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Detection of glucose via enzyme-coupling reaction based on a DT-diaphorase fluorescence probe

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ABSTRACT

Enzyme-coupling reactions play an important role in the assay of analytes. In this manuscript, we developed a new fluorescent probe for the detection of glucose through the enzyme-coupling reaction of DT-diaphorase (DTD). The probe was synthesized through a mild and simple synthetic procedure, and showed good fluorescence response to DTD. The reactions for the detection of glucose proceed as follows: glucose dehydrogenase oxidizes glucose to gluconolactone with NAD^+ as the electron acceptor to yield NADH, and NADH can be utilized by DTD to further react with the probe releasing resorufin. As a result of these tandem reactions, fluorescence off–on response will occur. The method showed high selectivity for glucose with a detection limit of 0.2 μM, which may provide a potential way for fluorescence detection of glucose through enzyme-coupling reactions. Furthermore, the applicability of the method has been demonstrated by detecting glucose in human urine samples.

1. Introduction

Diabetes mellitus, a worldwide public health problem, is a metabolic disorder caused by insulin deficiency and hyperglycemia and is reflected by blood glucose concentrations [1]. Therefore, it is of great importance to develop effective methods to monitor the level of glucose.

Untill now, various methods have been developed for the detection of glucose, which include electrochemical, optical or combination approaches [2,3]. Among these methods, the fluorescent ones currently available for glucose detection are mainly based on glucose oxidase–peroxidase–fluorescent indicators, which are carried out by detecting H_2O_2 generated through oxidation of glucose in the presence of O_2. However, this catalytic reaction is prone to interference by electron donors such as urea or bilirubin [4].

On the other hand, enzyme-coupling reactions play an important role in the assay of analytes, especially when the analyte cannot be measured directly or is difficult to be determined accurately. Thus, an alternative way for the detection of glucose is by virtue of the reductase-based enzyme-coupling reactions. Herein, we chose DT-diaphorase (DTD) as the reductase, which is coupled with glucose dehydrogenase to construct the detecting system. DTD is a cytosolic flavoprotein that catalyzes the two-electron reduction of a variety of quinones using nicotinamide adenine dinucleotide (NADH) as an electron donor [5,6]. The reactions for the detection of glucose proceed as follows: glucose dehydrogenase (GDH) oxidizes glucose to gluconolactone with NAD^+ as the electron acceptor to yield NADH, and NADH can be utilized by DTD to further react with the probe releasing resorufin and causing fluorescence off–on response. Therefore, the creation of an efficient DTD probe [7,8] is the key task for the detection of glucose via the enzyme-coupling reaction.

Till now, most of the fluorescent DTD probe use quinine–propionic acid as the recognition and trigger group [8–11], which however was synthesized via multiple time-consuming and complicated synthetic steps under harsh conditions. So it is still necessary to find an easily obtained and effective recognition group for DTD. Herein, 2-((3-oxo-3H-phenoxazin-7-yloxy)methyl)-cyclohexa-2,5-diene-1,4-dione (**1**, Scheme 1) has been developed as a DTD probe by just using a benzoquinone moiety as a new recognition moiety which is directly linked to the resorufin [12–14] through an ether linkage. Moreover, the probe can be prepared through a mild and simple synthetic procedure (for detailed characterization of probe **1**, see Fig. S1 and Fig. S2, ESI†). Reaction of **1** with DTD in the presence of NADH may cause the reduction of the quinone moiety, followed by the quinone–methide rearrangement reaction [15] and thereby the release of resorufin (Scheme 1). As a result, both color and fluorescence of resorufin would be recovered, which may provide an efficient method for the detection of glucose via the enzyme-coupling reaction.

