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ARTICLE TYPE

Polyfluorene based Zwitterionic Fluorescent Probe for Response Towards Biological Species in Aqueous Media

Chanchal Chakraborty, Manas Kumar Bera, Sudip Malik*

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Water soluble zwitterionic fluorescent conjugated boronic acid-bearing polyfluorene (PFBA) has been prepared from poly(9,9´-(6´´-bromohexyl)fluorene-co-alt-1,4-phenylene) (Polymer 3) through a post-polymerized quaternization with 3-pyridineboronic acid. Titration of diol containing monosaccharides (D-glucose and D-fructose), L-ascorbic acid and L-DOPA with PFBA polymer in 0.1M phosphate buffer (pH 7.4) solution results in significant concentration dependent quenching of the blue fluorescence of polymer due to static quenching as the bio-analytes form ground state boronic ester complex with PFBA. With D-glucose, emission colour of PFBA changes to greenish-yellow owing to the effective induced aggregated polymer structure. PFBA also exhibits maximum response to the biological analytes at pH 7.4 which provides a scope of PFBA to be used in biological systems.

Introduction

Fluorescence based conjugated polymer (CP) chemosensors have developed into an attractive research area as they offer real-time analysis, multiple sensing modes and higher sensitivities towards analytes than their small molecular counterparts.[1-6] The major advantages of these type of CPs are broad emission spectra across the full visible range, high photoluminescence quantum yield (PLQY), low synthetic cost, extent of solubility and ease of synthesis.[7] The higher sensitivity of CPs than small molecule based devices arises from collective optical response or its conducting properties as CPs contain a huge number of repeated light absorbing units. The transfer of excitation energy along the whole backbone of CPs to an acceptor results in the amplification of fluorescence signals.[1-5,8] Polyfluorene, which is the simplest regular stepladder-type conjugated polyphenylene, shows a bright blue fluorescence with high photoluminescence quantum yield both in solid and solution state.[9] The particular feature of polyfluorene is the ability to produce an amplified response to analytes, with high sensitivity and selectivity, which makes it highly attractive for the field of sensing application.[10-11] In particular, water-soluble conjugated polyfluorene based polymers show promising biosensing applications for DNA,[12-16] enzymes,[17] proteins,[18-19] and other biologically important species like heparin,[20] avidin,[21] organic phosphates,[22] cytochrome C[23] etc. Not only biomolecules, polyfluorene based CPs are very much pronounced for toxic metal cations[24-30] and toxic anions[31-33] sensing via 'turn off' or 'turn on' mechanism. Conjugated polyfluorene based polymers are usually made water-soluble by introducing hydrophilic ionic groups, such as sulfonic,[21,34] carboxylate,[35-36] ammonium[37] or phosphonate[27,38] groups to the polymer side chains to overcome π–π stacking interactions among the hydrophobic polymer backbones as well as the enhanced enthalpic interactions with water.

Saccharides, viz. monosaccharide, disaccharide, oligo- or polysaccharide, are one of the primary biological materials in living cells and a source of energy for animals. However, its uncontrolled levels in biological system may lead to some diseases like renal glycosuria, cystic fibrosis, diabetes and even cancer.[39-42] So, the detection of saccharides or sugar level has attracted interest in diagnostic analysis, food technology and biological science.[43] There are also a few non-sugar *cis*-1,2 diols such as L-DOPA (L-3,4-dihydroxyphenylalanine) and L-ascorbic acid which are very useful and closely related to biological system. Animals, including human, synthesize L-DOPA through biosynthesis from the amino acid L-tyrosine. L-DOPA can cross the protective blood–brain barrier so it is used to increase dopamine levels in the critical treatment of Parkinson's disease.[44] However, overdose or long time use of L-DOPA causes different side effects like hypertension, nausea, insomnia, auditory and/or visual hallucinations and dopamine dysregulation syndrome etc.[45] In contrast, L-ascorbic acid which is generally known as Vitamin C, is a naturally occurring organic biomolecule compound with antioxidant properties. Deficiency of L-ascorbic acid can cause Scurvy which leads even into death also. So, in diagnostic analysis and biotechnology, the detection and determination of L-DOPA and L-ascorbic acid is very important.

