Fluorogenic Biosensors Constructed via Aggregation-induced Emission Based on Enzyme-catalyzed Coupling Reactions for Detection of Hydrogen Peroxide

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Hydrogen peroxide ($H_2O_2$) is a main reactive oxygen by-product produced in the metabolism of organisms and a common biomarker of oxidative stress. Aggregation-induced emission (AIE) probes for $H_2O_2$ have been proposed. Such AIEgens mostly use benzeneboronic acid as a recognition group. Recently, a strategy involving enzyme-catalyzed polymerization of AIE compounds shows great potential in AIEgens design. We herein modify the AIE motif, tetraphenylethene (TPE) with $o$-phenylenediamine (TPE-TAF), which can be oxidated by $H_2O_2$ in HRP to form an intramolecular phenazine structure. Compared with a similar approach, the proposed strategy is simple and the TPE-TAF showed a sensitive “turn-on” fluorescence with $H_2O_2$. The detection limit (LOD) is 3.39 $\mu$M and the probe is highly specific against $H_2O_2$. We further verified the reaction mechanism of the enzyme-catalyzed coupling reaction. The probe is a promising candidate as a stable and safe fluorescent substrate in $H_2O_2$ sensing.

Keywords Hydrogen peroxide, aggregation-induced emission, fluorescence probe

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We further verified the reaction mechanism of the enzyme-catalyzed coupling reaction, and demonstrated that the probe is a promising candidate as a fluorescent substrate in H$_2$O$_2$ sensing.

Materials and Methods

Materials and instruments

3,4-Diaminobenzophenone, ferrous sulfate, sodium hypochlorite, sodium nitrite, sodium nitrate, sodium dihydrogen phosphate, disodium hydrogen phosphate were purchased from Shanghai Saen Chemical Technology Co., Ltd. Horseradish peroxidase (HRP) was purchased from Beijing Zhijie Fangyuan Technology Co., Ltd. 1,2-Dinitrobenzen and a 30% hydrogen peroxide (H$_2$O$_2$) solution were from Beijing Yinuokai Technology Co., Ltd. Diphenylamine and o-phenylenediamine were from Bailingwei Technology Co., Ltd. (Beijing, China).

The structure of synthesized compounds was validated by LC-MS (Agilent 6520 Q-TOF LC/MS, Agilent, USA) and 400 M NMR (Bruker, Germany). Characteristics of TPE-TAF were studied by using a fluorescence spectrophotometer (F4600, Hitachi) and transmission electron microscopy (Talos F200C, Czech Republic, USA). Dynamic size of TPE-TAF was measured by a Nanoparticle size analyzer (Nano-ZS, Malvern Instruments, UK). Fluorescence emission of TPE-TAF was observed by a laser scanning confocal microscope (TCS SP8, Leica, Germany).

Synthesis of TPE-TAF

Zinc powder (1.7 g, 26.5 mmol) was dissolved in 30 mL of anhydrous THF and reacted with 1.43 mL of TiCl$_4$ at –10°C under N$_2$ and refluxed for 2 h. Then, 3,4-diaminobenzophenone (1.40 g, 6 mmol) in 65 mL of THF was added and refluxed for 4 h, followed by adding 100 mL of a 10% K$_2$CO$_3$ solution (Fig. 1a). The mixture was vigorously stirred for 5 min, and then the crude product was filtrated to collect the organic layer. The aqueous layer was extracted by dichloromethane three times and combined with the organic aqueous. After being washed with water and dried with anhydrous Na$_2$SO$_4$, the organic solvent was removed to obtain a crude product. The crude product was purified by column chromatography (PE/EA = 1/2, v/v) to obtain a yellow solid compound with yielding 40%. $^1$H-NMR (400 MHz, MeOD) δ 4.74 (s, 8H), 6.13 – 6.15 (m, 2H), 6.27 – 6.31 (m, 4H), 6.92 (s, 10H); $^{13}$C-NMR (100 MHz, MeOD) δ 145.03, 139.64, 135.75, 133.51, 133.19, 131.01, 126.92, 125.36, 123.43, 119.74, 115.46. MS data, m/z: calculated, 392.2; found, 393.250 (M+H) (Fig. S1).

Morphology evaluation and working mechanism of TPE-TAF

The experiments were performed in two groups: (1) TPE-TAF (25 μM) dissolved in 10 mM PBS (pH 6.40, 25°C, 10 vol% DMSO) with HRP 0.001 mg/L; (2) TPE-TAF (25 μM) with hydrogen peroxide (100 μM) and HRP (0.001 g/L) in 10 mM PBS (pH 6.40, 25°C, 10 vol% DMSO) at 25°C. The particle size of the two TPE-TAF groups was then evaluated. To confirm the interaction mechanism of the probe molecule with hydrogen peroxide, we spiked o-phenylenediamine (OPD) and 1,2-dinitrobenzene (m-DNB) together, in which o-phenylenediamine was used as a competitive substrate and 1,2-dinitrobenzene as a control substrate.

