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Highly Selective Fluorogenic Multianalyte Biosensors Constructed via Enzyme-Catalyzed Coupling and Aggregation-Induced Emission

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Supporting Information

ABSTRACT: The development of a highly selective and fast responsive fluorogenic biosensor for diverse analytes ranging from bioactive small molecules to specific antigens is highly desirable but remains a considerable challenge. We herein propose a new approach by integrating substrate-selective enzymatic reactions with fluorogens exhibiting aggregation-induced emission feature. Tyrosine-functionalized tetraphenylethene, TPE-Tyr, molecularly dissolves in aqueous media with negligible fluorescence emission; upon addition of horseradish peroxidase (HRP) and H_2O_2, effective cross-linking occurs due to HRP-catalyzed oxidative coupling of tyrosine moieties in TPE-Tyr. This leads to fluorescence emission turn-on and fast detection of H_2O_2 with high sensitivity and selectivity. As a validation of the new strategy's generality, we further configure it into the biosensor design for glucose through cascade enzymatic reactions and for pathologically relevant antigens (e.g., human carcinoembryonic antigen) by combining with the ELISA kit.

Reactive oxygen species (ROS) normally generated from oxygen metabolism are recognized as a potential cause of cell damage when misregulated.[1] In particular, hydrogen peroxide (H_2O_2), known as one of the major ROS in living organisms and common biomarkers for oxidative stress, also serves as an indispensable signal molecule in mediating a variety of biological processes.[2] Therefore, it is of crucial importance and necessity to exploit advanced H_2O_2 sensing and imaging strategies applicable under complex biological milieu. Fluorometric H_2O_2 sensors offer distinct merits in terms of convenience, sensitivity, and spatiotemporal resolution. To date, a variety of reaction-based fluorogenic small molecule H_2O_2 probes, such as caged chromophores with H_2O_2-cleavable linkages (e.g., benzenesulfonyl ester, boronate ester, and benzil), have been developed.[1a−c,3] Nevertheless, most fluorescent H_2O_2 probes are subjected to limitations such as insufficient water solubility, low selectivity over competitive ROS species, and long incubation periods. High reactivity is desired for fast reaction-based fluorogenic detection of H_2O_2 but usually leads to unsatisfactory substrate stability in buffer or biological media. Thus, the invention of a fast responsive fluorogenic H_2O_2 biosensor, which is structurally stable during storage and intrinsically water-soluble, with high selectivity and sensitivity remains a considerable challenge.

As an alternative fluorogenic process, the concept of aggregation-induced emission (AIE) originally reported by Tang et al.[4] has recently emerged as a versatile and potent strategy for the construction of chemosensors and biosensors. Typical molecules exhibiting AIE features, such as tetraphenylethene (TPE), are nonemissive in molecularly dissolved state but undergo enhanced fluorescence emission in the aggregated state.[5] A number of fluorescent probes based on AIE effect have been elegantly designed and applied for sensing and imaging of diverse analytes ranging from pH,[6] gas,[7] metal ions,[8] explosives,[9] biomolecules,[10] and cell apoptosis processes.[11] Previous knowledge demonstrated that AIE-based sensors possess advantages including low background interference, improved imaging contrast, and superior resistance to photobleaching. On the other hand, it is well-known that enzyme-catalyzed reactions are highly selective and efficient toward specific metabolic substrates and can occur under extremely mild conditions.[12] Provided that the AIE process could be triggered and activated by virtue of H_2O_2-involved enzymatic reactions, we envisage that a novel fluorogenic H_2O_2 sensing platform with high specificity and fast response rate could be constructed via the delicate integration of AIE effect and enzyme-catalyzed reactions.

To verify our hypothesis, well-defined L-tyrosine-functionalized TPE, TPE-Tyr, consisting of a TPE core and four tyrosine moieties at the periphery was synthesized, where the TPE core serves as the AIE-active motif and peripheral tyrosine moieties confer water solubility and enzymatic coupling reactivity (Scheme 1). TPE-Tyr was expected to molecularly dissolve in aqueous solution, exhibiting negligible fluorescence emission. However, it can undergo covalent cross-linking via the formation of dityrosine linkages by exploiting horseradish peroxidase (HRP)-catalyzed phenol polymerization in the presence of H_2O_2, thereby activating the AIE process and switching on fluorescence emission of TPE core motif (Scheme 1). HRP-catalyzed transformation of TPE-Tyr from unimers to cross-linked aggregates, which is associated with prominent emission turn-on, provided an emerging H_2O_2 sensing strategy with high sensitivity and selectivity and fast response rate. The structural stability of TPE-Tyr is another built-in feature compared to conventional H_2O_2 probes based on caged chromophores.