2. Experimental

2.1. Apparatus and reagents

Fluorescence measurements were made on a Hitachi F-2500 fluorimeter (Tokyo, Japan). Fluorescence quantum yield (Φ) was

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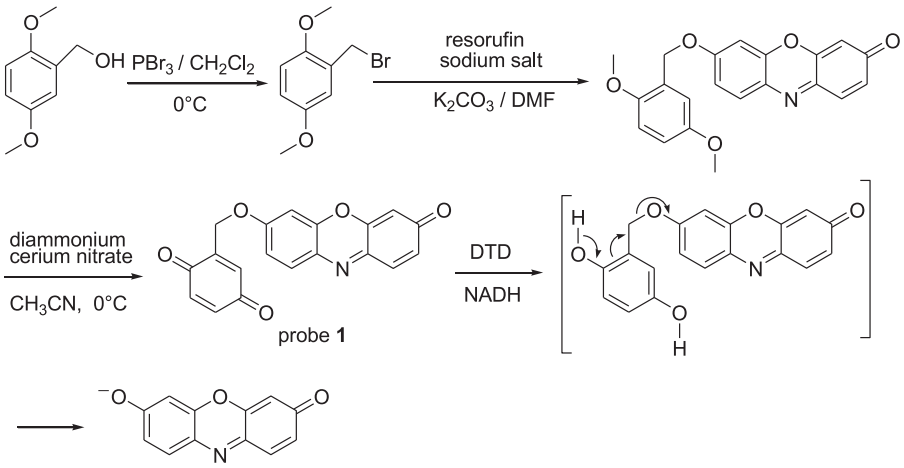


Scheme 1. Synthesis of probe 1 and its reaction with DTD.

determined by using resorufin (Φ=0.74 in water) as a standard. A model HI-98128 pH-meter (Hanna Instruments Inc., USA) was used for pH measurements. ^{1}H NMR and ^{13}C NMR spectra were measured with a Bruker DMX-400 spectrometer in CF_3COOD. HPLC analyses were carried out with LC-20AT pumps, SPD-20A UV–vis detector (Shimadzu, Japan) and Inertsil ODS-SP column (5 μm, 4.6 mm × 250 mm, GL Sciences Inc.). Electron impact time-of-flight mass spectra (EI-TOF MS) and high resolution EI-TOF mass spectra (HR-EI-TOF MS) were recorded with a GCT mass spectrometer (Micromass, Manchester, UK). Absorption spectra were recorded in 1-cm quartz cells with a TU-1900 spectrophotometer (Beijing, China).

Resorufin sodium salt, 2,5-dimethoxybenzyl alcohol, DT-diaphorase human (≥ 100 units/mg, E.C.1.6.99.2) expressed in *E. coli*, glucose dehydrogenase (≥ 200 units/mg, E.C. 1.1.1.47) from *Pseudomonas* sp., β-D-glucose, reduced nicotinamide adenine dinucleotide (NADH) and L-glutamic dehydrogenase (≥ 40 units/mg, E. C. 1.4.1.3) from bovine liver were purchased from Sigma-Aldrich. A 0.1 M phosphate buffer solution (PBS) of pH 7.4 was used. All other chemicals used were of analytical grade. Ultrapure water (over 18 MΩ cm) from a Milli-Q reference system (Millipore) was used throughout. The stock solution (1.0 mM) of probe **1** was prepared by dissolving requisite amount of it in deoxygenated DMSO, which should be used freshly.

