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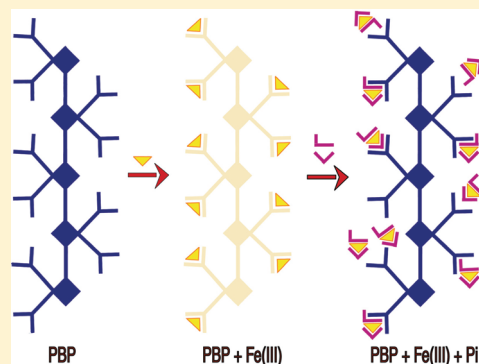
# A Remarkable Superquenching and Superdequenching Sensor for the Selective and Noninvasive Detection of Inorganic Phosphates in Saliva

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**S** Supporting Information

**ABSTRACT:** A neutral polyfluorene derivative, poly(9,9-bis(6'-benzimidazole)hexyl) fluorene-*alt*-1,4-phenylene (PBP), is synthesized and well characterized by  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, and GPC. PBP exhibits exemplary activity as noninvasive fluorescence sensor and accomplishes in situ monitoring of important biological targets like  $\text{Fe}^{3+}$  and inorganic phosphates in saliva. On binding  $\text{Fe}^{3+}$ , the fluorescence of PBP is quenched by 97% in label free conditions. The fluorescence of PBP is regained back on adding inorganic phosphate with a fluorescence enhancement of 106% due to the displacement of  $\text{Fe}^{3+}$  from the PBP. This PBP assay is further used to detect and estimate inorganic phosphate in fresh saliva samples which is also able to enhance the fluorescence by >94%. This ability of PBP to accomplish in situ monitoring and estimation of indispensable biological targets like  $\text{Fe}^{3+}$  and inorganic phosphates rapidly, at very low concentration with very high selectivity corroborates the extension of this assay system for safe clinical applications.



## 1. INTRODUCTION

The increasing need for accurate detection of inorganic phosphate ( $\text{P}_i$ ) remains an important research area in clinical analysis. In cells starved of glucose, the  $\text{P}_i$  quantity is found to be very high because intracellular glycolysis depends on the level of  $\text{P}_i$ .<sup>1</sup> As a consequence of the vital role of  $\text{P}_i$  in physiological system and medical diagnostics, few approaches such as ELISA<sup>2</sup> column chromatography<sup>3</sup> spectrometric assay,<sup>4</sup> potentiometric assay<sup>5</sup> and the conventional malachite green assay method<sup>6</sup> have been developed in the past. Although some of these techniques are still in use, they are less sensitive and have focused on measuring the  $\text{P}_i$  content present in blood. Since all these assays are invasive, require labeling and need longer duration, it is essential to develop rapid, sensitive and consistent method for the determination of  $\text{P}_i$ . In this regard, saliva monitoring offers a simple and noninvasive approach for rapidly evaluating it as a diagnostic fluid in real time.<sup>7</sup> Yet, neutral systems based on conjugated polymers (CPs) that are highly efficient, selective and sensitive to small alteration have not yet been developed for  $\text{P}_i$  detection noninvasively. Furthermore, saliva has lesser proteins compared to blood and the amount of  $\text{P}_i$  in saliva is 3 orders of magnitude more than blood serum. Because of these potential advantages and the ability to monitor systemic health and disease states easily, the development of saliva-based diagnostic assays that would be noninvasive, cheap, painless and patient friendly is extremely crucial and necessary.

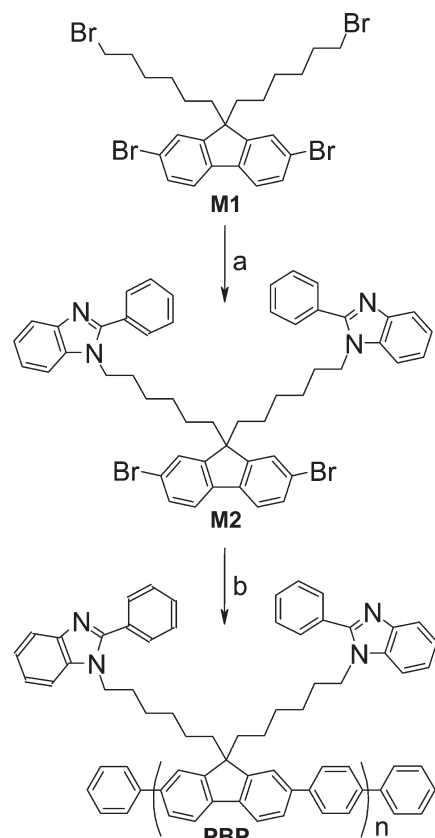
Similarly, iron is the most abundant and vital metal in physiological system and has a central role in metabolism, for oxygen transport and oxidative phosphorylation. Iron deficiency causes anemia and formation of hemoglobin ceases, whereas iron

