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A fluorescence glucose sensor based on pH induced conformational switch of i-motif DNA

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ABSTRACT

A facile fluorescence biosensor for the detection of glucose is proposed based on the pH-induced conformational switch of i-motif DNA in this paper. Glucose can be oxidized by oxygen (O_2) in the presence of glucose oxidase (GOD), and the generated gluconic acid can decrease the pH value of the solution and then induce the fluorophore- and quencher-labeled cytosine-rich single-stranded DNA to fold into a close-packed i-motif structure. As a result, the fluorescence quenching occurs because of the resonance energy transfer between fluorophore and quencher. Based on this working principle, the concentration of glucose can be detected by the decrease of fluorescence density. Under the optimal experimental conditions, the assay shows a linear response range of 5–100 μM for the glucose concentration with a detection limit of 4 μM. This glucose biosensor was applied to determine glucose in real samples successfully, suggesting its potential in the practical applicability.

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1. Introduction

Glucose is the most widely distributed monosaccharide in nature and plays a very important role in the field of biology. It is a major source of energy for living cells and acts as an indispensable metabolic intermediate in various metabolic processes of animal and plant. Furthermore, an abnormal glucose level in human blood or urine is commonly considered as a sign of diabetes or hypoglycemia. Therefore, accurate determination of glucose has great significance in food analysis and clinical diagnosis. A variety of methods such as electrochemical [1–3], colorimetric [4–6], Raman scattering [7], surface plasmon resonance (SPR) [8] methods have been applied to glucose detection. Recently, fluorescence [9–11] glucose biosensors have attracted much attention due to high sensitivity and practicality. However, most of these detection methods used nanoclusters [9] or quantum dots (QDs) [10,11] as probe and required complex modification and synthesis procedure. Moreover, the quantum dots (QDs) were poisonous and the cytotoxicity should be taken into consideration [12]. Thus, it is still urgent to develop new approaches to detect glucose with easy operation, high sensitivity and low toxicity.

DNA, due to its unique physical and chemical properties, especially its outstanding specificity of conformational polymorphism and programmable sequence recognition, plays an important role in bioanalysis in the past few years [13–15]. Another very attractive feature of DNA is that the conformation of DNA can reversibly convert with the external stimuli such as temperature, ionic conditions [16], DNA/RNA strands [17], acids/bases [18], enzymes [19], chemical oscillator [20]. As a response to external stimulus, the conformation of DNA may change in a controllable manner, forming peculiar structures such as G-quadruplex, hairpin structure, i-motif and so on [21]. The i-motif structure was first found in 1993. Under slightly acidic conditions, a cytosine (C)-rich single-stranded DNA (ssDNA) can form a four-stranded DNA structure called i-motif because the cytosine residues are partially protonated and form a intramolecular noncanonical C–CH(+) base pairs [20,22]. Various DNA-nanomachines have been developed based on the pH-induced conformational switch of i-motif DNA in the past few years, whereas most of which were focused on the conformational change of i-motif DNA but not the quantitative detection [20,23,24]. Recently, by the use of i-motif, a colorimetric method [25] for the quantitative colorimetric detection of glucose was proposed. However, the synthesis of AuNPs and salt-induced aggregation strategy made this assay complicated and time-consuming.

Herein, we proposed a simple fluorescence glucose sensor based on pH-induced conformational switch of i-motif DNA. Glucose can be oxidized by oxygen (O_2) in the presence of glucose oxidase (GOD). The generated gluconic acid can decrease pH of the solution and induce the fluorescence-labeled C-rich ssDNA (X) to fold into a close-packed i-motif structure, leading to the fluorescence quenching. As a result, the concentration of glucose could be determined

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by the decrease of fluorescence intensity. A complementary oligonucleotide (Y) was introduced to improve the sensitivity of this method. Different from previous method using gold nanoparticles [25], our glucose sensor used the fluorescence-labeled C-rich ssDNA to detect glucose directly, thus made the detection simple and time-saving.

