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Quantum dot-based FRET for sensitive determination of hydrogen peroxide and glucose using tyramide reaction

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ABSTRACT

In this paper, we report a new strategy for detection of hydrogen peroxide and glucose using quantum dot (QD)-based fluorescence resonance energy transfer (FRET) and tyramide reaction. The principle of FRET is based on highly sensitive reaction of a carbocyanine dye (Cy5) labeled tyramide and hydrogen peroxide catalyzed by horseradish peroxidase (HRP), and the fluorescence spectrum of QDs (EX_{max} 605 nm) partially overlaps with the absorption bands of Cy5. We firstly conjugated HRP to QDs, and then demonstrated an efficient FRET between HRP conjugated QDs (as energy donors) and tyramide labeled Cy5 (as energy acceptors) due to the formation of Cy5-labeled HRP–QDs assemblies in the presence of H_2O_2. We observed that the fluorescence Cy5 depended linearly on the H_2O_2 concentration within a range of concentration from 10 to 100 nM and the detection limit of this assay was 10 nM. Based on the principle for determination of H_2O_2, we develop a new strategy for assay of glucose by coupling with glucose oxidase-mediated reaction. The established methods were successfully used for determination of glucose levels in human sera, and the results obtained were in good agreement with commercially available method. Our method is at least 1 order of magnitude more sensitive than in the commercially available method. More importantly, our method described here can be extended to other assay designs using different oxidase enzymes, energy donors and energy acceptors, such as near-infrared (NIR)-to-visible upconversion nanoparticles and silicon and carbon QDs.

1. Introduction

Recently, hydrogen peroxide (H_2O_2) has attracted growing attentions in biochemical fields owing to its important role in biological systems. H_2O_2 is an important intermediate product of pathological processes in various diseases, such as cancer, cardiovascular disorders, and Alzheimer's disease [1]. It is of great importance to develop a simple and highly sensitive method for the determination of H_2O_2. Many methods are now available for the assay of H_2O_2 in biological samples including spectrophotometry, fluorometry, chemiluminescence and electrochemistry [2–5]. Among these methods, certain new probes [5–8] and nanoparticles [9–11] have been widely used for the assay of H_2O_2 because of their simplicity and high sensitivity. Fluorescence resonance energy transfer (FRET)-based approaches for the ratiometric fluorescence detections of H_2O_2 have been reported recently [12–14], while the synthesis of ratiometric fluorescence reporter is needed and the detection limit is usually about μmol/L level [13].

FRET is a powerful technique for probing very small changes in the distance between donor and acceptor fluorophores. In recent years, QDs have been favorably adopted in the FRET-based studies due to large Stokes shift, high quantum yield, good photo stability, and size-dependent emission-wavelength tunability. QDs are widely applied to FRET for probing DNA replication and telomerization [15,16], pH and ion sensing [17,18], photochromic switching [19], photodynamic medical therapy [20,21], sensing enzymatic activity [22,23], and single molecule fluorescence energy transfer [24].

Recently, we reported a new strategy for highly sensitive determination of H_2O_2 based on FRET using gold nanoparticles and tyramide reaction [25]. In the AuNPs-based FRET modes, only the FL emission of the donors appeared on the spectra. Since the acceptors have no emission, applications of these methods were limited in monitoring molecular interactions and measuring the conformational changes of biomolecules. In this study, we present a new FRET system for the high sensitive assay of H_2O_2 and glucose, and in this case the emissions of energy donors and acceptors both appeared on the spectra. The 605 QDs were used as fluorescent donors and tyramide labeled Cy5 were used as acceptors. Usually, tyramide labeled with fluorescent probe is utilized as reporter fluorescent substrate for HRP-catalyzed deposition that is signal amplification