Stable boronic acid based saccharide receptors have usually been used to create saccharide sensors as boronic acids are well known to bind diol biomolecules species with high affinities via reversible five- or six-membered cyclic boronate formation in non-aqueous or basic aqueous media.[46-47] The complexation results to a decrease of the pKa value and induces the formation of the anionic form of the boronic group at a definite pH.[48] The

anionic form of the boron group is electron-rich and possesses a sp^3-hybridized boron atom with a tetrahedral conformation. Boronic acid functionality has been widely introduced into small molecules as artificial receptors in fluorescent detection of carbohydrates however it is very less used in polymers.[49-52] Only a poly(aniline boronic acid)[53] has been prepared for potentiometric detection of saccharides and one boronic acid bearing polythiophene polymer is reported as fluoremmetric biological *cis*-diol sensor.[54] Recently, 4-vinylphenylboronic acid containing co-polymers have been reported for sensing glucose by utilizing micelle formation at the relatively high pH.[55-56] So, inclusion of boronic acid at the side chain residue in polyfluorene is expected to offer a highly sensitive fluorescent material for carbohydrates by amplification through cooperative multivalent interactions among carbohydrates and boronic acid residues. Again, it's very important to consider the biological aspect that the sensor system should be effective at the physiological pH. It does mean that sp^3-hybridized hydroxyboronate has to exist at physiological pH. It is only possible if the boronic acid has a pKa $\leq$ 7.0. Introduction of strong electron-withdrawing substituents on the bronic acid containing aromatic moiety is one of the possible ways to reduce the pKa of boronic acid derivatives.[57] In this present manuscript, we are reporting the synthesis of a water-soluble zwitterionic boronic acid-bearing conjugated polyfluorene (PFBA) (Scheme 1) having pKa $\leq$ 7.0 and explore its fluorescent responses towards *cis*-diol cointaining biomolecules like D-monosaccharides, L-ascorbic acid, L-DOPA at physiological pH.

Experimental

Materials

All these reagents (fluorene, 1,6-dibromohexane, 1,4-phenyldiboronic acid, neutral alumina, copper (II) bromide, $Pd(PPh_3)_4$, 3-bromopyridine, L-DOPA and L-ascorbic acid from Sigma-Aldrich Co. Ltd. and rest from Merck India Pvt. Ltd.) were used without further purification, and all the experiments were performed at room temperature (25 ^oC). All the solvents used for the synthesis were from Merck India Pvt. Ltd. and were used after distillation under N_2 environment. Poly(9,9′-(6′′-bromohexyl)fluorene-co-alt-1,4-phenylene) (Polymer 3) in Scheme1 was synthesized according to our previous reports.[58-59] All aqueous solutions were prepared in water (18 MΩ) obtained from a Millipore Milli-Q system.

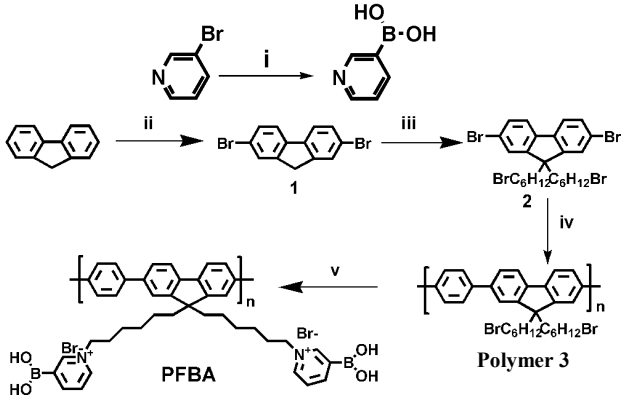
Instrumentation

The ^1H NMR and ^{13}C NMR spectra were acquired on 300 and 500 MHz Bruker DPX spectrometer, using $CDCl_3$ as solvent and TMS as standard reference, with chemical shift given in parts per million. UV-Vis spectra of all samples were studied with Hewlett-Packard UV-Vis spectrophotometer (model 8453). Photoluminescence (PL) studies of solution were recorded with a Horiba Jobin Yvon Fluoromax 3 spectrometer at an excitation wavelength 350 nm. Gel-permeation chromatography (GPC) was performed on a Shimadzu size-exclusion chromatographer (SEC) equipped with a guard column and a styragel HT-6E (7.8x300 mm, Waters) column with differential refractive index and UV/Vis detection by using THF as an eluent (1 mL/min at 35 ^oC) and polystyrene as a standard.

