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Highly specific-glucose fluorescence sensing based on boronic anthraquinone derivatives via the GOx enzymatic reaction

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ABSTRACT

The boronic acid anthraquinones, *o*-**HAQB** and *p*-**HAQB**, have been designed, and demonstrated to serve as fluorogenic biosensors for glucose. The sensory molecule, *o*-**HAQB**, has exhibited the specific-glucose sensing via the GOx enzymatic reaction. In this contribution, the fluorescence changes of *o*-**HAQB** reasonably correspond to the concentration of glucose upon the conversion of boronic acid to hydroxy based sensor by H_2O_2 generated from glucose along with GOx enzymatic reaction. Our sensing ensemble was then successfully applied to determine the glucose concentration in the range of 0.08–0.42 mM. The limit of detection (LOD) of *o*-**HAQB** for glucose detection using the GOx enzymatic probe is approximately 0.011 mM.

1. Introduction

Diabetes is a disease, which involves abnormal blood sugar metabolism due to inadequate insulin production. Blood sugar, especially glucose, has regularly been used to diagnose diabetes for over a century. Nowadays, the capability to accurately monitor blood glucose concentrations has proven essentially for diagnosing and curing many metabolic disorders, mainly diabetes.[1] Therefore, reliable and accurate saccharide sensors are continually developed in order to search for better ones for quantitative analysis and detection of saccharides and their derivatives in physiological media. Moreover, the indispensability of glucose monitoring in the incurable disease is a critical requirement for determination of the glucose levels in nondiabetic acute care patients because high variations in blood glucose level have also been observed in nondiabetic patients as a common sign of acute illness. Patients can modify their lifestyle to better control their diabetes and can use insulin in achieving effective dosing and timing.[2] The important demands for continuous, accurate, and relatively noninvasive glucose sensing methods have motivated methodologies as well as the design of various sensing materials.[3] For specific-glucose sensing, various analytical methods such as amperometry, potentiometry, spectrophotometry, or fluorometry are utilized for the enzymatic measurements of glucose concentration.[4] The traditional commercial glucose test is electrochemical, including a monitor based on the electrical wiring of glucose oxidase, which underlies the direct conversion of the concentration-dependent glucose flux to an electrical current. In this method, the generated hydrogen peroxide is oxidized under a constant working potential, and the extent of oxidation corresponds to the glucose concentration.[5] However, it needs careful calibration because other oxidized products may be easily adsorbed onto the metal electrode surfaces, causing a decrease in activity of the electrode surface and the sensitivity will dramatically decrease.[6]

The development of specific sensors for biochemically relevant analytes via chemical reaction has high impact and challenging. Fluorescence sensors with preferential binding to glucose have been developed in the past decade. The obvious advantages of a fluorescence sensor are the extreme sensitivity and lack of damage to the host system. The methodology involves the incorporation of synthetic based receptors, such as boronic acid. Boronic acid based sensors are the best candidate for saccharide probes due to the covalent binding properties of boronic acid with the diol group of sugars in water as widely reported for the last decade.[7] Moreover, most boronic acid derivatives based fluorophores showed the fluorescence emission band at a relatively long wavelength, which is an important role in detecting glucose in blood or serum samples. However, the disadvantages of some boronic acid based fluorescence sensor are the selectivity and a high pK_a values, which is not appropriate for physiological pH.[8] Moreover, a number of publications have reported the glucose-specific

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fluorescence changes with the interference of other saccharides especially fructose and ribose.[9] A few reports exist regarding the selective fluorogenic boronic acid for glucose sensing.[10] As the basic knowledge of boronic acid, aryl boronic acid and boronic ester react selectively with H_2O_2 to produce a phenolic compound.[11] To overcome the disadvantage of using a boronic-based fluorescence sensor for selective detection of glucose, we took an effort to combine the basic knowledge of the specific-glucose sensing by GOx enzymatic reaction with the new boronic-based anthraquinone for fluorescence changes. Our proposition for the specific-glucose fluorescence sensor via the GOx enzymatic reaction is schematically illustrated in Fig. 1.