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technique in immunoassay [26,27] and in situ hybridization of nucleic acids [28,29]. In the labeling process, tyramide generates 2, 2'-dihydroxydiphenyl derivatives via tyramide radical in the HRP-catalyzed oxidation with H_2O_2 at the high concentration of tyramide. However, in the presence of lower tyramide concentration, tyramide radical binds to the electron-rich moieties of protein such as a tyrosine residue, to generate tyramide-labeled protein [30]. Upon excitation with a wavelength of 488 nm, FRET occurred between 605 QDs and Cy5 in the assemblies due to the tyramide reaction. The fluorescence signals of 605QD and Cy5 were observed simultaneously. The 605 QD is an excellent energy donor with Cy5 for several reasons: no cross-talk between the emission spectra of 605 QD and that of Cy5, no direct excitation of Cy5 at the wavelength of 488 nm and multiple Cy5 efficiently coupling to a single 605QD, and this couple had been used in many FRET-based studies [24,31–34].

Under the optimized conditions, the fluorescence signal of Cy5 depended linearly on the H_2O_2 concentration within a range from 10 to 100 nM and the detection limit of this assay was 10 nM. In addition, H_2O_2 is generated in the oxidation reaction catalyzed by almost all oxidases. So the activity of oxidases, or the enzyme substrates, such as glucose, lactate, glutamate, urate, xanthine, choline, cholesterol and NADPH can be quantitatively assayed by the determination of the H_2O_2 concentration produced in the system. By coupling with glucose oxidase-mediated reaction, the present method was applicable to selective assay of glucose. The method was successfully used for determination of glucose levels in human sera, and the results obtained were in good agreement with commercially available methods.

2. Materials and methods

2.1. Chemicals and materials

QDs were obtained from Invitrogen (USA). Tyramide signal amplification (TSA) Plus Cy5 Kit was obtained from Perkin–Elmer (USA). Glucose was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glucose oxidase (GOx) was from BBI (UK). HRP, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and all other reagents were acquired from Sigma–Aldrich Chemical Co. (Milwaukee, USA). A glucose assay kit (hexokinase method) was purchased from Shanghai Kehua Bio-engineering Co., Ltd. (China). Ultra-pure water (18.2 M Ω) was obtained from the Millipore Simplicity System (Millipore, Bedford, MA, USA).

2.2. Preparation of QDs-HRP conjugates

The 48 nmol EDC (2000 equivalents) and 48 nmol NHS (2000 equivalents) were added into a mixture of 24 pmol of 605 nm quantum dots and 480 pmol of HRP (20 equivalents) in 30 μl phosphate buffer (20 mM, pH 6.0), and the mixture above was incubated at room temperature for 2 h. And then, bovine serum albumin (BSA) solution was added to 1 mg/mL as a blocking agent, and the reaction was continued for another 1 h at room temperature. The QD–HRP conjugates were purified by three washes using an ultra-filtration membrane (Micoron YM-100-100,000 NMWL, Millipore, USA) according to the instructions from the manufacture. Finally the purified products were kept in borate buffer at 4 °C.

A P/ACE MDQ capillary electrophoresis (CE) system (Beckman Colter Inc., Fullerton, CA, USA) was applied to characterize the QDs conjugates. $Na_2B_4O_7$ solution (25 mM, pH 8.0) was used as running buffer.

2.3. Assay of H_2O_2

The FRET-based assay for H_2O_2 was conducted by the following procedure. Typically, the reaction solution consisted of 900 nM tyramide-Cy5, 20 nM HRP–QD conjugates in 100 mM Tris buffer (pH 7.5). To 180 μL of the reaction solution, 20 μL of various concentrations of H_2O_2 solution (0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, and 4.0 μM) was added and incubated at room temperature for 10 min. The resultant mixture was poured into a $0.2 \times 1.0\ cm^2$ quartz cell and fluorescence spectra were measured as a function of the concentration of H_2O_2. FL spectra were recorded with an F-380 spectrometer (Tianjin Gangdong SCI & Tech. Development Co., Ltd., China). Control experiments were performed to confirm whether the FRET process occurred, which included incubation with no HRP–QD conjugates and replacing HRP–QD conjugates with BSA–QD conjugates at the same concentration. For each concentration of the H_2O_2 solution, the measurements were performed three times. Lifetime measurements were carried out using a QM/TM/IM fluorescence lifetime and steady state spectrometer (Photo Technology International, USA). Briefly, the reaction solution consisted of 900 nM tyramide-Cy5, 20 nM QD–HRP conjugates in 100 mM Tris buffer (pH 7.5) without or with 200 nM of H_2O_2 and it was incubated at room temperature for 10 min. Then the resultant mixture was poured into a $0.2 \times 1.0\ cm^2$ quartz cell and the nanosecond PL decay profiles were measured by the QM/TM/IM fluorescence lifetime and steady state spectrometer.