Synthesis of 3-pyridine boronic acid

Toluene (15 mL) in a two-necked flask, equipped with a stirrer, was cooled down to −60 °C. *n*BuLi (1.6 M in hexane, 8.7 mL, 13.9 mmol) was mixed with the toluene. After the internal temperature reached −60 °C, a solution of 3-bromopyridine (2 g, 12.60 mmol) in toluene (5 mL) was added. The internal temperature was maintained at <−50 °C by controlling the rate of addition. A yellow solid precipitated. The resulting slurry was aged for 15–30 min, then THF (10 mL) was added slowly, keeping the internal temperature at <−50 °C. The mixture was aged for 15 min, then trimethyl borate (1.68 mL, 15.12 mmol) was added over 2 min. The solids dissolved and a brown homogeneous solution was obtained. The reaction solution was warmed to −15°C and quenched with 2.7 N HCl (15 mL). The phases were separated and organic layer was washed with water (30 mL). The aqueous layers were combined, neutralized with 10N NaOH to pH 7, and extracted with THF (3×50 mL). The organic layers were combined and concentrated to dryness. The resulting solid was dissolved with THF (20 mL) and methanol (20 mL). This mixture was filtered to remove inorganic salts and the solids were washed with THF/MeOH (1:1; 25 mL). The combined filtrate was concentrated and dried to give 640 mg (61%) material as title product.

^1H NMR (500 MHz, CD_3OD) δ (ppm): 8.55 (1 H, s), 8.38 (1 H, d, J = 5 Hz), 8.21 (1 H, d, J = 7 Hz), 7.50 (1 H, m), 1.97 (2 H, s). HRMS. Calculated for $C_5H_6BNO_2$: 122.918. Found: 124.131 [M+H].



Scheme 1 Synthesis of the cationic poly(fluorene) PFBA. Reagent and condition used. i) n-BuLi, Trimethylborate, Aq. HCl ii) $CuBr_2$ on alumina, CCl_4, Refluxed, 5 h. iii) 1,6-dibromohexane, TBAB, KOH, H_2O, 75 ^oC, 15 mins. iv) $Pd(PPh_3)_4$, 2 M Na_2CO_3, toluene, 90 ^oC ; and v) 3-pyridine boronic acid, DMF, 70 ^oC, 3 days.

Synthesis of boronic acid bearing polyfluorene (PFBA)

3-Pyridineboronic acid (0.5 g) was added to a solution of polymer 3 (0.3 g) dissolved in a mixture of DMF (30 mL) and THF (20 mL). The mixture was stirred at 70^oC under a N_2 atmosphere for 48 h. After removing most of the solvent in rotary evaporator, the residue was precipitated in THF (50 mL). The precipitate was collected by filtration, washed with THF (100 mL) several times, and dried under vacuum at room temperature to obtain PFBA as a yellowish-brawn solid (500 mg).

^1H NMR (500 MHz, CD_3OD) δ (ppm): 8.55 (4 H, m), 8.26 (2 H, d), 7.96 (4 H, m), 7.42-7.43 (6 H, m), 4.50 (4 H, m), 2.64 (m,

4H), 2.10-0.8 (br, aliphatic H).As PFBA was sparingly soluble in CD_3OD, ^{13}C NMR study was not performed. It displays UV-Vis absorption maxima at λ_{max}=348 nm and emission maxima at λ_{max} =406 nm in aqueous solution.

