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An extremely rapid-response fluorescence probe for hydrogen peroxide and its applications in living cells

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A turn-on fluorescence probe ACF for rapid detection of H$_2$O$_2$ was constructed. The probe utilized 2-(azidomethyl)benzoyl group as new reaction site, which exhibited rapid response to H$_2$O$_2$ with a 118-fold fluorescence enhancement within 5 min. The biological application of ACF was confirmed by fluorescence imaging of H$_2$O$_2$ in living cells.

Hydrogen peroxide (H$_2$O$_2$), one of the most important reactive oxygen species (ROS), has been known as a harmful metabolic product and a component of the immune response to microbial invasion for a long time.$^1$ However, H$_2$O$_2$ functions as an ubiquitous intracellular second messenger when it is generated at a low concentration (< 0.7 μM)$^2$. It stimulates cell proliferation,$^3$ differentiation,$^4$ and migration$^5$ by activating the signalling pathways. H$_2$O$_2$ might be generated aberrantly and result in oxidative stress with stimulation by exogenous chemicals. However, H$_2$O$_2$ is an oxidant unlike the classical second messenger.$^6$ The resulting H$_2$O$_2$ and other ROS will attack cellular structures or biomolecules such as proteins,$^7$ liposomes,$^8$ and DNA,$^9$ which has been correlated with aging,$^{10}$ Alzheimer’s disease,$^{11}$ and cancer.$^{12}$ Obviously, location and timing of H$_2$O$_2$ generation in biological process is highly required and would provide important information to understand the function of H$_2$O$_2$.

Currently, several chemical methods have been developed to detect intracellular H$_2$O$_2$, including mass assays,$^{13}$ proteomics assays,$^{14}$ and fluorescence-based assays.$^{15}$ Among these methods, fluorescence-based assays were useful because of their non-destructive features. A few fluorescent probes designed for H$_2$O$_2$ detection have been reported since 2003.$^{16}$ The first developed and most popular probes were a kind of boronate ester,$^{17}$ which have occupied more than a half of all the H$_2$O$_2$ probes, including NIR, ratiometric, targetable, trappable and two-photon probes etc. Some of them have been successfully applied to monitor H$_2$O$_2$ at physiological levels in vitro and in vivo and others have been used to explore the cellular mechanisms associated with H$_2$O$_2$. Chang group contributed a lot in this field.$^{18}$ Another kind of fluorescent probes developed for H$_2$O$_2$ in the early stage contained arylsulfonyl esters as trap groups.$^{19}$ After that, several kinds of probes were designed based on unique H$_2$O$_2$-responsive sites such as diphenyolphosphine,$^{20}$ α-diketone groups,$^{21}$ metal complexes$^{22}$ and some Chalcogen.$^{23}$ The efforts to find novel H$_2$O$_2$-responsive sites were still in progress.

Although several H$_2$O$_2$-responsive sites have been developed and a lot of fluorescence probes have been constructed, the reaction rates and fluorescence background levels of some fluorescence probes were generally not satisfactory for biological applications. When treated with H$_2$O$_2$, most of them had a long response time which became an important and complex issue for monitoring the H$_2$O$_2$ concentration in living cells.$^{24}$ Only a few of them could respond to H$_2$O$_2$ fast and selectively. It is especially important to develop rapid-response probes to monitor the H$_2$O$_2$ in biological process.

Reduced fluorescent dyes such as 2’,7’-dichlorodihydrofluorescein diacetate were commonly used as fluorescence probes for H$_2$O$_2$.$^{25}$ However, it still showed non-fluorescence if 2’,7’-dichlorodihydrofluorescein diacetate was only oxidized to 2’,7’-dichlorofluorescein diacetate.$^{26}$ It is obvious that the ester bond was broken during or after the oxidation. In this process, H$_2$O$_2$ as a good nucleophile$^{27}$ might promote a nucleophilic substitution. So it is possible to construct a fluorescence probe for H$_2$O$_2$ based on breaking a special ester bond. Therefore, finding a special ester bond and linking it with a fluorophore may be a feasible strategy.

Based on this strategy, ACF was synthesized by the reaction of 2’,7’-dichlorofluorescein and 2-(azidomethyl)benzoyl acid (Scheme 1). ACF exhibited almost no fluorescence (fluorescence quantum yield: Ψ = 0.0024, in CH$_3$OH/PBS buffer, 10 mM, pH = 7.4, 5/95, ESI†).

Scheme 1 The synthesis of probe ACF and the response of ACF to H$_2$O$_2$.

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When treated with H$_2$O$_2$, ACF showed extremely rapid response. Only in 5 min, the fluorescent intensity was increased by 118-fold (Fig. 1). The fluorescent intensity increased quite fast in the first 4 min, after which the increase rate fell a little. After 10 min, the fluorescent intensity was almost linear to the time with a correlation coefficient of 0.9990. And when extended to 120 min, the fluorescent intensity even increased by 441-fold (fluorescence correlation coefficient of 0.9990). And when extended to 120 min, the fluorescent intensity even increased by 441-fold (fluorescence correlation coefficient of 0.9990). Then the effect of pH on the fluorescence of ACF was evaluated, which showed it was stable from pH 6 to 8, even in the presence of H$_2$O$_2$ (Fig. 51, ESI†).

To further estimate the selectivity of probe ACF for H$_2$O$_2$, time-dependent fluorescence changes to ROS and H$_2$S were recorded. As shown in Fig. 3, the fluorescence enhancements for other ROS were still negligible in 120 min. While the enhancement for H$_2$S in 30 min was obvious. However, it was not strong enough to obstruct the detection of H$_2$O$_2$ (the fluorescent intensity for H$_2$S to H$_2$O$_2$ was 1:9.6). What was more, the fluorescent intensity for H$_2$O$_2$ increased obviously with the time, while that for H$_2$S almost ceased. Thus, probe ACF showed high selectivity toward H$_2$O$_2$.