In addition, by taking advantage of H_2O_2 generation via glucose oxidase (GOx)-catalyzed glucose oxidation,[13] TPE-Tyr could also be configured into a glucose biosensor in combination with cascade GOx/HRP enzymatic catalysis (Scheme 1).

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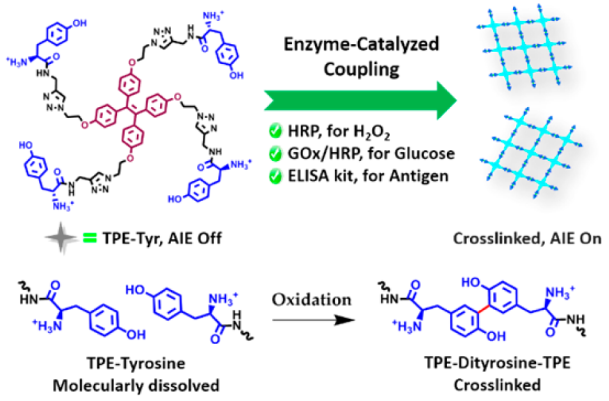
Scheme 1. Schematic Illustration of the Fabrication of Fluorometric Off−On Multianalyte Biosensor for H_2O_2, Glucose, and Specific Antigens via Enzyme-Catalyzed Coupling and Crosslinking of L-tyrosine (Tyr)-Functionalized TPE, TPE-Tyr, and the Corresponding AIE in Aqueous Media[a]



[a]HRP: horseradish peroxidase; GOx: glucose oxidase; ELISA: enzyme-linked immunosorbent assay.

Furthermore, HRP is typically involved in enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry due to its monomeric nature and the generation of colorimetric product in the presence of specific substrate such as 3,3′,5,5′-tetramethylbenzidine (TMB).[14] However, the shelf life of TMB and structural stability of the corresponding enzymatic product are unsatisfactory. We then demonstrate that TPE-Tyr probe was competent to integrate into commercial ELISA kit and serves as an optional fluorogenic substrate for HRP, exhibiting superior detection sensitivity against human carcinoembryonic antigen (CEA, a well-known biomarker for carcinoma; Scheme 1).

TPE-Tyr was synthesized via copper-catalyzed azide−alkyne click reaction between azide-functionalized TPE precursor and alkynyl-functionalized *N*-Boc-*O*-*tert*-butyl-L-tyrosine, followed by hydrolysis (Scheme S1). The chemical structure of TPE-Tyr and relevant precursors were verified by NMR, HPLC, and ESI-MS (Figures S1−S4). Potentiometric titration of TPE-Tyr in aqueous media revealed an apparent p*K*a of ∼6.4 (Figure S5). The pH-dependent fluorescence measurements revealed that TPE-Tyr aqueous solution (25 μM) exhibited negligible emission at pH < 6.2. At elevated pH, an abrupt emission intensity increase was observed, reaching a plateau at pH > 8.0 (Figure S6). In the range of pH 5−9, emission intensity exhibited a cumulative ∼50-fold increase, and notably, the most prominent intensity change occurred within the narrow range of pH 6−8. In addition, the TPE-Tyr aqueous solution was transparent at pH < 6.2, whereas it turned turbid at higher pH (Figure S6). The combined fluorescence and optical transmittance results revealed that TPE-Tyr molecularly dissolves in aqueous solution at mildly acidic pH (<6.2) and forms aggregates with prominently enhanced emission at elevated pH. To further verify the AIE feature, TPE-Tyr emission in buffer/DMSO mixture at varying DMSO contents was monitored, and dramatic emission enhancement was observed at DMSO (poor solvent for TPE-Tyr) content higher than ∼40 vol % (Figure S7). These results suggested that the anchoring of tyrosine moieties at the periphery of TPE did not alter the AIE feature of TPE core.[5a,b]