overload damages cells and tissues.<sup>8</sup> Hence, newer approaches for the selective detection of  $\text{Fe}^{3+}$  have appeared recently.<sup>9</sup> Because of their unique signal amplification properties, CPs have been widely investigated as optical transducers in highly sensitive chemical and biological sensors.<sup>10</sup> Polyfluorenes and its derivatives are an imperative class of CPs that have been extensively studied for blue LED's and photovoltaics due to their extraordinary optical, electrical, and thermal properties.<sup>11</sup> These properties can be altered and tuned as desired by preparing copolymers and composites and controlling the side chain length as well as incorporating different functional groups onto them.<sup>12</sup> This has helped achieve higher device performance and widened their applications in diverse interdisciplinary fields such as medical diagnostics, chemical and biological probing and energy conversion by harnessing the unique molecular wire effect and fluorescence amplifying capabilities.<sup>13</sup> CP-based fluorescent chemosensors for detecting physiologically and environmentally relevant alkali, alkaline earth metal ions, as well as heavy and transition metal ions has attracted considerable attention in recent years.<sup>14</sup> Yet neutral CP sensors for detecting analytes in biological fluids such as saliva, serum, etc. have not been developed. Hence, designing neutral CPs possessing functional groups capable of selectively binding vital biological analytes but does not exhibit responses from non specific binding remains a fascinating challenge. In this article, we report the synthesis of poly(9,9-bis(6'-benzimidazole)hexyl) fluorene-*alt*-1,4-phenylene (PBP), a

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Scheme 1. Synthetic Procedure for M2 and PBP<sup>a</sup>

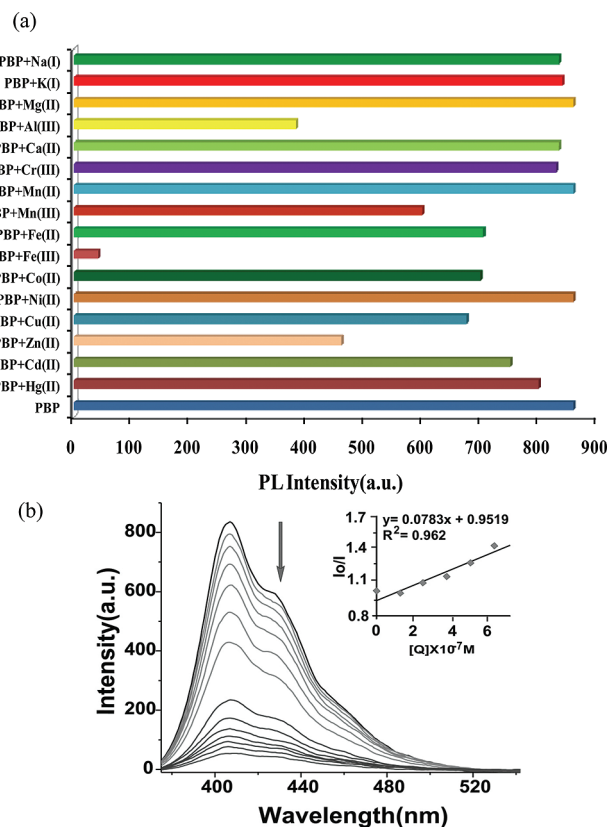
<sup>a</sup> Key: *a* = 2-Phenyl benzimidazole, sodium hydride, dry THF, reflux, 24 h, 94%. *b* = 1,4-phenylboronic acid, Pd(PPh<sub>3</sub>)<sub>4</sub>, K<sub>2</sub>CO<sub>3</sub> in THF/water mixture, Reflux, 36 h, 80%.

novel polyfluorene derivative, that performs selective recognition of Fe<sup>3+</sup> and P<sub>i</sub> in aqueous medium and saliva.