2.2. Synthesis of probe *1*

To a solution of 2,5-dimethoxybenzyl alcohol (168.9 mg, 1.0 mmol) in CH_2Cl_2 (15 mL), phosphorus tribromide (1.5 mL) was added dropwise at 0 °C. After stirring for 2 h, the mixture was washed three times with water. Then, the organic layer was separated, dried over Na_2SO_4 and concentrated in vacuo to give 2-(bromomethyl)-1,4-dimethoxy-benzene as a white residue, which was used directly in the next step. Then, to the solution of resorufin sodium salt (224 mg, 0.95 mmol) in DMF (55 mL), 2-(bromomethyl)-1,4-dimethoxybenzene (210 mg, 0.91 mmol) and K_2CO_3 (127 mg, 0.92 mmol) were added. The reaction mixture was stirred at 0 °C for 3 h, and then diluted with CH_2Cl_2 (50 mL). The organic layer was separated, washed three times with water (50 mL × 3), and then dried over Na_2SO_4. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography eluted with petroleum ether (b.p. 60–90 °C)/ethyl acetate (v/v, 10:1), affording 7-(2,5-dimethoxybenzyloxy)-3H-phenoxazin-3-one as a brick-red solid (182 mg, yield 55.3%). A solution of diammonium cerium nitrate (153 mg, 0.28 mmol) in water (3 mL) was slowly added to the solution of 7-(2,5-dimethoxybenzyloxy)-3H- phenoxazin-3-one (36 mg, 0.1 mmol) in CH_3CN (12 mL). The resulting mixture was stirred for 3 h at 0 °C, and diluted with CH_2Cl_2 (30 mL). Then, the organic layer was separated, washed with water (30 mL × 3), dried over Na_2SO_4 and concentrated in vacuo to give an orange solid, which was purified by silica gel chromatography (CH_2Cl_2/ethyl acetate, 10/1, v/v), obtaining probe **1** as an orange solid (16 mg, yield 49.2%). ^{1}H NMR (400 MHz, CF_3COOD, 298 K): δ=8.47−8.41 (*m*, 2H), 7.77−7.72 (*t*, *J*=10 Hz, 2H), 7.65 (*s*, 1H), 7.52 (*s*, 1H), 7.22 (*s*, 1H), 7.02–6.99 (*d*, *J*=10.4 Hz, 2H), 5.38(*s*, 2H). ^{13}C NMR (400 MHz, CF_3COOD): δ 189.4, 187.2, 175.6, 170.8, 151.0, 149.7, 142.4, 139.0, 136.9, 136.5, 135.1, 132.6, 125.,6, 123.4, 102.1, 99.7, 65.3. EI-TOF MS, *m*/*z*=333 $[M]^+$. HR-EI-TOF MS, calcd for $[M]^+$: *m*/*z*=333.0637; found: *m*/*z*=333.0642.

2.3. General procedure for DTD detection

Unless otherwise noted, all the measurements were made in 0.1 M PBS (pH 7.4) according to the following procedure with an excitation wavelength (λ_{ex}) of 550 nm and an emission wavelength (λ_{em}) of 585 nm. In a 2 mL tube, 1 mL of PBS and 10 μL of 1 mM probe were mixed, followed by addition of NADH (final concentration, 100 μM) and an appropriate volume of DTD sample solution. After incubation at 37 °C for 45 min in a thermostat, the reaction solution was transferred to a quartz cell of 1 cm optical length to measure absorbance or fluorescence with $\lambda_{ex/em}$=550/585 nm and both excitation and emission slit widths of 10 nm. In the meantime, a blank solution containing no DTD (control) was prepared and measured under the same conditions for comparison.

2.4. Fluorescence response of *1* to glucose based on the DTD-GDH coupling reaction

The solutions containing probe **1** (10 μM), DTD (7.5 μg/mL), GDH (37.5 μg/mL), NAD^+ (500 μM) and the corresponding glucose (at varied concentrations) in PBS with a final volume of 1 mL were incubated for 45 min at 37 °C. The fluorescence intensity of each solution after subtracting the baseline intensity ([analytes]=0) was plotted versus the analytes concentration.

3. Results and discussion

3.1. Fluorescence properties of *1*

The spectral properties of **1** in the absence and presence of DTD and NADH was firstly examined in 0.1 M phosphate-buffered

saline (PBS) of pH 7.4. As shown in Fig. 1(A) or (B), the probe itself is almost colorless and non-fluorescent with a quantum yield [16] of $\Phi \approx 0.01$; after reaction with DTD in the presence of NADH for 45 min, a new absorption at about 572 nm appears with a distinct color change from colorless to pink, and the reaction leads to a 16-fold fluorescence enhancement. In contrast, probe **1** in the absence of DTD (control) did not show significant change in fluorescence during the same period of time, suggesting that the probe is rather stable and DTD is indeed involved in the resorufin-releasing reaction. Interestingly, both the absorption and fluorescence spectra from the reaction system resemble those of resorufin, supporting the fact that the enzyme-triggered cleavage reaction causes the release of free resorufin.