2.4. Assay of glucose

The reaction solution consisted of 20 nM QD–HRP conjugates, 900 nM tyramide-Cy5, and 5 U/mL glucose oxidase in 100 mM Tris buffer (pH 7.5). To 180 μL of the reaction solution, 20 μL of various concentrations of glucose solution (0, 0.25, 0.5, 1.25, 2.5, 3.75, 5.0, 10.0 and 20.0 μM) was added and incubated at 37 °C for 5 min. FL spectra were recorded with an F-380 spectrometer. For each concentration of the glucose solution, the measurements were performed three times.

2.5. Determination of glucose in human serum samples

The reaction solution was same as that described in assay of glucose. Five human sera from healthy subjects were provided by Shanghai Jiaotong University Affiliated Hospital. The serum solutions were prepared by dilution with distilled water by a factor of 3000 for glucose detection. The reaction solution (180 μL) consisting of 20 nM QD–HRP conjugates, 900 nM tyramide-Cy5, and 5 U/mL glucose oxidase in 100 mM Tris buffer (pH 7.5) and the diluted serum solution (20 μL) were mixed and incubated at 37 °C for 5 min. Fluorescent spectra were recorded with an F-380 spectrometer, and the assay of each sample was repeated three times.

2.6. Colorimetric determination of glucose using commercially available kits

As a commercially available method for comparison with the present method, we used the glucose (hexokinase method) assay kit for measurements of glucose in serum. The measurements were performed according to the manufacturer's manual. We measured the absorbance of 340 nm for glucose using a UV/Vis-3501 spectrophotometer.

3. Results and discussion

3.1. Principle of detection for H_2O_2

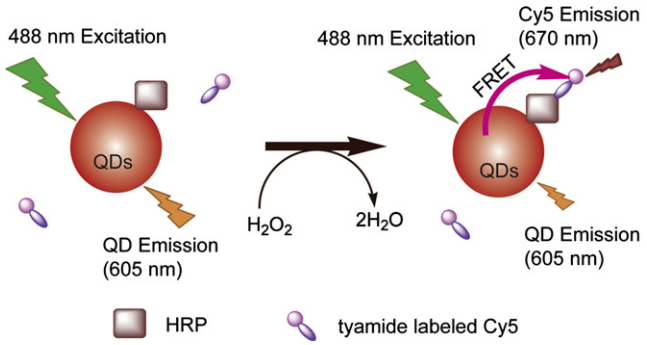
In this study, the design for determination of H_2O_2 is based on FRET principle. As shown in Scheme 1, in the design of FRET system, we choose HRP to catalyze the deposition of a tyramide labeled Cy5 amplification reagent onto QD surfaces that have been previously conjugated with HRP. This reaction is quick (usually 3–7 min) and results in the deposition of numerous Cy5 labels immediately adjacent to the immobilized HRP enzyme. Because the added labels are deposited proximal to the initial immobilized HRP enzyme site, there is minimal loss in resolution. And more importantly, the absorption spectrum of Cy5 partly overlaps with the emission spectrum of 605 QD. Fig. 1 shows the absorption and emission spectra of 605 QD and tyramide labeled Cy 5. Fig. 1a and Fig. 1b are the absorption and emission spectra of 605 QD, respectively. The absorption spectrum (UV) of Cy5 is Fig. 1c and the emission spectrum (PL) of Cy5 is Fig. 1d. The selection of 605 QD/Cy5 as a FRET pair permits only minimal spectral cross-talk. The broad absorption spectrum of the QD allows excitation at 488 nm, which is near the minimum of the absorption spectrum of Cy5, thereby nearly eliminating direct acceptor excitation.