2. Materials and methods

2.1. Reagents and materials

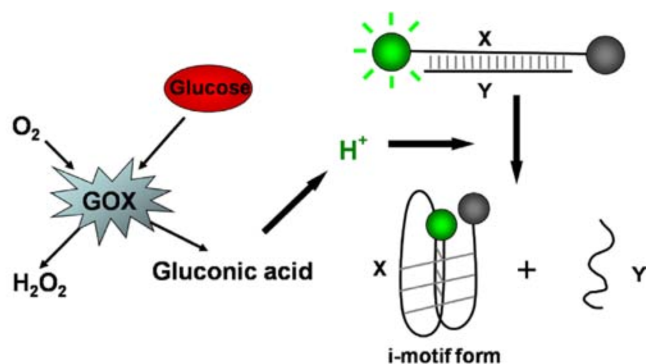
D-(+)-Glucose, Glucose oxidase (GOD) and D-Gluconic acid solution were bought from Sigma-Aldrich. Dopamine (DA) and uric acid (UA) were purchased from Acros Organics (NJ, USA). Fructose, lactose, sucrose and other salts were purchased from Beijing Chemical Company (China). The 21-mer oligonucleotide X: 5′-CY5-CCCTAACCCTAACCCTAACCC-BHQ-2-3′ and the 17-mer Complementary Strand Y: 5′-GTTAGTGTTAGTGTTAG-3′ were synthesized by Sangon Biotechnology Inc. (Shanghai, China). Before use, the glucose was dissolved in phosphate buffered saline (PBS) solution (0.1 mM, 100 mM NaCl). The concentration of the oligonucleotide was determined by measuring the UV absorbance at 260 nm. Human serum was obtained from The People's Liberation Army No. 461 Hospital. All other chemicals were of analytical grade and ultra-pure water (Milli-Q plus, Millipore Inc., Bedford, MA) was used throughout.

2.2. Instrumentations

The fluorescence intensity (FL) spectra were recorded on a LS-55 Luminescence Spectrophotometer (Perkin-Elmer Instruments U.K.). The emission spectra were recorded in the wavelength range of 620–800 nm upon excitation at 610 nm. The measurements of UV–vis absorption spectra were conducted on a Cary50 UV–vis Spectrophotometer (Varian, USA) with a 10 mm path length fused-silica cuvette. Circular dichroism (CD) spectral measurement was performed on a Jasco J-820 Circular Dichroism Spectropolarimeter (Tokyo, Japan).

2.3. The pretreatment of human serum

Fresh human serum samples were obtained from the local hospital and the serum was deproteinized before use [26]. The human serum was mixed with the same volume acetonitrile and shaken for 1 min. The precipitate was removed after centrifuging (15 min at 10,000 rpm). Then, the acetonitrile was removed by a rotary evaporator under reduced pressure in a water bath at 60 °C. After dissolving in certain volume of ultra-pure water, the deproteinized human serum was obtained.



Scheme 1. Schematic illustration of fluorescence detection of glucose using pH induced conformational switch of i-motif DNA.

2.4. Fluorescence measurements

A typical glucose detection procedure was conducted as follows: first, 8 μl GOD (1 mg/ml) and different concentrations of glucose were added into the phosphate buffered saline (PBS) solution (0.1 mM, pH=7.0, NaCl 100 mM) to make a total volume of 100 μl and the mixture was incubated at 37 °C in a water bath for 3 h. After that, DNA X (0.2 μM) and Y (0.04 μM) were mixed first and then added into the mixture. PBS was added to make the total volume 350 μl. The mixture was incubated for another 30 min. Finally, the fluorescence spectra were measured at room temperature (25 ± 1 °C). All of the fluorescence measurements were under the same conditions: the slit width for excitation was 5 nm and that for emission was 10 nm, and the excitation wavelength was set at 610 nm.

2.5. CD measurement of conformational switch of i-motif DNA

350 μl of 0.1 mM PBS buffer solution (pH 7.0) containing 1 μM X and 0.2 μM Y was put into a quartz cell (10 mm path length, 0.7 ml volume). Then, GOD alone and reaction mixture of GOD and glucose (the mixture had been kept at 37 °C in a water bath for 3 h before) was added, respectively. Before the measurement, the mixture was incubated for 30 min to reach equilibrium. The CD spectra were measured over the wavelength range from 230 nm to 380 nm and the scanning speed was 200 nm/min.