## 2. RESULTS AND DISCUSSION

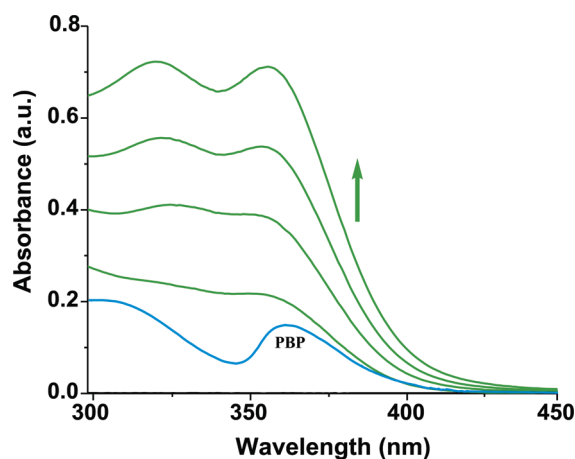
Scheme-1 depicts the synthesis of desired PBP polymer in high yield. In the first step of the synthetic route, 2-phenyl benzimidazole was *N*-alkylated with 2,7-dibromo-9,9-bis(6-bromohexyl)-9H-fluorene, M1, in the presence of sodium hydride to give M2 in 94% yield. Suzuki coupling of M2 with 1,4-phenylboronic acid resulted in the formation of desired copolymer, PBP, as yellow-brown solid in 80% yield. PBP was well characterized by <sup>1</sup>H and <sup>13</sup>C NMR and was found to have MW of 13964, PDI-2.01 (GPC in THF-PS as internal standard). Since PBP was soluble in several organic solvents including a combination of solvents such as THF/water, DMSO/water and DMF/water, the detection of several important analytes that are present in diagnostic assays such as saliva, serum etc. could be conveniently carried out.

The optical properties of CPs are modified when they associate with analytes, resulting in spectral changes that can be monitored by the shift in wavelength or quenching of the fluorescence intensity.<sup>15</sup> Since PBP possesses benzimidazole groups which are known to bind metal ions, we studied its interaction with a series of metal salts such as Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup>, Al<sup>3+</sup>, Ca<sup>2+</sup>, Cr<sup>3+</sup>, Mn<sup>2+</sup>, Mn<sup>3+</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, and Hg<sup>2+</sup> having concentrations 3.2 × 10<sup>-6</sup> M in water



**Figure 1.** (a) Bar diagram depicting effect of various metals on the fluorescence intensity of PBP in THF:water (4:1) (b) PL spectra of PBP (1.6 × 10<sup>-6</sup> M) in THF:water (4:1) with increasing concentration of Fe<sup>3+</sup> shows >97% quenching. Inset: Stern–Volmer quenching trend of PBP upon addition of Fe<sup>3+</sup>.

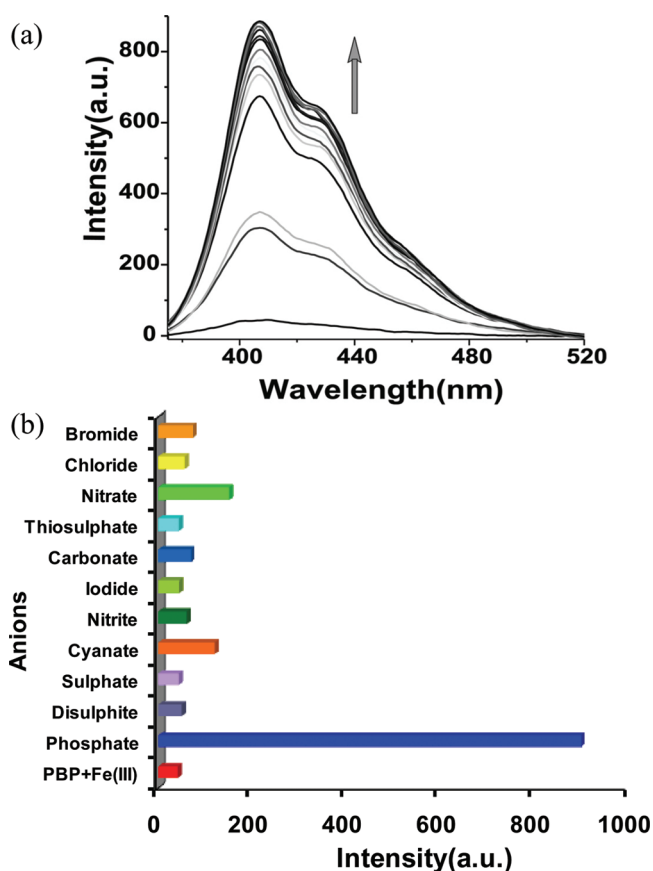
and monitored the changes by fluorescence spectroscopy. Metal salts like Zn<sup>2+</sup> and Al<sup>3+</sup> caused fluorescence quenching of PBP at very high concentrations whereas all other metal salts had negligible or virtually no effect on PBP (Figure 1a). Performing titration with aqueous solution of Fe<sup>3+</sup> metal salts (chloride and perchlorate) induced large quenching in the fluorescence of PBP (Figure 1b), and >97% reduction in fluorescence intensity occurred, clearly implying strong and selective association of PBP and Fe<sup>3+</sup>. Such remarkable quenching of the polymer PBP by Fe<sup>3+</sup> occurs because several factors such as transfer of electron, competent energy migration and exciton delocalization along the PBP occurs simultaneously.<sup>16</sup> The quenching behavior of PBP was studied using the Fe<sup>3+</sup> titration data. Figure 1b (inset) shows a Stern–Volmer plot (*K*<sub>SV</sub>) (*I*<sub>0</sub>/*I* vs [*Q*]), where *I*<sub>0</sub> is the fluorescence intensity of PBP, and *I* is the fluorescence intensity of PBP after addition of a given concentration of quencher [*Q*] where [*Q*] = Fe<sup>3+</sup>. The *K*<sub>SV</sub> value was found to be 7.8 × 10<sup>5</sup> M<sup>-1</sup>. The absorption maxima (360 nm) of PBP showed ~7 nm shift on titrating with Fe<sup>3+</sup> (Figure 2) confirming the static quenching mechanism.<sup>17</sup> Further, lifetime values of PBP were not modified on titration with Fe<sup>3+</sup>, signifying the static quenching mechanism. The enhancement in the 360 nm peak is because of the complex formation between the phenyl benzimidazole of the polymer PBP and Fe<sup>3+</sup> which was proved by performing similar experiments with phenyl benzimidazole (PBI) without being attached to any conjugated polymer (Supporting Information, Figure S10).



