Boronic Acid Functionalized Aza-Bodipy (azaBDPBA) based Fluorescence Optodes for the Analysis of Glucose in Whole Blood

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Ⓢ Supporting Information

ABSTRACT: A near-infrared fluorescent dye (aza-bodipy or azaBDPBA) functionalized with boronic acid groups was synthesized for the preparation of optodes to measure glucose in 40-fold diluted whole blood. Boronic acid groups as an electron deficient group on aza-bodipy was reacted with hydrogen peroxide into an electron-rich phenolic group leading to the red-shift of emission wavelength from 682 to 724 nm. The emission in near-infrared region offered a low level of background interference from whole blood. Also, the dual-wavelength emission guaranteed our probe to measure glucose in whole blood accurately after the conversion of glucose into hydrogen peroxide using glucose oxidase. The measuring range of glucose from 0.2 to 200 mM in the buffer was achieved with high selectivity. To facilitate the blood test, the probe was immobilized into thin hydrophobic polymer films to prepare the disposal glucose optode, which could detect glucose in the solution from 60 μM to 100 mM. The concentration of glucose in 40-fold diluted whole blood was determined using our optode and the reference method, respectively. The consistence in the concentration obtained from these two assays revealed that our azaBDPBA-based optodes were promising for the clinic assay of glucose in the whole blood.

KEYWORDS: fluorescence optode, dual-wavelength emission, near-infrared, glucose, whole blood, aza-bodipy
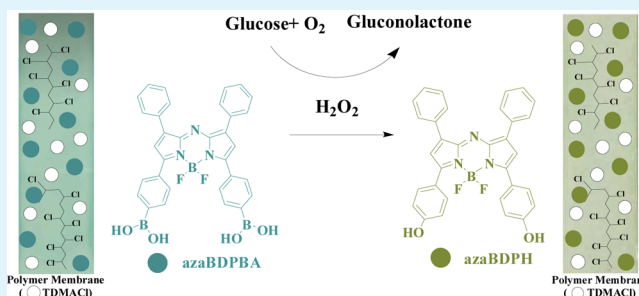
INTRODUCTION

The determination of glucose in whole blood has attracted much attention due to its importance in diagnosing diabetes.[1] The electrochemical glucose assays are popular;[2,3] however, electrochemical measurement often requires additional calibration, a special compartment, and service. Therefore, for more sophisticated clinical analyzers, most analyses are spectrophotometric. Also, an optical technique with the unique advantages of high throughput and avoiding strong electromagnetic situations in in vivo detection is needed for the clinic study. Because fluorescence technique offers high sensitivity and convenience, numerous fluorescent probes for the detection of glucose have been attempted.[4−6] The main detection strategy includes the generation of hydrogen peroxide from glucose using glucose oxidase and the following fluorescence measurement of hydrogen peroxide.[7,8] Although the fluorescence detection of glucose was achieved, further application in whole blood was restricted by the strong background absorption, autofluorescence, and scattering observed from whole blood.[9,10] Therefore, developing a novel fluorescence probe to realize direct measurements of glucose in whole blood is significant and challenging. Considering relatively low levels of background interference in the near-infrared (NIR, 700−1000 nm) region from whole blood and good penetration of NIR light through skin, a NIR fluorescence probe is critical.[11,12] Moreover, due to the complexity of whole blood, the NIR

fluorescence probe should provide the ratiometric or dual-wavelength fluorescence emission for more accurate detection.[13−16] Unfortunately, a NIR fluorescence probe that offers these signals has not been prepared, a sensor for the accurate fluorescence assaying of glucose in whole blood is missing.