Subsequently, we examined the reactivity of ACF towards different concentrations of H$_2$O$_2$ in CH$_3$OH/PBS buffer (10 mM, pH = 7.4, 5/95) at 25 °C. As expected, after incubation with H$_2$O$_2$, the fluorescence intensity of probe ACF increased gradually as the increase of the H$_2$O$_2$ amounts. Fig. 4 showed the fluorescence intensity of probe ACF increased almost linearly with the concentration of H$_2$O$_2$ in the range of 50–400 μM, and a correlation coefficient of 0.9901. Specifically, the detection limit of H$_2$O$_2$ was determined to be 6.5 nM based on the 3σ/slope method (ESI†), which was much lower than those of the reported probes. The good linearity indicated that probe ACF was able to qualitatively and quantitatively determine the level of H$_2$O$_2$.

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**Fig. 1 (A)** Time-dependent fluorescence spectra of ACF (5 μM) with H$_2$O$_2$ (80 eq, 400 μM) in CH$_3$OH/PBS buffer (10 mM, pH = 7.4, 5/95). (B) Line chart. Inset: enlarged view of time area in the first 6 min. λ$_{ex}$ = 450 nm, λ$_{em}$ = 527 nm. Slits: 5/5 nm.

To estimate the selectivity of probe ACF for H$_2$O$_2$, fluorescence responses to other ROS were examined. As shown in Fig. 2, significant fluorescence enhancement was observed after incubation with H$_2$O$_2$ for 5 min. Compared to H$_2$O$_2$, other ROS induced only negligible fluorescence enhancements under the same condition, including t-BuOOH, hydroxy radical, CH$_3$CO$_2$H, and so on. considering 2-azidomethylbenzoate had been used as a H$_2$S trap, H$_2$S was also examined. It is true that fluorescence enhancement is observed after incubation with H$_2$S. But the increment was far less than that of H$_2$O$_2$ (Fig. 2).

**Fig. 2 (A)** Fluorescence response of ACF (5 μM) incubated with ROS and H$_2$S (80 eq) in CH$_3$OH/PBS buffer (10 mM, pH = 7.4, 5/95) for 5 min. (B) Bar graph. 1. Blank; 2. H$_2$O$_2$; 3. Na$_2$S; 4. tBuOOH; 5. CH$_3$CO$_2$H; 6. tBuOOH; 7. HO·; 8. NaOCl. λ$_{ex}$ = 519 nm, λ$_{em}$ = 527 nm. Slits: 5/5 nm.

**Fig. 3** Time-dependent fluorescence spectra of ACF (5 μM) with ROS and H$_2$S (80 eq, 400 μM) in CH$_3$OH/PBS buffer (10 mM, pH = 7.4, 5/95). Time points represent 30, 60, 90, and 120 min. λ$_{ex}$ = 450 nm, λ$_{em}$ = 527 nm. Slits: 5/5 nm.
D and E of HeLa cells incubated with Bright-field (D), fluorescence image (E) and the overlay (F) of Figure probe imaging of H implying that the probe was suitable for bioimaging of H almost no cytotoxicity in 0.1–30 μM range to both of them, Fig. 4 nucleophilic substitution induced by H incubated with H pre-treated with showed almost no fluorescence (Fig. 5B). However, if the cells were incubated with H (10 μM) in culture medium for 15 min, strong fluorescence was observed (Fig. 5A). A controlled experiment showed that the probe would completely lose its effect if the azido group was instead by a hydrogen atom, which proved the importance of the azido group. Though the exact mechanism and the special effect of azido group were not clear, the in-depth mechanism study is in progress.

Encouraged by the above excellent results, we subsequently explored the potential applications of ACF for monitoring and imaging of H2O2 in living cells. Firstly, the cytoxicity of ACF was evaluated using A-549 cells and Raw 264.7 cells (obtained from the College of Life Science, Nankai University, Tianjin, China; Serum was purchased from Gibco) by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Fig. S4, ES1]. Probe ACF showed almost no cytoxicity in 0.1–30 μM range to both of them, implying that the probe was suitable for bioimaging of H2O2 in living cells. Finally, we assessed the application of the probe for monitoring and imaging of H2O2 in living cells. HeLa cells (obtained from the College of Life Science, Nankai University, Tianjin, China) incubated with ACF (10 μM) in culture medium for 15 min at 37 °C, showed almost no fluorescence (Fig. S5B), however, if the cells were pre-treated with ACF (10 μM) for 15 min and then incubated with H2O2 (10 eq, 100 μM) for 15 min, strong fluorescence was observed (Fig. S5E). The obvious fluorescent enhancement indicated that probe ACF could image H2O2 in living cells. ACF responded to H2O2 only in 15 min, making the detection get close to real-time monitoring.

Conclusions

In conclusion, aiming at finding rapid-response fluorescent probes for detection of H2O2, a new probe 2',7'-dichloro-3',6'-bis(2-(azidomethyl)benzoate)fluorescein (ACF) was developed. ACF can rapidly respond to H2O2 and offer highly sensitivity and selectivity by utilizing the unique chemical reactivity of the ester bonds and H2O2. Preliminary fluorescence imaging experiments indicate that ACF is a good fluorescent tool for rapidly monitoring H2O2 in living cells. We believe the novel H2O2 response site will be broadly used for quantitatively monitoring of H2O2 in biological systems. Relevant studies on this strategy and its biological applications are underway.

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An extremely rapid-response fluorescence probe for \( \text{H}_2\text{O}_2 \) was constructed for monitoring the \( \text{H}_2\text{O}_2 \) levels in biological process.

[Diagram of the probe]