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A single dual-emissive nanofluorophore test paper for highly sensitive colorimetry-based quantification of blood glucose

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ABSTRACT

Fluorescent test papers are promising for the wide applications in the assays of diagnosis, environments and foods, but unlike classical dye-absorption-based pH test paper, they are usually limited in the qualitative yes/no type of detection by fluorescent brightness, and the colorimetry-based quantification remains a challenging task. Here, we report a single dual-emissive nanofluorophore probe to achieve the consecutive color variations from blue to red for the quantification of blood glucose on its as-prepared test papers. Red quantum dots were embedded into silica nanoparticles as a stable internal standard emission, and blue carbon dots (CDs) were further covalently linked onto the surface of silica, in which the ratiometric fluorescence intensity of blue to red is controlled at 5:1. While the oxidation of glucose induced the formation of Fe^{3+} ions, the blue emission of CDs was thus quenched by the electron transfer from CDs to Fe^{3+}, displaying a serial of consecutive color variations from blue to red with the dosage of glucose. The high-quality test papers printed by the probe ink exhibited a dosage-sensitive allochromatic capability with the clear differentiations of ~5, 7, 9, 11 mM glucose in human serum (normal: 3–8 mM). The blood glucose determined by the test paper was almost in accordance with that measured by a standard glucometer. The method reported here opens a window to the wide applications of fluorescent test paper in biological assays.

1. Introduction

Several hundred billions of dollars are annually cost by governments and persons in the diagnosis of biological medicines due to the expensive disbursements in experimental instruments and manpower. As an example, about 300 million people suffer from diabetes in worldwide and this number is estimated to almost double in 2030 (Shaw et al., 2010; Whiting et al., 2011), who need the daily monitoring of blood glucose. The expensive consumption has always attracted huge efforts in the development of miniature sensors for the biological assays with low cost and easy operation. With high sensitivity and simplicity, fluorescent sensors have been considered as a powerful tool in biological label (Grossi et al., 2001; Jiang et al., 2016) and imaging (Kamiyama et al., 2016), and clinical diagnosis (Sandanaraj et al., 2010; Zheng et al., 2015). A general methodology is to link a recognition element onto a fluorescent signal unit to prepare the fluorescent probes that can produce the fluorescent "turn-off" and "turn-on" responses to the molecules of interest, e.g. dye probe for the monitoring of reactive oxygen species (Zhang et al., 2016), quantum dots (QDs) for assays of DNA (Zhang et al., 2005; Peng et al., 2007), carbon dots (CDs) and graphene oxides for the detections of proteins (Chai et al., 2015; Mei and Zhang, 2012). On the basis of various fluorescent probes, the development of fluorescent test papers is promising for the simple and inexpensive assays of biological molecules (Mei and Zhang, 2012), metal ions (Yuan et al., 2012), explosives (Zhang et al., 2011) and pesticides (Zhang et al., 2014). Unlike classical pH test paper, however, most of reported fluorescent test papers only can provide the yes/no detection by fluorescent brightness. The accurate quantification by dosage-dependent color responses on fluorescent test papers still has not been achieved.

In principle, the use of two different colorful fluorescent probes

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may solve the problem on the color variations with the dosages of target species, in which one acts as an internal standard emission and another is sensing element with fluorescent response (Domaille et al., 2010; Qiao et al., 2015). When the fluorescence of sensing probe is quenched by analyte, a serial of color evolution can be displayed. Unfortunately, the simple mixing of two colorful probes leads to the formation of an intermediate color, greatly suppressing the range of color variations. On the other hand, the instability of mixing probes by the interactions with biological molecules in samples will adversely affect the accurate ratiometric fluorescence in bioassays. These factors may seriously limit their colorimetric effect and quantitative capability. In this present study, we report a single dual-emissive ratiometric fluorescent probe and its as-prepared test paper for colorimetry-based quantification of glucose in human serum. The control of emissive intensity on the blue and red fluorescences in a single probe allows the color evolution from blue to red with the concentrations of blood glucose. Moreover, the background fluorescence of serum can be overcome by the dilution of 100 times due to the supersensitivity of probe to glucose.