Boron-dipyrromethene (Bodipy) dyes are well-known to be highly fluorescent under UV light, very stable, and amenable to structure modification.[17] The substitution of the methine-bridged carbon atom by nitrogen leads to a so-called aza-bodipy with a bathochromically shifted absorption of about 90 nm with respect to the analogous derivative, which has a NIR excitation wavelength.[18] To achieve the specific biosensing, electron-deficient groups were modified on aza-bodipy.[19] Upon reacting with the targets, the electron-deficient groups were converted into electron-rich groups, resulting in a different absorbance/excitation energy level from aza-bodipy.[20] Therefore, aza-bodipy could be an ideal base to provide ratiometric or dual-wavelength fluorescence emission in NIR for glucose assay. In this study, boronic acid groups were incorporated as the electron-deficient group on aza-bodipy. As proposed in Figure 1A, the presence of hydrogen peroxide converted phenyl-boronic acid groups into electron rich phenolic group so that
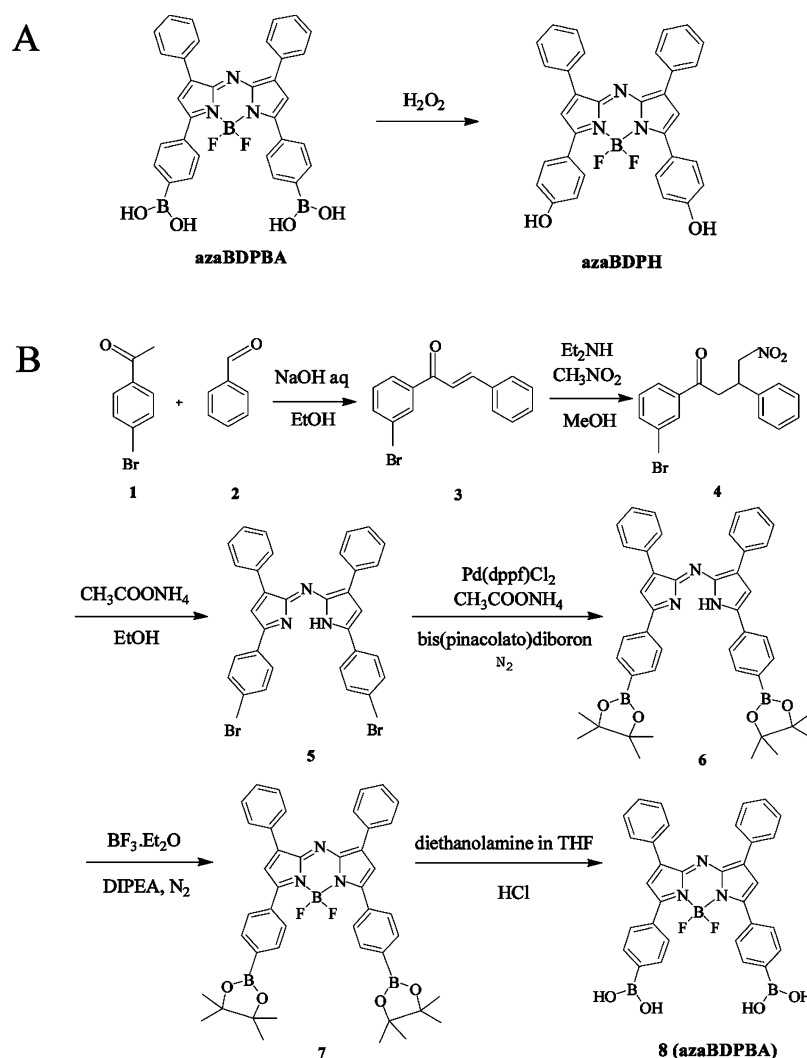
Figure 1. (A) The detection mechanism and (B) synthesis scheme of azaBDPBA.

the emission wavelength could be shifted to provide dual-wavelength emission spectrum for the analysis of hydrogen peroxide. Because glucose oxidase (GOx) generated hydrogen peroxide from glucose, this probe should offer the accurate measurement of glucose in whole blood. As compared with the direct analysis of glucose using a boronic-acid-based probe,[21] our probe offered a higher selectivity because the hydrogen peroxide detected was generated from glucose only by glucose oxidase. Moreover, considering the advantages of polymeric optodes over homogeneous system, such as continuous monitoring and miniaturized sensors eliminating interferences,[22] the probe was immobilized into thin hydrophobic polymer films to prepare disposal optode for the assay of glucose in whole blood.

