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COMMUNICATION

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_2O_2 was constructed. The probe utilized 2-(azidomethyl)benzoyl group as new reaction site, which exhibited rapid response to H_2O_2 with a 118-fold fluorescence enhancement within 5 min. The biological application of ACF was confirmed by fluorescence imaging H_2O_2 in living cells.

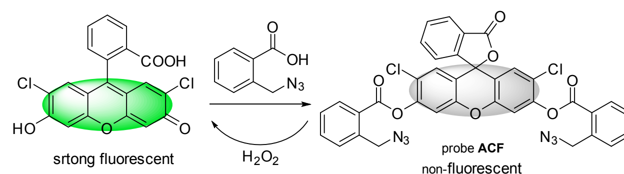
Hydrogen peroxide (H_2O_2), one of the most important reactive oxygen species (ROS), has been known as a harmful metabolic product and a component of the immune response to microbial invasion for a long time.[1] However, H_2O_2 functions as an ubiquitous intracellular second messenger when it is generated at a low concentration (< 0.7 μM).[2] It stimulates cell proliferation,[3] differentiation,[4] and migration[5] by activating the signalling pathways. H_2O_2 might be generated aberrantly and result in oxidative stress with stimulation by exogenous chemicals. However, H_2O_2 is an oxidant unlike the classical second messenger.[6] The resulting H_2O_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_2O_2 generation in biological process is highly required and would provide important information to understand the function of H_2O_2.

Currently, several chemical methods have been developed to detect intracellular H_2O_2, including mass assays,[13] proteomics assays,[14] and fluorescence-based assays.[15-21] Among these methods, fluorescence-based assays were useful because of their non-destructive features. A few fluorescent probes designed for H_2O_2 detection have been reported since 2003.[16a] The first developed and most popular probes were a kind of boronate ester,[16] which have occupied more than a half of all the H_2O_2 probes, including NIR, ratiometric, targetable, trappable and two-photon probes etc. Some of them have been successfully applied to monitor H_2O_2 at physiological levels in *vitro* and in *vivo* and others have been used to explore the cellular mechanisms associated with H_2O_2. Chang group contributed a lot in this field.[16b] Another kind of fluorescent probes developed for H_2O_2 in the early stage contained arylsulfonyl esters as trap groups.[17] After that, several kinds of probes were designed based on unique H_2O_2-responsive sites such as diphenylphosphine,[18] α-diketone groups,[19] metal complexes[20] and some Chalcogen.[21] The efforts to find novel H_2O_2-responsive sites were still in progress.

Although several H_2O_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_2O_2, most of them had a long response time which became an important and complex issue for monitoring the H_2O_2 concentration in living cells.[22] Only a few of them could respond to H_2O_2 fast and selectively. It is especially important to develop rapid-response probes to monitor the H_2O_2 in biological process.

Reduced fluorescent dyes such as 2',7'-dichlorodihydrofluorescein diacetate were commonly used as fluorescence probes for H_2O_2.[23] However, it still showed non-fluorescence if 2',7'- dichlorodihydrofluorescein diacetate was only oxidized to 2',7'-dichlorofluorescin diacetate.[24] It is obvious that the ester bond was broken during or after the oxidation. In this process, H_2O_2 as a good nucleophile,[16b] might promote a nucleophilic substitution. So it is possible to construct a fluorescence probe for H_2O_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: Φ = 0.0024, in CH_3OH/PBS buffer, 10 mM, pH = 7.4, 5/95, ESI†).



Scheme 1 The synthesis of probe **ACF** and the response of **ACF** to H_2O_2

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When treated with H_2O_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 quantum yield: Φ = 0.6780, in CH_3OH/PBS buffer, 10 mM, pH = 7.4, 5/95, ESI†). The fluorescent intensities in the following experiments were recorded in 5 min due to the rapid-response of **ACF** for H_2O_2. 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_2O_2 (Fig. S1, ESI†).