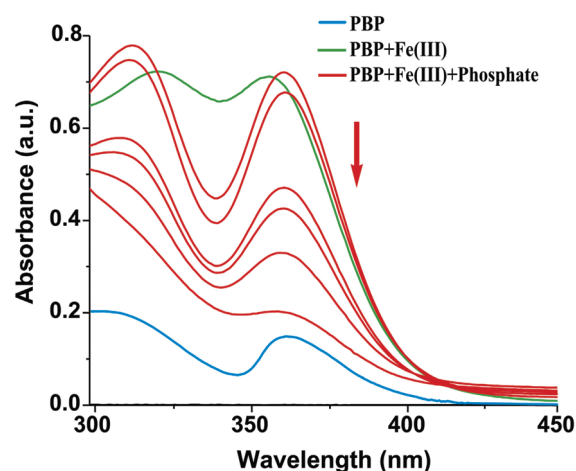
**Figure 2.** UV-vis titration of PBP in THF:water (4:1) with increasing concentration of  $\text{Fe}^{3+}$ .

Figure 1b indicates that maximum quenching of PBP ( $\sim 97\%$ ) occurs at total concentration as low as  $3.38 \times 10^{-6}$  M of  $\text{Fe}^{3+}$ . This significant capability of PBP to detect aqueous solution of  $\text{Fe}^{3+}$  irrespective of various salts widens its utility in probing  $\text{Fe}^{3+}$  in biological systems with a extensive range of possible applications that comprise iron metabolism, anemia etc. Recently, there has been growing interest in using  $\text{Fe}^{3+}$  compounds as binders of phosphate<sup>18</sup> in blood serum, whole blood and dietary phosphates, instead of traditional binders like salts of calcium, aluminum,<sup>19</sup> and lanthanum,<sup>20</sup> which have numerous drawbacks like toxicity, intolerance to patients, expensive and unidentified side effects.<sup>21</sup> Yet, noninvasive techniques utilizing  $\text{Fe}^{3+}$  and CP-based phosphate binders, that are safe and simple for patients, remains undeveloped and merits investigation. Employing the PBP- $\text{Fe}^{3+}$  assay system, we examined the binding of  $\text{P}_i$  at pH-7.4 by fluorescence spectroscopy. Since  $\text{HPO}_4^{2-}$ :  $\text{H}_2\text{PO}_4^{1-}$  exists in a 61:39 ratio at pH 7.4, we have used the term “inorganic phosphates or only  $\text{P}_i$ ” in this article.

