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Ultra-Sensitive Hydrogen Peroxide Sensor Based on Peroxiredoxin and Fluorescence Resonance Energy Transfer

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Abstract: In this paper, a fluorescence resonance energy transfer (FRET)-based sensor for ultra-sensitive detection of H₂O₂ was developed by utilizing the unique enzymatic properties of peroxiredoxin (Prx) to H₂O₂. Cyan and yellow fluorescent protein (CFP and YFP) were fused to Prx and mutant thioredoxin (mTrx), respectively. In the presence of H₂O₂, Prx was oxidized into covalent homodimer through disulfide bonds, which were further reduced by mTrx to form a stable mixed disulfide bond intermediate between CFP-Prx and mTrx-YFP, inducing FRET. A linear quantification range of 10–320 nM was obtained according to the applied protein concentrations and the detection limit (LOD) was determined to be as low as 4 nM. By the assistance of glucose oxidase to transform glucose into H₂O₂, the CFP-Prx/mTrx-YFP system (CPmTY) was further exploited for the detection of glucose in real sample with good performance, suggesting this CPmTY protein sensor is highly practical.

Keywords: hydrogen peroxide; biosensors; peroxiredoxin; thioredoxin

1. Introduction

Hydrogen peroxide (H₂O₂) is a strong oxidant, which is widely used in bleaching agents [1,2] and disinfectant [3,4]. In vivo, it is a main component of reactive oxygen species (ROS), which has long been considered to be harmful to cells [5] and involved in the development of many diseases [6,7]. Nonetheless, it is also found to participate in many signaling pathways [8,9] and to help defend against microbe infection [10,11] and abiotic stress [12]. Moreover, it is a by-product of many enzymatic reactions [13,14], with glucose oxidase (GOX) as a typical example [15,16]. The unique and significant role of H₂O₂ attracts great research interest in biosensor development because one can know the amount of specific enzyme substrate indirectly by measuring its quantity. Therefore, the detection of H₂O₂ is of practical significance.

At this moment, quite a lot of methods exist to detect H₂O₂, and they can be classified into four categories: colorimetric [17], spectrophotometry [18], electrochemistry [19], and fluorescence [20]. Among these, fluorescence-based methods have shown advantages like high sensitivity, fast response, and ability to fulfill in situ measurement in organelles within the cell. Besides fluorescent small molecules [21] and various fluorescent nano materials [22], fluorescent protein (FP) is another frequently utilized constituent in H₂O₂ biosensors.

Many proteins in cell recognize H_2O_2 with high selectivity and sensitivity by the advantage of specific activity of biological enzymes towards their substrate. One can easily fuse these recognition elements to FPs through regular genetic manipulations to construct H_2O_2 probes. HyPer [23,24] and roGFP2-Orp1 [25,26] are two prototypes of this kind, which involve a single FP to measure the excitation ratio, and both are frequently exploited in H_2O_2 -related biochemical research. Enyedi et al. [27] developed two fluorescence resonance energy transfer (FRET)-based H_2O_2 probes, which were different from Hyper and roGFP2-Orp1, involving two FPs and measuring emission ratio other than excitation. Compared to single FP, FRET-based protein sensors are easier to construct, and it is unnecessary to optimize the insertion site of response element into FP, and their ratiometric readouts can eliminate background interference. To the best of our knowledge, these are the only two pioneers in this type, therefore we would like to explore more. Peroxiredoxin (Prx) is a ubiquitous antioxidant protein [28]. As a member of the Prx family, Prx2 reacts with H_2O_2 to form two intermolecular disulfide bonds in a homodimer, which can be reduced by thioredoxin/thioredoxin reductase (Trx/TrxR) system [29]. Both Prx and Trx were fused to a single FP to construct two efficient genetic redox probes, roGFP2-Prx [30] and TrxRFP [31], respectively. It was reported that transient mixed disulfide bond existed between Prx and Trx during the PRx reduction process [32], as verified by the observation of stable intermediate of mixed disulfide dimer between Trx active-site cysteine mutant (mTrx) and Prx [33,34]. This stable intermediate inspired us to construct a Prx/mTrx and FRET-based H_2O_2 probe. In fact, at the same time as conducting this study, we also employed this Prx/mTrx combination to develop a cpYFP-based H_2O_2 sensor and have recently proved its feasibility [35].

