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

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Hydrogen peroxide (H₂O₂) 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 H₂O₂ have been proposed. Such AIEgens mostly use benzenboronic 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 H₂O₂ 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 H₂O₂. The detection limit (LOD) is 3.39 μM and the probe is highly specific against H₂O₂. 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 H₂O₂ sensing.

Keywords Hydrogen peroxide, aggregation-induced emission, fluorescence probe

(Received December 29, 2020; Accepted February 18, 2021; Advance Publication Released Online by J-STAGE April 23, 2021)

Introduction

Hydrogen peroxide (H₂O₂) (biological level is about 0.48×10^{-8} – 0.57×10^{-8} M), is a main reactive oxygen by-product produced in the metabolism of organisms and a common biomarker of oxidative stress.^{1–4} Many methods such as colorimetry, surface enhanced Raman spectroscopy, chromatographic analysis, chemiluminescence, fluorescence and electrochemical analysis have been developed.^{1–12} Among them, fluorescent probes have attracted wide concern, since they are cost-effective, highly sensitive and convenient to operate. However, most of fluorescent compounds are not photostable, and aggregation caused quenching (ACQ) often occurs in biological environments.

In recent years, fluorogens with a AIE (aggregation-induced emission) feature have been proposed in a construction of biochemical sensors. AIEgens are greatly emissive in an

aggregated state, and therefore have significant resistance against photobleaching.^{13–18} The reported strategies for H₂O₂ AIEgens design have usually employed benzenboronic acid to respond with hydrogen peroxide.^{19–22} Recently, approaches involving enzyme-catalyzed polymerization of AIEgen were proposed for H₂O₂ sensing. Wang *et al.* designed tyrosine-functionalized tetraphenylethene (TPE-Tyr), with a TPE core as an AIE motif and four peripheral tyrosines as an enzyme substrate. Intermolecular cross-linking could occur under the HRP-catalyzed oxidative coupling of tyrosine moieties in the presence of hydrogen peroxide, leading to a “turn-on” AIE property.²³ Yuan *et al.* used a similar strategy to develop enzyme-catalyzed AIE-active substrates by copper-mediated azide-alkyne click (CuAAC) polymerization.²⁴ These strategies improve the aggregation of the AIEgens, but their synthesis usually requires multiple processes. We herein modify the AIE motif, tetraphenylethene (TPE), with *o*-phenylenediamine (TPE-TAF), which can be oxidated by H₂O₂ in HRP and form an intramolecular phenazine structure.²⁵ Compared with a similar approach, the proposed strategy is simple and the TPE-TAF has shown a sensitive “turn-on” fluorescence in response to H₂O₂. The detection range is 10 – 100 μM, with a detection limit (LOD) of 3.39 μM, and the probe is highly specific against H₂O₂.

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Fig. 1 Synthetic route of TPE-TAF (a); fluorescence emission spectra (b); fluorescence intensity (c) of TPE-TAF in DMSO with a varying water fraction ($\lambda_{\text{ex}} = 320 \text{ nm}$).

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_2O_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-Dinitrobenzene and a 30% hydrogen peroxide (H_2O_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_2CO_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_2SO_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%. $^1\text{H-NMR}$ (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.

Fig. 2 (a) Fluorescence emission spectra of TPE-TAF (25 μM) with hydrogen peroxide (50 μM) and HRP (1 mg/mL) at 450 nm under different pH conditions; (b) fluorescence intensity of TPE-TAF (25 μM) with hydrogen peroxide (50 μM) and different concentrations of HRP.

Fig. 3 (a) Fluorescence-emission spectra of TPE-TAF (25 μM) with different concentrations of hydrogen peroxide; (b) fluorescence intensity of TPE-TAF (5 μM) incubated with hydrogen peroxide (0, 25, 35, 40, 55 μM) for different period of times; (c) fluorescence emission spectra of TPE-TAF with different reactive oxygen species; (d) fluorescence intensity of TPE-TAF with different reactive oxygen species.

Characteristics of H_2O_2 sensing by TPE-TAF

The optimal pH value for H_2O_2 sensing was investigated as follows: TPE-TAF (25 μM) was incubated with HRP (1 mg/L) and H_2O_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_2O_2

in biological environments.

To detect H_2O_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_2O_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_2O_2 and

Fig. 4 (a) Particle size of dissolved TPE-TAF (25 μM) and aggregated TPE-TAF in the presence of hydrogen peroxide (100 μM); (b) TEM and confocal fluorescent image of the polymerized TPE-TAF aggregates ($\lambda_{\text{ex}} = 320 \text{ nm}$).

Fig. 5 Mechanism validation of TPE-TAF to H₂O₂. (a) Chemical structure of the competitive substrate *o*-phenylenediamine (OPD) and control substrate 1,2-dinitrobenzene (*m*-DNB); (b) fluorescence emission spectra of TPE-TAF (25 μM) with hydrogen peroxide (100 μM) in different concentrations of OPD (0, 25, 50, 150, 200 μM); (c) fluorescence emission spectra of TPE-TAF (25 μM) with hydrogen peroxide (100 μM) in different concentrations of *m*-DNB (0, 25, 50, 150, 200 μM); (d) fluorescence emission spectra of TPE-TAF (25 μM) with hydrogen peroxide (100 μM) in different concentrations of *m*-DNB and OPD (0, 25, 50, 150, 200 μM).

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 (·O^tBu), 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_2O_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_2O_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_2O_2 sensing, which can be oxidated by H_2O_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_2O_2 is 10 – 100 μM with a detection limit (LOD) of 3.39 μM , and the probe is highly specific against H_2O_2 . We finally demonstrated the reaction mechanism of the enzyme-catalyzed coupling reaction. As a summary, the probe is a promising candidate as a fluorescent substrate in H_2O_2 sensing.

Conflicts of Interest

All other authors declare no competing interests.

Acknowledgements

This work was supported by the Natural Science Foundation of China of Tianjin City (18JCYBJC24800), National Natural Science Foundation of China (No. 81573390), Beijing Natural Science Foundation (7194312), Beijing Nova Program (Z201100006820127), Tsinghua University-Peking Union Medical College Hospital Collaboration Project (PTQH201911583), Beijing Key Laboratory for Mechanisms Research and Precision Diagnosis of Invasive Fungal Diseases.

Supporting Information

Supplementary material related to this article can be found in the online version.

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