Fluorescence ratiometric assays of hydrogen peroxide and glucose in serum using conjugated polyelectrolytes†

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A new water-soluble cationic polyfluorene with boronate-protected fluorescein (peroxyfluor-1) covalently linking to the polymer backbone (PF-FB) was synthesized as a fluorescence probe to optically detect hydrogen peroxide (H_2O_2) and glucose in serum. The peroxyfluor-1 exists as a lactone form which is colorless and non-fluorescent. Without addition of H_2O_2, the fluorescence resonance energy transfer (FRET) from fluorene units (donor) to peroxyfluor-1 (acceptor) is absent and only blue donor emission is observed upon excitation of the fluorene units. In the presence of H_2O_2, the peroxyfluor-1 can specifically react with H_2O_2 to deprotect the boronate protecting groups and to generate green fluorescent fluorescein. The absorption of fluorescein overlaps the emission of polyfluorene, which encourages efficient FRET from the fluorene units to the fluorescein. By triggering the shift in emission color and the ratio change of blue to green emission intensities, it is possible to assay H_2O_2 and its concentration change in buffer solution and in serum. The PF-FB probe can detect H_2O_2 in the range from 4.4 to 530 μM. Since glucose oxidases (GOx) can specifically catalyze the oxidation of β-D-(+)-glucose to generate H_2O_2, glucose detection is also realized with the PF-FB probe as the signal transducer. Thus, an optical assay for hydrogen peroxide (H_2O_2) and glucose in serum was created which combines a fluorescent ratiometric technique based on FRET with the light-harvesting properties of conjugated polymers.

Introduction

Hydrogen peroxide is a key reactive oxygen species (ROS) in living systems. It plays an important role in physiological and pathological processes.[1] Emerging evidence proves the role of H_2O_2 as a second messenger in cellular signal transduction.[2] Moreover, oxidative damage resulting from the cellular imbalance of H_2O_2 is closely related to several human diseases including Alzheimer's disease,[3] cardiovascular disorders[4] and cancers.[5] H_2O_2 is generated by almost all oxidases, and so the activity of oxidases, or the enzyme substrates, such as glucose, can be quantitatively assayed by determining the H_2O_2 produced.[6] Thus new diagnostic methods for detecting and quantifying H_2O_2 are needed.

Since fluorescence spectroscopy is one of the most useful analytical tools in bioanalysis,[7] many fluorescent probes have been developed for the detection of H_2O_2. Fluorescent probes, such as dihydro analogues of fluorescent dyes,[8] phosphine-based fluorophores[9] and lanthanide coordination complexes,[10] have been designed, however, the lack of water compatibility, requirement of external activating enzymes, low selectivity toward H_2O_2 over other ROS or narrow pH working range (6.6–7.2) limit their practical application. Recently, probes with high selectivity toward H_2O_2 based on the deprotection mechanism of fluoresceins with ROS-cleavable protecting groups (such as pentafluorobenzenesulfonyl fluoresceins and peroxyfluor-1) have been developed,[11,12] however these intensity-based probes can not quantitatively measure the concentration changes of H_2O_2 in heterogeneous biological samples. In comparison to intensity-based methods, the fluorescence resonance energy transfer (FRET) technique exhibits a large shift in emission profiles and provides a ratiometric fluorescence measurement,[13] which is not affected by external non-specific events. To date few studies have been demonstrated to detect H_2O_2 using ratiometric probes.[14]

In comparison to small molecule counterparts, the electronic structure of conjugated polymers coordinates the action of a large number of absorbing units. The excitation energy along the backbone of the conjugated polymer is transferred to an energy/electron acceptor, resulting in an amplified detection signal.[15] Therefore, water-soluble conjugated polymers have attracted more attention as the optical platforms of sensitive chemical and biological sensors.[16] Recently, we have designed a new ratiometric system based on a conjugated polymer to detect H_2O_2 and glucose with high sensitivity and broad pH working range.[17] However, this sensing system is unworkable with the increase of ion strength of the assay buffer solution because the main driving force is electrostatic interaction, which restricts its application in blood samples. In this contribution, we design a new conjugated polymer (PF-FB) with boronate-protected fluorescein covalently linked to the polymer backbone to eliminate the electrostatic interaction. As expected, this new polymer probe shows good potential to detect H_2O_2 and glucose both in buffer solution and in serum.