3.2. Determination of H_2O_2 concentrations

Recently, we systematically investigated the conjugation of quantum dots (QDs) with certain biomolecules such as HRP using CE and found that the pIs of bio-macromolecules played an important role in the conjugation reaction [35]. In this study, the 655 QD–HRP bioconjugates were characterized by CE with laser-induced fluorescent detector because there is not matched bandpass filter for 605 QD on our CE system, which may be helpful for application of 605 QD–HRP conjugates.

We examined that the reaction completed within 1 min (data not shown). The incubation time of 10 min was used in this study. The number of assemblies formed depends on the concentrations of HRP–QDs, tyrimade-Cy5, and H_2O_2 and on the tyramide radicals binding affinity to electron-rich moieties of QDs–conjugated HRP. Thus, reducing the concentration of HRP–conjugated QDs leads to a more sensitive assay. In practice, the minimum concentration of HRP–conjugated QDs may be limited by the photodetectors or the fluorescence background of the sample. Up to a certain value, increasing the concentrations of HRP–QD conjugates leads to broaden the dynamic range of the assay. Therefore, the concentrations of HRP–QDs were studied systematically in order to build optimal conditions for the FRET detection of H_2O_2. Fig. 2C shows the relationship between the concentration of HRP–QDs and ratio of the Cy5 fluorescence intensity at 670 nm and the QDs fluorescence intensity at 605 nm. The ratio increased when the concentration of HRP–QDs increased from 1.0×10^{-8} M to 2.0×10^{-8} M, and then decreased when the concentration of HRP–QDs increased from 2.0×10^{-8} M to 5.0×10^{-8} M. This might be contributed to the number of Cy5 coupling to a single 605QD decreasing along with increase of the concentration of HRP–QDs. The FRET efficiency is the highest when concentration of HRP–QDs is 2.0×10^{-8} M. Hence this concentration was used in further experiments.

In the tyramide reaction process, in the presence of lower tyramide concentrations, tyramide radical binds to the electron-rich moieties of protein such as a tyrosine residue, to generate tyramide-labeled protein [30]. However, tyramide generates 2, 2'-dihydroxydiphenyl derivatives via tyramide radical in the HRP-catalyzed oxidation with H_2O_2 when it is at high concentrations. So the concentrations of Cy5-tyramide were studied systematically in order to establish optimal conditions for the FRET detection of H_2O_2. Fig. 3 shows the relationship between the concentration of Cy5-tyramide and the Cy5 fluorescence intensity at 670 nm. The Cy5 fluorescence intensity increased when the concentration of Cy5-tyramide increased from 5.0×10^{-8} to 9.0×10^{-7} M and the Cy5 fluorescence intensity reached highest when 9.0×10^{-7} M of Cy5-tyramide was used. Then the Cy5



Scheme 1. Schematic illustration for the FRET process between HRP–605QD (donor) and tyramide labeled Cy5 (acceptor).

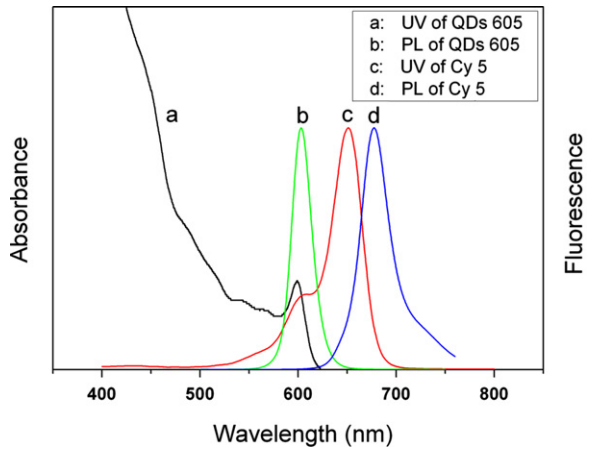


Fig. 1. The normalized UV–vis absorption and emission spectra of 605QD and tyramide labeled Cy5.