2. Experimental section

2.1. Reagents and instruments

All chemicals used were analytical grade. Tetraethylorthosilicate (TEOS), 3-mercaptopropionic acid (MPA), 3-mercaptopropyltrimethoxysilane (MPS), N-hydroxysuccinimide (NHS), 3-aminopropyltriethoxysilane (APTS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and glucose oxidase were obtained from Sigma-Aldrich. Te powder, $NaBH_4$, $CdCl_2 \cdot 2.5H_2O$, sulfuric acid (98%), NaOH, ammonium hydroxide (25%), ethanol, citric acid, ethylenediamine, hydrogen peroxide (H_2O_2) and glucose were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Blood was collected by venous sampling from a healthy volunteer. Ultrapure water (18.2 MΩ cm) was prepared by a Millipore water purification system.

UV–vis absorption and fluorescence spectra were recorded by Shimadzu UV-2550 spectrometer and Cary Eclipse fluorescence spectrophotometer, respectively. A JEOL 2010 transmission electron microscope was used to observe the shapes of samples. Fluorescent photos were taken under an AGL-9406 UV lamp (365 nm excitation) with a Canon 600D digital camera.

2.2. Synthesis of carbon dots

Carbon dots (CDs) were prepared by the previously reported method in literature (Zhu et al., 2013). 1.05 g of citric acid and 0.34 mL of ethylenediamine were first dissolved in 20 mL ultrapure water. Then, the solution was transferred to a polytetrafluoroethylene autoclave (30 mL) and heated at 200 °C for 5 h. After cooling down, the resultant CDs were purified by dialysis for 24 h.

2.3. Synthesis of CdTe QDs

CdTe quantum dots (QDs) were prepared by the previous method (Zhang et al., 2011). 0.06 g Te powder and 0.10 g $NaBH_4$ were mixed in 2 mL ultrapure water under nitrogen atmosphere in an ice bath, then stirred for 6 h to get the NaHTe solution. Meanwhile, 0.23 g of $CdCl_2 \cdot 2.5H_2O$ and 210 μL of MPA were dissolved in 100 mL of ultrapure water and adjusted pH to 9 with 1.0 M NaOH, and the solution was deoxygenated by bubbling nitrogen for 30 min. Then, 5 mL of H_2SO_4 (0.5 M) was injected into the as-prepared NaHTe solution and the timely generated H_2Te gas was input into the above solution of $CdCl_2$ and MPA until the solution color changed from colorless to orange. After refluxing for 48 h, red CdTe QDs with an emission at 630 nm were obtained. Finally, the QDs were purified by precipitation with acetone and dispersed in 100 mL ultrapure water.

2.4. Synthesis of QDs@SiO_2

The as-synthesized QDs were embedded in silica nanoparticles through the hydrolysis of TEOS. 40 mL of ethanol, 5 mL of QDs solution, and 15 mL of ultrapure water were mixed under stirring in a 100 mL flask for 10 min, and 20 μL of MPS was added into the flask and stirred in the dark for 12 h. Then, 500 μL of TEOS and 500 μL of ammonium hydroxide were sequentially added, and the mixture was stirred for another 12 h. Subsequently, 50 μL of APTS was mixed to the above solution which was vigorously stirred for 12 h again. The resultant QDs@SiO_2 nanoparticles were washed with ethanol three times and further dispersed in 30 mL of ultrapure water for future use.

2.5. Synthesis of ratiometric fluorescent probe QDs@SiO_2-CDs

200 μL of CDs solution, 2.0 mg EDC and 2.0 mg NHS were dissolved into 8 mL of ultrapure water and stirred for 30 min in the dark. Then, 240 μL of QDs@SiO_2 was added into the above mixture and stirred for 12 h at room temperature. Finally, the QDs@SiO_2-CDs probes were collected by centrifugation (2000 rpm, 40 min), and redispersed in 8 mL of ultrapure water for future use.

2.6. Preparation of test paper

A commercial ink cartridge was washed thoroughly with ultrapure water. Then 2 mg of the ratiometric probe in 10 mL of ultrapure water was injected into the vacant cartridge. The commercial microporous membranes without any background fluorescence were sticked on a piece of A4 paper. Afterwards, the probe was printed on the microporous membranes through an inkjet printer connected with a computer. The printing process was repeated 30 times to enhance the amount of probe and make the probe evenly adhere onto the porous membranes. Finally, the filter membranes displayed strong blue fluorescence color under a 365 nm UV lamp.