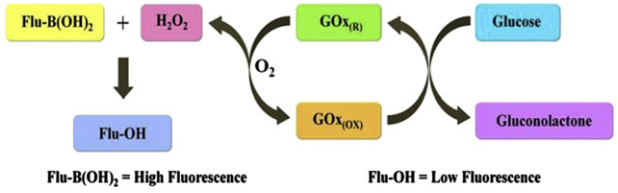


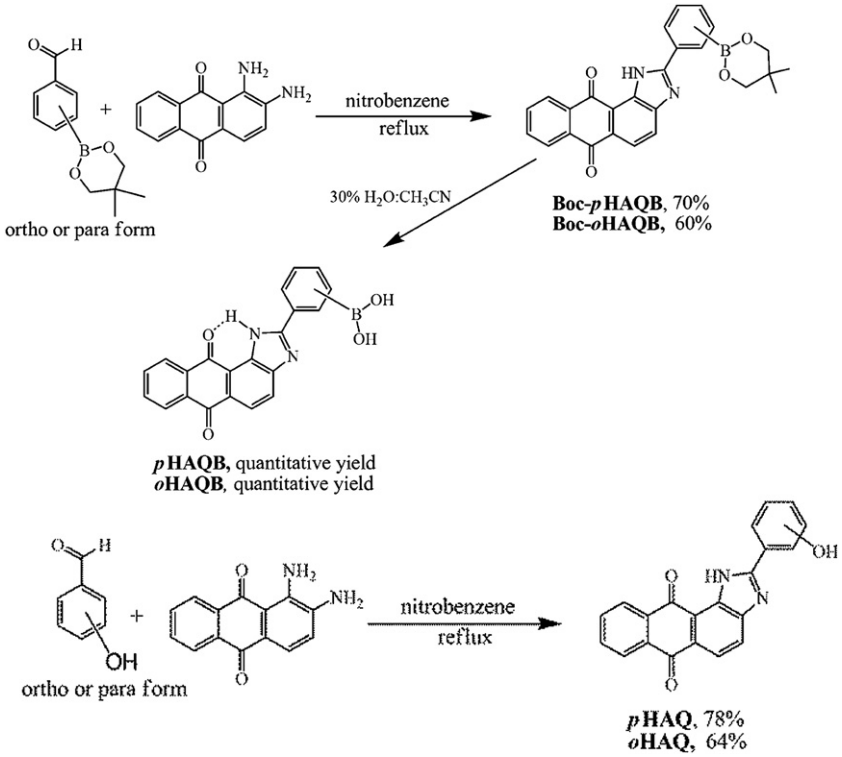
Fig. 1. Schematic illustration of the GOx enzymatic mechanism for glucose detection using boronic-based fluorescence sensors.

directly. Herein, new anthraquinone based fluorosensors, ***o*HAQB** and ***p*HAQB** containing boronic acid as the enzymatic probe for glucose has been designed and synthesized. The sensing affinity for glucose was investigated by fluorescence spectrophotometry. Among both sensors, ***o*HAQB** permits the selective detection of glucose upon the GOx enzymatic reaction with a detection limit of 11.4 μM and average % recovery of 104, which is indicative of powerful glucose sensing.

2. Results and discussion

2.1. Synthesis and physical properties of sensors *p*HAQB and *o*HAQB

Synthetic methodologies for the new probes, ***p*HAQB** and ***o*HAQB** are shown in Scheme 1. The heterocyclic precursors, **Boc-*p*HAQB** and **Boc-*o*HAQB** were achieved from oxidative condensation between diamino anthraquinone and formyl phenylboronated ester with 70% and 60% yields, respectively. The characterization of all sensors was carried out using ^1H as well as ^{13}C NMR and HRMS. For ^1H NMR spectra, the appearance of a broad singlet signal at around 13.76 and 12.97 ppm for ***p*HAQB** and ***o*HAQB**, respectively, supports the formation of intramolecular hydrogen bonding between the NH of the imidazole ring and the neighboring quinone carbonyl group.[12]



Scheme 1. Synthesis of sensory molecules, ***p*HAQB**, ***o*HAQB**, ***p*HAQ** and ***o*HAQ**.