Preparation of the solutions

A stock solution of PFBA (1 mg/100 mL) was prepared in 0.1 M phosphate buffer solution (pH=7.4) (H_2O:EtOH = 4:1). 10 mM aqueous solutions of different bio-analytes (D-glucose, D-fructose, L-ascorbic acid and L-DOPA) were prepared by dissolving into water.

Result and Discussions

It is stated earlier that incorporation of boronic acid functionality to polyfluorene side chain may effectively produce a brilliant fluorescent probe for *cis*-diols. To achieve this, boronic acid bearing cationic polyfluorene polymer was synthesized according to Scheme 1. Starting from fluorene, we prepared poly(9,9′-(6′′-bromohexyl)fluorene-*co*-*alt*-1,4-phenylene) (Polymer 3) by Pd-catalyzed Suzuki cross-coupling polymerization technique using monomer 2 and 1,4-phenylbisboronic acid.[58-59] Water soluble zwitterionic fluorescent conjugated boronic acid-bearing polyfluorene (PFBA) was prepared from Polymer 3 through a postpolymerization quaternization with 3-pyridineboronic acid. The molecular weight of precursor Polymer 3 was determined from GPC study using polystyrene as standard, and it yielded the following values M_w~8000 and PDI~1.75. Because of polymer aggregation with the column fillers induced by the ionic nature of side chains, it was difficult to determine the molecular weight of the cationic polyfluorene (PFBA).[60]

The solubility of zwitterionic boronic acid-bearing PFBA is different from its precursor Polymer 3 that is readily soluble in common organic solvents, such as chloroform, THF, DCM, and moderately soluble in DMF and DMSO. It is insoluble in polar solvents like ethanol, methanol, acetone and water. Unlike polymer 3, PFBA is insoluble in common organic solvents like THF, chloroform, methylene chloride, and toluene, soluble in DMSO, ethanol, methanol etc. and sparingly soluble in water. Water solubility of PFBA arises from the hydrophilic feature of its cationic pyridinium groups which readily overcome the π–π stacking interactions among the hydrophobic polymer backbones and enhance solubility in polar solvent like water.

Photophysical analysis

To examine the optical properties of the boronic acid-bearing polyfluorene co-polymers, UV-Vis and PL experiments have been carried out using the stock solutions of PFBA in 0.1 M PBS. The optical properties of PFBA polymer get significantly affected if its boronic acid part associates with the *cis*-diol binding sites of bio-analytes. In the present manuscript, we have used four *cis*-diol containing bio-analytes (chemical structures in Fig. 1a). 1 mM of each analyte solutions are added to 2 mL of stock PFBA solution and corresponding UV-Vis and fluorescence spectroscopy are monitored. After the addition of analytes to PFBA solution, there are no distinct colour changes observed with naked eye. However after irradiation of 365 nm UV light on PFBA and PFBA with analyte solutions (Fig. 1b), quenching of the bright blue fluorescence of PFBA has been observed. In addition of D-glucose, the fluorogenic response of PFBA turns greenish-yellow from bright blue emission in PFBA.

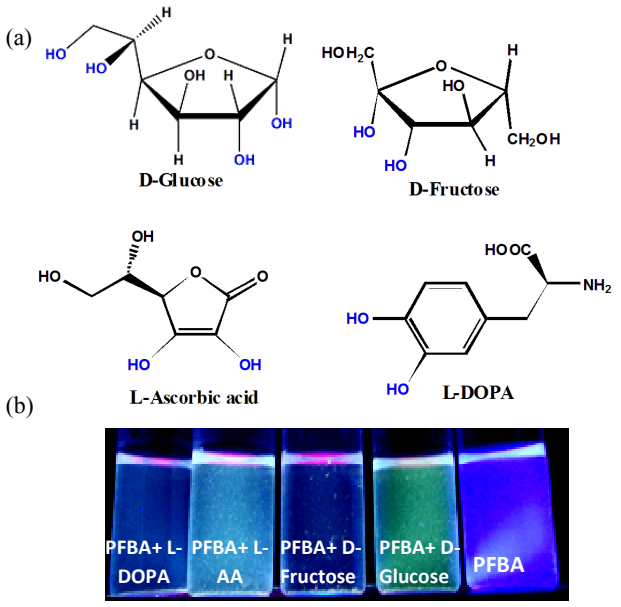


Fig. 1 (a) Chemical structures of four bio-analytes and their diol binding sites. (b) Fluorogenic response of probe PFBA (1 mg/100 mL in water:ethanol (4:1) mixture) upon addition of bio-analytes (1 mM).