In the present study, we chose cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) as the energy donor and acceptor, respectively, to construct two fusion proteins of CFP-Prx and mTrx-YFP. After the addition of H_2O_2 to the mixture of these two fusion proteins (CPmTY), the FRET ratio increased immediately as a result of the CFP-Prx/mTrx-YFP heterodimer formation through a mixed disulfide bond, showing the feasibility of CPmTY as a H_2O_2 probe. CPmTY exhibited high sensitivity and good selectivity towards H_2O_2 . The maximum emission ratio increase is much greater than precedent ones [27] and thus much more sensitive. Further research also indicates good performance of CPmTY in glucose detection by the transformation of glucose into H_2O_2 with GOX.

2. Materials and Methods

2.1. Reagents

GOX (from *Aspergillus niger*) and dithiothreitol (DTT) were purchased from Aladdin Industrial Corporation. Hydrogen peroxide (35 wt% solution in water, stabilized) was bought from Acros Organics. Glucose and other reagents supplied by Sinopharm Chemical Reagent co. Ltd. are of analytical grade and used without further purification. Tert-butyl hydroperoxide (t-BOOH), cystine, oxidized glutathione (GSSG), xanthine, xanthine oxidase and 3-morpholinocarbonyl-L-proline (SIN-1) were all obtained from Sigma-Aldrich.

2.2. Protein Expression and Purification

Trx (NCBI ACCESSION: XM_652682.1) and Prx (NCBI ACCESSION: XM_676869.1) utilized in this study were both from *Aspergillus nidulans*. Cysteine to serine mutation (C39S) in Trx was incorporated by site-directed mutagenesis according to the instruction manual of QuikChange™ Site-Directed Mutagenesis Kit from Stratagene, and the PCR kit of Premix PrimeSTAR HS (Takara, Code No.: R040A) was used in the experiment. CFP (mTurquoise2) was cloned into pET-28a (+) plasmid and the intermediate plasmid P1 obtained. Then, Prx was cloned into P1 at the 3' end of CFP by restriction enzyme digestion and ligation with linker sequence between them and the final expression plasmid P2 (for chimeric protein CFP-Prx) was obtained. YFP (mNeonGreen) and mTrx (TrxC39S) were cloned into pET-28a (+) by similar method to get expression plasmid P4 (for chimeric protein mTRX-YFP). Expression plasmid (P2 or P4) was transformed in to competent *E. coli* BL21 (DE3) through heat shock at 42 °C for 90 s, and protein synthesis was induced with 0.1 mM isopropyl

thiogalactoside (IPTG) at 30 °C. Protein purification was conducted by affinity chromatography with Ni-NTA agarose according to the QIAexpressionist™ handbook (QIAGEN, Cat No.: 30210).

2.3. SDS-PAGE

Protein was analyzed by nonreducing 12% SDS-PAGE and gels were made up in our own laboratory and stained by Coomassie brilliant blue. CFP-PRX and mTrx-YFP were reduced by adding DTT and passed through a desalting column pre-equilibrated with appropriate buffer. Subsequently, they were mixed together with equimolar ratio and treated with indicated concentration of H₂O₂ at 30 °C for 5 min. At last excessive amount of N-Ethylmaleimide (NEM) was added after treatments to block remaining thiol groups prior to dilution in gel-loading buffer.

2.4. Fluorescence Measurement

Emission spectra were measure on an F-4600 fluorescence spectrophotometer (HITACHI, Tokyo, Japan) in a cuvette with excitation at 400 nm and emission from 460 to 560 nm. The FRET ratio (518 nm/476 nm) was then calculated from specific emission spectrum.

2.5. Selectivity Test

Oxidized glutathione, cysteine, tert-butyl hydroperoxide (t-BOOH), superoxide radicals and peroxynitrite anion (ONOO⁻) were exploited to test the selectivity of this detection method versus H₂O₂. Superoxide radicals were generated by xanthine–xanthine oxidase system and ONOO⁻ by SIN-1.

2.6. Glucose Detection

An amount of 2 µL GOX (1 U/mL) and 10 µL glucose solution with various concentrations were mixed together. Then, 988 µL of CFP-Prx and mTrx-YFP mixture of equal molar ratio was added to GOX-glucose reaction system and the FRET ratio was measured. In this approach, the relation between glucose concentration and FRET ratio was established.