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Experimental

Materials and measurements

2,7-Dibromofluorene, bis(pinacolato)diboron and the catalyst $Pd(dppf)Cl_2$ were purchased from the Pacific Chemsource Inc (China). 3-Iodophenol (**4**) and benzene-1,2,4-tricarboxylic acid (**5**) were from Alfa. Di-*tert*-butyl dicarbonate was from Acros. *N*-Hydroxysuccinimide was from Aldrich. 1-(6′-Bromohexyloxy)-2,5-dibromo-4-methoxybenzene (**1**),[18] 2,7-dibromo-9,9-bis(6′-bromohexyl)-fluorene (**8**)[19] and 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzene (**9**)[20] were synthesized according to the procedures in the literature. The synthetic details of the monomers and polymers are described in the ESI.† ^1H NMR and ^{13}C NMR were collected on a 400 MHz AC Bruker spectrometer. Mass spectra were recorded on an AEI-M850-MS or an APEXII-electrospray ionization (ESI) instrument. Elemental analyses were carried out with a Flash EA1112 instrument. GPC was performed on a Water Styragel system using polystyrene as a calibration standard with THF as the eluent. UV-vis absorption spectra were taken on a JASCO V-550 spectrophotometer and the fluorescence spectra were measured using a Hitachi F-4500 fluorometer with a Xe lamp as excitation source.

Assays for H_2O_2 and glucose

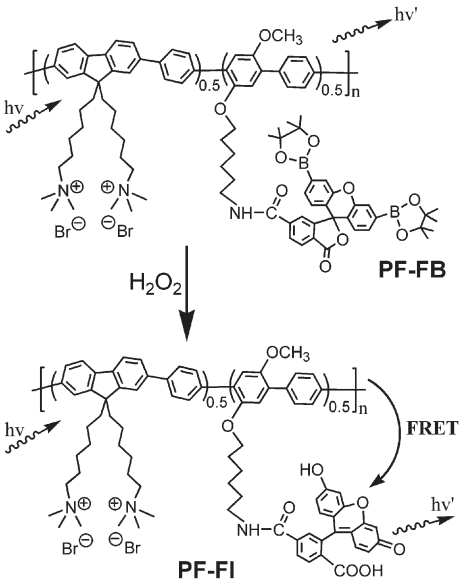
To a solution of PF-FB in phosphate buffer (50 mM, pH = 7.2) or in serum was added H_2O_2 at room temperature. After incubating for 20 minutes, the fluorescence spectra were measured with an excitation wavelength of 360 nm.

For the detection of glucose in serum, the serum was saturated with oxygen gas for 30 minutes. To a solution of PF-FB in serum were added glucose and glucose oxidase (GOx), respectively. After incubating for 1 hour, the fluorescence spectra were measured with an excitation wavelength of 360 nm.

Results and discussion

Design of the fluorescent probe PF-FB for H_2O_2

Our new H_2O_2 assay is illustrated in Scheme 1. The boronate-protected fluorescein (peroxyfluor-1) is covalently linked to the side chain of water-soluble polyfluorene (PF-FB). The peroxyfluor-1 exists as a lactone form which is colorless and non-fluorescent.[12,14] FRET from the fluorene units (donor) to the peroxyfluor-1 (acceptor) is absent and only blue donor emission is observed upon excitation of the fluorene units. In the presence of H_2O_2, the peroxyfluor-1 can specifically react with H_2O_2 to deprotect the boronate protecting groups and to generate fluorescent fluorescein (Fl)[12,14] which acts as the FRET acceptor.[16l] In this case, efficient intramolecular FRET from fluorene units to fluorescein is observed. In comparison to our previous cationic PF/peroxyfluor-1 system,[17] the new PF-FB probe eliminates the effect of electrostatic interactions on the FRET efficiency. By triggering the shift in emission color and the ratio change of blue to green emission intensities, it is possible to assay H_2O_2 and its concentration change in buffer solution and in blood samples.