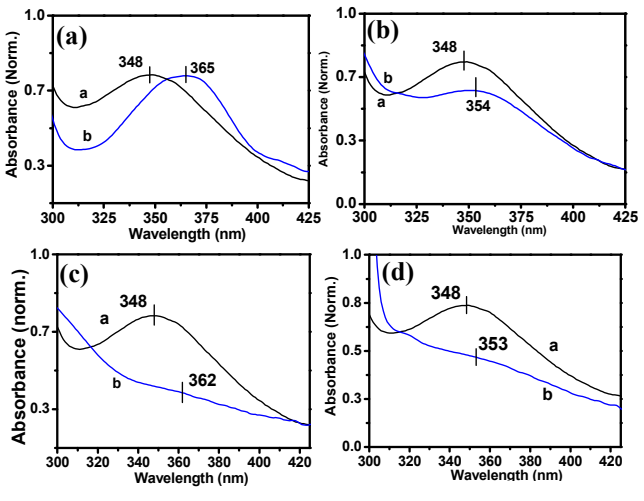


Fig. 2 UV-Vis spectra of PFBA (1 mg/100 mL in water:ethanol (4:1) mixture) in 0.1 M phosphate buffer (pH 7.4) in absence and presence of different concentrations of (A) D-glucose,(B) D-fructose, (C) L-ascorbic acid and (D) L-DOPA. In each case spectra a for blank PFBA and b for PFBA with analyte (1 mM).

In UV-Vis spectroscopy, PFBA exhibits absorption maxima at 348 nm that is the characteristic absorption for π-π* transition of polyfluorene conjugated backbone (Fig. 2a-d). When the *cis*-diol analyte solutions are added (1 mM each), the characteristic absorption maxima of polyfluorene is red shifted to higher wave length. For D-glucose the red shift is maximum (17 nm) whereas for D-fructose, L-ascorbic acid and L-DOPA, the value of red shifting are 6, 14 and 5 nm respectively. This red shift of λ_{max} (5-

17 nm) after addition of analyte solutions clearly indicates the boronate ester formation by *cis*-diol analyte and boronic acid functionality of PFBA polymer. Though D-glucose retains the absorption intensity, there is decrease of absorption intensity in case of D-fructose, L-ascorbic acid and L-DOPA.

To get further insight into the fluorescence quenching by addition of different bio-analytes, the decrease in fluorescence intensity has been investigated by adding successive aliquots of analytes stock solutions to the diluted 2 mL of PFBA solution in 0.1 M PBS buffer (pH 7.4). As shown in Fig. 3a-d, quenching of photoluminescence intensity is observed even upon addition of very low level of analyte and the fluorescence intensity decreases rapidly upon the further addition of the added concentration of analytes. During quenching by step wise addition of analyte solutions, the λ_{max} of emission at 406 nm does not change its position, indicating that a static quenching is predominant here rather than the dynamic quenching.[54,61]

In PL spectra of PFBA with D-glucose (Fig 3a), it is very interesting to note that a greenish-yellow emission band ~525 nm is enhanced upon stepwise addition of D-glucose solution to PFBA. This emission is exclusively due to the aggregation of polyfluorene polymer chain, owing to the formation of boronate ester between PFBA and D-glucose. From Fig. 1a it is evident that two diol functionalities present in D-glucose are capable to form complex with boronic acids of PFBA. So, there is a possibility of the formation of boronate ester with two successive polymer chains that are coming closer to form an induced aggregated polymer structure.[62] As a result of this, an overall greenish-yellow emission is prominent after addition of D-glucose in PFBA (Fig. 1b).