Results and Discussion

AIE properties of TPE-TAF

Using a one-step McMurry reaction, we synthesized TPE-TAF with 3,4-diaminobenzophenone. The TPE-TAF showed good water solubility. Therefore, we detected the AIE features of the probe by increasing the fraction of DMSO. As the fraction increased, the fluorescence intensity of TPE-TAF at 450 nm was gradually elevated (Fig. 1b), which demonstrated a typical AIE behavior of TPE-TAF. Due to the low fluorescence in 10% DMSO, TPE-TAF was dissolved in 10% DMSO for subsequent experiments.
Characteristics of H$_2$O$_2$ sensing by TPE-TAF

The optimal pH value for H$_2$O$_2$ sensing was investigated as follows: TPE-TAF (25 μM) was incubated with HRP (1 mg/mL) and H$_2$O$_2$ (50 μM) with 10% DMSO in 10 mM PBS. The fluorescence emission spectra showed a low FL intensity from pH 3.5 to 5.1, but displayed high intensity starting from pH 5.1. The highest FL intensity could be observed in the range of pH 6.4 – 7.4 (Fig. 3a, Fig. S2), probably attributed to the remarkable crosslinking of TPE-TAF by HRP under the neutral pH range. These results showed that the probe can stably respond to H$_2$O$_2$ in biological environments.

To detect H$_2$O$_2$, we then optimized the HRP concentration. A PBS buffer (pH 6.4, 10 mM, 10% DMSO) containing 25 μM TPE-TAF, 50 μM H$_2$O$_2$ and different concentrations of HRP were reacted for 30 min. As can be seen in Fig. 2b, there was nearly no fluorescent emission in the absence of HRP, indicating a negligible background interference. When the concentration of HRP reached 0.001 g/L, the fluorescence emission was the highest (Fig. S3). We next evaluated the dynamic sensing process by mixing 25 μM TPE-TAF with 50 μM H$_2$O$_2$ and...
1 mg/L HRP. The fluorescence spectra of the reaction were measured every 5 min. As shown in Fig. 3, with a prolongation of the reaction time, the fluorescence intensity increased and reached a stable state after 30 min.

Based on the optimal conditions, we further studied the fluorescence intensity changes for different concentrations of H₂O₂. As shown in Fig. 3, for a blank group without H₂O₂, the intensity of TPE-TFA was very low after incubation for 20 min. As the concentrations of H₂O₂ increased, the FL intensity was gradually enhanced. The FL intensity at 455 nm correlated well with the concentration of hydrogen peroxide from 0 – 100 μM (\(R^2 = 0.996\)). The detection limit (LOD) was calculated to be 3.39 μM (S/N = 3, n = 11) (Fig. S4).

To evaluate the sensing specificity, we tested the fluorescence changes of the probe interacting with various active oxides, such as hydroxyl radicals (HO·), peroxy-tert-butanol (TBHP), tert-butoxy (·OBu), peroxy anion (O₂⁻), as well as hypochlorite anion (ClO⁻), nitrite (NO₂⁻) and nitrate (NO₃⁻) (Fig. 3). The reaction of hydrogen peroxide with the TPE-TFA caused the most obvious increment of the fluorescence intensity compared to other compounds, demonstrating an excellent specificity of the probe with little interference by other active molecules.
Morphology and reaction mechanism for the H$_2$O$_2$ sensing

We then investigated the morphology changes of TPE-TAF (25 μM) after binding with hydrogen peroxide. Under the reaction of 1 mg/L HRP and 100 μM H$_2$O$_2$, the particle size of the probe increased from 5 nm to 1 μm (Fig. 4a), and the aggregated particles were highly emissive (Fig. 4b).

We further validated the proposed mechanism for the enzyme-catalyzed crosslinking of TPE-TAF. Substrate competition experiments were conducted by adding OPD or m-DNB into the sensing system. As can be seen in Fig. 5, the fluorescence intensity of TPE-TFA decreased with an increase of the OPD concentration, but did not change with the concentration of m-DNB being elevated. This result indicated that OPD might compete with the TPE-TFA, and thus inhibited the crosslinking reaction and reduced the fluorescence intensity induced by TPE-TFA aggregation.

Conclusions

In this work, we modified tetraphenylethene (TPE) with o-phenylenediamine (TPE-TAF) for H$_2$O$_2$ sensing, which can be oxidated by H$_2$O$_2$ in HRP and form an intramolecular phenazine structure, which thus exhibited a “turn-on” AIE feature. Compared with similar approaches, the proposed strategy is simple and convenient. The detection range for H$_2$O$_2$ is 10 – 100 μM with a detection limit (LOD) of 3.39 μM. The fluorescence intensity of TPE-TFA decreased with an increase of the OPD concentration, but did not change with the concentration of m-DNB being elevated. This result indicated that OPD might compete with the TPE-TFA, and thus inhibited the crosslinking reaction and reduced the fluorescence intensity induced by TPE-TFA aggregation.

Conflicts of Interest

All other authors declare no competing interests.

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Supporing Information

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