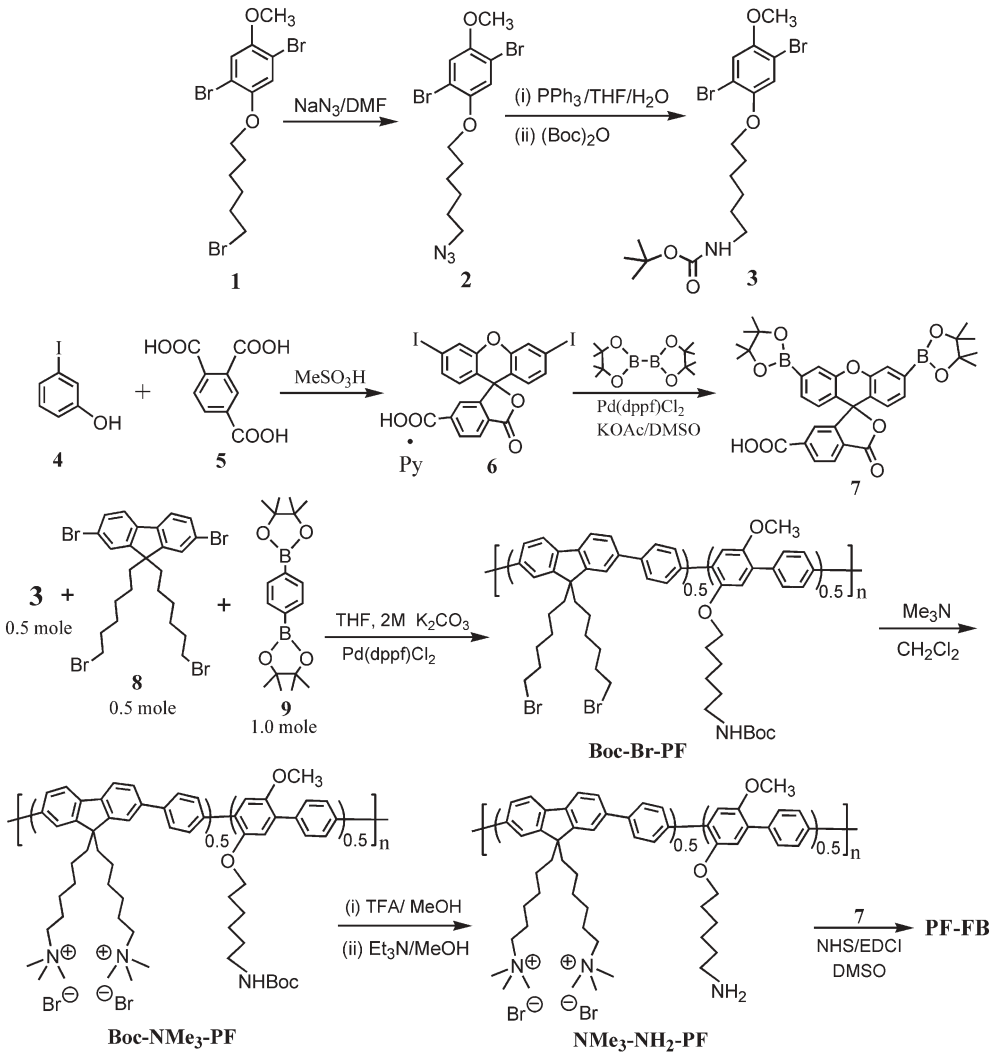


Scheme 1 Schematic representation of the H_2O_2 assays.