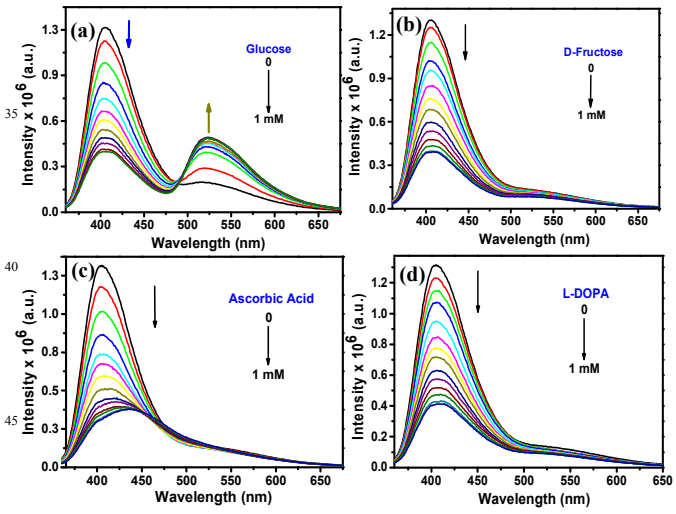


Fig. 3 Photoluminescence spectra of PFBA (1 mg/100 mL in water:ethanol (4:1) mixture) in 0.1 M phosphate buffer (pH 7.4) in presence of different concentrations of (a) D-glucose,(b) D-fructose, (c) L-ascorbic acid and (d) L-DOPA.

Quenching mechanism

PL intensities with respect to addition of different analyst concentrations are plotted in Fig. 4a-d. From the plots, it is clearly seen that the highest quenching occurs after addition of ~850-900 μM of different analyte to 2 mL of PFBA solution at pH 7.4. In static quenching, the quencher (here the *cis*-diol analytes) forms a ground-state complex with the fluorophore which is subsequently quenched after excitation: quenching constant K_{sv} in static quenching equals the apparent complex formation constant of quencher to fluorophore.[54] With the use of the Stern–Volmer equation, we can determine binding constants by simple way (Fig. 4a-d inset).[63] A quantitative measure of the fluorescent quenching are achieved by determining the well known Stern–Volmer constant (K_{sv}):

$$I_0/I = 1 + K_{sv}[Q],$$

Where, I_0 is the initial unquenched fluorescence intensity, I is the fluorescence intensity in the presence of quencher, K_{sv} is the static quenching constant, [Q] is the quencher concentration. The equation discloses that I_0/I increases in direct proportion to the concentration of the quencher (Fig. 4a-d inset) and K_{sv}, which is the Stern–Volmer constant, defining the efficiency of quenching, can be determined from slope of the plots. The Stern–Volmer quenching constants of PFBA owing to the formation of complex with these four bio-analytes are calculated as well as listed in Table 1.

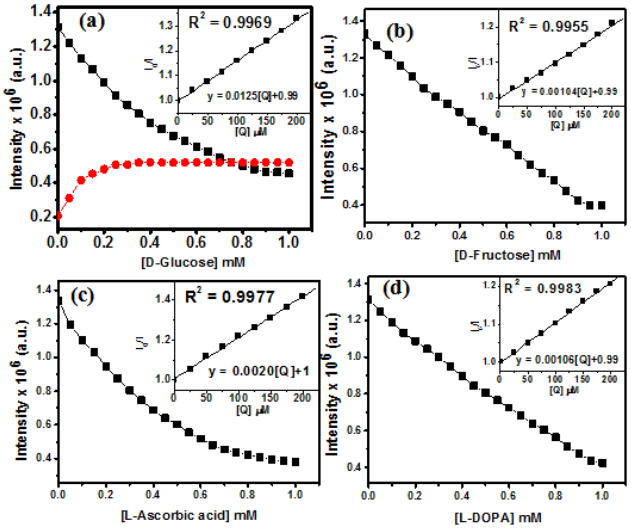


Fig. 4 The fluorogenic titration profile of PFBA (1 mg/100 mL in water:ethanol (4:1) mixture) at 406 nm upon addition of different biomolecules analytes (Black line), (a) D-glucose, (b) D-fructose, (c) L-ascorbic acid and (d) L-DOPA. Florescent titration profile of PFBA at 525 nm upon addition of D-glucose (Red line) in (a). In inset, The Stern–Volmer curves of PFBA with different concentrations of biological species in 0.1 M phosphate buffer (pH 7.4) is given in the inset.

