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Selective and Sensitive Sensing of Free Bilirubin in Human Serum Using Water-Soluble Polyfluorene as Fluorescent Probe

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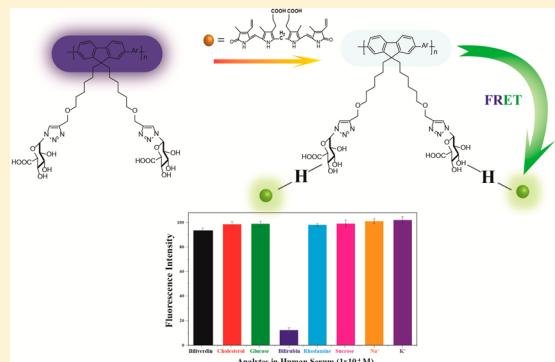
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Supporting Information

ABSTRACT: The adherence of serum protein on conjugated polymer is a major bottleneck in the application of the latter for selective sensing of small biomolecules in blood serum. In this report, we present new polyfluorenes with D-glucuronic acid appendage that is a nonreceptor for any serum protein, thereby providing a platform for selective sensing of free bilirubin in the clinically relevant range of <25 to >50 $\mu\text{mol/L}$ in human blood serum. The appended D-glucuronic acid formed noncovalent interactions with bilirubin, which in conjunction with favorable spectral overlap between the polymers and bilirubin facilitated efficient FRET process in aqueous solutions. Addition of bilirubin resulted in the quenching of the polyfluorene emission with simultaneous appearance of bilirubin emission exhibiting visual emission color change from blue to light green. The polymer remained stable in serum even under severe basic conditions and exhibited high selectivity with visual sensitivity only toward free bilirubin in human serum in the presence of crucial interferences such as hemoglobin, proteins, biliverdin, glucose, cholesterol, and metal ions. Nanomolar sensing of bilirubin could also be demonstrated successfully using one of the D-glucuronic acid appended polymer (PF-Ph-GlcA), which could sense ~150 nm of bilirubin in human serum. The combined role of energy transfer and noncovalent interaction highlights the potential of the new polymer design for highly selective sensing activity in complex biofluids.



INTRODUCTION

Exploration of water-soluble conjugated polymers remains an active area of research for their applicability in chemo-^{1–3} and biosensors,^{4–10} targeted delivery,^{11,12} cell imaging,^{13–15} and logic gates.^{16,17} Solubility in aqueous medium is the prime prerequisite for a conjugated polymer to find desired potential in bioapplications. Generally, the rigid conjugated polymers are functionalized with either charged side chains or nonionic neutral side chains with high polarity to achieve solubility in aqueous medium. The hydrophilic pendant groups aid in compensating the hydrophobic nature of the polymer backbone, thereby enhancing water solubility and also facilitating interactions with specific targeted analytes.¹⁸ Functionalization of ionic pendants^{19–21} induces remarkable water solubility, but selectivity is compromised in the presence of multiple biological and nonbiological substances such as proteins²² and nucleic acids²³ due to nonspecific electrostatic interactions.²⁴ Conjugated polymers with charged pendants are known to show differential binding toward serum proteins that are used to develop sensing array for proteins.²⁵ Substitution of neutral side chains of sugar units such as glucose, mannose, etc., also act as receptors toward proteins.^{26,27} Consequently, selective sensing of small biomolecules like free bilirubin in the presence of serum proteins poses significant challenges to a conjugate polymer based probe.

The detection of free bilirubin in serum is considered as one of the potential routes for diagnosing liver disorders.²⁸ Liver disorders result in increment of free bilirubin concentration from normal level of <25 μmol (<1.2 mg/dL) to >50 $\mu\text{mol/L}$ (>2.5 mg) in blood indicative of jaundice condition.²⁹ Excess level of free bilirubin is severely toxic to human body because it can accumulate in body organs and causes brain hemorrhage by crossing the blood–brain barrier.³⁰ Considering the drawbacks in the currently practiced diazo test method for free bilirubin estimation,³¹ fluorometric biosensors offering amplified sensitivity and selectivity with a wide window of analyte concentration range are highly desirable. Reports on fluorometric detection of bilirubin in human blood serum are very sparse. However, very recently, Au nanoparticle conjugated HSA protein as fluorometric biosensor for the sensing of bilirubin in blood serum was reported.³² They made use of quenching of the emission of human serum albumin (HSA) stabilized gold nanoclusters upon interaction with bilirubin for detection of free bilirubin. In our current report we present a FRET based visual fluorescence color change (from blue to light green) method of detection of free bilirubin in human

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blood serum sample. In our previous work, pentadecylphenol (PDP)-functionalized polyfluorenes were employed as a model for fluorometric detection of bilirubin in organic (THF) medium.³³ Our preliminary work highlighted the potential of developing a water-soluble polyfluorene which could take advantage of the favorable photophysical properties of polyfluorene–bilirubin combination for the detection of bilirubin. The advantage is expected to be twofold if the moiety incorporated into polyfluorene backbone could also additionally act as an interaction site for bilirubin.