Considering that TPE-Tyr contains four tyrosine moieties and phenol functionality can undergo HRP-catalyzed polymerization in the presence of H_2O_2, the enzymatic process will also lead to the covalent cross-linking of TPE-Tyr with enhanced emission due to the AIE feature of TPE. Thus, the HRP/H_2O_2-actuated fluorogenic process can be further exploited to design TPE-Tyr based biosensors for H_2O_2 or HRP. To achieve optimized sensing performance and maintain HRP activity, pH 6.0 buffer solution is used in all subsequent experiments. Control experiments revealed that the addition of HRP, H_2O_2, GOx, glucose or the combination of them into TPE-Tyr solution in PBS buffer did not affect solution pH (Figure S8). TPE-Tyr (25 μM in 10 mM PBS buffer, pH 6.0) exhibited negligible emission in the presence or absence of HRP (Figure S9). However, upon HRP/H_2O_2 addition, prominently enhanced emission at ∼470 nm was observed, which was ascribed to HRP-catalyzed cross-linking of TPE-Tyr (Scheme 1 and Figure S9).

Specifically, upon addition of H_2O_2 (50 μM) into the aqueous mixture of TPE-Tyr and HRP, the fluorescence emission increased rapidly and reached a plateau within ∼5 min, resulting in a cumulative ∼14-fold intensity increase at ∼470 nm (Figure 1a). In comparison with previously reported fluorogenic H_2O_2 probes with H_2O_2-cleavable linkages (>1 h incubation time),[1a−c,3,15] the enzymatic AIE-based H_2O_2 probe exhibited much faster response rate. Enzymatic reaction kinetics were then examined at varying H_2O_2 concentrations (0−50 μM; Figure S10), and it was found that at lower H_2O_2 concentration, shorter incubation duration was needed to reach the final steady state. For instance, less than 0.5 min was needed for assaying H_2O_2 at a concentration of 4.0 μM. On the other hand, upon addition of H_2O_2 at a concentration higher than 50 μM, the fluorescence emission did not exhibit any further increase (Figure S11), indicating that the enzymatic coupling reaction was already completed at 50 μM H_2O_2. At fixed TPE-Tyr and H_2O_2 concentrations (25 μM and 50 μM, respectively), HRP

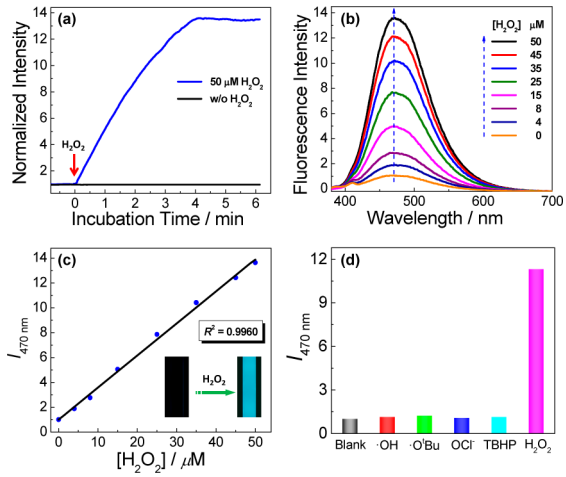


Figure 1. (a) Incubation time dependence of normalized fluorescence intensity of TPE-Tyr in the presence and absence of H_2O_2. (b) Normalized emission spectra of TPE-Tyr aqueous solution in the presence of H_2O_2 at different concentrations. (c) H_2O_2 concentration dependence of normalized emission intensities at 470 nm. The inset in (c) shows optical photographs recorded under 365 nm UV light irradiation for TPE-Tyr aqueous solution in the absence and presence of 50 μM H_2O_2, respectively. (d) Response of TPE-Tyr to various ROS species and normalized emission intensities at 470 nm are shown. For details, see Supporting Information. TPE-Tyr 25 μM, HRP 0.01 g/L; PBS buffer (pH 6.0, 10 mM, 25 °C); λ_{ex} = 360 nm, λ_{em} = 470 nm; [H_2O_2] 0−50 μM; the reaction duration was fixed at 5 min in all cases.