3. Results and Discussion

3.1. Construction of the Proposed H₂O₂ Probe

We employed CFP and YFP to construct the proposed H₂O₂ probe, as a FRET donor and acceptor, respectively. As shown in Figure 1, CFP was linked to Prx (to form Prx-CFP) and YFP to mutant Trx (to form mTrx-YFP). Two intermolecular disulfide bonds formed in Prx homodimer in the presence of H₂O₂, and then mTrx reacted with the homodimer to generate mixed disulfide dimers, bringing the tethered CFP and YFP close enough to generate FRET signals, which in reverse could indicate H₂O₂ concentration in the medium. We named this new probe CPmTY, the abbreviation of the essential mixed disulfide dimer CFP-Prx/mTrx-YFP.

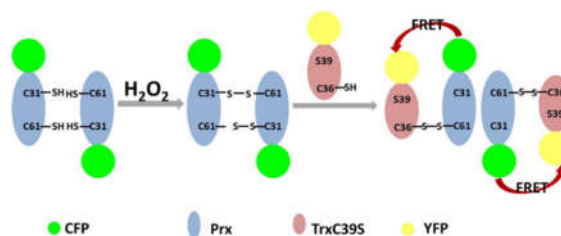


Figure 1. Schematic illustration of the working principle of proposed H₂O₂ probe CFP-Prx/mTrx-YFP. CFP—cyan fluorescent protein, Prx—peroxiredoxin, mTrx—mutant thioredoxin, YFP—yellow fluorescent protein.

3.2. FRET Signals Respond to H_2O_2 Concentration as a Result of CFP-Prx/mTrx-YFP Conjugation through Disulfide Bond

CFP-Prx and mTrx-YFP were successfully expressed and purified in this work. The fluorescence properties of both FPs were not interfered in each chimeric protein. At the same time, CFP-Prx retained the ability to react with H_2O_2 to form dimer as free Prx did. When mixing and adding H_2O_2 into the mixture, CFP-Prx/mTrx-YFP heterodimer formed as verified by SDS-PAGE (Figure 2a). If one disulfide bond in Prx dimer remained, an mTRX and a PRX-CFP dimer formed a covalent heterotrimer. Otherwise, this heterotrimer further resolved by another mTrx-YFP and decomposed into two CFP-Prx/mTrx-YFP heterodimers. Therefore, there were five kinds of entities in the mixture with the presence of H_2O_2 corresponding to the five bands in each lane of SDS-PAGE image from top to bottom: (1) CFP-Prx/CFP-Prx/mTrx-YFP heterotrimer, (2) CFP-Prx/CFP-Prx homodimer, (3) CFP-Prx/mTrx-YFP heterodimer, (4) monomer CFP-Prx, and (5) monomer mTrx-YFP, where (2) and (3) were very close to each other. Moreover, as the H_2O_2 amount increased, the monomers decreased in concentration while the trimer and dimer increased. FRET happened between CFP-Prx and mTrx-YFP in hetero dimer and trimer, as revealed in the fluorescence spectra (Figure 2b). Each spectrum corresponds to one lane in the SDS-PAGE image and more hetero dimer and trimer lead to higher FRET signals (decrease of CFP fluorescence and increase of YFP's). In this way, the H_2O_2 concentration can be reflected by the FRET signal intensity.