D-Glucuronic acid is a vital water-soluble carbohydrate that performs the key function of removing several insoluble toxic substances through glucuronidation and subsequent urinal excretion as glucuronides in human metabolism.³⁴ In addition, D-glucuronic acid is also a nonreceptor carbohydrate for the serum proteins, thereby providing a platform for receptor-free biosensing applications. In the liver, glucuronidation of bilirubin (insoluble in water) plays a vital role in forming bilirubin glucuronides (soluble in water) to avoid accumulation of toxic unconjugated (free) bilirubin in body fluids.³⁵ Thus, incorporation of D-glucuronic acid into polyfluorenes is expected to serve the dual purpose of imparting water solubility to the otherwise fully hydrophobic polyfluorenes while additionally acting as an interacting site for bilirubin. As the competitive interference of proteins can be eliminated via D-glucuronic acid appendage, selective sensing of bilirubin in serum is expected to be feasible. A polymer appended with the sugar unit D-glucose was also designed as a nonbilirubin interacting reference polymer.

In this report, new water-soluble conjugated polyfluorene appended with D-glucuronic acid and D-glucose were successfully designed and demonstrated for biosensing application in human blood serum for the first time. The structure and photophysical properties of the polymers were characterized by relevant techniques. The appendage of glucuronic acid imparted water solubility and amphiphilic nature that could aid in the self-organization of the polymers. Unfortunately, the water solubility of the glucose appended polyfluorene was not very good. The polymers exhibited good spectral overlap with bilirubin, thus fulfilling the criteria for energy transfer process. Fluorescence sensing experiments were carried out in human serum which contained hemoglobin, proteins, cholesterol, glucose, and metal ions as crucial interferences. The glucuronic acid appended polymer exhibited high degree of selectivity toward bilirubin that was verified from the poor quenching efficiencies of competing interferences such as biliverdin, cholesterol, sugars, and metal ions.

EXPERIMENTAL SECTION

Materials and Methods. 2,7-Dibromo-9H-fluorene, D-glucuronic acid, D-glucose pentaacetate, trifluoroacetic acid (TFA), bilirubin, biliverdin, phosphate buffered saline (PBS), human serum, azidotrimethylsilane, SnCl₄, propargyl alcohol, Pd(PPh₃)₄, 1,4-benzene-diboronic bis(pinacol ester), sodium methoxide, sodium hydride, Pd₂(dba)₃, CsF, quinine sulfate, tetrabutylammonium bromide, 1,6-dibromohexane, uridine diphosphate glucuronic acid sodium salt, and bis(pinacolato)diboron were purchased from Aldrich Company Ltd. and were used as received. Copper sulfate, sodium ascorbate, HCl, acetic anhydride, iodine, sodium thiosulfate, potassium hydroxide, glucose, sucrose, sodium bicarbonate, K₂CO₃, and ethanol were purchased from Merck Chemicals Ltd. Tetrahydrofuran (THF), acetone, petroleum ether, ethyl acetate, DCM, and methanol were purchased locally and were purified using standard procedures. ¹H, ¹³C NMR and ¹H–¹H COSY spectra were analyzed using a Bruker-