For our hypothesis, the H_2O_2 generated by the reaction between glucose and glucose oxidase would convert the boronic acid to the phenol based fluorescence sensor and the fluorescence change would be related to the concentration of glucose. Although, most organic fluorophores can exhibit fluorescence quenching by H_2O_2, we designed the organic fluorophore containing boronic acid for specific detection of glucose upon the GOx enzymatic reaction without the interference of glucose binding to the boronic acid

Sensors ***o*HAQB** and ***p*HAQB** in 10% DMSO with 0.1 M HEPES buffer at pH 7.4 showed a strong fluorescence intensity at 609 and 589 nm, respectively, with quantum yields of 0.322 and 0.231 with respect to a typical quinone compound as shown in Fig. 2. As expected, fluorescence responses of ***p*HAQ** and ***o*HAQ** consisting of hydroxy groups were relatively low in aqueous media and consequently their quantum yields could not be determined.

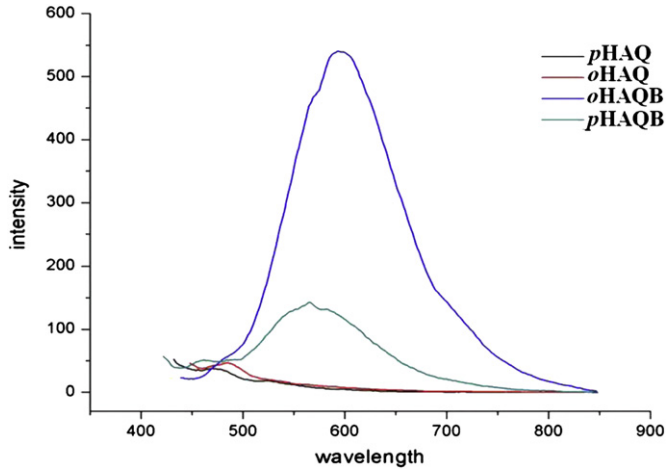


Fig. 2. Emission spectra of *p***HAQB**, *o***HAQB**, *p***HAQ** and *o***HAQ** in 10% DMSO with 0.1 M HEPES buffer at pH 7.4.

These positive preliminary results encouraged us to investigate the boronic acid based sensors as a glucose sensing under the enzymatic mechanism mentioned above.

To study the binding properties between sensors *p***HAQB** and *o***HAQB** toward glucose without glucose oxidase, the fluorescence titration curves exhibited large changes of fluorescence quenching for *p***HAQB** but the fluorescence quenching of *o***HAQB** in the presence of glucose displayed insignificant change as shown in Fig. 3. As a result of steric hindrance of boronic binding site of *o***HAQB** toward glucose, *p***HAQB** preferred to bind glucose over *o***HAQB**. Moreover, the binding constants of *o***HAQB** and glucose cannot be determined.

We, therefore, reasoned that *o***HAQB** was a better candidate for enzymatic activation as it would be no competition from direct binding.