2.7. Detections of glucose

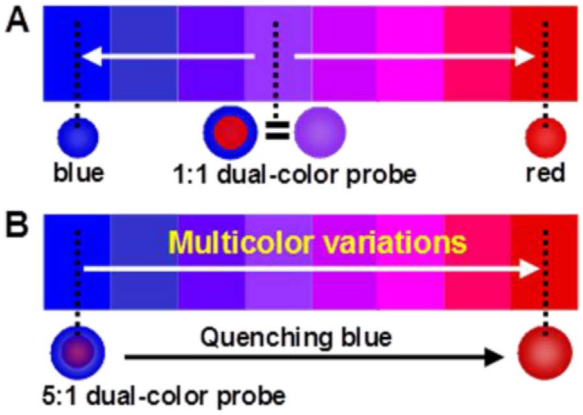
Fluorescent detection of glucose: Different concentrations of glucose and 0.2 mg/mL glucose oxidase were first mixed in HEPES buffer (pH 7.0) at 37 °C for 40 min. Then, the reacted solution was dropped into the mixture of probe and 350 μM Fe^{2+}, and the fluorescent spectra were recorded with a spectrometer.

Detections of blood glucose on test paper: The fresh blood was collected from a healthy volunteer and the serum was obtained by centrifugation to remove blood cells. The serum was diluted and then treated as above procedure by the oxidization of glucose using glucose oxidase with the addition of Fe^{2+}. The reacting mixture was dropped onto the as-prepared test paper, and subsequently the color changes of test paper were observed under a 365 nm UV lamp.

3. Results and discussion

3.1. Colorimetric principles using a dual-color probe

In general, the available ranges of color variation are governed under the red-green-blue (RGB) principle. Scheme 1 illustrates the



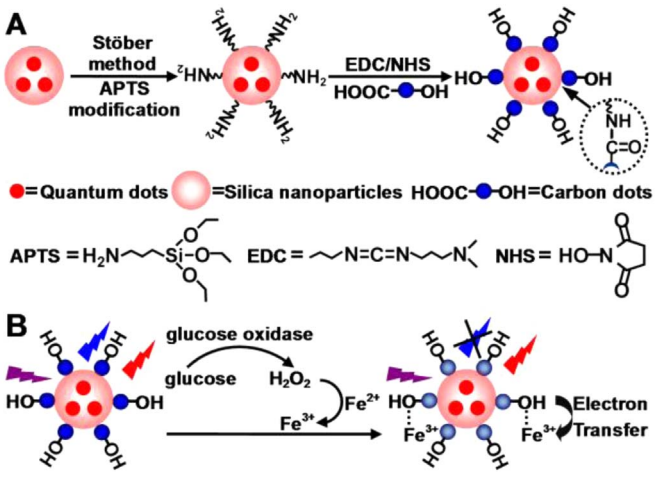
Scheme 1. Two typical color proportions of blue to red in a single dual-emissive fluorescent probe and resultant color-varying ranges in a fluorescent sensor: (A) 1:1 dual-color probe (B) 5:1 dual-color probe. The white arrows indicate the ranges of color variation.

two typical color proportions of blue to red in a single dual-emissive fluorescent probe with a core-shell structure and the resultant color-varying ranges in a fluorescent sensor. The composite proportions of 1:1 blue/red in fluorescent intensity will lead to an intermediate composite purple and thus the maximal range of color variations is either "from purple to red" or "from purple to blue" by fluorescent response to an analyte, as indicated with white arrow in Scheme 1A. That is to say, the formation of intermediate composite color greatly suppresses the theoretical color-varying range of a dual-color probe, which makes the wider range "from blue to red" or "from red to blue" not achieved. When the proportion of blue to red is adjusted to 5:1, the probe still displays a blue, avoiding the formation of intermediate purple (Zhou et al., 2016). Upon quenching the blue with analyte, a wide/consecutive multicolor variation from blue to red can be observed on the as-prepared fluorescent test papers with the dual-emissive probe (Scheme 1B), which will extend the color-varying range in colorimetric assays. In the present work, we have demonstrated the above mechanism by integrating red QDs and blue CDs in a single probe, and achieved the semi-quantitative detection of blood glucose on its as-prepared fluorescent test paper.