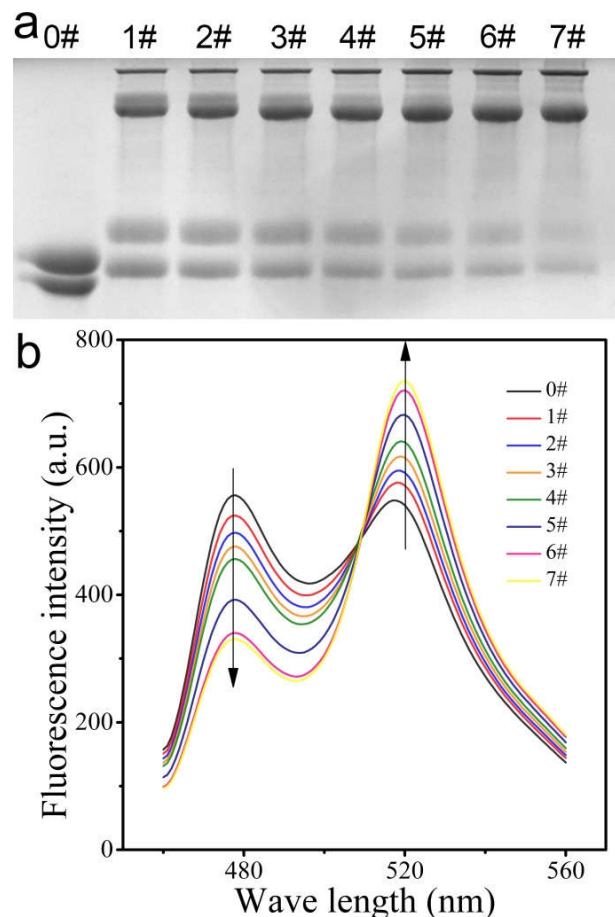


Figure 2. Demonstrations of H_2O_2 induced homo/hetero dimerization between CFP-Prx and mTrx-YFP and corresponding fluorescence resonance energy transfer (FRET) signal changes. (a) SDS-PAGE of CFP-Prx and mTrx-YFP mixtures after reacting with H_2O_2 , in 0# sample no H_2O_2 existed, from 1# to 7# sample H_2O_2 concentration increased gradually. (b) Fluorescence spectra of the same sample as indicated in panel (a).

To further prove that intermediate disulfide bonds was the cause for the FRET signal, we add DTT, which can destroy the bond, after H_2O_2 and FRET signal diminished gradually in six minutes (Figure 3a). The reaction between DTT and protein disulfide bond was rather slow compared with that between Prx and H_2O_2 , which completed in seconds. Thus we can measure the FRET signal immediately after H_2O_2 addition, which is a benefit of this detection method. We also constructed wild-type Trx fused with YFP (wtTrx-YFP), which cannot induce FRET with CFP-Prx in the presence of H_2O_2 (Figure 3b), as the resolving cysteine in wtTrx destroys the intermediate disulfide bond.

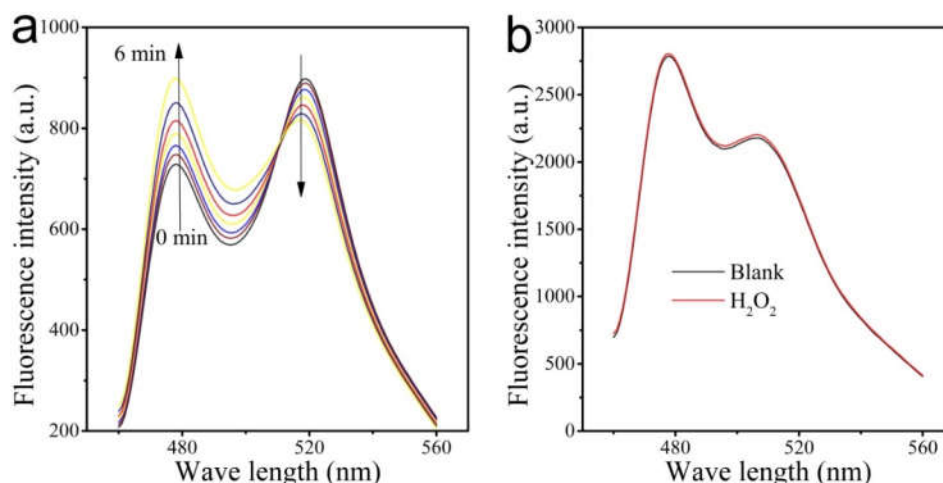


Figure 3. Confirmation of disulfide bonds formation as the mechanism of H_2O_2 induced FRET in CFP-Prx/mTrx-YFP system (CPmTY). (a) Fluorescence spectrum variation in 6 min when excess amount of DTT was added after CPmTY reaction with and H_2O_2 . (b) Fluorescence spectra of CFP-Prx/wtTrx-YFP (wild-type Trx that retained the resolving cysteine residue, C39) mixture in the absence (black line) and presence (red line) of abundant H_2O_2 .