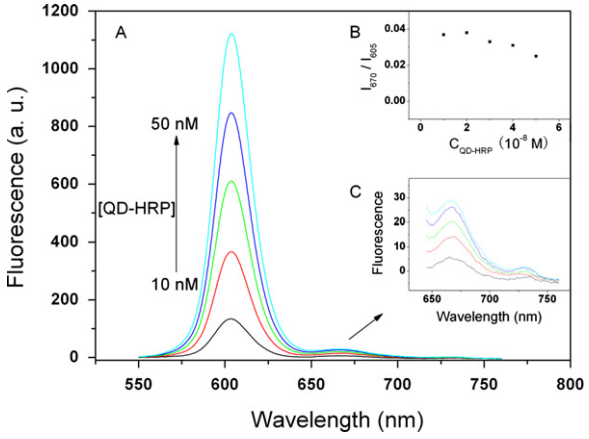


Fig. 2. (A) Fluorescence spectra of the 605 QDs and Cy5 based FRET system at different concentrations of 605 QD–HRP conjugates. The 100 mM Tris buffer (pH 7.5) solutions included 900 nM Cy5-tyramide and 25 nM H_2O_2. (B) The amplificatory fluorescence spectra of the Cy5 based FRET system at different concentrations of 605 QD–HRP conjugates. (C) The relationship between the concentration of 605 QD–HRP conjugates and ratio of the Cy5 fluorescence intensity at 670 nm and the QDs fluorescence intensity at 605 nm (I_{670}/ I_{605}).

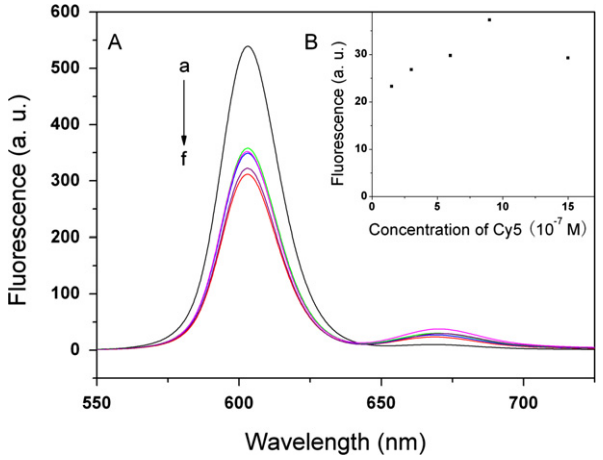


Fig. 3. (A) Fluorescence spectra of the 605 QDs and Cy5 based FRET system at different concentrations of Cy5-tyramide. The 100 mM Tris buffer (pH 7.5) solutions included 20 nM 605 QD–HRP, without (a) or with 100 nM H_2O_2 (b, c, d, e and f). (B) The relationship between the concentration of Cy5 and the Cy5 fluorescence intensity at 670 nm.