3. Results and discussion

3.1. Principle of the fluorescence detection using i-motif DNA as probe

The mechanism of our designed glucose biosensor is shown in Scheme 1. In this work, a 21-mer ssDNA strand (sequence X) was used in fabrication of biosensor. The DNA was labeled with a pH-insensitive fluorophore CY5 at the 5′ end and a quencher BHQ at the 3′ end. Before the addition of glucose, the C-rich ssDNA X was hybridized with the 17-mer complementary strand Y. The fluorescence intensity was high because the fluorophore and quencher were separated from each other effectively due to the formation of rigid double-stranded DNA (dsDNA). Glucose could be oxidized by oxygen (O_2) in the presence of GOD via the following reaction, $\text{glucose} + O_2 + H_2O \xrightarrow{GOD} \text{gluconic acid} + H_2O_2$. Upon the addition of the reaction mixture of glucose and GOD, the generated gluconic acid could decrease the pH value of the system. Under slightly acidic conditions, the C-rich ssDNA could switch into a four-stranded, close-packed DNA structure through C–CH(+) base pairing (i-motif DNA) which was more stable than the dsDNA, [22] and the complementary strand Y was thus released. In that way, the fluorophore and quencher were in close proximity and the fluorescence of CY5 was quenched because of resonance energy transfer between neighbouring quencher [20], leading to the decrease of fluorescence intensity. Based on this working principle, the concentration of glucose could be determined by the decrease of fluorescence intensity.

To confirm the hypothesis, fluorescence spectra of the proposed i-motif DNA system which contains the C-rich ssDNA X and complementary strand Y were measured under different experimental conditions. The results indicated that the generated gluconic acid was responsible for the conformational transition. As shown in Fig. 1, the addition of gluconic acid could lead to the

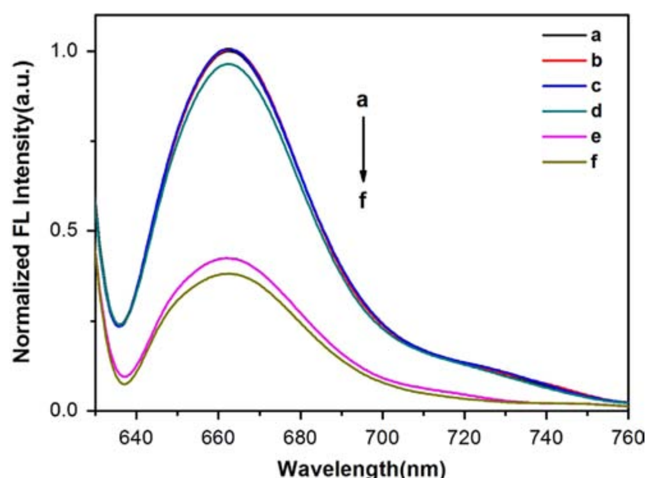


Fig. 1. Fluorescence spectra of i-motif DNA system in the presence of blank (a), 100 μM glucose (b), 8 μg/ml GOD (c), 100 μM H_2O_2 (d), 8 μg/ml GOD+50 μM glucose (the mixture is kept at 37 °C in a water bath for 3 h in advance) (e), 50 μM gluconic acid (f). Experiment conditions are: C_X=0.2 μM, C_Y=0.04 μM, C_{PBS}=0.1 mM, pH=7.0.

Table 1
The pH value of reaction mixture contained GOD and different concentrations of glucose after incubation at 37 °C for 3 h. Experiment conditions are: C_{GOD}=8 μg/ml, C_{PBS}=0.1 mM, pH=7.0.

$C_{glucose}$ (μM)	0	1	5	15	30	50	100	150
pH	7.0	6.95	6.92	6.82	6.67	6.52	5.89	4.91

significant decrease of fluorescence intensity whereas the sole addition of either glucose or GOD could not. Upon the addition of gluconic acid, the change of fluorescence spectra was similar to that caused by the same concentration glucose in the presence of GOD. Except gluconic acid, H_2O_2 is another product of oxidation of glucose. We also investigated the effect of H_2O_2 on the conformational change by sole addition of H_2O_2 (with the same concentration as glucose) to this system. The fluorescence intensity remained almost unchanged, indicating that H_2O_2 could not lead to conformational switch of i-motif DNA. These results proved that the generated gluconic acid induced the conformational change of i-motif DNA and the fluorescence of the system could sensitively respond toward glucose.