3.3. Ability of CPmTY to Detect H_2O_2 with Low limit and Wide Range by Optimal Protein Concentration

First, we conducted the detection experiment in 20 mM Tris-HCl (pH 8.0) with 150 mM NaCl, the same buffer as used for protein purification. In this condition, the FRET ratio was very small, even after adding an excess amount of H_2O_2 (much like Figure 3b). We then removed NaCl from the buffer and found the FRET ratio increased significantly in response to H_2O_2 (much like Figure 2b). Salt ions may bind on protein interfaces to destroy hydrophobic interactions between protein dimer and prevent proteins from getting close to each other by electrostatic repulsion. Thus, NaCl may decrease the FRET ratio by impeding CFP-Prx dimerization and separating CFP and YFP in the CFP-Prx/mTrx-YFP conjugate. Moreover, YFP used in this study is pH sensitive, and shows stronger fluorescence intensity in pH 6–8. Therefore, we finally chose to examine the detection performance of CPmTY in 1 mM phosphate buffer (pH 7.0). Based on the working principle, the detection range of this method depends on the concentration of the two chimeric proteins. Here, we set the molar ratio as 1:1. With 200 nM CFP-Prx and 200 nM mTrx-YFP, the FRET ratio of CPmTY increased linearly in the range from 0 to 80 nM of H_2O_2 . At a concentration four times of the former (i.e., 800 nM CFP-Prx and 800 nM mTrx-YFP) the linear range expanded to 120–320 nM. Based on these results, it could be concluded that H_2O_2 detection range by CPmTY varies with CFP-Prx and mTrx-YFP protein concentrations. However, the protein concentrations should neither be too high nor too low. As the concentration increased, spontaneous FRET occurred between separate CFP-Prx and mTRX-YFP, diminishing detection sensitivity (i.e., the difference between largest and smallest FRET ratio). As shown in Figure 4, the slope of the left fitting curve (i.e., 0.01) is greater than four times of the right (i.e., 0.0015). If the protein concentrations continue to increase, slope value of the fitting curve would tend to be zero, meaning that spontaneous FRET between separate CFP-Prx and mTrx-YFP equals to that within the CFP-Prx/mTrx-YFP conjugate. On the other hand, too low protein concentrations also damage detection performance, as the fluorescence intensity is similar to the background

interference, and at the same time both reaction possibilities between reduced CFP-Prx and H_2O_2 and between oxidized CFP-Prx dimer and mTrx-YFP decrease drastically.

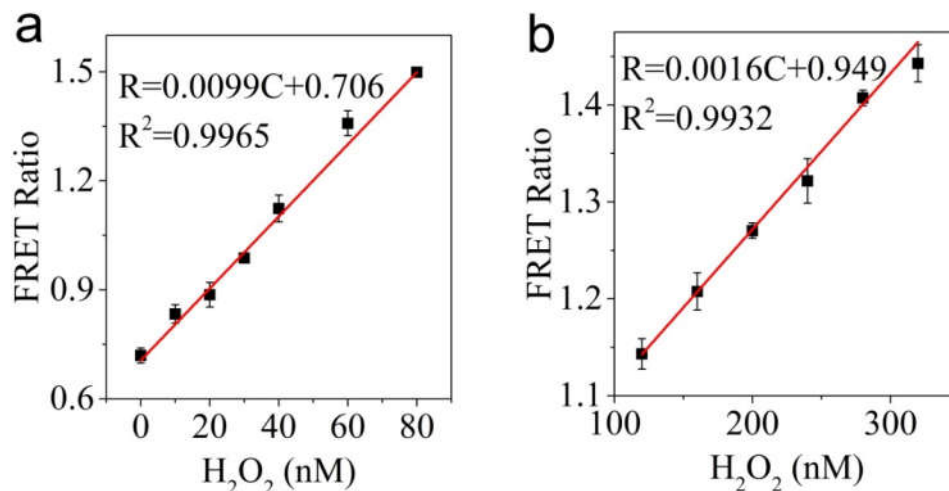


Figure 4. Titration curve of FRET in CPmTY over a serial of H_2O_2 concentrations. (a) 200 nM CFP-Prx and 200 nM mTrx-YFP with 0, 10, 20, 30, 40, 60, 80 nM H_2O_2 . (b) 800 nM CFP-Prx and 800 nM mTrx-YFP with 120, 160, 200, 240, 280, 320 nM H_2O_2 .