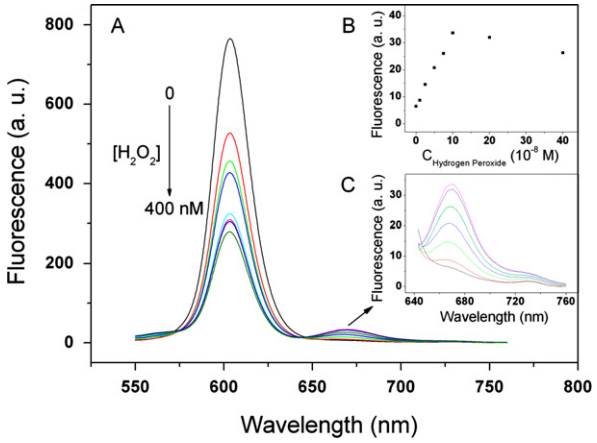


Fig. 5. (A) Fluorescence spectra of the 605 QDs and Cy5 based FRET system at different concentrations of H_2O_2. (B) The amplificatory fluorescence spectra of the Cy5 based FRET system at different concentrations of H_2O_2. (C) The relationship between the concentration of H_2O_2 and the Cy5 fluorescence intensity at 670 nm. The reaction solution consisted of 900 nM tyramide-Cy5, 20 nM HRP-QD and various concentrations of H_2O_2 in 100 mM Tris buffer (*pH* 7.5).

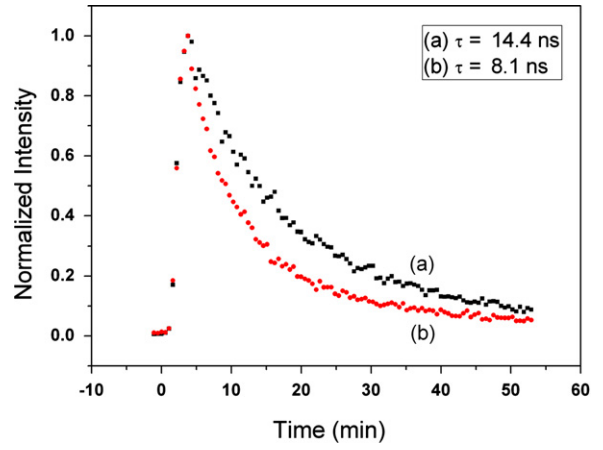


Fig. 4. Fluorescence lifetime decay curves and τ-values of the QD emission (605 nm, excitation at 488 nm). (a) 20 nM QD-HRP, (b) 20 nM QD-HRP and 200 nM H_2O_2 added. Both solutions included 900 nM Cy5-labeled tyramide.

fluorescence intensity decreased when increased the concentration of Cy5-tyramide further. This is mainly attributed to the formation of 2, 2'-dihydroxydiphenyl derivatives via tyramide radical when it is at high concentrations [30] instead of the formation of QD–HRP–tyramide-Cy5 assemblies. Therefore, the concentration of 9.0×10^{-7} M for Cy5-tyramide was used in further experiments.

We characterized the change in the excited-state lifetime for donors caused by the energy-transfer process. Fig. 4 shows the nanosecond PL decay profiles of 605 QDs prepared without or with H_2O_2. The average lifetime of 605 QDs decreased from 14.4 ns to 8.1 ns when the concentration of H_2O_2 increased from 0 nM to 200 nM. The decrease in PL lifetime is mainly attributed to its linking with Cy5 through the tyramide reaction and following FRET between 605 QDs and Cy5.

On the basis of the principle of FRET-based sensing, the amount of H_2O_2 was detected by employing the FRET system consisting of HRP–conjugated 605 QDs (donor), and tyramide-Cy5 (acceptor). As shown in Fig. 5, the Cy5 fluorescence intensity is increased gradually with the increasing amount of H_2O_2. The fluorescence increasing is related to the concentration of H_2O_2 because when more H_2O_2 is added, more acceptors (Cy5) will be combined with the donors (QD–HRP–tyramide-Cy5 conjugates) to reach a spacing suitable for FRET to occur, which will in turn cause the transfer of more energy from the donor to the acceptor and the more increasing of Cy5 fluorescence intensity (Scheme 1). As shown in Fig. 5, a linear relation between H_2O_2 concentration (X) and the Cy5 fluorescence intensity (Y) was obtained in the range of 10–100 nM H_2O_2. The linear regression equation is described as $Y=6.96+2.66X$, and the correlation coefficient (R) is 0.996. The relationships between the H_2O_2 concentration and the 605 QD fluorescence intensity or I_{670}/I_{605} are shown in Fig. S2 and S3. The linear range of H_2O_2 is 1.0×10^{-8} M -1.0×10^{-7} M ($Y=536.05\ -24.47X$, $R=0.972$) when used the 605QD fluorescence intensity as Y-axis and is 1.0×10^{-8} M -1.0×10^{-7} M ($Y=0.0043+0.0102X$, $R=0.994$) when used I_{670}/I_{605} as Y-axis. Since the correlation coefficient of the relation between H_2O_2 concentration and the Cy5 fluorescence intensity is better, it is used in the following research.