Figure 3a depicts the rapid fluorescence dequenching of 106% on titrating aqueous  $\text{P}_i$  with PBP- $\text{Fe}^{3+}$  assay (pH 7.4 in water). It is clearly observed that the largest spectral enhancement occurs at concentration of  $5.3 \times 10^{-7}$  M of  $\text{P}_i$ , which gradually leveled off at a concentration of  $6.6 \times 10^{-6}$  M. To determine the effect of other anions on the dequenching of PBP- $\text{Fe}^{3+}$  spectra, similar titration experiments were carried out with several anions such as bromide, chloride, nitrate, thiosulfate, carbonate, iodide, nitrite, cyanate, sulfate, disulfite anions. As observed in Figure 3b, the fluorescence of PBP- $\text{Fe}^{3+}$  assay is barely dequenched on addition of these anions. The  $>100\%$  dequenching of PBP- $\text{Fe}^{3+}$  assay on adding aqueous  $\text{P}_i$  to the quenched PBP- $\text{Fe}^{3+}$  assay indicates that the  $\text{Fe}^{3+}$  attached to PBP has been displaced that leads to recovery of the fluorescence. The mechanism for the remarkable fluorescence dequenching is proved by examining the changes in the UV/vis spectra of the PBP polymer on adding  $\text{Fe}^{3+}$  and the spectral changes occurring thereafter in the presence of  $\text{P}_i$ . As shown in Figure 2 (green), it is clearly observed that there is a 7 nm shift in absorption and significant spectral enhancement on addition of  $\text{Fe}^{3+}$  to PBP. However, on addition of  $\text{P}_i$  to the cuvette containing the PBP- $\text{Fe}^{3+}$  complex, the PBP- $\text{Fe}^{3+}$  spectra is modified (red, Figure 4) to an extent of having similarity with that of the original PBP spectra. (blue, Figure 4) This clearly suggests that the addition of  $\text{P}_i$  to a solution



**Figure 3.** (a) PL spectra of PBP- $\text{Fe}^{3+}$  with increasing concentration of  $\text{P}_i$  (pH-7.4 in water) shows 106% dequenching. (b) Bar diagram depicting effect of various anions on the fluorescence intensity of PBP- $\text{Fe}^{3+}$ .

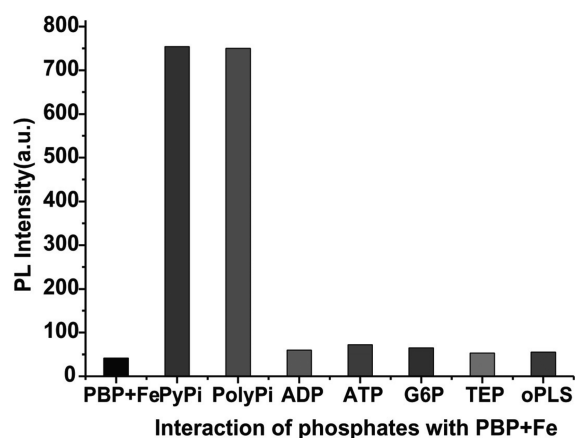


**Figure 4.** UV-vis spectra of PBP- $\text{Fe}^{3+}$  with increasing concentration of  $\text{P}_i$  (pH 7.4 in water).

of PBP- $\text{Fe}^{3+}$  results in the displacement of  $\text{Fe}^{3+}$  from the polymer PBP.

The interaction of PBP- $\text{Fe}^{3+}$  with few representative inorganic and organic phosphates was also performed and is represented in Figure 5. The fluorescence dequenching of PBP- $\text{Fe}^{3+}$  by pyrophosphate ( $\text{PyP}_i$ ) (Figure S1, Supporting Information)



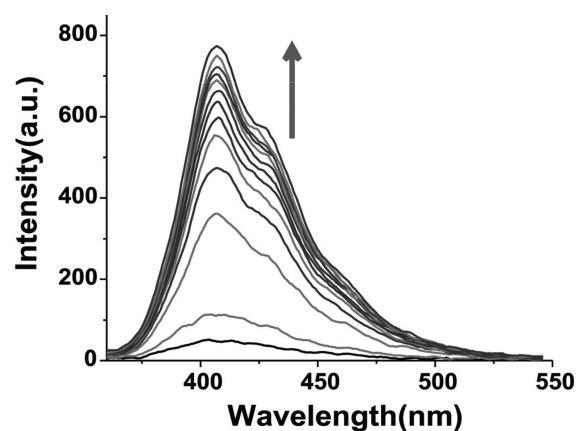


**Figure 5.** Bar diagram depicting effect of organic/inorganic phosphates on the fluorescence intensity of PBP-Fe<sup>3+</sup>.

and polyphosphate (PolyP<sub>i</sub>) was 93% and 90%, whereas, adenosine diphosphate (ADP), adenosine triphosphate (ATP), glucose-6-phosphate (G6P), triethyl phosphate (TEP) and *o*-phospho-L-serine (oPLS) cause no changes to the fluorescence spectra of PBP-Fe<sup>3+</sup>. The above dequenching results are clear indications that PBP-based assay can be selectively used for the detection of P<sub>i</sub> in aqueous medium. The outstanding “super-dequenching” of PBP-Fe<sup>3+</sup> assay on adding P<sub>i</sub> presents an exceptional, exceedingly sensitive and homogeneous assay exclusively for P<sub>i</sub> detection.