3.2. *QDs@SiO_2-CDs fluorescent nanoprobe and detection mechanism of glucose*

Scheme 2A illustrates the synthesis of the dual-emissive ratiometric fluorescent probe QDs@SiO_2-CDs nanoparticles (NPs). The surface of red CdTe QDs was first modified with 3-mercaptopropyltrimethoxysilane (MPS) to direct the growth of SiO_2 at the surface of QDs by the hydrolysis of tetraethylorthosilicate (TEOS), which embedded CdTe QDs into SiO_2 NPs. Subsequently, the QDs@SiO_2 was further functionalized with 3-aminopropyltriethoxysilane (APTS) to obtain a large amount of $-NH_2$ groups at surface. The blue CDs with surface –COOH groups were synthesized by the hydrothermal method using citric acid and ethylenediamine as starting materials (Zhu et al., 2013). The CDs were covalently linked onto the surface of the QDs@SiO_2 by the dehydrated reaction between the surface –COOH and $-NH_2$ groups through the catalysis of EDC and NHS. Here, the used CdTe QDs have a size of 7–8 nm and a red emission at 630 nm (Zhang et al., 2011), and the CDs have a size of 2–3 nm and a blue emissive peak at 445 nm. The ratiometric intensity of blue to red fluorescences can be adjusted to the ratio of 5:1 by controlling the amounts of CDs and QDs. The as-synthesized QDs@SiO_2-CDs probes have an average size of ~80 nm, as revealed by TEM in Fig. S1.

In the structure of QDs@SiO_2-CDs probe, the red fluorescence of



Scheme 2. Schematic illustrations of (A) the synthesis of the dual-emissive ratiometric fluorescent probe QDs@SiO_2-CDs nanoparticles, and (B) the visual detection of glucose by quenching blue emission via Fe^{3+} with the aids of glucose oxidase and Fe^{2+}.

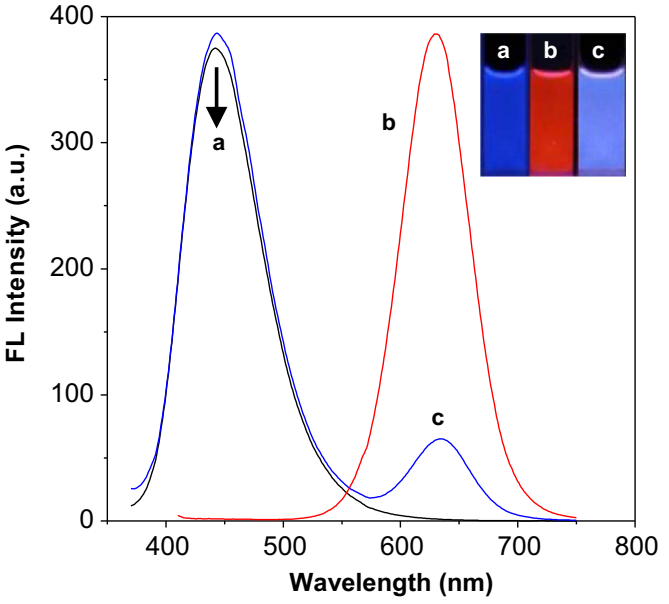


Fig. 1. Fluorescent spectra of (a) CDs, (b) QDs and (c) QDs@SiO_2-CDs. The inset photos were taken under a 365 nm UV lamp.

QDs is stabilized in the interior of SiO_2 and thus plays a role of internal standard, and meantime the blue fluorescence of CDs at the surface of SiO_2 acts as a signaling unit, as drawn in Scheme 2B. It is well known that the glucose can be oxidized under the catalysis of glucose oxidase to produce H_2O_2 that transforms Fe^{2+} into Fe^{3+} ions (Lai et al., 2014). The phenolic hydroxyl groups at the surface of CDs can efficiently capture Fe^{3+} by a coordination reaction. Because the outer electronic structure of Fe^{3+} is $4s^23d^5$, and the d orbits are half filled, the electrons in the excited state of CDs will be easily transferred to the half-filled 3d orbits of Fe^{3+} (Zhu et al., 2013). The two above steps lead to the fluorescence quenching of CDs. Moreover, the emission spectrum of CDs and the absorption spectrum of Fe^{3+} show little spectral overlap with each other (Fig. S2), so the resonance energy transfer should not be the dominant mechanistic pathway (Mei et al., 2012). The quenching of blue fluorescence of CDs and the stability of red fluorescence of QDs cause a gradual color evolution from blue to red for the visualization assay of glucose.