In order to further prove this concept, we also measured the pH value of the reaction mixture of glucose and GOD with the glucose concentration range from 0 μM to 150 μM. Table 1 showed that as the concentration of glucose increased, the pH decreased in a concentration-dependent manner. When the concentration of glucose was 150 μM, the pH value greatly decreased from 7.0 to 4.91. The glucose or H_2O_2 alone could not induce change of pH value. These results clearly showed that it was gluconic acid which decreased the pH value. What's more, fluorescence spectra of i-motif DNA system (X+Y) were measured with PBS solutions of different pH values. As shown in Fig. 2A, with the decrease of pH value, the fluorescence intensity decreased substantially, indicating a sensitive response of this system to pH change. A sharp decrease of fluorescence intensity was observed in the pH range of 7.0 to 6.0, suggesting a significant conformational transition in this pH range. This result was consistent with the previous report that the formation of the i-motif structure showed a sharp transition at approximate pH 6.5 [27].

3.2. Conformational switch of i-motif DNA characterized by CD

CD spectra in the UV region can be used to distinguish different DNA structures [28]. In this work, CD technique was used to monitor the conformational change of the C-rich ssDNA. As clearly shown in Fig. 3, in the absence of glucose, there was a positive peak near 275 nm and a negative peak near 245 nm, suggesting a duplex DNA structure. Upon addition of glucose, the CD spectra showed a dramatic change, with a positive peak near 287 nm and a negative peak near 260 nm, indicating the formation of a typical i-motif conformation [24]. The CD experiment results were consistent with our concept that the glucose oxidation reaction triggered conformational change of i-motif DNA and induced the fluorescence quenching of fluorophore-modified DNA probes.

3.3. Optimization of experimental conditions for fluorescence detection

In this work, the relative fluorescence intensity decrease, $[(F_0-F)/F_0]$ (F_0 and F are the fluorescence intensity of i-motif DNA system at 662 nm in the absence and presence of glucose), was introduced to investigate the effect of several factors, such as the ratio of X and Y, the concentration of PBS solution, the pH environment and the equilibrium time, on the conformational switch of DNA probe.

In this system, besides the C-rich ssDNA X, a 17-mer complementary DNA strand Y was also used. Upon the addition of Y, the fluorescence intensity increased dramatically due to the formation of DNA duplex, whose rigid structure separated the fluorophore and quencher more efficiently compared with random-coiled ssDNA X. Fig. 4 revealed that there was no obvious difference among the three ratio of X to Y with regards to their effects on the relative fluorescence intensity along with the concentration of glucose. In order to save raw materials, a ratio of X to Y as 5:1 was chosen in this experiment.

An appropriate PBS solution concentration is crucial in this pH induced fluorescence assay. The higher the concentration of the PBS, the larger the buffer capacity. If a high-concentration PBS was used in the enzyme reaction mixture, due to its high buffer capacity, the enzymatically generated gluconic acid could not change the solution pH obviously and the sensitivity of the assay would be decreased. To select the optimal PBS concentration, we recorded the fluorescence response of the i-motif DNA system to 50 μM glucose with different concentration of PBS solution in the range of 5 mM to 0.1 mM. It could be seen in Fig. 5 that the detection sensitivity dramatically decreased with the increase of PBS concentration and the assay achieved the highest sensitivity at 0.1 mM. Therefore, 0.1 mM was chosen as the optimal PBS concentration.

Besides the concentration of PBS, the pH value of the buffer is another important factor in this work since the conformational switch of i-motif DNA is highly pH-dependent [20,24,25]. Fig. 6 shows the effect of pH value over the range of 6.5 to 8.0. It was found that with the pH value decreased, the detection sensitivity increased. However, at pH 6.5, the fluorescence was almost completely quenched when concentration of glucose was 50 μM, exhibiting a narrow detection range. Furthermore, at pH 6.5, the fluorescence intensity of the system was relatively weak as shown in Fig. 2, resulting in a narrow linear response range of the detection. So PBS buffer with pH 7.0 was used in our system.

In the detection system, on the one side, the complementary strand Y hybridizes with the C-rich strand X, forming DNA duplex. On the other side, the C-rich DNA X folds into a close-packed i-motif due to the acidity decrease of the solution upon mixing with GOD-glucose reaction mixture. To elucidate the reaction kinetics of i-motif DNA-glucose system, the time scan of the fluorescence intensity of fluorophore modified i-motif DNA in the presence of glucose was carried out as shown in Fig. 7. After the addition of glucose-GOD reaction mixture, the fluorescence intensity decreased immediately and the conformational switch of i-motif DNA system reached equilibrium in about 30 min. As a