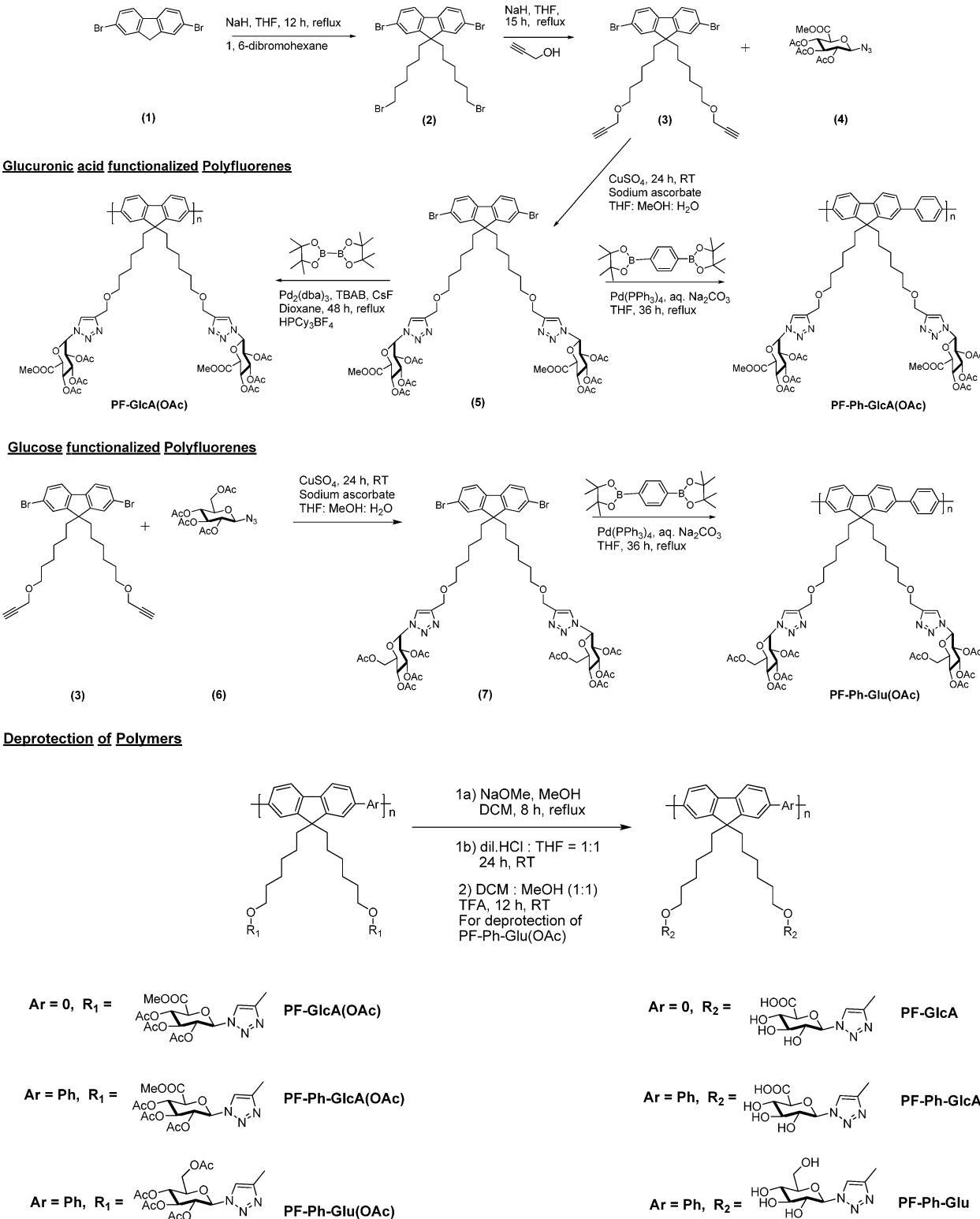
AVENS 200 and 400 MHz spectrometer. Chemical shifts are reported in ppm at 25 °C using CDCl₃ and MeOH-d₄ as solvents containing a trace quantity of tetramethylsilane (TMS) as internal standard. The purity of the compounds was determined by HR-MS or MALDI-TOF in combination with elemental analysis. The MALDI-TOF analysis was done on Voyager-De-STR MALDI-TOF (Applied Biosystems, Framingham, MA) equipped with 337 nm pulsed nitrogen laser for the purpose of desorption and ionization. A 2 μM solution of sample was premixed with DHB (2,5-dihydroxybenzoic acid) matrix in THF and mixed well before spotting on 96-well stainless steel MALDI plate through dried droplet method for the analysis. Mass spectra were measured with ESI ionization in MSQ LCMS mass spectrometer. Elemental analysis was done by Thermo Finnigan flash EA 1112 series CHN analyzer. HRMS (ESI) were recorded on ORBITRAP mass analyzer (Thermo Scientific, Q Exactive). The molecular weights of the polymers were determined by size exclusion chromatography (SEC), which was performed using a Viscotek VE 1122 pump, a Viscotek VE 3580 RI detector, and a Viscotek VE 3210 UV/vis detector in DMF using polystyrene as standards. DLS measurements were carried out on a Zetasizer ZS-90 apparatus, utilizing 633 nm red laser (at 90° angle) from Malvern Instruments. The reproducibility of the data was checked at least three times using independent polymer solutions.