Fig. 1 shows the fluorescent spectra of CDs, QDs and QDs@SiO_2-

CDs, in which the two emissive peaks of QDs@SiO_2-CDs probe at 445 and 630 nm completely corresponds to those of individual CDs and QDs, respectively. In the fluorescent spectra of probe, we can see that the emissive intensity of blue fluorescence is 5 times that of red fluorescence. Under a UV lamp, CDs and QDs emit strong blue and red fluorescences, respectively, and QDs@SiO_2-CDs display a sky blue. These confirm that QDs were successfully embedded into SiO_2 NPs and CDs were linked onto the surface of SiO_2 NPs, and the ratiometric fluorescence was controlled at a proportion of 5:1 of blue to red.

Before the use of probe, the stability of fluorescence of probe was investigated by the successive exposure to 365 nm ultraviolet light for 15 min each time. The value of I_{445}/I_{630} almost kept constant, suggesting the good photostability of the ratiometric fluorescent probe (Fig. S3).

3.3. Fluorescent response and selectivity of QDs@SiO_2-CDs to Fe^{3+}

Here, we use the QDs@SiO_2-CDs probe with the 5:1 fluorescence intensity ratio of blue to red for this current investigation. QDs@SiO_2-CDs probes display blue color in aqueous solution as the fluorescence of CDs is much stronger than that of QDs (the inset of Fig. 2A). Upon the addition of different concentrations of Fe^{3+}, the blue fluorescence of the probe gradually decreases, whereas the red fluorescence keeps constant (Fig. 2A). With the concentration of Fe^{3+} up to 300 μM, the fluorescence intensity of CDs becomes weaker than that of QDs, which leads to the remarkable change of color from blue to red under a UV lamp. Although the blue fluorescence is not completely quenched, our eye is more sensitive to red. The fluorescence ratio I_{445}/I_{630} is linearly correlated with the concentration of Fe^{3+} in the range of 20 − 100 μM (R^2=0.991) (Fig. S4). The detection limit of Fe^{3+} is calculated to be 2.3 μM by 3σ rule. In addition, similar fluorescence quenching of pure CDs was also observed by the addition of Fe^{3+} (Fig. S5), confirming the above fluorescent response mechanism. The dynamic response of QDs@SiO_2-CDs to Fe^{3+} revealed that the reaction was rapidly completed in 3 min (Fig. S6).