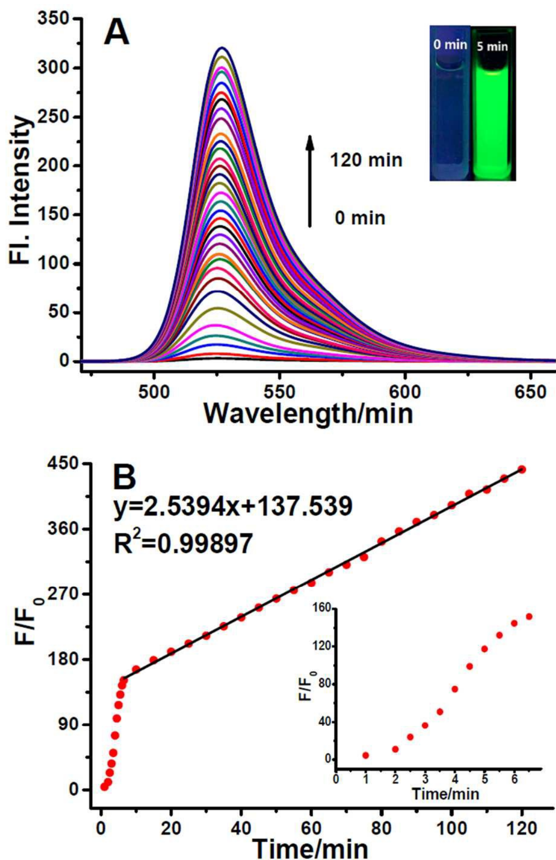

Fig. 1 (A) Time-dependent fluorescence spectra of **ACF** (5 μM) with H_2O_2 (80 eq, 400 μM) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95). (B) Line chart. Inset: enlarged view of time area in the first 6 min. λ_{ex} = 450 nm, λ_{em} = 527 nm. Slits: 5/5 nm.

To estimate the selectivity of probe **ACF** for H_2O_2, fluorescence responses to other ROS were examined. As shown in Fig. 2, significant fluorescence enhancement was observed after incubation with H_2O_2 for 5 min. Compared to H_2O_2, other ROS induced only negligible fluorescence enhancements under the same condition, including *t*-BuOOH, hydroxy radical, CH_3CO_3H, and so on. Considering 2-azidomethylbenzoate had been used as a H_2S trap,[25] H_2S was also examined. It is true that fluorescence enhancement is observed after incubation with H_2S. But the increment was far less than that of H_2O_2 (Fig. 2).

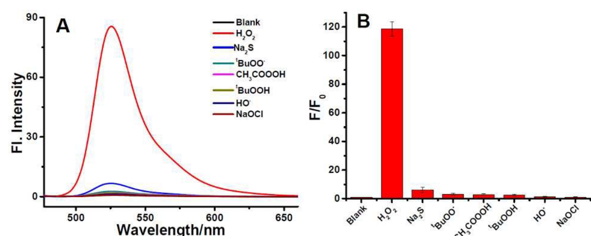

Fig. 2 (A) Fluorescence response of **ACF** (5 μM) incubated with ROS and H_2S (80 eq) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95) for 5 min. (B) Bar graph. 1. Blank; 2. H_2O_2; 3. Na_2S; 4. *t*BuOO·; 5. CH_3CO_3H; 6. *t*BuOOH; 7. HO·; 8. NaOCl. λ_{ex} = 519 nm, λ_{em} = 527 nm. Slits: 5/5 nm.

To further estimate the selectivity of probe **ACF** for H_2O_2, time-dependent fluorescence changes to ROS and H_2S were recorded. As shown in Fig. 3, the fluorescence enhancements for other ROS were still negligible in 120 min. While the enhancement for H_2S in 30 min was obvious. However, it was not strong enough to obstruct the detection of H_2O_2 (the fluorescent intensity for H_2S to H_2O_2 was 1:9.6). What was more, the fluorescent intensity for H_2O_2 increased obviously with the time, while that for H_2S almost ceased. Thus, probe **ACF** showed high selectivity toward H_2O_2.

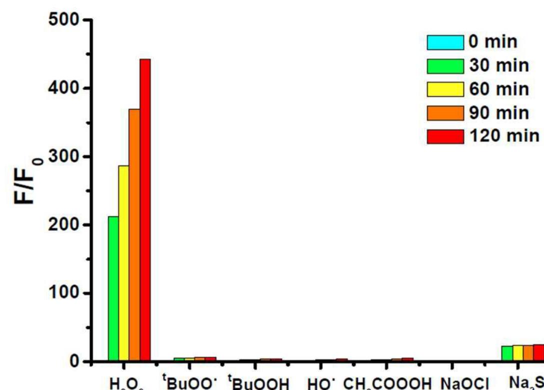

Fig. 3 Time-dependent fluorescence spectra of **ACF** (5 μM) with ROS and H_2S (80 eq, 400 μM) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95). Time points represent 30, 60, 90, and 120 min. λ_{ex} = 450 nm, λ_{em} = 527 nm. Slits: 5/5 nm.

Subsequently, we examined the reactivity of **ACF** towards different concentrations of H_2O_2 in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95) at 25 °C. As expected, after incubation with H_2O_2, the fluorescence intensity of probe **ACF** increased gradually as the increase of the H_2O_2 amounts. Fig. 4 showed the fluorescence intensity of probe **ACF** increased almost linearly with the concentration of H_2O_2 in the range of 50−400 μM, and a correlation coefficient of 0.9901. Specifically, the detection limit of H_2O_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_2O_2.