The optimal detection limit of CPmTY was determined to be 4 nM (calculated as $3S_b/m$, S_b is the standard deviation of 20 blank responses and m the slope of calibration curve), which was much lower than most of the present H_2O_2 detection methods (as shown in Table 1). In the general detection test, we preferred to set the protein concentrations to a relatively low level to increase detection sensitivity. Hence, when applying a low detection range, the dilution of samples with high H_2O_2 concentration can eliminate the interference of other compositions such as salts in the sample.

Table 1. Comparison of the performance of various H_2O_2 sensors.

Method	Detection Limit	Linear Range	Reference
Enzymatic colorimetric detection	2.5 μM	0.05–0.50 mM	[36]
Hybrid microflow enzymatic amperometric detection	50 μM	100 μM –100 mM	[19]
Small-molecule fluorescence detection	5.3 μM		[37]
Small-molecule fluorescence detection	0.07 μM	0.5–200 μM	[18]
Small-molecule fluorescence detection	21 nM	3–500 μM	[38]
Small-molecule fluorescence detection	25 nM	1–60 μM	[39]
Small-molecule fluorescence detection	160 nM	5–20 μM	[40]
Nanocomposite colorimetric detection	14 nM	0.01–30 μM	[17]
Nanocomposite ratiometric fluorescence Detection	112 μM	60–600 μM	[22]
Nanocomposite nonenzymatic colorimetric detection	168 nM	0.5–10 μM	[22]
Nanocomposite nonenzymatic colorimetric detection	3.87 nM	10 nM–10 mM	[41]
Nanocomposite nonenzymatic fluorescence detection	22 nM	30–300 nM	[42]
Nanocomposite nonenzymatic fluorescence detection	11 nM	30–110 nM	[42]
Nanocomposite nonenzymatic ratiometric fluorescence detection	10 nM	0.025–5.0 μM	[43]
Nanocomposite nonenzymatic amperometric detection	1.6 μM	Up to 14 mM	[44]
	2.2 μM	Up to 15 mM	
	0.1 μM	Up to 20 mM	
Nanocomposite nonenzymatic amperometric detection	0.5 μM	0.002–1.0 mM	[45]

Nanocomposite nonenzymatic amperometric detection	1 μ M	10 μ M–15 mM	[46]
Nanocomposite nonenzymatic amperometric detection	2 nM	12.64 nM–2104 μ M	[47]
Nanocomposite nonenzymatic photoelectrochemical detection	1.2 μ M	5–250 μ M	[48]
CFP-Prx/mTrx-YFP ratiometric fluorescence Detection	4 nM	10–320 nM	This study

3.4. Detection Selectivity

The detection of H_2O_2 in cells or other biological samples is frequently interfered with other oxidants, so the performance of CPmTY was tested in the presence of potential interfering species. The results were shown in Figure 5. CPmTY did not react with most of them (superoxide anion, cysteine, oxidized glutathione, and peroxyxynitrite) and showed excellent selectivity to H_2O_2 . The only exception is T-BOOTH, which is synthetic and does not exist in real samples. This selectivity may be ascribed to the high specificity of Prx to H_2O_2 .

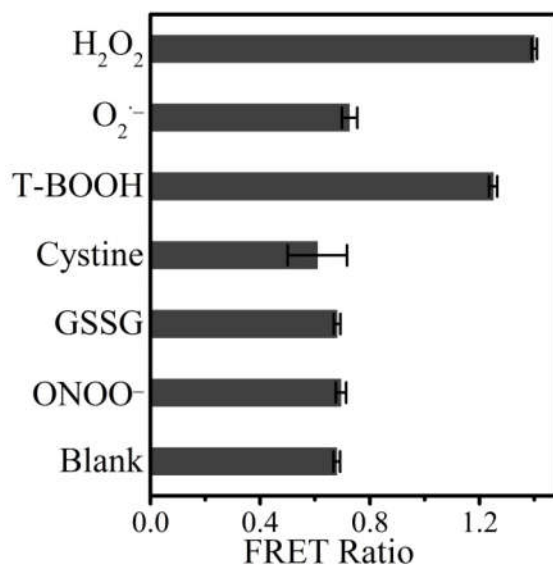


Figure 5. Interference of common oxidant on FRET signal of CPmTY. Concentration of oxidized glutathione (GSSG), cysteine, tert-butyl hydroperoxide (t-BOOH): 1 μ M, H_2O_2 : 100 nM, superoxide radicals (O_2^-) and peroxyxynitrite anion (ONOO^-) were produced in situ by specific enzymatic reactions as described in experimental section.