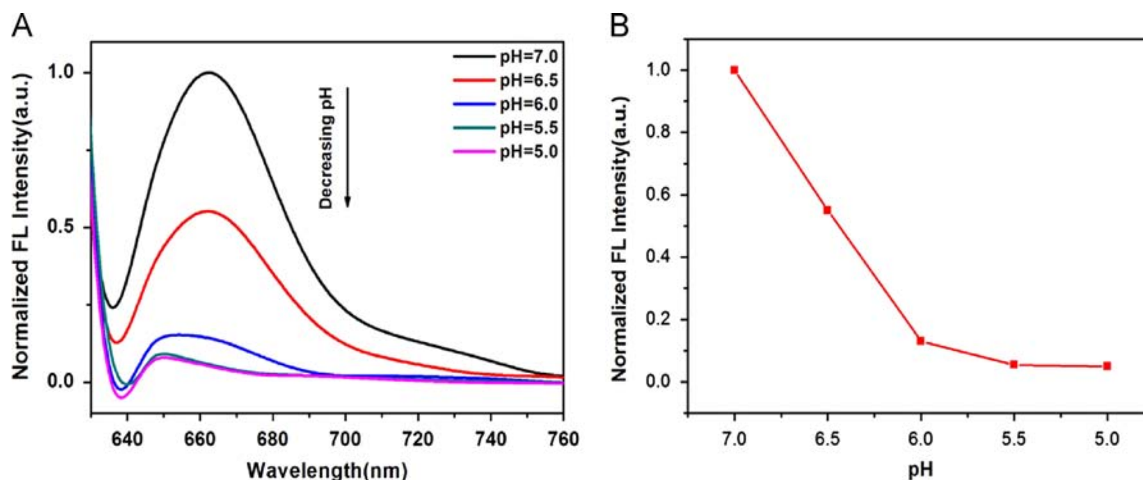


Fig. 2. (A) Fluorescence spectra of i-motif DNA system in PBS solutions with different pH values. (B) Plot of the relative fluorescence intensity (F/F_0) of i-motif DNA system at 662 nm versus pH value. F_0 and F are the fluorescence intensity of i-motif DNA system at 662 nm in the absence and presence of glucose, respectively. Experiment conditions are: $C_X=0.2$ μM, $C_Y=0.04$ μM, $C_{PBS}=0.1$ mM.

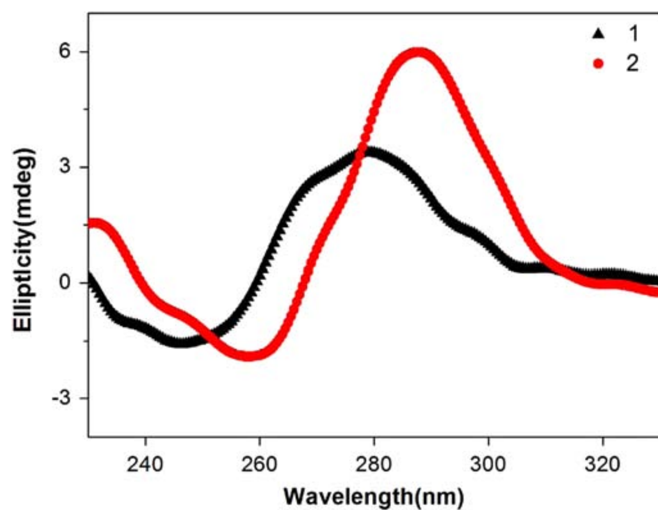


Fig. 3. CD spectra of i-motif DNA system in the presence of blank (1), 8 μg/ml GOD+50 μM glucose (2). Experiment conditions are: $C_X=1$ μM, $C_Y=0.2$ μM, $C_{PBS}=0.1$ mM, pH=7.0.

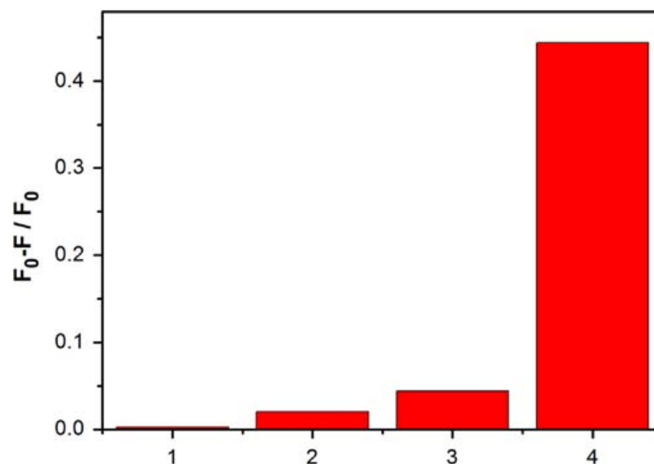


Fig. 5. The fluorescence response of the i-motif DNA system to 50 μM glucose with different concentration of PBS solution (1) 5 mM, (2) 1 mM, (3) 0.5 mM, (4) 0.1 mM. Experimental conditions are: $C_X=0.2$ μM, $C_Y=0.04$ μM, pH=7.0.