The relative standard deviation (RSD) of this method is 3.2% (obtained from a series of nine standard samples with each containing 1.0×10^{-7} M of H_2O_2). These results demonstrate that the FRET system consisting of HRP–biofunctionalized QDs and tyramide-Cy5 is a promising approach for highly sensitive detection of H_2O_2.

3.3. Determination of glucose concentrations

It is well known that the determination of β-D-glucose is very important in food analysis, and in the monitoring of bioreactor processes as well as in clinical diagnosis and treatment of diabetes [36]. Although various methods have been reported for the determination of glucose, enzymatic methods using GOx are widely used due to their simplicity and selectivity. Therefore, we attempted to develop a highly sensitive method for the determination of glucose by coupling our QDs-based FERT method with GOx-catalyzed oxidation of glucose, and the principle of this assay is described in Fig. 6. Fig. 6 shows binding between tyramide labeled with Cy5 and HRP–QD conjugates by H_2O_2. Tyramide radical in the HRP-catalyzed oxidation with H_2O_2 binds to the electron-rich moieties of protein such as a tyrosine residue, to generate tyramide-labeled HRP. Because the H_2O_2 concentrations correspond to the increase of tyramide-Cy5 fluorescence signal, we can determine the concentrations of H_2O_2 by FRET. Further, because H_2O_2 is produced by the reaction between glucose and oxygen in the presence of glucose oxidase, the concentrations of

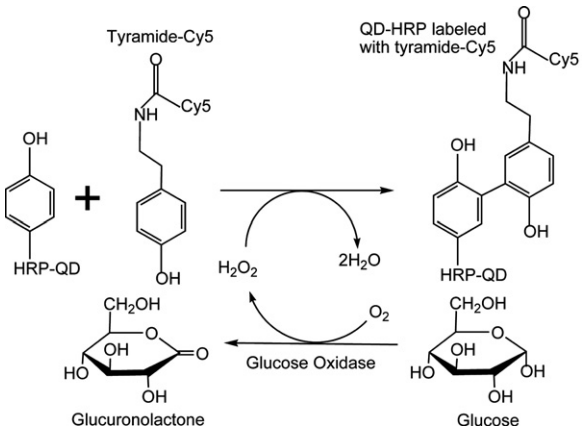


Fig. 6. Scheme for the enzymatic reaction of glucose.