3.2. Optimization of experimental conditions and fluorescence kinetic curves

To optimize the conditions for DTD assay, the effects of pH and temperature on the fluorescence of **1** were studied. Fig. 2 (A) showed that in the range of pH 4–8, the fluorescence intensity increased with the increase of pH, and reached to a plateau at around pH 8.0, which may be due to the fact that resorufin fluoresces only in their anionic forms (pK_a=5.8) [17,18]. Considering the applicability of the probe in biosystems, pH 7.4 is chosen in our study. In addition, it can be seen from Fig. 2(B) that with the increase of temperature, the fluorescence intensity increased until 37 °C, and further increase of temperature led to the decrease of fluorescence intensity. Since the fluorescence intensity of resorufin is stable in the above temperature range (data not shown), it is considered that this change in fluorescence intensity may be ascribed to the change of DTD activity with temperature: the enzyme activity increased with temperature raise until 37 °C, which however, was inhibited at higher temperature. Thus, 37 °C was used for the present system.

Reaction kinetic studies showed that the fluorescence of **1** at 585 nm increased gradually upon addition of DTD, and this increase reached a maximum after 45 min (Fig. S3, ESI†). Moreover, higher concentrations of DTD resulted in faster reaction and more fluorescence enhancement. It should be pointed out that a strange phenomenon was observed, that is, no fluorescence was detected during the first 20 min of reaction (Fig. S3, ESI†). To understand this phenomenon, the effect of DTD on fluorescence needs to be studied in the following studies.

3.3. Effect of DTD on fluorescence of resorufin in the presence of NADH

Previous studies indicated that diaphorases including DTD are able to reduce resorufin to a non-fluorescent product [19,20]. If this is the case, resorufin released from **1** would be reduced to a non-fluorescent product by DTD, leading to the non-fluorescence phenomenon. Moreover, if the reduction were irreversible, this reaction would cause negative results in our enzyme activity assay. To explore the influence of this reduction, a control experiment was carried out by reacting DTD with resorufin in the presence of NADH, and an interesting phenomenon occurred: the solution of resorufin immediately became colorless within 3 s upon addition of DTD; 2 min later, however, the color of the solution gradually reappeared, and after 4 min the color was almost recovered to the initial pink (Fig. S4, ESI†). This recovery was further confirmed by the absorption spectral measurement, which revealed that the absorption spectrum of resorufin in the long wavelength region overlapped well with that from its reaction solution with DTD for 4 min (Fig. S4, ESI†). The exact reason for this is unclear. However, these results indicate that the redox reaction of resorufin is fast

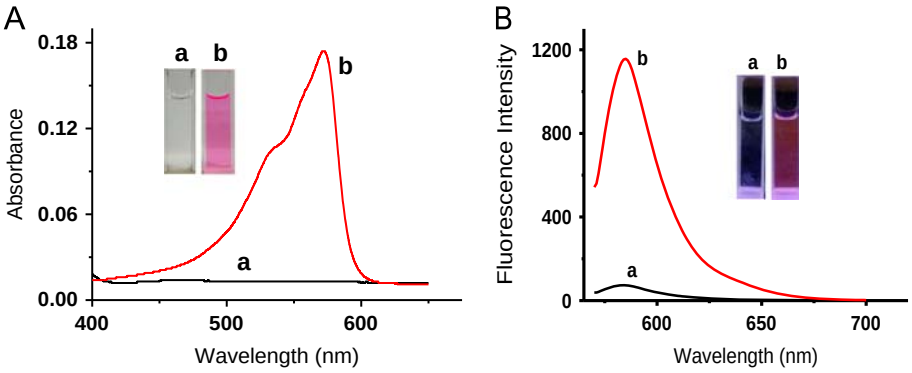


Fig. 1. (A) Absorption and (B) fluorescence emission (λ_{ex}=550 nm) spectra of **1** (5 μM) (a) before and (b) after reaction with DTD (7.5 μg/mL) in the presence of 100 μM NADH at 37 °C for 45 min. The color and fluorescence changes of **1** before and after the reaction are shown in the insets of the corresponding figures.