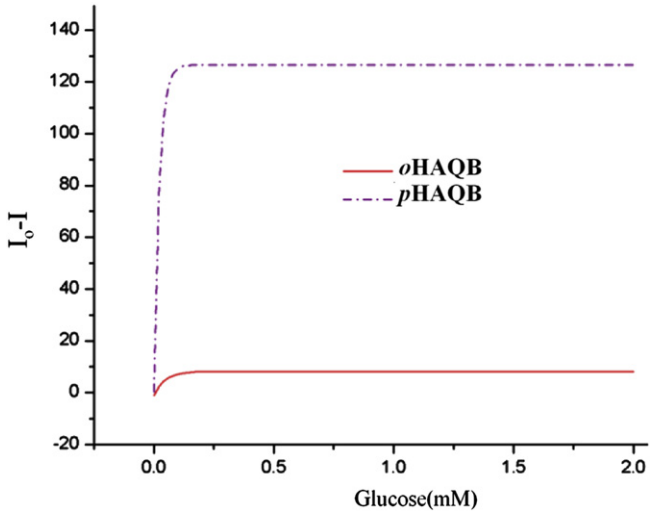


Fig. 3. Fluorescence titration curves of 20 μM *o***HAQB** and *p***HAQB** at 590 and 560 nm, respectively, with glucose in 100 mM HEPES buffer (pH 7.4).

2.2. Enzymatic detection of glucose using *o*HAQB

Prior to the use of enzymatic mechanism of GOx and glucose, the optimized conditions of the system were investigated in terms of amount of GOx unit, temperature and reaction time. From the fluorescence results as shown in ESI, the appropriate condition for the high efficiency of enzymatic probe was performed by six units of GOx at a temperature of 37 °C and reaction time of 60 min. These appropriate conditions were utilized for all manipulations related to the enzymatic reaction of GOx.

From the literature review[13] reporting the binding ability between GOx and boronic acid, we thus tested the binding ability of *o***HAQB** and GOx by fluorescence spectrophotometry. We found unchanged emission spectra of *o***HAQB** in the presence of GOx as shown in Fig. S5. This indicated unbound *o***HAQB** with GOx suggesting no influence of the complexation of *o***HAQB** and GOx toward emission spectral changes. Hence, we have focused on studying the use of *o***HAQB** for detection of glucose. Hypothetically, H_2O_2 generated from the reaction of glucose and glucose oxidase would convert the boronic acid based sensor to the hydroxy group and subsequently induced the fluorescent quenching of anthraquinone derivatives. To confirm this hypothesis, the product of the boronate-based fluorescent probe (*o***HAQB**) hydrolyzed by H_2O_2 generated from the reaction of the glucose and GOx was investigated by ESI-MS (shown in ESI), which showed the peak of *m*/*z* 340.08 corresponding to the structure of *o***HAQ** (the hydrolyzed boronate-based *o***HAQB**).

We first examined the comparison of fluorescence changes of *o***HAQB** upon direct addition of H_2O_2 and the addition of glucose along with GOx as shown in Fig. 4.

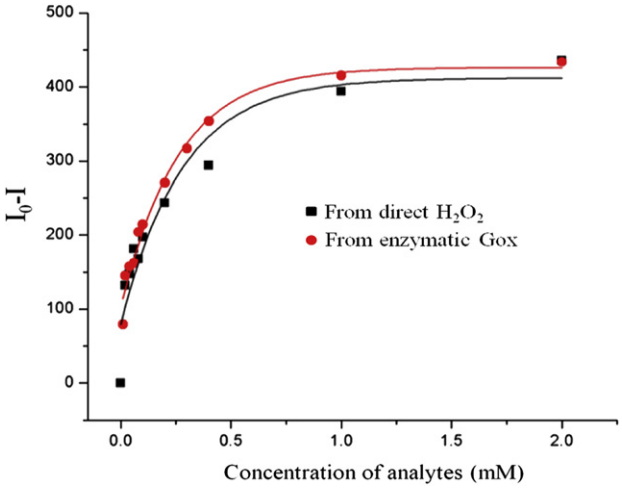


Fig. 4. Fluorescence titration curves (at 609 nm) of 20 μM *o***HAQB** upon addition of direct H_2O_2 and addition of glucose and GOx in 100 mM HEPES buffer (pH 7.4).