■ EXPERIMENTAL SECTION

Materials and Instrumentation. All chemicals used for synthesis and detection were commercially available and used as received. Rabbit plasma and sheep whole blood were both purchased from Nanjing Bianzhen Biotechnology (China). PerkinElmer LS50B fluorescence spectrometer and Enspire Multimode Reader (PerkinElmer) were utilized for fluorescence measurements.

Synthesis of azaBDPBA. The detailed synthesis procedure and characterization of azaBDPBA are shown in Supporting Information.

Preparation of Glucose Optode. First, 100 mg of membrane components containing 1 mmol/kg azaBDPBA, 1 mmol/kg TDMACl, PVC, and plasticizer DOS (1:2 by weight) was dissolved in 1.0 mL of THF. After complete dissolution, an 80 μL aliquot of the cocktail was pipetted onto a quartz plate (45 × 11 mm) uniformly, and the solvent was evaporated thoroughly to get the glucose optode.

Assay of glucose in buffer and whole blood. To measure glucose in solution, phosphate buffer saline (PBS, pH 7.4)/ethanol (v/v 1:1) solution containing 10 μM azaBDPBA, glucose oxidase (5 U mL^{-1}) and glucose were prepared. Then, 200 μL of the above solutions were transferred into U-bottomed 96-microwell polypropylene plates. The plates were covered with highly transparent film (VIEWseal, Greiner, Germany) to prevent solvent evaporation. The sealed wells were monitored successively between requisite intervals of shaking (shake mode, orbital; speed, 60 rpm; diameter, 10 mm; T, 38 °C). The excitation wavelength was 655 nm. Each data point was the average of at least six determinations.

For the assay of glucose in whole blood, the blood was 40-fold diluted in volume with required buffer solutions and used immediately. Different amounts of glucose were spiked separately into the sample (4 mL) containing 10 μM azaBDPBA and 5 U mL^{-1} GOx. After being conditioned at 38 °C for 10 min, the mixture was transferred into a 4 mL quartz cuvette for fluorescence measurements. The top of the quartz cuvette was sealed by pearl film to obtain a closed system. For comparison, plasma and whole blood were tested on the same day by automatic biochemical analyzer (HITACHI 7080, Japan) and Roche glucose meter (ACCU-CHEK Performa, Germany), respectively.

Assay of Glucose Using Optode. The quartz plates with the prepared film were inserted into custom-made quartz cuvette with two slots. The slots were designed to fix the plate to obtain the stable fluorescence signals. The films were incubated in PBS (pH 7.4, 2.5 mL)/ethanol (v/v 1:1) at 38 °C in the presence of glucose oxidase (5 U mL^{-1}) until the stable fluorescence intensities were obtained. The standard addition experiments were carried out by successively adding glucose to rabbit plasma (2.5 mL) with glucose oxidase in quartz cuvette. The samples were incubated at 38 °C for 10 min before the fluorescence measurement.

■ RESULTS AND DISCUSSION

Synthesis of azaBDPBA. Boronic acid functionalized aza-Bodipy (azaBDPBA, compound 8) was prepared according to Figure 1B. The conversion of bromide atoms in aza-bodipy structure into boronic acid group was achieved through Suzuki coupling.[23] The hydrolysis of azabodipy-boronic acid ester using conventional method with HCl gave the final product with a high yield of 73%. The ^{1}H NMR, ^{13}C NMR and mass spectra of azaBDPBA are shown in Figure S1 (Supporting Information). The maximum excitation and emission wavelengths were determined to be 655 and 682 nm, respectively. The quantum yield in methanol reached 0.60 using Nile blue as the reference dye. As compared with other aza-bodipy derivatives,[20,24−26] the relatively high quantum yield guaranteed strong fluorescence, which benefited the following glucose detection in whole blood.