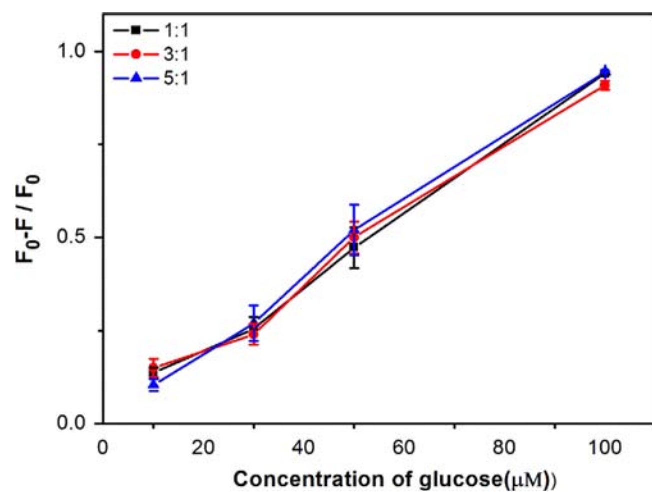


Fig. 4. The relative fluorescence intensity decrease, [$(F_0-F)/F_0$], versus the concentration of glucose with different ratio of strand X to strand Y. Experimental conditions are: $C_X=0.2$ μM, $C_{PBS}=0.1$ mM, pH=7.0. The error bar represents the standard deviation from the mean of three repeated measurements.

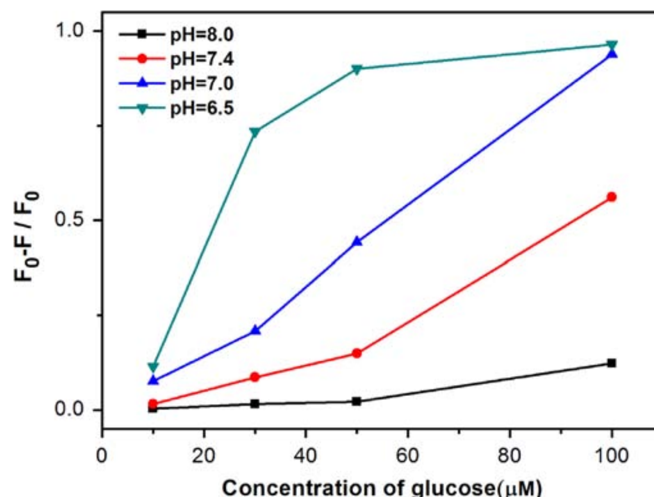


Fig. 6. The pH-dependent [$(F_0-F)/F_0$] value as a function of glucose concentration. Experimental conditions are: $C_X=0.2$ μM, $C_Y=0.04$ μM, $C_{PBS}=0.1$ mM.

result, 30 min was selected in our following measurement as the equilibrium time.

3.4. Quantitative detection of glucose

Under the optimal experimental conditions (equilibrium time: 30 min, buffer pH: 7.0, C_{PBS}: 0.1 mM, the ratio of X to Y is 5:1), the fluorescence spectra of the sensor were recorded in the presence of various concentrations of glucose. As can be observed in Fig. 8A, with the increase of glucose concentration, the fluorescence intensity decreased. Fig. 8B depicts the derived calibration curve. A linear relationship between the [$(F_0-F)/F_0$] and glucose concentration is obtained over the range of 5–100 μM ($R^2=0.995$). This is consistent with Fig. 2B, in which the fluorescence intensity of the C-rich DNA system decreased linearly with the decrease of pH from 7.0 to 6.0, corresponding to the pH range of the system in the glucose concentration range of 0–100 μM (Table 1). The limit of detection (LOD) of the assay is 4 μM (3σ). Our method exhibited a better sensitivity towards glucose compared to other reported methods [29,30]. Table 2 shows the analytical features of several determination methods of glucose [4,7,9,30,31].