Synthesis and optical properties of the polymer PF-FB

The procedures for the synthesis of the monomers and polymers are shown in Scheme 2. Reaction of **1** with excess sodium azide in anhydrous DMF gives **2** in 95% yield. Reduction of **2** with PPh_3 in THF–H_2O (v/v 44 : 6) and subsequent protection with di-*tert*-butyldicarbonate yields **3** in 86% yield. Compound **6** is obtained by reaction of 3-iodophenol (**4**) with benzene-1,2,4-tricarboxylic acid (**5**) in methanesulfonic acid in 36% yield. Reaction of **6** with bis(pinacolato)diboron in the presence of potassium acetate and $Pd(dppf)Cl_2$ in DMSO yields boronic ester **7** in 27% yield. The Boc-protected copolymer Boc-Br-PF is prepared by palladium-catalyzed Suzuki cross-coupling reaction[21] between one equivalent of monomers **3** and **8** with 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzene (**9**) in the presence of 2.0 M aqueous K_2CO_3 and $Pd(dppf)Cl_2$ in THF (43% yield). The gel permeation chromatography (GPC) analyses show that the weight-average molecular weight (M_w) and number-average molecular weight (M_n) of Boc-Br-PF are 60210 and 24660, respectively, with the polydispersity index (PDI) of 2.44. The polymer Boc-Br-PF is treated with 30% trimethylamine–ethanol solution to obtain water-soluble Boc-NMe_3-PF followed by Boc-deprotecting with trifluoroacetic acid (TFA) to give NMe_3-NH_2-PF containing free primary amino groups. Compound **7** is linked covalently to the side chain of NMe_3-NH_2-PF in the presence of *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) to afford target copolymer PF-FB.

The photophysical properties of PF-FB were investigated in water. As shown in Fig. 1, the UV-vis absorption spectrum of PF-FB exhibits a maximum peak at 375 nm, which is attributed to the π–π* transition of the polymer backbone. Upon excitation at 360 nm, the emission spectrum shows a maximum peak at 420 nm with a shoulder peak at 445 nm, which is characteristic of polyfluorenes.[22] As expected, the



Scheme 2 Synthetic route to the monomers and copolymers.

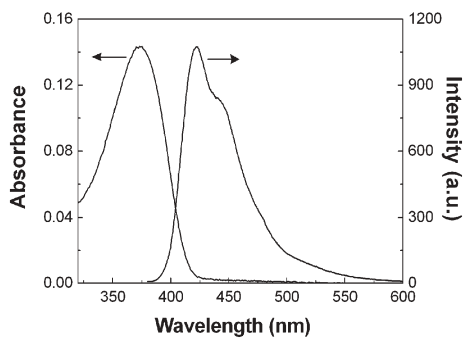


Fig. 1 UV-vis absorption and emission spectra of PF-FB in water. The excitation wavelength is 360 nm.

typical fluorescein absorption band centered at 480 nm and emission band centered at 530 nm were not observed, which proves that no FRET occurs from the fluorene units to boronate-protected fluorescein. The fluorescence quantum yield (QY) of PF-FB is calculated to be 0.165 with 9,10-diphenylanthracene in cyclohexane as the standard.[13]

Assays for H_2O_2

The emission maximum of PF-FB itself ([PF-FB] = 1.5×10^{-6} M in repeat units (RU)) in phosphate buffer solution (50 mM, pH 7.2) appeared at around 420 nm and no emission of the peroxyfluor-1 unit was observed at the excitation wavelength of 360 nm (Fig. 2a). Adding H_2O_2 ([H_2O_2] = 1.76 mM) into the solution of PF-FB led to a significant quenching of fluorene unit emission at 420 nm and the appearance of the peroxyfluor-1 unit emission at 527 nm. The efficient FRET confirmed that a fluorescent fluorescein as energy acceptor was generated from the reaction of peroxyfluor-1 and H_2O_2. Noted that the FRET ratio of fluorescein to fluorene emission intensities (I_{527nm}/I_{420nm}) increased from 0.17 in the absence of H_2O_2 to 1.01 after H_2O_2 treatment, where the 6-fold FRET ratio was enhanced due to the FRET.