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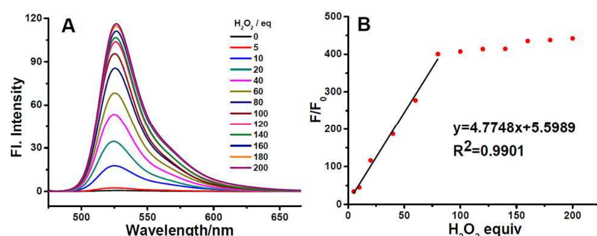

Fig. 4 (A) Fluorescence spectra of **ACF** (5 μM) incubated with different concentrations of H_2O_2 (0–200 eq, 0–1.0 mM) in CH_3OH/PBS buffer (10 mM, pH = 7.4, 5/95) for 5 min. (B) Linear fitting chart. λ_{ex} = 450 nm, λ_{em} = 527 nm. Slits: 5/5 nm.

Next, we carried out competition experiments in the presence of ROS and H_2S (Fig. S2, ESI†). **ACF** was still able to respond to H_2O_2 with strong fluorescence enhancements in the prescence of the interfering species. Moreover, the process of **ACF** for detection of H_2O_2 was confirmed by the HRMS-ESI spectra. The mass signal for 2',7'-dichlorofluorescin ($[M + H]^+$ calcd. for $C_{20}H_{11}Cl_2O_5^+$, 400.9978, found: 400.9962, Fig. S3, ESI†), was detected after probe **ACF** was incubated with H_2O_2. So the ester bond was possible broken by the nucleophilic substitution induced by H_2O_2. 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 H_2O_2 in living cells. Firstly, the cytotoxicity 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, ESI†]. Probe **ACF** showed almost no cytotoxicity in 0.1–30 μM range to both of them, implying that the probe was suitable for bioimaging of H_2O_2 in living cells. Finally, we assessed the application of the probe for monitoring and imaging of H_2O_2 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. 5B). However, if the cells were pre-treated with **ACF** (10 μM) for 15 min and then incubated with H_2O_2 (10 eq, 100 μM) for 15 min, strong fluorescence was observed (Fig. 5E). The obvious fluorescent enhancement indicated that probe **ACF** could image H_2O_2 in living cells. **ACF** responded to H_2O_2 only in 15 min, making the detection get close to real-time monitoring.

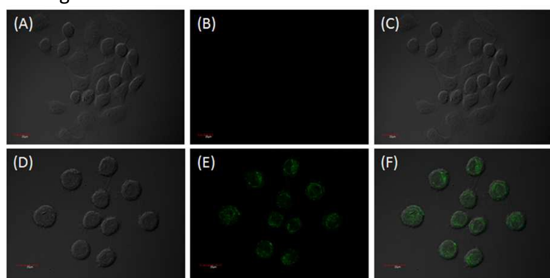

Fig. 5 Bright-field (A), fluorescence image (B) and the overlay (C) of Figure A and B of HeLa cells incubated with **ACF** (10 μM) for 15 min. Bright-field (D), fluorescence image (E) and the overlay (F) of Figure D and E of HeLa cells incubated with **ACF** (10 μM) for 15 min and washed with PBS three times. After replacement of the medium, cells were incubated with H_2O_2 (10 eq, 100 μM) for another 15 min.

Conclusions

In conclusion, aiming at finding rapid-response fluorescent probes for detection of H_2O_2, a new probe 2',7'-dichloro-3',6'-bis(2-(azidomethyl)benzoate)fluorescein (**ACF**) was developed. **ACF** can rapidly respond to H_2O_2 and offer highly sensitivity and selectivity by utilizing the unique chemical reactivity of the ester bonds and H_2O_2. Preliminary fluorescence imaging experiments indicate that **ACF** is a good fluorescent tool for rapidly monitoring H_2O_2 in living cells. We believe the novel H_2O_2 response site will be broadly used for quantitatively monitoring of H_2O_2 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 hydrogen peroxide and its applications in living cells

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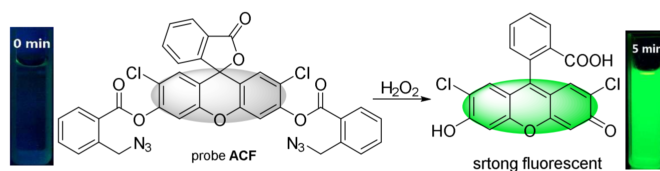
An extremely rapid-response fluorescence probe for H_2O_2 was constructed for monitoring the H_2O_2 levels in biological process.



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