Table 1 The Stern–Volmer quenching constants of polymer PFBA by biological species.

Species	D-glucose	D-fructose	L-ascorbic acid	L-DOPA
Quenching Constants	1.25×10^4	1.04×10^3	2.0×10^3	1.06×10^3

To evaluate the details about the nature of quenching mechanism, we have performed the time-correlated single photon counting (TCSPC) measurement with PFBA in 0.1 M phosphate buffer (pH 7.4) in presence of different concentrations of D-glucose. From Fig. 5a-b, it is evident that average life time of PFBA does not change with addition of quencher analyte. The corresponding time constants, amplitudes and average life times are given in Table 2. As the average life time is constant even

after addition of D-glucose to PFBA, we can conclude that static quenching is prominent after addition of bio-analytes.[61]

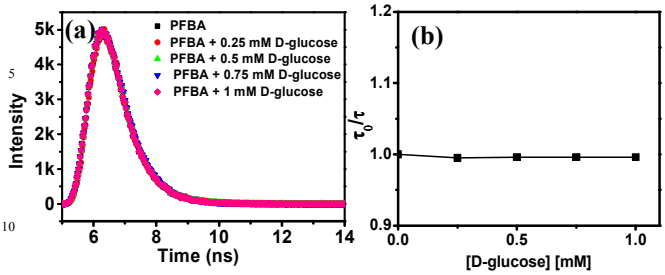


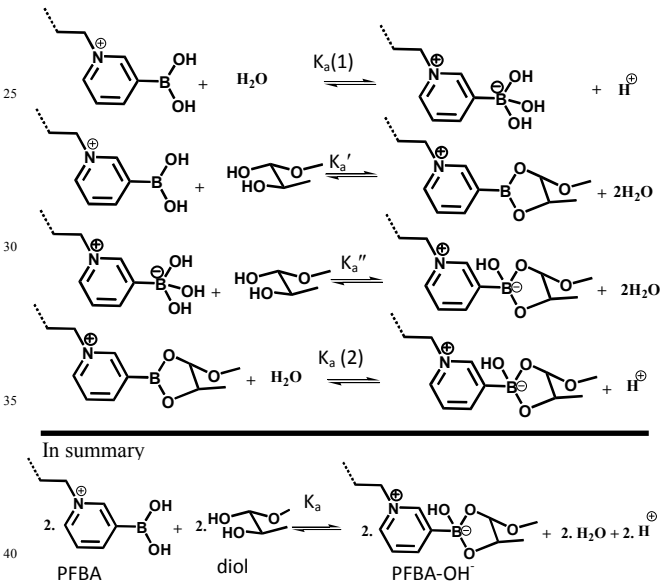
Fig. 5 (a) TCSPC plot of PFBA (1 mg/100 mL in water:ethanol (4:1) mixture) in 0.1 M phosphate buffer (pH 7.4) in presence of different concentrations of D-glucose, (b) corresponding τ_0/τ vs. D-glucose concentration plot.

Table 2 Fluorescence lifetime data for PFBA and PFBA with D-glucose.

Sample	τ_1 [ns]	α_1	τ_2 [ns]	α_2	$\langle\tau\rangle$ [ps]
PFBA	0.449	62.05	0.726	37.95	550
PFBA+0.25mM D-glucose	0.442	66.75	0.778	33.25	553
PFBA+0.5mM D-glucose	0.445	67.46	0.775	32.54	552
PFBA+0.5mM D-glucose	0.446	67.04	0.770	32.96	552
PFBA+1mM D-glucose	0.448	66.54	0.760	33.46	552

Effect of pH on fluorogenic response

pH has tremendous influence on boronate ester formation between boronic acids and *cis*-diols in aqueous medium according to the equilibrium reactions in Scheme 2.



$$K_a = \frac{[PFBA-OH^-][H^+]}{[PFBA][diol]} \quad ----(1)$$

Scheme 2: Equilibrium between boronic acids of PFBA and *cis*-diol.