For meeting the detection requirement in blood samples (for example, serum), the sensing probe needs to be efficient for large ion strength and a wide pH range. To examine the effect of ionic strength on the FRET from the fluorene units to the fluorescein, the fluorescence spectra of PF-FB ([PF-FB] = 1.5×10^{-6} M) were studied in the presence of H_2O_2

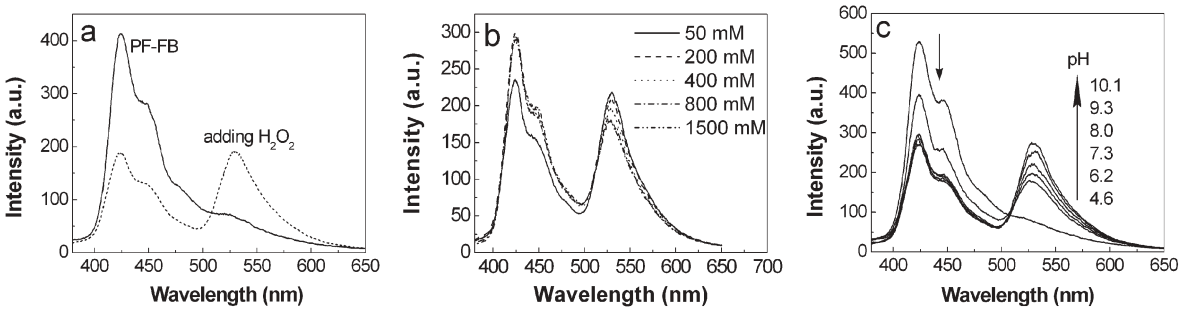


Fig. 2 (a) Emission spectra of PF-FB in phosphate buffer (50 mM, pH 7.2) in the absence and presence of H_2O_2. (b) Emission spectra of PF-FB in the presence of H_2O_2 in phosphate buffer (pH 7.2) with varying ion strength from 50 to 1500 mM. (c) Emission spectra of PF-FB in the presence of H_2O_2 in phosphate buffer (50 mM) with varying pH value from 4.6 to 10.1. [PF-FB] = 1.5×10^{-6} M, [H_2O_2] = 1.76 mM. The excitation wavelength was 360 nm.

([H_2O_2] = 1.76 mM) at a pH value of 7.2 with varying ion strengths. Fig. 2b shows that there was only a slight decrease in the FRET efficiency upon varying the buffer ionic strength from 50 to 1500 mM, which confirmed that the fluoran pendant was covalently linked to the polymer side chain and the emission of fluorescein originated from the intramolecular FRET. To check for the working pH range of the PF-FB probe, the effect of medium pH values on the fluorescence spectra of PF-FB ([PF-FB] = 1.5×10^{-6} M) was investigated in the presence of H_2O_2 ([H_2O_2] = 1.76 mM) in phosphate buffer (50 mM). Since the absorption and fluorescence of fluorescein are both pH dependent,[23] a pH dependent FRET ratio was also observed (Fig. 2c). At pH 4.6, inefficient FRET from fluorene units to fluorescein was observed. The FRET ratio (I_{527nm}/I_{420nm}) was 0.46 at pH 6.2 and 0.68 at pH 10.1, that is, only an approximate 30% difference for the FRET ratio was exhibited over a pH range from 6.2 to 10.1. The above results exhibit that PF-FB can detect H_2O_2 for large ion strength and a wide pH range. Thus, PF-FB affords the chance to detect H_2O_2 in biological samples, for example, in serum.

To examine the ability of PF-FB to detect H_2O_2 in biological samples, assay experiments were performed in serum solution. Fig. 3a shows the fluorescence spectral changes of PF-FB as a function of H_2O_2 incubating time. In these experiments, H_2O_2 ([H_2O_2] = 4.4 mM) was added to a solution of PF-FB ([PF-FB] = 1.5×10^{-6} M) in phosphate buffer (50 mM, pH 7.2) containing 20% serum, and the emission spectra were measured at 1 min intervals over 13 minutes by the excitation at 360 nm. The initial solution of PF-FB showed less FRET from fluorene units to fluorescein. After adding H_2O_2, the intensity of emission band centered at 420 nm was gradually decreased and that centered at 527 nm was gradually increased with the incubating time from 0 to 10 minutes and the FRET ratio (I_{527nm}/I_{420nm}) reached a plateau after 10 minutes (Fig. 3b). These observations indicated that PF-FB can be utilized as a probe to detect H_2O_2 in real time.