Response of azaBDPBA to Glucose in Buffer. The key for the application of azaBDPBA to glucose assay was that the probe was responsive for hydrogen peroxide. Therefore, the fluorescence change of azaBDPBA upon the reaction with hydrogen peroxide was studied. Due to the limited solubility of the probe, PBS (pH 7.4)/ ethenol (v/v 1:1) solution was used to contain 10 μM probe. In presence of hydrogen peroxide, the emission at 682 nm from azaBDPBA decreased, while a new peak at 724 nm increased (Figure 2A). The LC-mass spectrum results confirmed the conversion of boronic acid to phenol

group (Figure S2, Supporting Information), which supported that the generation of phenol group on aza-bodipy. Two emission wavelengths at 682 and 724 nm revealed that our probe could provide dual-wavelength fluorescence signal for the detection of hydrogen peroxide. The detection range of hydrogen peroxide was determined to be 0.2−500 mM (Figure S3, Supporting Information). The successful detection of hydrogen peroxide in the NIR region offered the feasibility of glucose assay in whole blood by applying glucose oxidase.

Figure 2B show that the addition of glucose oxidase into the solution with azaBDPBA and glucose gave the similar fluorescence changes of two emission peaks at 682 and 724 nm, respectively. The fluorescence change revealed that the reaction of glucose and glucose oxidase generated hydrogen peroxide resulting in the fluorescence recognition by azaBDPBA. Because the fluorescence intensities depended on the amounts of probe and product, a gradual decrease in the ratio of fluorescence intensity at 682 and 724 nm was observed with time, as shown in Figure S4 (Supporting Information). When the time was over 8 min, stable fluorescence signals in pH of 7.4 at 38 °C were obtained. The response time of 8 min was much shorter than the time reported (typically 15−60 min) by the other fluorescence probes.[27,28] The faster reaction rate will minimize the time for the whole assay and thus be significant for the clinic test. The calibration curve of glucose in solution exhibited the measuring range of glucose from 0.2 to 200 mM, as shown in Figure 2C. Considering the normal blood glucose concentration in the range of 3−8 mM and abnormal glucose levels between 9 and 40 mM,[29,30] the detection ability of azaBDPBA was suitable for the assay of glucose in whole blood. Also, the pre-existing hydrogen peroxide generated from the biological process in the body was reported to be as low as micromolar[31] and should not induce the significant deviation on our measurement.

The selectivity of the probe was studied by measuring the fluorescence of the solution in the presence of various saccharides including fructose, galactose, sorbitol, and sucrose. Although the boronic acid group was capable of reacting with saccharides (fructose mainly) to form borate ester, the ester formation led to the emission maximum change of the probe from 682 to 695 nm (Figure S5, Supporting Information). The distinct difference from the fluorescence change caused by hydrogen peroxide or glucose provided high selectivity of our probe. Figure 2D shows that the selectivity toward glucose was more than 9-fold greater than other saccharides by monitoring the intensity ratio at 682 and 724 nm. It was noted that the concentrations of interfering saccharides, such as fructose and galactose, in blood were much lower than glucose so that the accuracy of our assay was guaranteed. Also, the fluorescence responses to glucose in the absence and presence of saccharides were compared. As shown in Figure S6 (Supporting Information), similar responses were observed after 8 min, which supported that saccharides did not interfere with the assay. Other interferences commonly existed in electrochemical assay such as ascorbic acid, acetaminophen, tyramine, and uric acid were introduced into the solution. According to the high selectivity of azaBDPBA toward hydrogen peroxide, no effect on the fluorescence spectrum was observed. All these results supported that the proposed assay can be applicable for highly sensitive glucose analysis in whole blood.