Given that PBP-Fe<sup>3+</sup> assay is capable to detect P<sub>i</sub> at such low concentrations, we employed this platform for fluorometric detection and estimation of P<sub>i</sub> in saliva. Fresh saliva sample was used in the study and was always obtained after cleaning the mouth with water. In a typical collection procedure, saliva was allowed to accumulate in the base of mouth for ~3 min and collected in a sample vial. This was repeated until approximately 5 mL was obtained and which was used immediately for analysis. Since the procedure adopted for obtaining saliva is totally non-invasive and painless to a patient and fresh samples are available whenever desired, this method provides a safe and alternative investigative fluid to serum for diagnostic application. This freshly obtained whole saliva was directly used in the titration experiments with PBP-Fe<sup>3+</sup> assay. On increasing the quantity of saliva (2  $\mu$ L aliquot) in the cuvette, maximum fluorescence dequenching taking place was 94% (Figure 6). Importantly the spectral characteristics observed on adding saliva to the PBP-Fe<sup>3+</sup> was identical to that observed with P<sub>i</sub> indicating that the fluorescent enhancement occurring is due to the P<sub>i</sub> present in saliva.

With the above spectral data we have also performed the estimation of P<sub>i</sub> in saliva using this fluorometric titration method since the PBP-based assay shows remarkable dequenching in the presence of P<sub>i</sub> at very low concentration. From the spectral changes depicted in Figure 6, the minimum quantity of P<sub>i</sub> that could be estimated accurately in saliva (4  $\mu$ L) is found to be 1.44 mmol/L that was in agreement with the standard titration plots (Figure 3a) values (Supporting Information, Figure S2), implying that extremely low quantities of P<sub>i</sub> could be estimated using this assay. The maximum fluorescence enhancement observed was 94% implying the presence of 8.64 mmol/L ( $\pm 0.09$ ) of P<sub>i</sub> in saliva, analyzed by performing four titration experiments. With the help of the above assay, the analysis of P<sub>i</sub> in saliva can be performed within 30 min and has the advantage of



**Figure 6.** Enhancement in PL spectra of PBP + Fe<sup>3+</sup> on addition of 2  $\mu$ L aliquots of saliva.

being very precise and uncomplicated. To calculate the correctness of these values, estimation of supernatant P<sub>i</sub> was carried out using the literature method.<sup>22</sup> The P<sub>i</sub> quantity estimated by this method was 3.22–5.90 mmol/L, well within the fluorometric estimation results range. The results obtained by PBP-Fe<sup>3+</sup> assay for P<sub>i</sub> detection in saliva has several advantages over serum-based clinically permitted method,<sup>23</sup> such as higher order of sensitivity, noninvasive, rapid, label-free, and direct sampling of fresh saliva as and when required. Furthermore, since the absorbance (280 nm) and fluorescence (340 nm) peaks of saliva<sup>24</sup> do not overlap or interfere with the PBP-Fe<sup>3+</sup> assay system, PBP-based assays can be applied for the detection of vital saliva components.

Experiments with this neutral polymer PBP suggest that detection of biologically essential and indispensable cations and anions such as Fe<sup>3+</sup> and P<sub>i</sub> can be performed accurately by the above assay. PBP exhibits very high fluorescence quenching and dequenching in the presence of Fe<sup>3+</sup> and P<sub>i</sub> without requiring labeling and in a patient friendly method using saliva as a detection fluid. The most important obstacle to use saliva as a diagnostic fluid has been the fact that several vital analytes are generally present in lower quantities in saliva than in serum. However, this is not the case with P<sub>i</sub>, which is usually present in much higher quantities in saliva than in serum. Yet, there are no available methods for the detection of P<sub>i</sub> from whole saliva. From practical point, since this PBP-based assay detects P<sub>i</sub> in saliva (noninvasively) with exceedingly high selectivity and sensitivity than currently used clinical methods from serum, it can have immediate use in diagnostic application, forensic science and dental monitoring. These studies also demonstrate that it is possible for identifying many more vital disease-associated salivary biomarkers with emerging technology platforms such as the fluorimetric estimation method in combination with neutral CPs.