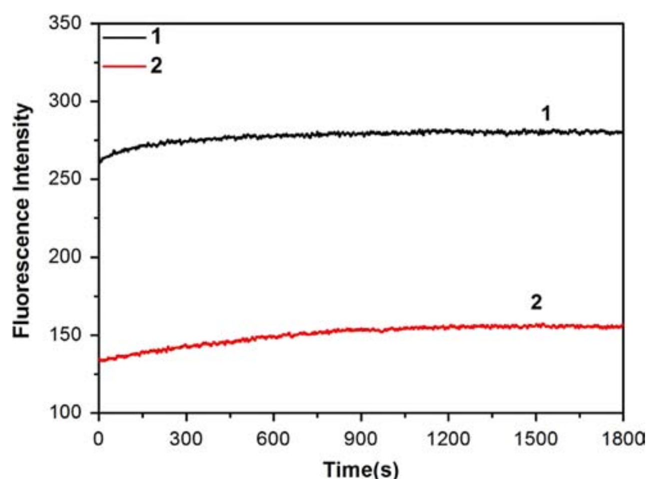


Fig. 7. Fluorescence time course of i-motif DNA system (1) in the absence of glucose and (2) in the presence of 50 μM glucose with excitation at 610 nm and emission at 662.5 nm. Experimental conditions are: $C_X=0.2$ μM, $C_Y=0.04$ μM, $C_{PBS}=0.1$ mM, pH=7.0.

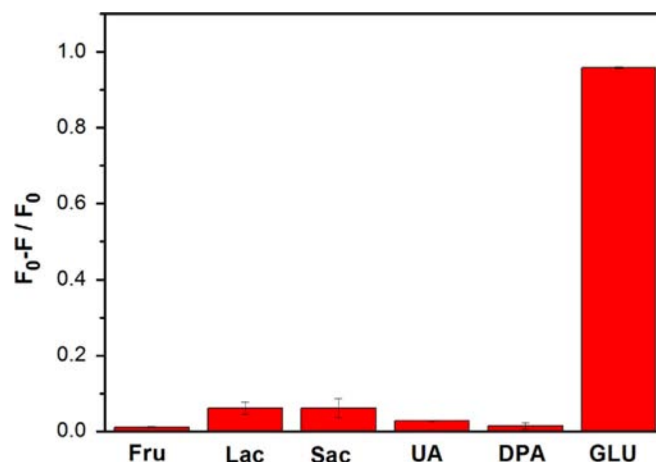


Fig. 9. Interference test with 1 mM fructose, 1 mM lactose, 1 mM saccharose, 100 μM dopamine (DPA), 100 μM uric acid (UA) and 100 μM glucose, respectively. Experimental conditions are: $C_X=0.2$ μM, $C_Y=0.04$ μM, $C_{PBS}=0.1$ mM, pH=7.0.

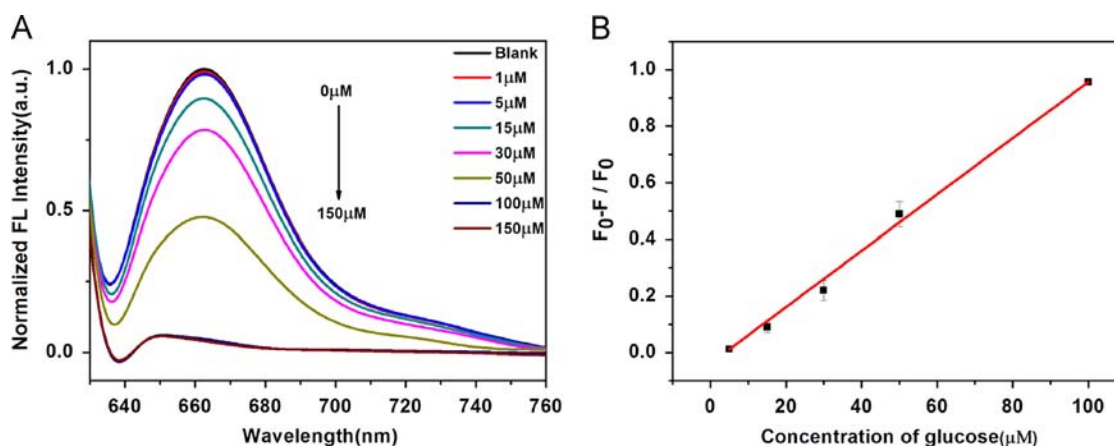


Fig. 8. (A) Fluorescence spectra of i-motif DNA system with different concentrations of glucose in the range of 1–150 μM. (B) Derived calibration curve of relative fluorescence intensity of i-motif DNA system versus the concentration of glucose in the range of 5–100 μM. Experimental conditions are: $C_X=0.2$ μM, $C_Y=0.04$ μM, $C_{PBS}=0.1$ mM, pH=7.0.