As anticipated, the fluorescence responses of *o***HAQB** in the presence of H_2O_2 by direct addition, and H_2O_2 generated by the reaction of glucose and glucose oxidase were consistent and almost overlapped. The small difference of fluorescence responses from both systems is acceptable. In this contribution, monitoring based on the fluorescent changes of *o***HAQB** stemmed from the direct conversion of the glucose by glucose oxidase corresponding to the schematic illustration in Fig. 1. Therefore, we envisaged the use of *o***HAQB** as the enzymatic GOx probe for glucose. To prove the selectivity of *o***HAQB** for specific-glucose sensing, the effects of interfering saccharides (2.0 mM) including galactose, fructose, sucrose and maltose were evaluated in the determination of 0.2 mM of glucose with and without GOx in 100 mM HEPES buffer (pH 7.4).

Regarding the fluorescence responses of *o***HAQB** shown in Fig. 5, poor responses were readily observed for galactose and fructose with fluorescent enhancement as well as sucrose and maltose with fluorescence quenching. On the other hand, the observation of a large fluorescent enhancement of *o***HAQB** in the presence of

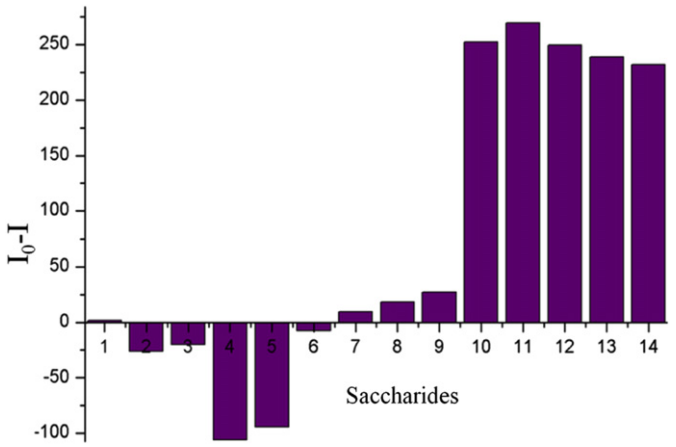


Fig. 5. Fluorescent responses of *o***HAQB** (20 μM) in the presence of 0.2 mM glucose [1] or 2 mM of the competitive saccharides: galactose [2], galactose+GOx [3], fructose [4], fructose+GOx [5], sucrose [6], sucrose+GOx [7], maltose [8], maltose+GOx [9], glucose+GOx [10] and the mixture of glu+GOx with galactose [11], fructose [12], sucrose [13], and maltose [14].

glucose along with GOx as shown in Fig. 5 could indicate a highly specific recognition for glucose. Interestingly, other saccharides did not affect the glucose detection under the enzymatic GOx mechanism.

To verify the sensing application of the boronic anthraquinone based sensors (*o***HAQB**), the fluorescence titration between *o***HAQB** and glucose in the presence of glucose oxidase was investigated in the optimum conditions. As depicted in Fig. 6, the emission band at 609 nm of *o***HAQB** was gradually decreased upon the increment of glucose. To normalize the fluorescence titration of enzymatic probe system of *o***HAQB**, the calibration curve was plotted between I_0-I and the glucose concentration (shown in inset of Fig. 6). The fluorescence response was linear in the range of 0.08–0.42 mM with an acceptable correlation coefficient (R^2) of 0.99082. Therefore, the range of concentration for glucose sensing by the GOx enzymatic probe system is 0.08–0.42 mM. The limit of detection (LOD) of *o***HAQB** for glucose detection using GOx enzymatic probe is approximately 0.0114 mM or 11.4 μM.