Fig. 4a also shows the dependence of emission spectra of PF-FB on the concentration of H_2O_2 with a fixed PF-FB concentration in 20% serum solution ([PF-FB] = 1.5×10^{-6} M in RU). It shows that the FRET efficiency increased gradually with successive addition of H_2O_2. A linear relationship between the FRET ratio (I_{527nm}/I_{420nm}) and H_2O_2

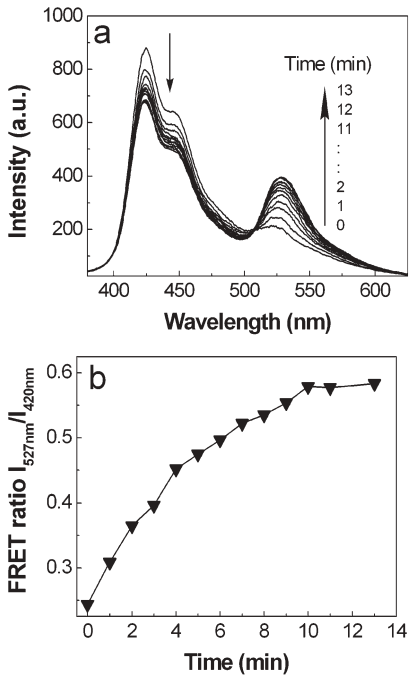


Fig. 3 (a) Emission spectra of PF-FB and (b) FRET ratio (I_{527nm}/I_{420nm}) as a function of the H_2O_2 incubating time. [PF-FB] = 1.5×10^{-6} M, [H_2O_2] = 4.4 mM. The experiments were performed in phosphate buffer (50 mM, pH 7.2) containing 20% serum. The excitation wavelength was 360 nm.

concentration was observed over the H_2O_2 concentration range from 4.4 to 530 μM, where the emission intensity was obtained at the H_2O_2 incubating time of 20 minutes with a standard commercial fluorometer (Hitachi F-4500, Japan) equipped with a xenon lamp excitation source and a photomultiplier tube (Fig. 4b). This indicates that the PF-FB can also be utilized as a probe to assay the H_2O_2 concentration quantitatively.

Assays for glucose

Many oxidases can catalyze the oxidation of their respective substrates in the presence of oxygen to generate H_2O_2,[6] which suggests that PF-FB could be employed as the signal transducer for the fluorescent sensing of the respective

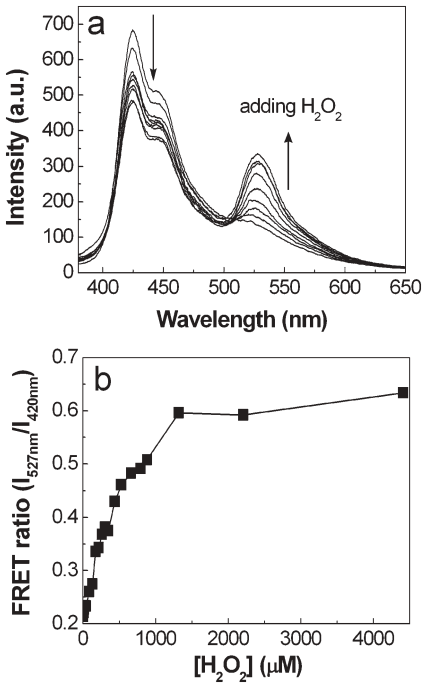


Fig. 4 (a) Emission spectra of PF-FB as a function of the H_2O_2 concentration. (b) FRET ratio (I_{527nm}/I_{420nm}) as a function of the H_2O_2 concentration. [PF-FB] = 1.5 × 10^{-6} M, [H_2O_2] = 0–4.4 mM. The experiments were performed in phosphate buffer (50 mM, pH 7.2) containing 20% serum. The excitation wavelength was 360 nm.