Response of azaBDPBA to Glucose in the Diluted Whole Blood. Direct fluorescence measurement of glucose in 40-fold diluted sheep whole blood was performed by spiking
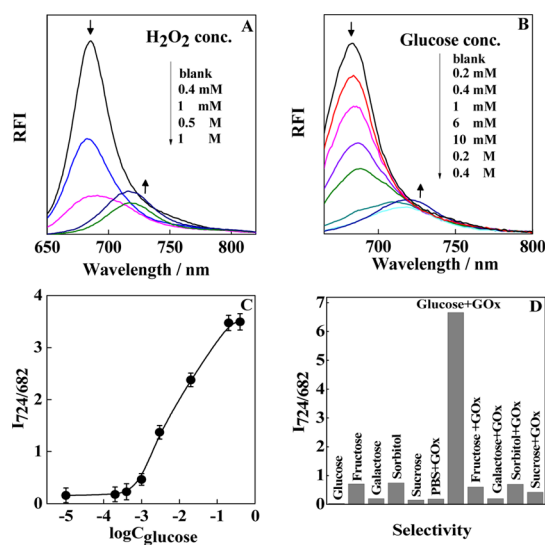

Figure 2. Relative fluorescence intensity (RFI) of azaBDPBA to (A) H$_2$O$_2$ at concentrations of 0, 0.4 mM, 1 mM, 0.5 Mn and 1M; (B) glucose at concentrations of 0, 0.2 mM, 0.4 mM, 1 mM, 6 mM, 10 mM, 0.2 M, and 0.4 M; (C) titration curve relation to aqueous glucose from 0 to 0.4 M; and (D) the selectivity over competitive saccharides (20 mM) in the absence and presence of GOx.

Table 1. Determination of Glucose Level in Rabbit Plasma and Sheep Whole Blood Samples with azaBDPBA-based Optodes and Comparison with Reference Methods

	sample	results by reference methods (mM)	results by azaBDPBA-based assays (mM)	recovery (%)	RSD (%)
homogeneous assay	plasma[a]	0.80	0.78 ± 0.03	97.5	3.2
		4.82	4.73 ± 0.98	98.1	3.9
		8.78	8.69 ± 0.25	99.0	2.8
		9.70	9.72 ± 0.18	100.2	1.8
	whole blood[b]	2.1 ± 0.1	2.18 ± 0.01	109.2	0.6
		4.0 ± 0.2	4.10 ± 0.10	107.9	2.1
		6.3 ± 0.6	6.22 ± 0.29	101.9	4.6
		8.4 ± 0.7	8.51 ± 0.11	101.4	1.3
optodes	whole blood[b]	1.8 ± 0.0	1.78 ± 0.09	98.9	5.0
		2.5 ± 0.1	2.64 ± 0.03	101.5	1.0
		3.6 ± 0.1	3.69 ± 0.15	105.5	4.0
		6.2 ± 0.6	6.28 ± 0.05	100.7	0.8

[a]Plasma samples were analyzed by HITACHI 7080 (Japan); all data are the average of three measurements. [b]Whole blood samples were analyzed by Roche glucose meter (ACCU-CHEK Performa, Germany); all data are the average of three measurements.

the glucose oxidase in the blood with azaBDPBA. Compared with the fluorescence spectrum of blood in absence of glucose oxidase, the emission peak at 686 nm decreased, and the new peak came out at 716 nm after the addition of oxidase, as shown in Figure S7 (Supporting Information). The new emission wavelength at 716 nm was a little different from that at 724 nm observed from the previous aqueous reaction, which might be attributed to the complex biological background, for example, the existence of large amounts of hemoglobin (about 20 mM) in the red cells.[32] The explanation was supported by the restoration of emission peaks at 686 and 724 nm in plasma without red cells, as shown in Figure S8 (Supporting Information). To establish the calibration curve for quantitative measurement of glucose in whole blood, various concentrations of glucose were injected into the blood. Because the linear increase of intensity ratio at 686 and 716 nm was obtained (Figure S9, Supporting Information), the concentration of glucose in whole blood can be determined from the calibration curve. To investigate the accuracy of our assay, the concentrations of glucose in whole blood and plasma using azaBDPBA and reference method were measured, respectively. As summarized in Table 1, good consistency in the concentration and recovery revealed the success of azaBDPBA in the assay of glucose in the diluted whole blood.