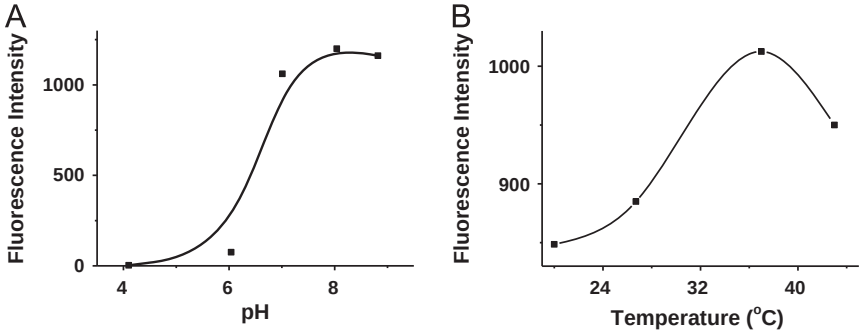


Fig. 2. Effects of (A) pH and (B) temperature on the reaction of 1 (10 μM) with DTD (7.5 μg/mL) in the presence of 100 μM NADH. λex/em=550/585 nm.

and reversible, and thus would not affect the detection of DTD when the reaction time longer than 4 min is used (e.g., 45 min in the present study).

3.4. Linearity of DTD

Under the optimal conditions that were ascertained above, the fluorescence response of **1** to DTD at varied concentrations is shown in Fig. 3. As is seen, the fluorescence intensity of the reaction system increases with the DTD concentration from 0 to 12.5 μg/mL, and an about 16-fold fluorescence increase could be produced with the usage of 12.5 μg/mL DTD. Moreover, a good linear equation of $\Delta F = 226.9 \times C$ (μg/mL) + 45.2 ($R = 0.992$) can be obtained in the range of 0.5–4 μg/mL DTD. The detection limit ($3S/m$, in which S is the standard deviation of blank measurements, $n = 11$, and m is the slope of the linear equation) is determined to be 6.34×10^{-3} μg/mL, featuring a high sensitivity.

3.5. Docking and inhibitor study

Because of the special non-fluorescence phenomenon of the reaction system in the first 20 min, the initial reaction rate and thus the Michaelis constant of the enzyme-catalyzed reaction cannot be determined by measuring the increase of fluorescence intensity. However, a docking study (Fig. 4) was performed to evaluate the binding ability of **1** for DTD. The docking score ($-\lg K_d$) is 5.80, indicating that probe **1** has a good binding affinity for DTD, which is also supported by the result of the ribbon model created by Pymol. As is seen from Fig. 4(B), there are two potential hydrogen bonds in the resulting complex, which may contribute to the good binding affinity as well.

To prove that the fluorescence enhancement is caused by DTD, the effect of dicoumarin (an inhibitor of DTD [21]) on the reaction was also investigated (Fig. S5, ESI†). The results showed that the introduction of dicoumarin largely decreased the fluorescence intensity of the reaction system, suggesting that the activity of DTD can be effectively suppressed by the inhibitor and the fluorescence increase indeed arises from the action of DTD. Moreover, this result also demonstrates that the probe may be used as a screening tool for the inhibitor of DTD.

3.6. Reaction mechanism study

To ensure that the fuorescence response of probe **1** to DTD is caused by the enzyme-catalyzed reduction of the quinone moiety, followed by the release of resorufin, the reaction solution of **1** with DTD was subjected to HPLC analyses to explore the enzyme-catalyzed reaction mechanism. As shown in Fig. S6 (ESI†), the chromatographic peaks of NADH, DTD, resorufin and **1** are located at 2.0, 2.1, 4.2, and 12.1 min, respectively. After reaction of probe **1** with DTD for 45 min in the presence of NADH, the chromatographic peak of **1** decreases remarkably, concomitant with the appearance of a peak at 4.2 min indicative of resorufin. This clearly indicates that, after reaction, the recovery of both color and fluorescence arises from the generation of resorufin.