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concentration can also affect the extent of emission enhancement (Figure S12). In subsequent biosensor assays, the incubation duration of enzyme-catalyzed reactions was set at 5 min, and HRP concentration was fixed at 0.01 g/L to achieve rapid and reliable sensing performance. For the aqueous mixture (10 mM PBS buffer, pH 6.0) of TPE-Tyr and HRP, the emission intensity at ~470 nm increases linearly with H_2O_2 concentrations in the range of 0−50 μM (R^2 = 0.996; Figure 1b,c). The H_2O_2 detection limit was determined to be ~0.35 μM if we define the detection limit as the H_2O_2 concentration at which 10% emission enhancement could be observed.

We further probed the underlying mechanism and morphological evolution during enzyme-catalyzed cross-linking of TPE-Tyr and emission turn-on. Dynamic light scattering (DLS) measurements revealed that the scattering intensity of aqueous TPE-Tyr solution considerably increased and leveled off within ~5 min upon H_2O_2 addition (Figure S13a). This is in line with the aggregate formation and fluorescence emission evolution kinetics (Figure 1a). The enzymatic generation of TPE-Tyr aggregates was also verified by the evolution of intensity-averaged hydrodynamic diameters, $\langle D_h \rangle$, being ~2 and ~400 nm before and after HRP/H_2O_2 addition (Figure S13b). The sole addition of H_2O_2 or HRP resulted in negligible changes in $\langle D_h \rangle$. In addition, transmission electron microscopy (TEM) images revealed the coexistence of both spherical and irregular morphologies for cross-linked TPE-Tyr aggregates (Figure S14),[12f] which were intensely emissive as evidenced by confocal laser scanning microscopy (CLSM) studies (Figure S14c). In order to verify that HRP-catalyzed coupling of TPE-Tyr led to aggregate formation, substrate competition experiments were also conducted by externally adding L-tyrosine or *O*-*tert*-butyl ether protected L-tyrosine (Figure S15).

Considering that H_2O_2 is a highly selective substrate for HRP, we speculate that the TPE-Tyr based enzymatic probe for H_2O_2 should exhibit high selectively and specificity over other ROS species. As expected, aqueous TPE-Tyr solution at pH 6.0 showed no apparent fluorescence enhancement in response to other ROS including hydroxyl radical (•OH), *tert*-butoxy radical (•O*t*Bu), hypochlorite (OCl^-), and *tert*-butyl hydroperoxide (TBHP) (Figures 1d and S16). This provides to be a great advantage as compared to previously reported nonenzymatic reaction-based fluorogenic H_2O_2 probes.[1a−c,3,15]

Profiting from the inherent multicomponent feature of enzyme-catalyzed tandem reactions, the fluorogenic TPE-Tyr probe based on activated AIE process can be further configured into biosensors for other analytes of interest such as glucose and specific antigens (i.e., human CEA) (Figure 2a and Figure S17a). GOx can selectively oxidize glucose in the presence of oxygen to yield gluconic acid and H_2O_2. The as-generated H_2O_2 could be successively utilized for HRP-catalyzed coupling reaction of TPE-Tyr, generating highly emissive cross-linked aggregates. Therefore, the TPE-Tyr probe could be also employed for glucose sensing. As shown in Figure S18a, TPE-Tyr exhibited negligible emission in the absence and presence of GOx or HRP enzymes, whereas considerably enhanced emission was observed upon addition of glucose in the presence of both enzymes. Again, the enzymatic reaction was completed within ~5 min, resulting in ~10-fold emission enhancement (Figure S18b).