### 3. CONCLUSION

In conclusion, we have described for the first time the use of a neutral fluorescent polymer PBP as a rapid, sensitive and practical technique for the detection of P<sub>i</sub> in saliva. Systems such as PBP with such robust fluorescence “superquenching and dequenching” activity are unexplored, providing unique platform for a plethora of safe diagnostic applications. Additionally as a compelling reason, saliva as a diagnostic fluid has several advantages over serum, most prominently, the noninvasive collecting techniques dramatically reduces anxiety and discomfort among

patients and procurement of samples repeatedly over time for accurate monitoring. In general, application of neutral CP-based assays for saliva monitoring can revolutionize healthcare and diagnostic procedure that presently rely mainly on painful and unsafe venepuncture techniques. Through these studies we have thus demonstrated that it is possible to identify and estimate vital disease-associated salivary biomarkers by using emerging technology platforms such as neutral CP materials along with patient friendly and simple to perform methods.

## 4. EXPERIMENTAL SECTION

**4.1. Reagents and Materials.** All chemicals and solvents were purchased from Aldrich Chemicals (India), Merck (India), or Ranbaxy (India) and were used as received. THF distilled over sodium benzo-phenone and Milli-Q water is used.

**4.2. Instrumentation.** Compounds were characterized by FT-IR,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectroscopy. Molecular weight was determined by using gel permeation chromatography (Waters) and found to have MW of 13964 PDI 2.01 (GPC-PS as standard in THF).  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR analysis were recorded in a Varian-AS400 (Oxford) 400 MHz instrument, and the chemical shifts were recorded in parts per million (ppm) on the scale using tetramethylsilane (TMS) as a reference. FT-IR analysis was carried out on air-dried samples with a Perkin-Elmer-Spectrum One FT-IR Spectrometer from 4000 to  $450\text{ cm}^{-1}$ . UV–visible spectra were recorded, by dissolving a calculated amount of the sample in an appropriate solvent, on a Hitachi UV–visible U–2001 Spectrophotometer or on a Perkin-Elmer Lambda UV–visible Spectrophotometer. Photoluminescence spectra were recorded using a Varian spectrometer by excitation of the polymer at 370 nm.

**4.3. Synthesis and Characterization of Monomer (M2) and Polymer (PBP).** Synthesis of the monomers: Monomer M1 was prepared by previously reported method. For M2, a mixture of 2,7-dibromo-9,9-bis(6-bromohexyl)-9H-fluorene (1.0 g, 1.54 mmol) (M1), 2-phenyl-1H-benzimidazole (1.21 g, 6.19 mmol), and sodium hydride (0.15 g, 6.19 mmol) was stirred in dry THF for 24 h under nitrogen atmosphere. Reaction progress was monitored by TLC (1:9 EtOAc: hexane). After completion of the reaction, the base was removed by filtering through a Celite pad. The compound was purified by recrystallization from EtOH to obtain pure compound (1.21 g, Yield = 94%), confirmed by FTIR, NMR, and mass spectroscopy. Supporting Information (Figure S3–S6)

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ),  $\delta$  (ppm): 7.81 (d), 7.61 (d), 7.45(m), 7.26 (t), 4.09 (t), 1.78 (m), 1.60 (t), 0.95 (q), 0.46 (q).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ),  $\delta$  (ppm): 153.7, 152.0, 142.8, 139.1, 135.4, 130.5, 129.8, 129.3, 128.8, 126.0, 122.8, 122.5, 121.7, 121.4, 119.8, 110.2, 55.4, 44.5, 40.0, 29.6, 29.1, 26.2, 23.4. FTIR ( $\nu_{\text{max}}$  KBr pellete): 3417, 2928, 1643, 1520, 1450, 1394, 1155, 1060, 812, 743, 698  $\text{cm}^{-1}$ . MS for  $\text{C}_{37}\text{H}_{40}\text{Br}_2\text{O}_2$ : calculated, 675.80; found, 675.3.)

