dihyroxy-1,3-benzenedisulfonic acid (Tiron, a cell-permeable $O_2^{-\bullet}$ scavenger) as well as BESSo-AM, and then stimulated with butyric acid, the mean fluorescence intensity value was reduced to 482 au. The phenomena observed through flow cytometry of Jurkat T cells could be clearly visualized by means of fluorescence microscopy. Stimulation with butyric acid increased the number of cells stained by the fluorescence product tetrafluorofluorescein. Tiron markedly inhibited cell staining induced by stimulation with butyric acid. Because Tiron clearly functioned as an intracellular scavenger of $O_2^{-\bullet}$ in these experiments, the results demonstrate that BESSo-AM can serve as a fluorescent probe for detecting intracellular $O_2^{-\bullet}$. This intracellular assay with BESSo-AM thus revealed that ROS production in the cytosol of Jurkat T cells originates from the generation of $O_2^{-\bullet}$.

Conclusion

Most ROS function as oxidants, and hence oxidative mechanisms have been involved in the fluorescence production from ROS probes. Probe design based on such redox reactions has borne highly sensitive ROS fluorescent probes and yet suffered from conferring those probes with specificity toward an individual ROS to be monitored. Thus, future work should capitalize on other chemical properties of ROS besides their oxidizing nature, in the hope of designing fluorescent probes yielding highly specific responses for a target ROS. This concept is realized in the proposed strategy based on protection–deprotection chemistry. Deprotection of the pentafluoro-BES group in $BESH_2O_2$ brings about perhydrolysis, which is a characteristic reactivity of H_2O_2. With high specificity, $O_2^{-\bullet}$ transforms BESSo into its deprotected fluorescent compound. We believe that our strategy has a further potential for designing near-infrared fluorescent probes as well as new probes allowing fluorescent imaging of other biological molecules. We are working on the development of these probes. However, high specificity is just one of the requirements for fluorescent probes. No one doubts that ROS probes with reversibility provide more useful information on the cellular redox balance related to various types of diseases. The design of reversible ROS probes to visualize both ROS generation and disappearance under cellular reducing conditions is a markedly challenging field and will be one of our ongoing research projects.

Conflict of Interest

The author declares no conflicts of interest.

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