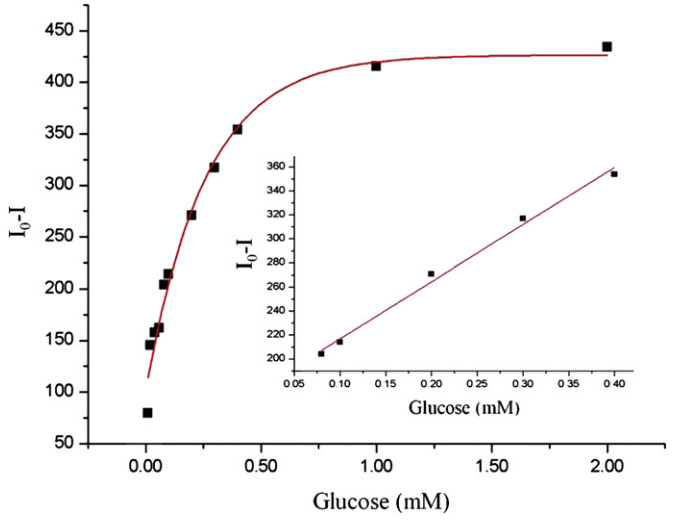


Fig. 6. Fluorescence titration curve of 20 μM *o***HAQB** and six units of glucose oxidase titrated with glucose in 10% DMSO with 0.1 M HEPES buffer at pH 7.4. The excitation wavelength was 415 nm. The reaction was performed at 37 °C and 1 h and inset: liner calibration curve of *o***HAQB** in the same condition.

In analytical applications using this spectrofluorometric method, *o***HAQB** was applied to detect glucose in drinking water. The % recoveries (shown in Table 1) of the spike samples of **oHAQB** were in the range of 91–116 and the average % recovery of 104, which is acceptable for analytical application.

Table 1
Analysis of glucose using *o***HAQB** with the enzymatic reaction of GOx in drinking water

Added glucose (mM)	Found (mM)	% Recovery
0.05	0.51	102
0.10	0.091	91
0.15	0.175	116
0.20	0.216	108
0.30	0.313	104

3. Conclusions

A fluorescence sensory molecule, *o***HAQB**, has been successfully synthesized, and its glucose sensing capability has been developed under enzymatic mechanism of GOx. All corresponding results are consistent with the hypothesis that H_2O_2 generated from the enzymatic reaction of glucose and GOx showed high affinity to hydrolyze the boronic acid group to the hydroxy moiety with the proportional fluorescence changes of the sensor, *o***HAQB**. To the best of our knowledge, this is the first report of the use of boronic-based fluorescence biosensor to detect glucose under the GOx enzymatic reaction. Consequently, *o***HAQB** serves as an excellent biosensor for glucose sensing with high selectivity. The proposed mechanism highlighted the benefits of further use of the simple fluorescence sensor as the GOx enzymatic probe for detection of glucose in real-time analysis in the field of medical diagnosis and food safety.

4. Experimental section

4.1. Materials and general methods

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Mercury Plus 400 NMR spectrometer. All chemical shifts were reported in parts per million (ppm) using the residual proton or carbon signal in deuterated solvents as internal references. MALDI-TOF mass spectra were carried out on Bruker Daltonics MALDI-TOF using 2-cyano-4-hydroxy cinnamic acid (CCA) as matrix.

All materials and solvents chemicals were purchased from Aldrich, Fluka and Merck as standard analytical grade and were used without further purification. Commercial grade solvents such as dichloromethane, ethyl acetate, hexane, and methanol were purified by distillation. Anhydrous solvents such as dichloromethane were dried over CaH_2 and freshly distillation under nitrogen atmosphere. Column chromatography was carried out on silica gel (Kieselgel 60, 0.063–0.200 mm, Merck). Thin layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60, F_{254}, 1 mm). Compounds on TLC plates were detected by the UV-light.

For UV/vis and Fluorescence analysis, compounds were dissolved in dimethyl sulfoxide (DMSO, fluorometric grade, Dojindo) to obtain 1 mM stock solutions. These stock solutions were diluted with buffer as specified in the figure legends to the desired concentration. For determination of the quantum efficiency of fluorescence (Φ_f), were calculated using the integrated emission intensity of quinine sulfate as standard. All absorption spectra were obtained with Hewlett Packard 8452A Diode Array Spectrometer.