substrates. The oxidase and substrate chosen as a proof of concept are glucose oxidase (GOx) and its specific β-D-(+)-glucose. Fig. 5a compares the spectra before and after the addition of glucose to the solution of PF-FB ([PF-FB] = 1.54 × 10^{-6} M in RUs) and GOx (3.3 mg mL^{-1}) in oxygen saturated phosphate buffer (50 mM) containing 20% of serum. Upon adding glucose ([glucose] = 15.4 mM) and allowing incubating at 37 °C for 1 hour to allow for complete oxidation by GOx, efficient FRET from fluorene units to fluorescein was observed. The control experiments showed that glucose itself did not change the emission spectra of PF-FB. GOx itself slightly increased the FRET efficiency, which resulted from the cofactor of GOx, flavin adenine dinucleotide (FAD).[24] These results indicated that the GOx catalyzed the oxidation of glucose to generate H_2O_2, leading to the efficient FRET response of PF-FB. Fig. 5b shows the FRET ratio (I_{527nm}/I_{420nm}) changes of PF-FB as a function of glucose concentration in the concentration range from 0 to 15.4 mM. With an increase of the glucose concentration from 0 to 7.7 mM, the value of I_{527nm}/I_{420nm} increases gradually and reaches a plateau for glucose concentrations higher than 9.0 mM. It is well known that the normal clinical range for glucose in blood is between 3.5 and 6.1 mM, and abnormal glucose levels can reach as high as 20 mM.[25] Thus, PF-FB shows good potential to assay glucose in blood samples. Fig. 5c shows the time curves of glucose detection. It is found that the FRET ratio (I_{527nm}/I_{420nm}) values gradually increased with the incubating time from 0 to 20 minutes and did not change much after 20 minutes. It is still possible to detect glucose even within five minutes, which demonstrates the rapid response of the PF-FB to glucose in the presence of glucose oxidase.

Conclusions

In summary, a new conjugated polyfluorene containing a boronate-protected fluoran pendent (PF-FB) is designed and synthesized by the palladium-catalyzed Suzuki coupling reaction. Optical assays for hydrogen peroxide and glucose in serum are created which combine the fluorescent ratiometric technique based on FRET with the light-harvesting properties of conjugated polymers. In comparison to our previous cationic PF/peroxyfluor-1 system,[17] the new PF-FB probe eliminates the effect of electrostatic interactions on the intramolecular FRET. It shows a wide working pH range and the intramolecular FRET was only slightly influenced by the ion intensity of the assay buffer. This new polymer probe shows good potential to detect H_2O_2 and glucose both in buffer solution and in serum.

Acknowledgements

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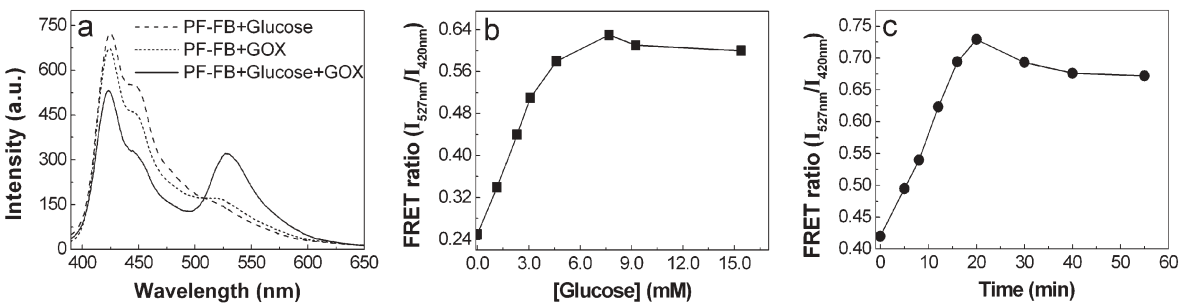

Fig. 5 (a) Emission spectra of PF-FB/GOx in the absence and presence of glucose. [PF-FB] = 1.54 × 10^{-6} M, [GOx] = 3.3 mg mL^{-1}, [glucose] = 15.4 mM. (b) FRET ratio (I_{527nm}/I_{420nm}) as a function of the glucose concentration. [PF-FB] = 1.54 × 10^{-6} M, [GOx] = 3.3 mg mL^{-1}, [glucose] = 0–15.4 mM. (c) FRET ratio (I_{527nm}/I_{420nm}) as a function of incubating time. [PF-FB] = 1.54 × 10^{-6} M, [GOx] = 3.3 mg mL^{-1}, [glucose] = 7.7 mM. The measurements were performed in oxygen saturated phosphate buffer (50 mM) containing 20% of serum and the excitation wavelength was 360 nm.

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