3.7. Fluorescence response of *1* to glucose based on the DTD-GDH coupling reaction

Finally, we assessed the utility of probe **1** as a fluorescent probe for the detection of glucose [22] via the DTD/GDH coupling reaction [23]. The mechanism of this detection is shown in

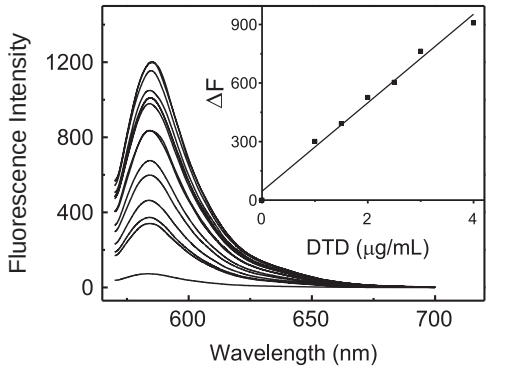
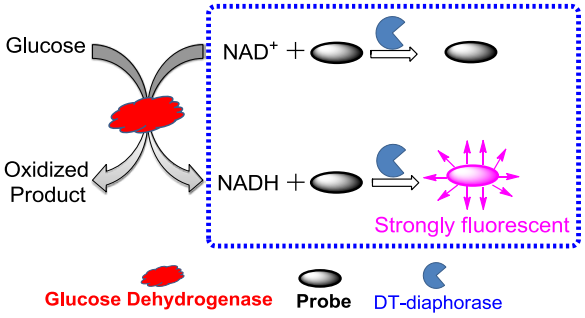


Fig. 3. Fluorescence response of **1** (10 μM) to DTD at varied concentrations (from bottom to top: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7.5, 10, 11, and 12.5 μg/mL). The inset shows the plot of ΔF against the DTD concentration; ΔF is the difference of fluorescence intensity of the probe with and without DTD. $\lambda_{ex} = 550$ nm.



Scheme 2. Assay of glucose via the DTD/GDH coupling reaction.

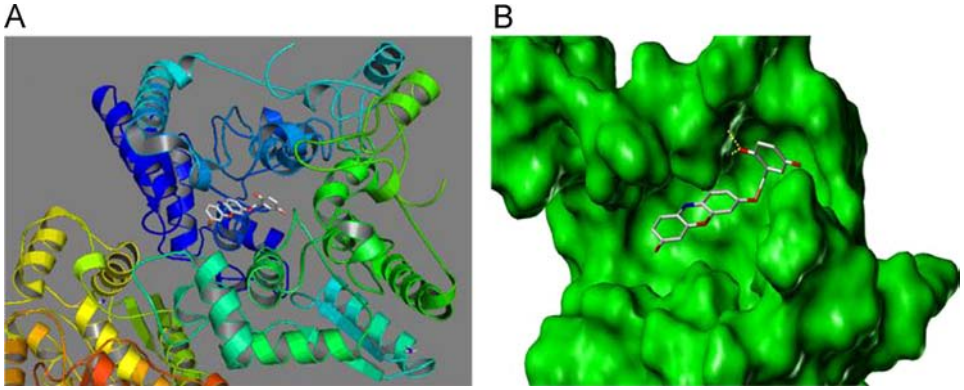


Fig. 4. (A) The docked conformer of 1 at the binding cleft of DTD (generated via Surflex docking-scoring combinations); (B) the details of the binding of 1 to DTD domains showing the potential hydrogen bonds in yellow dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