All fluorescence spectra were obtained with Varian Cary Eclipse Fluorescence Spectrophotometer. The boronate esters were synthesized according to the previously published procedure.[14]

4.2. Synthesis

Preparation of 2-(*R*-(5,5-*dimethyl*-1,3,2-*dioxaborinan*-2-*yl*)*phenyl*)-1*H*-*anthra*[1,2-*d*]*imidazole*-6,11-*dione* (*R*=2, 4). Into a two-neck round bottom flask equipped with a magnetic bar, the corresponding formyl phenylboronate ester (1 mmol) in nitrobenzene (25 mL) was added dropwise to a solution of 1,2-diamino-1,4-anthraquinone (1 mmol) in nitrobenzene (75 mL).

The reaction mixture was slowly heated to 150 °C for 1 day under nitrogen atmosphere. The solution was cooled to room temperature and then the precipitate slowly formed. The precipitate was filtered and washed with diethylether to give a brown solid of the protecting products (**Boc**-*o***HAQB**=45%, **Boc**-*p***HAQB**=60%). ^{1}H NMR (DMSO, 400 MHz) **Boc**-*o***HAQB**, δ (ppm): 13.56 (s, 1H), 8.41 (d, 1H, *J*=6.4 Hz), 8.25–8.13 (m, 4H), 7.94–7.92 (m, 2H), 7.57–7.50 (m, 3H), 3.70 (s, 4H), 1.07 (s, 6H). **Boc**-*p***HAQB**, δ (ppm): 10.18 (s, 1H), 8.40 (t, 1H, *J*=8.2 Hz), 8.29–8.21 (m, 4H), 8.13–8.05 (m, 2H), 7.97–7.93 (m, 3H), 3.78 (s, 2H), 3.11 (s, 2H), 0.97 (s, 3H) 0.72 (s, 3H).

Preparation of R-(6,11-*dioxo*-6,11-*dihydro*-1*H*-*anthra*[1,2-*d*]*imidazol*-2-*yl*)*phenylboronic acid* (*R*=2: *o***HAQB**, 4: *p***HAQB**). Protecting groups of 2-(*R*-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)phenyl)-1*H*-anthra[1,2-*d*]imidazole-6,11-dione (*R*=2:2o, 4: 2p) (1 mmol) were removed by refluxing in 30 mL 0f 30% H_2O:CH_3CN. The yellow solid of product *o***HAQB** and brown solid of product *p***HAQB** was filtered off and washed with diethylether to provide the desired product in a quantitative yield.

*Characterization data for o***HAQB**: Melting point 350–354 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 13.76 (s, 1H), 8.26–8.21 (m, 2H), 8.14 (d, 2H, *J*=6.8 Hz), 7.99 (d, 2H, *J*=6.0 Hz), 7.94–7.92 (m, 1H), 7.75 (d, 1H, *J*=8.4 Hz), 7.68 (d, 2H, *J*=6.8 Hz). ^{13}C NMR (100.6 MHz, DMSO-d_6) δ=182.36, 182.00, 181.50, 159.60, 148.58, 133.60, 132.59, 132.33, 131.91, 129.68, 129.23, 128.49, 128.04, 126.84, 126.66, 126.23, 126.12, 124.74, 121.60, 120.93, 118.58. IR spectrum (KBr, (cm^{-1})): 3421 (N–H (2°-amines) stretching, wk), (2935 (C=C, stretching) 1657 (C=O stretching, str), 1584, 1490 (C=C, stretching, str), 1300 (C–N) stretching). HRESI-MS calculated for $C_{21}H_{13}N_2O_4$: 369.12 [M], observed: 387.09 [M+H_2O]=419.123.