To shift the equilibrium towards right side, the formation of the hydroxyboronate (PFBA-OH$^-$), which has a sp^3 hybridized tetrahedral boron atom, is required. The value of pKa(2) should be 1–2 units lower than pKa(1) which ranges from 8 to 10,[64,65] It is stated earlier that, PFBA-OH$^-$ will only exist in fair amounts at neutral pH if the boronic acid has a pKa ≤ 7.0. It means that more alkaline condition is required to form strong complexes (Scheme 2). In our design, we have kept a strong electron withdrawing pyridinium cation attached with the aromatic moiety-bearing boronic acid so that pKa of boronic acid is ≤ 7.0.

Our approach to detect carbohydrates at neutral pH is possible due to the formation of a zwitterionic pyridinium hydroxyboronate having low pKa value of ~4.0,[54,64,66] which is obtained through post-polymerization functionalization of bromide bearing polyfluorene with 3-pyridineboronic acid. Alkylation of the nitrogen in 3-pyridineboronic acid results a pyridinium salt, which simultaneously functions as an electron withdrawing group as well as the solubilising unit in polar solvent. Moreover, the pH effect on fluorescent responses of PFBA to *cis*-diols analytes (Fig. 6) has been checked. This plot reveals that PFBA shown optimum fluorescence response at pH 7.4 for all analyte biomolecules.

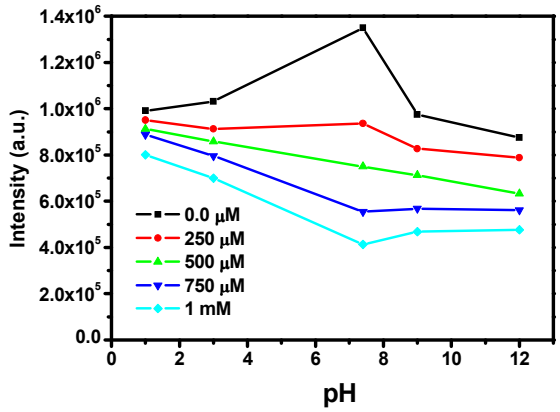


Fig. 6 Fluorogenic responses of 406 nm peak of polymer to D-glucose at different pH values. Concentrations of D-glucose are given in the plot.

Conclusions

In summary, a novel, zwitterionic, boronic acid bearing conjugated polyfluorene polymer (PFBA) has been designed, synthesized and characterized and its brilliant blue fluorescence is quenched after complexation with diol bearing biomolecules like monosaccharides, L-DOPA and L-ascorbic acid. The quenching of fluorescence of PFBA after addition of *cis*-diol analytes is exclusively due to static quenching which is confirmed by photoluminescence and TCSPC studies. The static quenching has occurred due to ground state boronic ester formation with bio-analytes. In presence of D-glucose only, the PFBA probe produces a greenish yellow emission owing to the formation of induced aggregated polymer structure. The quenching constants are very high for D-glucose and the value is highest as it contains two diol for binding with boronic acids. The conjugated polymer based fluorescent probe (PFBA) gives maximum response with bio-analyte from aqueous solution at physiological pH 7.4 and succinctly envisages the use of the polymer in biological system.

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Notes and references

Polymer Science Unit, Indian Association for the Cultivation of Science, 2A & 2B Raja S. C. Mullick Road., Jadavpur, Kolkata – 700032, India
E-mail: psusm2@iacs.res.in

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For 'Table of Content' Use

Water soluble, boronic acid bearing polyfluorene has been synthesized for the detection of diol containg biomolecules through effective fluorescence 'turn off' at pH 7.4.