**4.4. Synthesis of the Polymer, PBP.** A mixture of M2 (0.50 g, 0.57 mmol), 1,4-phenylenediboric acid (0.11 g, 0.64 mmol), potassium carbonate (0.20 g, 1.10 mmol), THF (10 mL)/water (5 mL), and Pd(0) (0.033 g, 0.028 mmol) was carefully degassed and stirred at 70 °C for 24 h. After 24 h iodobenzene (0.02 g, 0.11 mmol) was added and allowed to continue at 70 °C for 12 h followed by addition of 0.05 g of phenylboronic acid. The solution was cooled to room temperature; it was precipitated in methanol to remove monomers. The filtrate was concentrated, precipitated into methanol again, and the obtained solid was then washed with 200 mL of acetone. The resultant polymer PBP was obtained as powder, 0.36 g, Yield = 80%. Supporting Information (Figure S7–S9)

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ),  $\delta$  (ppm): 7.71(d), 7.51(d), 7.38(m), 7.26(s), 4.04(m), 1.93(m), 1.59(m), 0.98(m), 0.65(d).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ),  $\delta$  (ppm): 153.8, 143.2, 140.5, 135.7, 130.8,

129.8, 129.1, 128.8, 127.7, 122.8, 122.5, 121.4, 120.12, 110.2, 55.4, 44.7, 40.5, 29.7, 29.4, 26.4, 23.7.

**UV Titration of Polymer with Metal.** A stock solution of PBP ( $5 \times 10^{-5}\text{ M}$ ) in THF/water and a stock solution of Fe(III) ( $2 \times 10^{-4}\text{ M}$ ) in Milli-Q water were prepared. Then 30  $\mu\text{L}$  of PBP solution was diluted with 3 mL of THF and this was titrated with Fe(III) metal solution up to a 1:2 concentration. The PBP absorption maximum ( $\lambda_{\text{max}}$ ) was found at 370 nm.

**4.5. UV Titration of Polymer + Metal with Phosphate.** Stock solution of  $\text{P}_i$  was prepared ( $2 \times 10^{-4}\text{ M}$ ) in Milli-Q water. The stock solution was diluted up to ten times and titrated against PBP + Fe(III) as above.

**4.6. PL Titration of PBP with Metal.** A PBP stock solution of  $5 \times 10^{-5}\text{ M}$  in THF/water and metal  $2 \times 10^{-4}\text{ M}$  solution in Milli-Q water was prepared. The concentration of PBP in cuvette was adjusted to be  $1.6 \times 10^{-6}\text{ M}$ . The fluorescence response with various metals was performed in aqueous solution until PBP and metal ratio in cuvette reached 1:2 under the excitation wavelength of 370 nm. This was found to be highly sensitive toward Fe(III), as when the concentration ratio reached 1:2, 97% quenching was observed.

**4.7. PL Titration of PBP + Fe(III) Assay with Anions.** A solution of PBP ( $5.0 \times 10^{-5}\text{ M}$ ) was prepared in THF/water and metal ( $2 \times 10^{-4}\text{ M}$ ) solution in Milli-Q water. The solutions of NaOH were prepared in Milli-Q water and was used to adjust the pH to 7.4. A solution of PBP was placed in a quartz cell and the fluorescence spectrum was recorded. First  $\text{Fe}^{3+}$  was added to quench the fluorescence, and titration was performed with various anions up to 1:2 ratio of PBP + Fe(III) and anions to dequench the fluorescence. The fluorescence intensity changes were recorded at room temperature each time (excitation wavelength: 370 nm).

**4.8. PL Titration of PBP + Fe(III) Assay with Phosphates.** A solution of PBP ( $5.0 \times 10^{-5}\text{ M}$ ) was prepared in THF/water. The solution of inorganic phosphates were prepared in Milli-Q water. A solution of PBP was placed in a quartz cell (10.0 mm width) and the fluorescence spectrum was recorded. After the addition of solution of Fe(III) ( $3.2 \times 10^{-6}\text{ M}$ ) to quench the fluorescence of PBP, the solution of phosphate ( $6.24 \times 10^{-6}\text{ M}$ ) was introduced in portions and the fluorescence intensity changes were recorded at room temperature each time (excitation wavelength: 370 nm).

**4.9. PL Titration of PBP + Fe(III) Assay with Pyrophosphate.** Same procedure as above was used.

**4.10. Fluorescence Response of PBP + Fe(III) with Human Saliva.** Fresh saliva was always used for analysis after rinsing the mouth with Milli-Q water. In a typical collection procedure, saliva was allowed to accumulate in the base of mouth for 3 min and collected in a sample vial. This was repeated until approximately 5 mL was obtained and was used immediately for analysis. Saliva was added in 2  $\mu\text{L}$  aliquots to the cuvette and changes were recorded in the PL spectra.

## ■ ASSOCIATED CONTENT

Supporting Information. PL and UV–vis titrations and FT IR and  $^1\text{H}$  and  $^{13}\text{C}$  NMR, and mass spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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