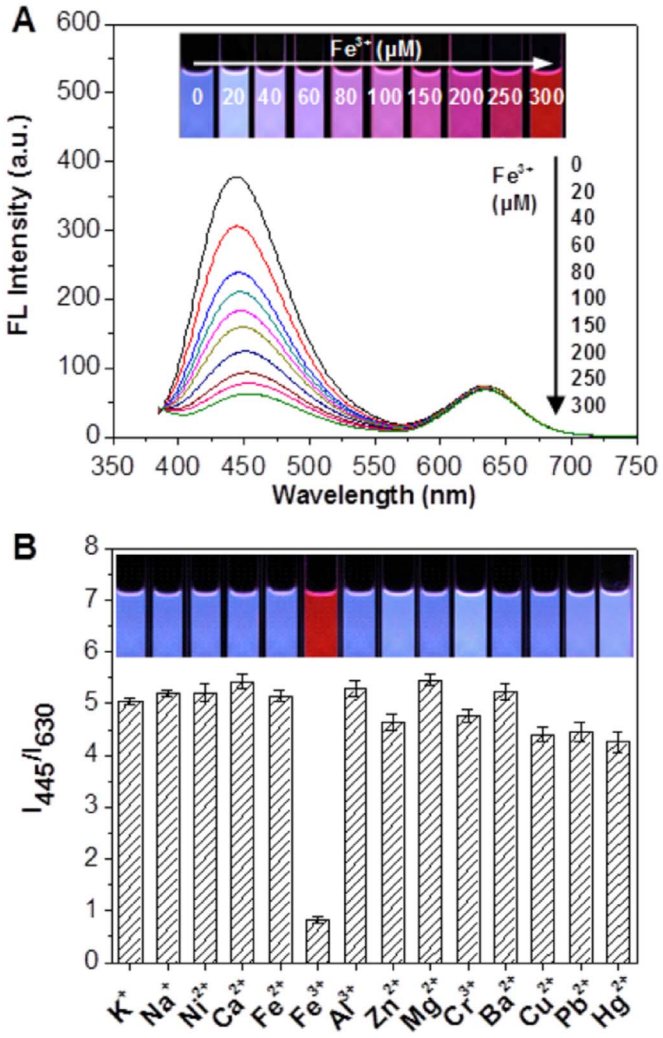


Fig. 2. (A) The fluorescent spectra (λ_{ex}=360 nm) of the ratiometric probe as a function of the Fe^{3+} concentration. (B) The selectivity of the ratiometric probe to various metal ions (300 μM). The insets show the corresponding fluorescence colors under a 365 nm UV lamp. Here, I_{445}/I_{630} is the ratio of fluorescence intensities of CDs to QDs.

The selectivity of probe to Fe^{3+} was carefully examined by the comparison with other metal ions. As shown in Fig. 2B, the fluorescence intensity I_{445}/I_{630} of probe can be quenched by 85% in the presence of 300 μM Fe^{3+}, while the other metal ions do not have significant influence on I_{445}/I_{630} in the same condition. Meanwhile, the colors of solution keep blue with the addition of other metal ions, but the addition of Fe^{3+} transforms the color into bright red under a UV lamp (the inset of Fig. 2B). Furthermore, the fluorescent response of probe to Fe^{3+} is not influenced by the coexistence of other metal ions (Fig. S7). These above results indicate excellent selectivities in both ratiometric fluorescence and color change to Fe^{3+}.

3.4. Fluorescent response and selectivity of QDs@SiO_2-CDs to glucose

According to the glucose detection mechanism in Scheme 2B, we tested the ratiometric fluorescent response of probe to the oxidization reaction of glucose through the following procedure. Glucose was first oxidized in HEPES buffer at 37 °C for 40 min (Fig. S8) in the presence of glucose oxidase that kept its activity in 2 months in the −20 °C refrigerator (Fig. S9). Then, the reacted solution was dropped into the mixture of probe and Fe^{2+}. The H_2O_2 resulting from the oxidization of glucose transformed Fe^{2+} into Fe^{3+} that quenched the fluorescence of CDs, however, individual H_2O_2 or Fe^{2+} did not quench the fluorescence of CDs (Fig. S10). Here, we further determined the optimized dosage of glucose oxidase by the test of half maximum response (HMR) of the ratiometric probe (Fig. S11).

Fig. 3A shows that the fluorescent peak from the CDs of QDs@SiO_2-CDs probe gradually decreases with the increase of glucose concentration. Meanwhile, the fluorescent peak from QDs of probe keeps almost unchanged. The fluorescence intensity ratio I_{445}/I_{630} is linearly related to the glucose concentration in the range of 15 − 75 μM (R^2=0.989) (Fig. S12). The calculated detection limit is 3.0 μM according to the 3σ rule. Consecutive color variations from blue to red with the dosage of glucose can be seen under a 365 nm UV lamp. That is to say, the ratiometric fluorescent probe can be used for the detection of glucose. It should be noted that although the HMR to glucose kept unchanged at different ratios of blue to red in the composite probe (Fig. S13), only could the 5:1 ratio of blue to red achieve the widest and consecutive color evolution from blue to red, as illustrated in Scheme 1B.

The selectivity of the probe for glucose detection was also studied by monitoring the change of ratiometric fluorescence intensity of the solution by the addition of various physiological compounds, including saccharides, vitamin, amino acids, carbamide, and various anions (200 μM) (Fig. 3B). While the I_{445}/I_{630} kept almost unchanged, the fluorescent color of solution was still the original blue under a UV lamp (the inset of Fig. 3B). This is mainly because glucose oxidase only specifically catalyzes the oxidization of glucose to release H_2O_2.