At constant TPE-Tyr and GOx/HRP concentrations, the fluorescence emission spectra collected upon adding glucose and incubating for 5 min are shown in Figure S17b. It is intriguing to note that the emission intensity at 470 nm increase linearly with glucose concentrations in the range of 0−1 mM (R^2 = 0.999;

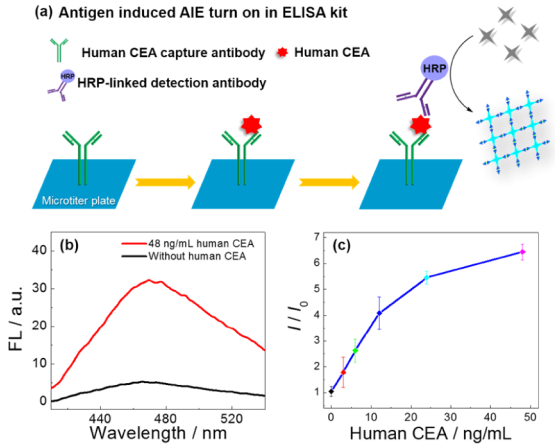

Figure 2. (a) Schematics of the sandwich ELISA format for quantitative analysis of human CEA based on HRP-catalyzed oxidative coupling and aggregation-induced emission. (b) Typical fluorescence spectra recorded in 10 mM PBS buffer (pH 6.0, 37 °C) in the absence and presence of human CEA (TPE-Tyr 25 μM, H_2O_2 200 μM), respectively. (c) Human CEA-dependent changes in emission intensities obtained via the ELISA kit by following standard protocols using fluorogenic TPE-Tyr as the substrate molecules instead of the colorimetric TMB.

Figure S17c). The detection limit of glucose was determined to be ~10 μM. Since GOx is highly selective for the glucose substrate, thus, the sensing selectivity can be guaranteed. As shown in Figure S17d, aqueous mixture of TPE-Tyr and GOx/HRP exhibited negligible emission upon treating with D-fructose (Fru), D-galactose (Gal), and D-mannose (Man), respectively. Moreover, the coexistence of these saccharides did not adversely affect the sensing performance for glucose in terms of both detection sensitivity and selectivity. The above results suggested that TPE-Tyr could also serve as an excellent biosensor for glucose, in addition to H_2O_2 sensing.

To further validate the new strategy's utility and explore its potential applications in clinical diagnosis, we also integrate the AIE-based TPE-Tyr probe into the commercial ELISA kit. As a proof of concept, TPE-Tyr was exploited as a novel fluorogenic substrate to replace the conventional colorimetric TMB substrate in a commercial human CEA ELISA kit (Figure 2a).[14] According to standard protocols, the CEA detection limit by the commercial ELISA kit was estimated to be ~5.0 ng/mL on the basis of colorimetric TMB substrate (Figure S19). When using TPE-Tyr/H_2O_2 mixture as the fluorogenic reporter for HRP-linked detection antibody by using CEA-spiked sample, the drastically enhanced emission implied that TPE-Tyr can successfully serve as an optional substrate for CEA detection via ELISA (Figure 2b). The CEA calibration curve determined via ELISA using TPE-Tyr/H_2O_2 is shown in Figure 2c. Specifically, in the CEA concentration range of 0−12 ng/mL, the normalized emission intensities vary linearly with CEA levels. Moreover, the CEA detection limit was estimated to be ~2.0 ng/mL. Besides better sensitivity and CEA detection limit based on fluorogenic TPE-Tyr substrate, the structural stability of TPE-Tyr and emission stability of enzymatically cross-linked aggregates can provide additional advantages, as both the colorimetric TMB substrate and its enzymatic product suffer from limitations such as insufficient stability. The use of TPE-Tyr allows for more flexible design of ELISA protocols.

In summary, well-defined tyrosine-functionalized TPE-Tyr was synthesized and in the presence of HRP/H_2O_2 or GOx/HRP/glucose, it underwent covalent cross-linking associated

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with fluorescence emission turn-on due to activated AIE process by virtue of enzyme-catalyzed coupling. Thus, TPE-Tyr can serve as an enzymatic fluorogenic biosensor for H_2O_2 and glucose with improved structural stability, enhanced detection sensitivity and selectivity, and faster response rate, as compared to previous reaction-based small molecule fluorogenic H_2O_2/glucose biosensors. We further demonstrate that TPE-Tyr probe can be configured into a commercial ELISA kit for human CEA sensing, serving as a novel fluorogenic enzymatic substrate for HRP and offering superior storage and emission stability and improved detection limit. Overall, the conceptual integration of enzymatic reactions with AIE processes represent a general and potent strategy toward the construction of novel types of fluorogenic biosensors with optimized sensitivity/selectivity and rapid detection features.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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