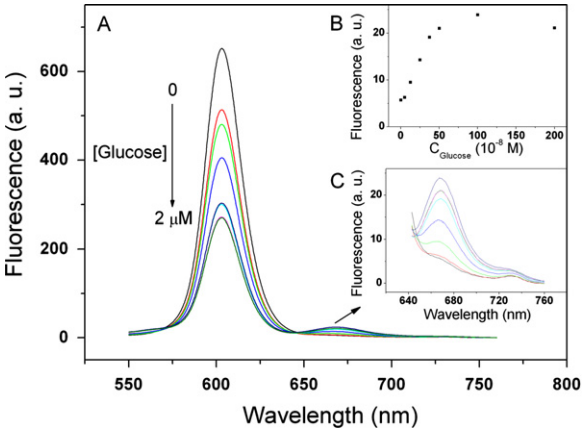


Fig. 7. (A) Fluorescence spectra of the 605 QDs and Cy5 based FRET system at different concentrations of glucose. (B) The amplificatory fluorescence spectra of Cy5 based FRET system at different concentrations of glucose. (C) The relationship between the concentration of glucose and the Cy5 fluorescence intensity at 670 nm. The reaction solution consisted of 900 nM tyramide-Cy5, 20 nM HRP-QD and 5 U/mL GOx in 100 mM Tris buffer (pH 7.5). To the reaction solution (180 μL), 20 μL of various concentrations of glucose solution was added and incubated at 37 °C for 5 min.

H_2O_2 can be determined, and thus the concentrations of glucose can be deduced. Under the optimized conditions, a linear relation between glucose concentrations and Cy5 fluorescence intensity was obtained in the range of 5.0×10^{-8}–5.0×10^{-7} M glucose with the detection limit of 5.0×10^{-8} M as shown in Fig. 7. The linear regression equation is described as $Y=5.28+0.34X$, and the correlation coefficient (R) is 0.989.

In contrast, a commercially available method using glucose/hexokinase assay kit showed a detection limit of 2.15×10^{-6} M and a range of determination of 2.15×10^{-6}–2.15×10^{-4} M ($Y=0.031X-0.005$, $R=0.999$) as shown in Fig. S4. The detection limit and the minimum value of determination for glucose in our system were at least 1 order of magnitude more sensitive than the commercially available method.

3.4. Assay of glucose concentrations in human serum

This method was applied for determination of glucose levels in human sera, which has compared with the commercially available method. The results are shown in Table 1. Our results are in good agreement with commercially available method as shown in column two and column three of Table 1. The relative standard deviations of assays are about 2.9%–9.3% as shown in column four. The recovery results further demonstrate the reliability of this method because the data are higher than 85% as shown in column seven.

Table 1
Glucose levels in serum samples measured by QD-based FRET and glucose kit.

	Glucose kit			Proposed method		
	Glucose (M)	Glucose (M)	RSD (%)	Added (M)	Found (M)	Recovery (%)
1	7.77×10^{-3}	7.22×10^{-3}	7.1	1.50×10^{-7}	3.79×10^{-7}	92
2	7.75×10^{-3}	7.12×10^{-3}	8.1	1.50×10^{-7}	3.72×10^{-7}	90
3	7.22×10^{-3}	7.01×10^{-3}	2.9	1.50×10^{-7}	3.68×10^{-7}	89
4	6.14×10^{-3}	5.57×10^{-3}	9.3	1.50×10^{-7}	3.20×10^{-7}	89
5	5.79×10^{-3}	5.46×10^{-3}	5.7	1.50×10^{-7}	3.10×10^{-7}	85

4. Conclusion

In summary, we described a new method for the determination of hydrogen peroxide and glucose based on FRET system using 605 QD as energy donor and Cy5 as energy acceptor, and demonstrated an efficient FRET between HRP conjugated 605 QDs (as energy donors) and Tyramide labeled Cy5 (as energy acceptors) based on the HRP-catalyzed deposition, tyramide radical binds to an electron-rich moieties of HRP such as a tyrosine residue. The tyramide reaction initiated the conjugation between the HRP conjugated 605 QDs and tyramide labeled Cy5 in the presence of H_2O_2, and in turn served as a bridge to make the distance between the donors and the acceptors short enough for FRET to occur. The presence of H_2O_2 in the system results in the occurrence of FRET, which enables the determination of the concentration of the H_2O_2. The analysis results suggested that the FRET system was simple and applicable for the detection of H_2O_2 with a low detection limit. Our method was successfully applied for determination of glucose levels in human sera by coupling with glucose oxidase-mediated reaction. In addition, the concepts discussed here are also applicable to other assay designs using different enzymes, energy donors and energy acceptors, such as near-infrared (NIR)-to-visible upconversion nanoparticles and silicon and carbon QDs.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.12.014.

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