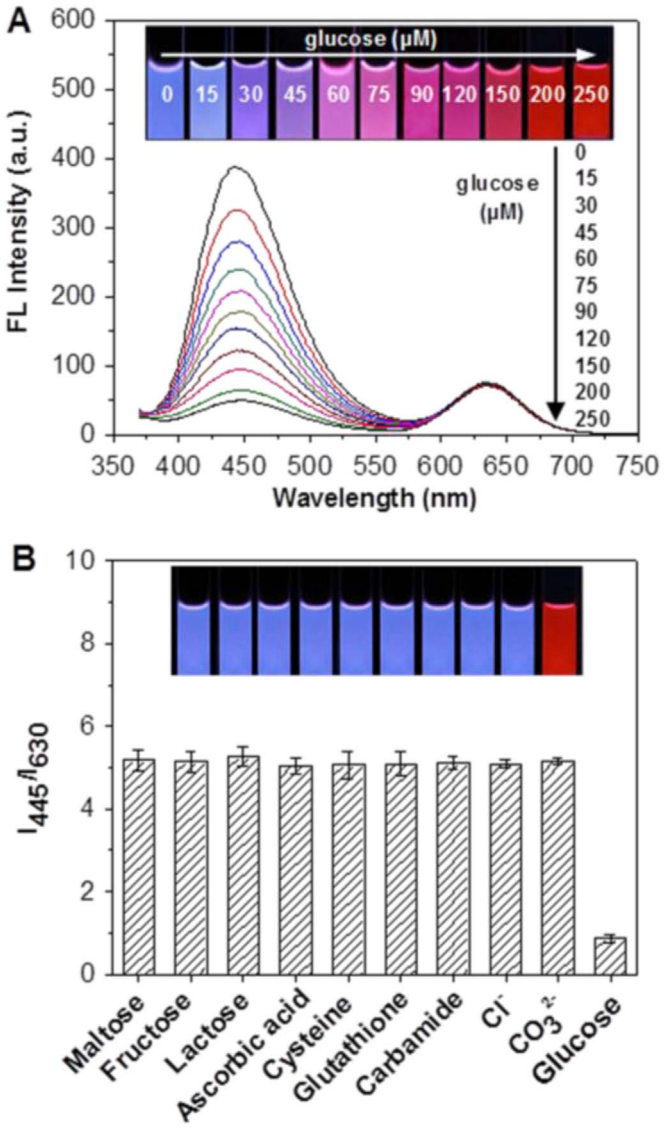
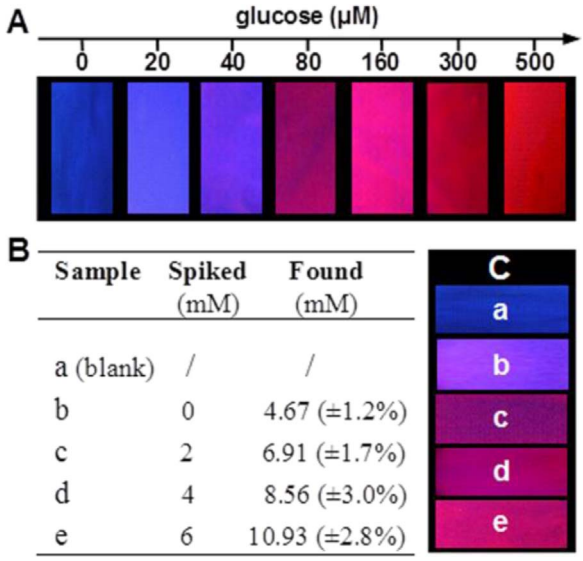


Fig. 3. (A) The fluorescent spectra (λ_{ex}=360 nm) of the ratiometric probe as a function of the glucose concentration. (B) The I_{445}/I_{630} of the ratiometric probe with the additions of 200 μM maltose, fructose, lactose, ascorbic acid, cysteine, glutathione, carbamide, Cl^-, CO_3^{2-} and glucose. The insets show the corresponding fluorescence colors under a 365 nm UV lamp. I_{445}/I_{630} is the ratio of the fluorescence intensities of CDs to QDs.



Sample	Spiked (mM)	Found (mM)
a (blank)	/	/
b	0	4.67 (±1.2%)
c	2	6.91 (±1.7%)
d	4	8.56 (±3.0%)
e	6	10.93 (±2.8%)

Fig. 4. (A) The visual detection of glucose using the fluorescent test papers. The photos were taken under a 365 nm UV lamp. (B) The recovery tests of glucose in human serum, a: blank, b: fresh human serum, c–e: human serum spiked with 2, 4, 6 mM glucose, respectively. Each sample was diluted 200-fold before measurements. The recovery of glucose was determined by the linear relation of I_{445}/I_{630} with the concentration of glucose. (C) The corresponding paper-based visual detection of above human serum samples. Each sample was diluted 100-fold before the addition onto test papers. The photos were taken under a 365 nm UV lamp.