3.5. Detection of Glucose

Detection of glucose involves glucose oxidation by glucose oxidase to generate H_2O_2 and the ensuing H_2O_2 measurement. First, the quantitative relationship between glucose concentration and the FRET ratio was calibrated. The glucose solution was diluted to a serial of concentrations (0, 2, 4, 6, 8, 10, 12 μ M), and reacted with GOX. Subsequently, CFP-Prx and mTrx-YFP mixture, both 150 nM, was added to the glucose oxidation system and fluorescence spectra were measured. The glucose oxidation was conducted in a small volume in order to obtain relatively higher enzyme and substrate concentration for fast and complete reaction. Figure 6 showed the corresponding fluorescence spectrum from each measurement, labeled by the glucose concentration. The FRET ratio (518 nm/476 nm) was calculated and plotted versus glucose concentration as a calibration curve (inset in Figure 6). Finally, the calibration equation of $R = 0.0039C + 0.822$ ($R^2 = 0.9809$) was obtained for the glucose analysis. Herein, R is the FRET ratio (518 nm/476 nm), R^2 is correlation coefficient and C is the concentration of glucose (nM).

To verify the applicability of CPmTY for glucose detection, Dulbecco's modified eagle medium (DMEM, Corning Cat. No. 10-013) was tested with labeled glucose concentration of 4500 mg/L, that is, 25 mM. The result was 24.58 mM, indicating a recovery of $98.32 \pm 0.47\%$ and confirming the applicability of CPmTY.

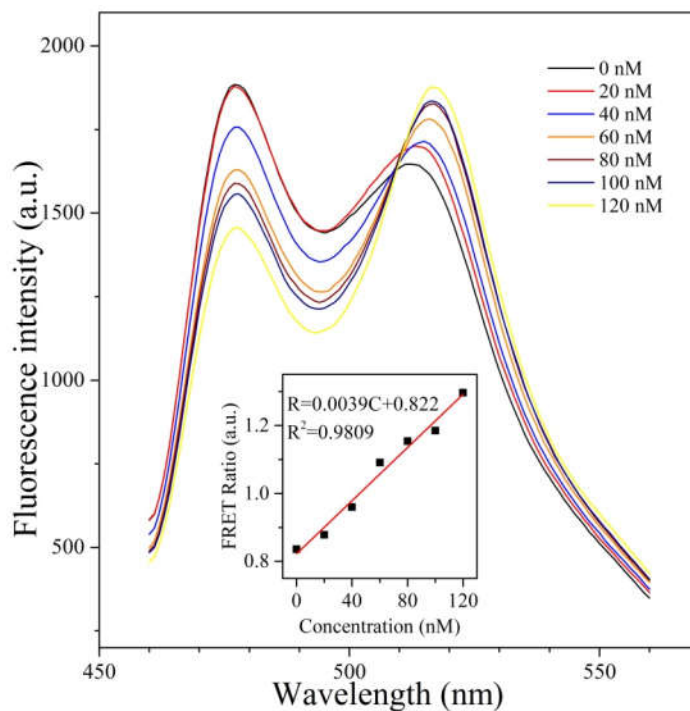


Figure 6. Detection of glucose with CPmTY. A series of glucose solution reacted with glucose oxidase (GOX) and then the reaction products were added to 300 nM CFP-Prx and 300 nM mTrx-YFP mixtures, glucose concentrations indicated were the final ones in the detection mixtures.

4. Conclusions

In this work, we developed a mild, sensitive, and fast detection method for H_2O_2 by the distinctive reaction among H_2O_2 , Prx, and mTrx, which can bring tethered CFP and YFP together to induce FRET. The detection limit was determined to be as low as 4 nM. This sensitive method shows satisfactory selectivity toward H_2O_2 over other interfering oxidants. Moreover, this method can be applied to detect glucose content in DMEM with the aid of GOX and the result is very close to the labeled data. As there are numerous H_2O_2 reacting proteins, such as Prx and H_2O_2 transforming reactions, like GOX in nature, this study provides a novel idea to develop biosensors for H_2O_2 and substances that can be transformed to H_2O_2 by suitable enzymes.

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Conflicts of Interest: The authors declare no conflict of interest.

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