Table 2
Analytical features of different determination methods of glucose. [a] Present study.

Methods	System	pH	Response	Detection limits (μM)	Real samples
Fuorescence[9]	Au nanoclusters/GOD	7	H_2O_2	5	Human serum
Fuorescence[30]	FITC-CdSe QDs/GOD	7.2	H_2O_2	1000	–
Electrochemical[31]	GOD/BSA/PtNP-SWCNT	7.4	H_2O_2	40	–
Colorimetric[4]	$C_{60}[C(COOH)_2]_2$/GOD/TMB	3.5	H_2O_2	0.5	Human serum
SERS[7]	AgFON	–	Glucose	–	–
Fuorescence[a]	DNA/GOD	7	Gluconic acid	4	Human serum, urine

Table 3
Glucose detection in urine samples.

Samples	Found amount (μM)	Standard added (μM)	Found value (μM)	Recovery (n=3, %)
Urine	ND	30	29.28	97.60
		50	51.02	102.04

Urine sample was diluted 300-fold for detection. ND: not detected.

Table 4
Glucose detection in serum samples.

Samples	Referenced values[a] (mM)	Determined values[b] (mM)	Relative deviation (%)
Serum 1	5.39	5.16	4.26
Serum 2	6.33	6.12	3.32

Serum samples were diluted 300-fold for detection.

[a] Value provided by the local hospital.
[b] Value determined by our fluorescence biosensor.

3.5. Selectivity test

The selectivity of this fluorescence biosensor for glucose was tested with some glucose analogues such as fructose, lactose and sucrose, as well as some common interferents, such as dopamine (DA) and uric acid (UA). For the selectivity test, the concentrations of glucose analogues were ten times of glucose concentration, and the concentrations of other interferents were identical with the glucose concentration, which were close to their physiological levels [32]. As shown in Fig. 9, compared to glucose, none of the analogues or interferents caused a significant change in fluorescence intensity of i-motif DNA system. Therefore, it can be inferred that our method has a good selectivity for glucose.

3.6. Detection of glucose in real samples

In order to evaluate the applicability of the fluorescence biosensor in real samples, we tested this assay with human urine. According to the glucose concentration in urine of diabetics, the fresh urine sample from healthy volunteer was diluted 300 times with PBS solution [6]. The experimental results showed that blank urine sample of healthy volunteer gave no obvious fluorescence response, indicating that our sensing system was applicable in human urine sample. The standard addition experiments results were given in Table 3. The recoveries of glucose in diluted human urine were in the range of 97.60–102.04%. Similarly, our method was also applied to analyze the intrinsic glucose level in human serum. The measured results were listed in Table 4. It can be seen that the glucose concentrations measured with our method were close to the values provided by local hospital. These results demonstrated that the glucose sensor has great potential for quantitative determination of glucose in real clinical samples.

4. Conclusion

In summary, on the basis of pH-induced conformational switch of i-motif DNA, we have successfully developed a facile fluorescence method for the detection of glucose. To the best of knowledge, the use of fluorescence-labeled C-rich ssDNA for quantitative detection of glucose has not been reported before. This assay exhibited several excellent features. First, in comparison with previous fluorescence approaches, this method required no complicated synthesis or modification procedure and used the fluorescence-labeled DNA as glucose probe directly, making the sensing facile and time-saving. Second, most of glucose detection methods mentioned above were based on the response to enzymatically generated H_2O_2. Our glucose sensor used a pH-sensitive C-rich DNA to detect another product of glucose oxidase reaction, gluconic acid, which induced the acidity change of solution. It opens up a new path to detect some other biomolecules whose reaction product can also induce the acidity change of solution. Third, the proposed method can also detect glucose effectively in real samples such as urine and human serum. Thus, we believe it may have great potential in the practical applicability.

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