3.5. Detection of blood glucose on test papers

In the present work, the fluorescent test papers were prepared using QDs@SiO_2-CDs probe as fluorescent ink through an inkjet printer connected with a computer. The repeating printing 30 times led to the enough/even coverage of probe on a piece of cellulose acetate membrane. The test paper was stored in the 4 °C refrigerator and could be stable in 30 days (Fig. S14). After glucose was oxidized in the presence of glucose oxidase, Fe^{2+} ions were added into the system. Subsequently, the mixing solution was dropped onto the as-prepared test papers. After 3 min (Fig. S15), the fluorescent colors of test papers were observed under a UV lamp (Fig. 4A). Apparently, the color change from blue to cornflower blue to purple to purplish red to pink to rose red to deep red was observed with the dosage increase of glucose from 0 to 500 μM. The consecutive color variations were easily distinguished at these typical dosages of glucose with our eye.

Moreover, we examined the effectiveness and reliability of test paper in the application for the detections of blood glucose in human serum. The fresh blood was collected from a healthy volunteer and the serum was obtained by centrifugation to remove blood cells. The content of glucose in the serum sample is 4.8 mM measured with Beckman Coulter AU5800 in hospital. The ranges of blood glucose concentration in healthy people and diabetics are 3–8 and 9–40 mM, respectively (Badugu et al., 2004; Shi et al., 2011). After the addition of glucose with different concentrations, the serum was diluted and then treated as above procedure with oxidization of glucose. The recoveries of glucose by the ratiometric fluorescence I_{445}/I_{630} of probe are in good agreement with the amounts of glucose in the serum samples (Fig. 4B). The relative standard deviations are all around 3%.

The same samples were tested with the fluorescent test papers, and observed under a UV lamp. As shown in Fig. 4C, the colors of test papers gradually changed from the original blue to pink with the increase of glucose in serum. Surprisingly, the concentrations of glucose, 0, ~5, ~7, ~9, ~11 mM correspond to blue, purple, purplish red, scarlet and pink, respectively, indicating a very sensitive dosage-discerning capability by our naked eye. Moreover, the spontaneous glucose (4.8 mM) in the original serum of a healthy volunteer makes the test paper display a purple (photo b in Fig. 4C). Importantly, the test paper can give a clear judgment whether our blood glucose is at a normal or abnormal level for the clinical diagnosis of diabetes.

It should be noted that the background Fe^{2+} in human serum may produce the interference on the detection of blood glucose. In general, Fe^{2+} concentration in human serum is at the level of 7–14 μM. In our experiments, the human serum was diluted at least 100 times before test, and thus the background Fe^{2+} was only 0.07–0.14 μM, which was much lower than our used Fe^{2+} (350 μM) in the reaction system. Accordingly, the interference of background Fe^{2+} in serum can almost completely be omitted.

4. Conclusions

In summary, we have designed a single dual-emissive ratiometric fluorescent probe with blue and red emissions, and

proposed a novel mechanism of glucose detection by the change of ratiometric fluorescence and the discernable color responses with the dosage of glucose. The probe integrated the high hydrophilic property, supersensitive surface, stable internal standard emission, and optimized dual-emissive intensity ratio to achieve the visual color response to glucose. By the comparison with previous reports for the detection of glucose, the advantages of this assay in the present work are listed as below. (1) Our design of dual-emissive ratiometric probe greatly improves the sensitivity of detection, in which the glucose in normal blood even diluted 100 times also can be clearly detected. (2) The dual-emissive ratiometric fluorescence achieves a dosage-sensitive consecutive color variation for the clear visualization of glucose. (3) The high-quality test paper fabricated by printing has a satisfying performance for the detection of blood glucose in real samples, which can give a clear judgment whether our blood glucose is at a normal or abnormal level. The results reported here prospect the bright future of fluorescent test paper for the feasible and inexpensive applications in clinical medical diagnosis.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2016.07.021.

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