Response of azaBDPBA Based Optodes to Glucose in the Diluted Whole Blood. To achieve azaBDPBA based optodes for the measurement of glucose in whole blood, we further immobilized azaBDPBA into thin hydrophobic polymer films to prepare disposal optode. Figure 3A showed the
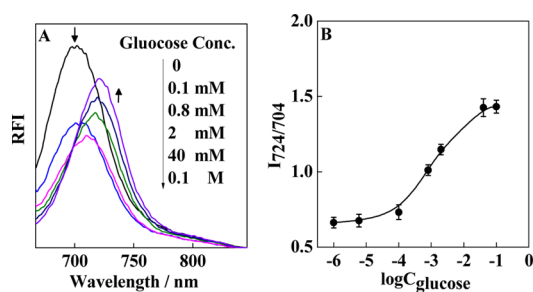
emission peak at 704 nm from the polymeric optode upon the excitation of 655 nm. After the exposure of the optode to the solution with glucose and glucose oxidase, the fluorescence intensity with peak wavelength at 704 nm decreased, while, the intensity at 724 nm increased. Compared with the emission wavelengths in aqueous solution, the wavelength discrepancy could be ascribed to the different polarity of the surrounding matrix.[9] To investigate the influence of the film thickness on the assay kinetics, the films with different thickness were prepared by dropping various amounts of cocktails on the plates. The minimal amount of cocktail to cover the plate was 20 μL. As shown in Figure S10 (Supporting Information), the thinner film offered the shorter time to reach the steady state response. Therefore, 20 μL was applied to prepare the optode for the following experiments. Similar to the previous aqueous assay, glucose concentration in a dynamic range from 60 μM to 100 mM was determined, as shown in Figure 3B. The lower detection limit from the optode indicated that the reaction between azaBDPBA and hydrogen peroxide was more preferred in organic phase than in the aqueous solution. The relative standard deviations were less than 4%, calculated from three spots on the optode exposed to the same concentration of aqueous glucose. These identical responses will permit the future high-throughput glucose screening using our optode. The blood samples were remeasured by the prepared optodes, and the results were compared with those from commercial glucose meters, as shown in Table 1. The analysis accuracy, recoveries, and relative standard deviation for whole blood agreed with the clinically allowable percentage (%) error, which exhibited that the optode could analyze glucose in the diluted whole blood accurately.

CONCLUSIONS

To summarize, an optode based on a new type of NIR fluorescence probe for glucose assay was developed. This fluorimetric detection system utilizing enzymatic mechanism and boronic acid oxidation offers excellent selectivity for glucose over competing saccharides owing to the double-wavelength fluorescence emission and enzyme specificity. The good consistency in the determination of glucose in the diluted whole blood was obtained using our optode and the reference method, which exhibited that our optode could analyze blood glucose accurately. Future work will focus on the optimization of molecular structure to achieve the ratiometic measurement

Figure 3. (A) Fluorescence spectra of optode to glucose at the concentration of 0, 0.1 mM, 0.8 mM, 2 mM, 40 mM and 0.1 M; (B) titration curve of optode to glucose.

of glucose in whole blood. Also, our optode will be applied on the high-throughput detection of blood glucose by imaging the blood samples on the film. The achievement of glucose assay in whole blood using fluorescence signal will eventually speed up the clinic test.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis procedure and characterization of azaBDPBA; LC-mass spectrum of azaBDPH; and additional figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b00265.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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