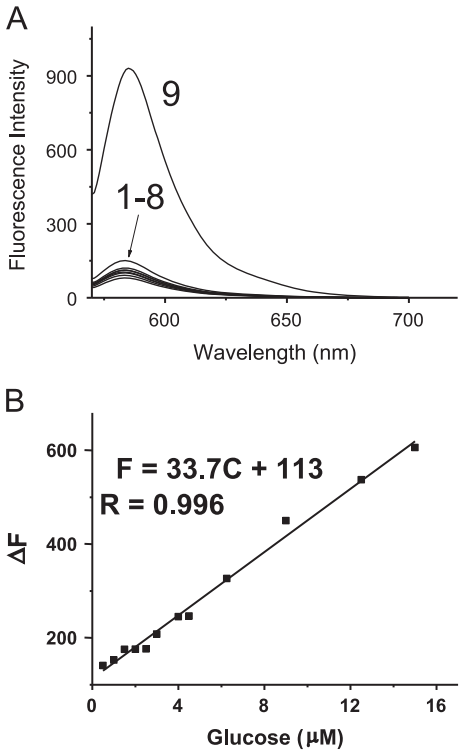


Fig. 5. (A) Fluorescence emission spectra (λ_{ex}=550 nm) of **1** (10 μM) in PBS of pH 7.4 with different reaction substances: (1) probe **1** only (control); (2) GDH (37.5 μg/mL); (3) DTD (7.5 μg/mL); (4) NAD^+ (500 μM); (5) NADH (500 μM); (6) glucose (200 μM); (7) DTD (7.5 μg/mL)+NAD^+ (500 μM); (8) GDH (37.5 μg/mL)+DTD (7.5 μg/mL)+NAD^+ (500 μM); and (9) GDH (37.5 μg/mL)+DTD (7.5 μg/mL)+NAD^+ (500 μM)+glucose (200 μM). (B) The plot of ΔF against the glucose concentration.

Table 1
Determination of glucose in human urine samples.[a]

Sample	Glucose added (μM)	Glucose found (μM)	Recovery (%)
A	0	0.38	
B	4	4.2 ± 0.18	95.5 ± 4.5
C	10	10.2 ± 0.81	98.2 ± 8.1
D	13	12.8 ± 0.88	95.5 ± 6.7

[a] Mean of three determinations ± standard deviation. The detection system contains probe **1** (10 μM), GDH (37.5 μg/mL), DTD (7.5 μg/mL), NAD^+ (500 μM), urine with different concentrations of glucose added in PBS of pH 7.4.

Scheme 2. GDH oxidizes glucose to gluconolactone with NAD^+ as the electron acceptor to yield NADH [24], and NADH can be utilized by DTD to further react with the probe releasing resorufin. Based on this tandem-reaction mechanism, the fluorescence off–on response is anticipated. As shown in Fig. 5(A), a strong fluorescence can be produced only in the presence of all the reactants, that is, the reaction system in the absence of any single reactant does not generate significant fluorescence. This clearly shows that the fluorescence enhancement results from the tandem enzyme-coupling reaction.

Moreover, there exists a good linearity between the fluorescence increase and the glucose concentration in the range of 0.5–15 μM (Fig. 5(B)), with a linear equation of $\Delta F = 33.7 \times C$ (μM)+113 (R=0.996). The detection limit is determined to be 0.2 μM, which is one of the lowest detection limits for glucose to our knowledge [25,26]. In addition, any enzyme that can convert NAD^+ to NADH may be coupled with DTD, and this coupling reaction may be employed to detect the related analyte, as further demonstrated for glutamic acid assay with glutamate dehydrogenase (Fig. S7, ESI†).

3.8. Determination of glucose in human urine samples

Clinically, the ratio of glucose excretion in urine is also used to evaluate the extent of diabetes mellitus. Thus, in order to evaluate the practicability of the proposed method, the detection of glucose in human urine was performed. Different concentrations of glucose were added into the urine sample, and the recoveries of glucose determined by present method are given in Table 1. It can be seen that the recoveries of glucose range from 95.5% to 98.2%, and the relative errors are no more than 9%, suggesting that the proposed approach is of potential application for clinical diagnosis.

4. Conclusions

In summary, we have developed a new reductase-based fluorescent probe for the detection of glucose through the enzyme-coupling reaction. The probe was synthesized through a mild and simple synthetic procedure, and displayed a distinct color and fluorescence off–on response to glucose. The reactions for the detection of glucose proceed as follows: glucose dehydrogenase oxidizes glucose to gluconolactone with NAD^+ as the electron acceptor to yield NADH, and NADH can be utilized by DTD to further react with the probe releasing resorufin. As a result of these tandem reactions, fluorescence off–on response occurs. The probe and the DTD-based enzyme-coupling reaction may provide a potential way for fluorescence detection of glucose for clinical diagnostics.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.12.032.

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