Characterization data for *p***HAQB**: Melting point 366–367 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ=12.97 (s, 1H), 8.34 (d, 1H, *J*=7.6 Hz), 8.26 (s, 2H), 8.06–8.25 (m, 2H), 7.96 (m, 4H), 7.80 (m, 2H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ=183.37, 182.56, 158.60, 137.53, 134.76, 134.54, 133.36, 133.23, 133.160, 128.33, 127.47, 127.09, 126.50, 125.27, 121.33, 118.91; IR spectrum (KBr, (cm^{-1})): 3415 (N–H (2°-amines) stretching, wk), (1658 (C=O stretching, str), 1523, 1470 (C=C, stretching, str), 1298 (C–N) stretching, med), 723 (C–H bending, str); Elemental analysis: Anal. Calcd for: $C_{21}H_{13}N_2O_4$: C, 68.51; H, 3.56; N, 7.61; found: C, 68.57; H, 3.56; N, 7.62. MALDI-TOF: *m*/*z* for $(M+3H)^+$=382.11.

4.3. Glucose sensing studies

4.3.1. The complexation studies of the boronic anthraquinone based sensors with glucose using fluorescence titration experiments. Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The stock solutions of glucose were prepared at concentrations of 6.00×10^{-3} mol/L in 0.1 M HEPES buffer pH 7.4. In a 5.0 mL beaker, 0.30 mL of the stock solution of sensor was mixed with the portions of stock solution of glucose to give final concentration of glucose. After volumes were adjusted to 3.00 mL with 0.1 M HEPES buffer (pH 7.4) to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution, the mixtures was stirred at room temperature for 10 min and placed in a 100.0 mm width quartz cell and then fluorescence spectra were recorded at 25 °C.

*4.3.2. Fluorescence measurement of boronic anthraquinone based sensors (o**HAQB**) in optimum condition of the enzymatic probe system for glucose detection by glucose oxidase.* Typically, the sensors were prepared in spectroscopic grade DMSO at concentrations of 2.00×10^{-4} mol/L as a stock solution. The stock solutions of glucose were prepared at concentrations of 6.00×10^{-3} mol/L in 0.1 M HEPES buffer pH 7.4. In a 10.0 mL vial, 0.30 mL of the stock solution of sensor was mixed with the portions of stock solution of glucose to give final concentration of glucose. After volumes were adjusted to 3.00 mL with 0.1 M HEPES buffer pH 7.4 to give a final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution, then six units of glucose oxidase were added to the solution mixture of the sensor and the glucose. The mixtures was stirred at 37 °C for 60 min and placed in a 100.0 mm width quartz cell and then, the fluorescence spectra were recorded at 25 °C. For kinetic studies, the fluorescence spectra were recorded at 25 °C every 5 min.

4.3.3. Competitive studies of other saccharides. In a 10.0 mL vial, 0.30 mL of 2.00×10^{-4} mol/L of a sensor in spectroscopic DMSO was mixed with 100 μL of 6.00×10^{-2} mol/L of stock solutions including D-glucose, D-galactose, D-fructose, D-sucrose and D-maltose in 0.1 M HEPES buffer pH 7.4 and all solutions was adjusted to 3.00 mL to give final concentration of sensors at 2.00×10^{-5} mol/L in 10% DMSO aqueous solution. Then six units of glucose oxidase were added to the solution mixture of the sensor and the saccharides. The mixture solution was stirred at 37 °C for 60 min and placed in a 100.0 mm width quartz cell and then fluorescence spectra were recorded at room temperature.

4.3.4. Glucose detection of drinking water sample. The commercially drinking water sample was used for volume adjustment with 0.1 M HEPES buffer pH 7.4 to give a final concentration of 10% DMSO aqueous solution of sample. The glucose stock solution was spiked to the mixture solution of *o***HAQB** to give the final concentration of 0.05–0.3 mM of glucose and 2.00×10^{-4} M of *o***HAQB** and then 6 units of glucose oxidase was added to the solution mixture of the sensor and the glucose. After stirring the mixture solution at temperature of 37 °C for 60 min, the sample mixture was then recorded at the room temperature.

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Supplementary data

^{1}H NMR spectrum of *o***HAQB** and *p***HAQB**. Fluorescence spectra and Mass spectroscopy. Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.tet.2012.08.037.

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