Photophysical Studies. Absorption spectra were measured using PerkinElmer Lambda 35 UV-vis spectrophotometer. Steady-state fluorescence studies and time-resolved fluorescence lifetime measurements were recorded using Horiba Jobin Yvon Fluorolog 3 spectrophotometer having a 450 W xenon lamp for steady-state fluorescence, and fluorescence lifetime decays were collected by a time-correlated single photon counting (TCSPC) setup from IBH Horiba Jobin Yvon (U.S.) using a diode laser (IBH, U.K., NanoLED-320 and 375 L, with a $\lambda_{\text{max}} = 320$ and 375 nm) having a full width at half-maximum of 89 ps as a sample excitation source. The emission and excitation slit width was maintained at 1 nm throughout the experiments, and the data were obtained in “S1c/R1” mode (to account for the variations in lamp intensity). All the absorption and emission studies were conducted in dim light at the temperature of 15 °C in order to avoid structural isomerization of bilirubin, and the stock solutions were stored below the temperature of 10 °C. Because of the insolubility of bilirubin in water at pH = 7.4, all the bilirubin stock solutions were prepared at pH = 10 using PBS buffer.

Isothermal Titration Calorimeter Experiment. ITC experiments were carried out in Micro cal iTC-200 instrument. The titration was carried out in PBS buffer in alkaline medium at 25 °C using an isothermal titration calorimeter (Microcal iTC-200) with stirring at 1000 rpm. Polymer (200 μL with concentration of 2 mM) was taken in the sample cell, and bilirubin (~20 mM) was taken in the syringe. A typical titration experiment consisting of 19 consecutive injections each of 2 μL volume in the duration of 20 s at an interval of 180 s was conducted. Heat of dilution (T) upon addition of bilirubin in solvent was determined by performing a blank titration by injecting the bilirubin solution into the solvent. To obtain the heat of binding between polymer and bilirubin, the value of heat of dilution was subtracted from the total value of heat of binding. A single set of binding model was fitted with the binding isotherm which was used to extract binding constant (K), binding stoichiometry (N), change of enthalpy (ΔH) and the change of entropy (ΔS) for the binding.

Sensing of Free Bilirubin in Water. The stock solutions of the polymers were prepared at 1 μM in water at pH = 10 using PBS buffer. Bilirubin solutions of concentrations ranging from 1×10^{-6} to 1×10^{-4} M in PBS buffer at pH = 10 were prepared and kept in dark at 15 °C. The bilirubin solution was added to the 1 μM solution of polymer in water (pH = 10). The changes in the absorption and emission of the polymer on addition of different concentrations of bilirubin were recorded at 15 °C with the slit width of 1 nm.

Sensing of Free Bilirubin in Human Serum. Human blood serum was purchased from Sigma-Aldrich (Heat Inactivated from male AB clotted whole blood, USA origin and sterile-filtered, CAS no-HS667) and used as such without any further treatment. Human serum is the preferred part of blood containing vital components such

Scheme 1. Synthesis of Glucuronic Acid- and Glucose-Functionalized Polyfluorenes

as hemoglobin (≤ 25 mg/dL), total proteins (4–9 g/dL), iron (40% UG), cholesterol (80–200 mg/dL), glucose (50–180 mg/dL), and sodium (100–160 mg/dL). The absorption and fluorescence measurements were conducted using a fixed amount of $100 \mu\text{L}$ of human blood serum. The analyte, free bilirubin was added directly to serum in the concentration range of 1×10^{-6} to 1×10^{-4} M, and this mixture was added to the $1 \mu\text{M}$ solution of polymer in water (pH = 10). The final volume of test solution was adjusted to the fixed volume

of 2.5 mL by using PBS buffer at pH = 10. A control experiment was performed in the same volume of serum and polymer without the addition of bilirubin. The changes in the absorption and emission of the polymer on addition of different concentrations of bilirubin in the presence of serum were recorded at 15°C with the slit width of 1 nm.

Preparation of Conjugated Bilirubin. An aliquot of the freshly prepared liver homogenate was mixed thoroughly but gently with stock solutions of free bilirubin and uridine diphosphateglucuronide

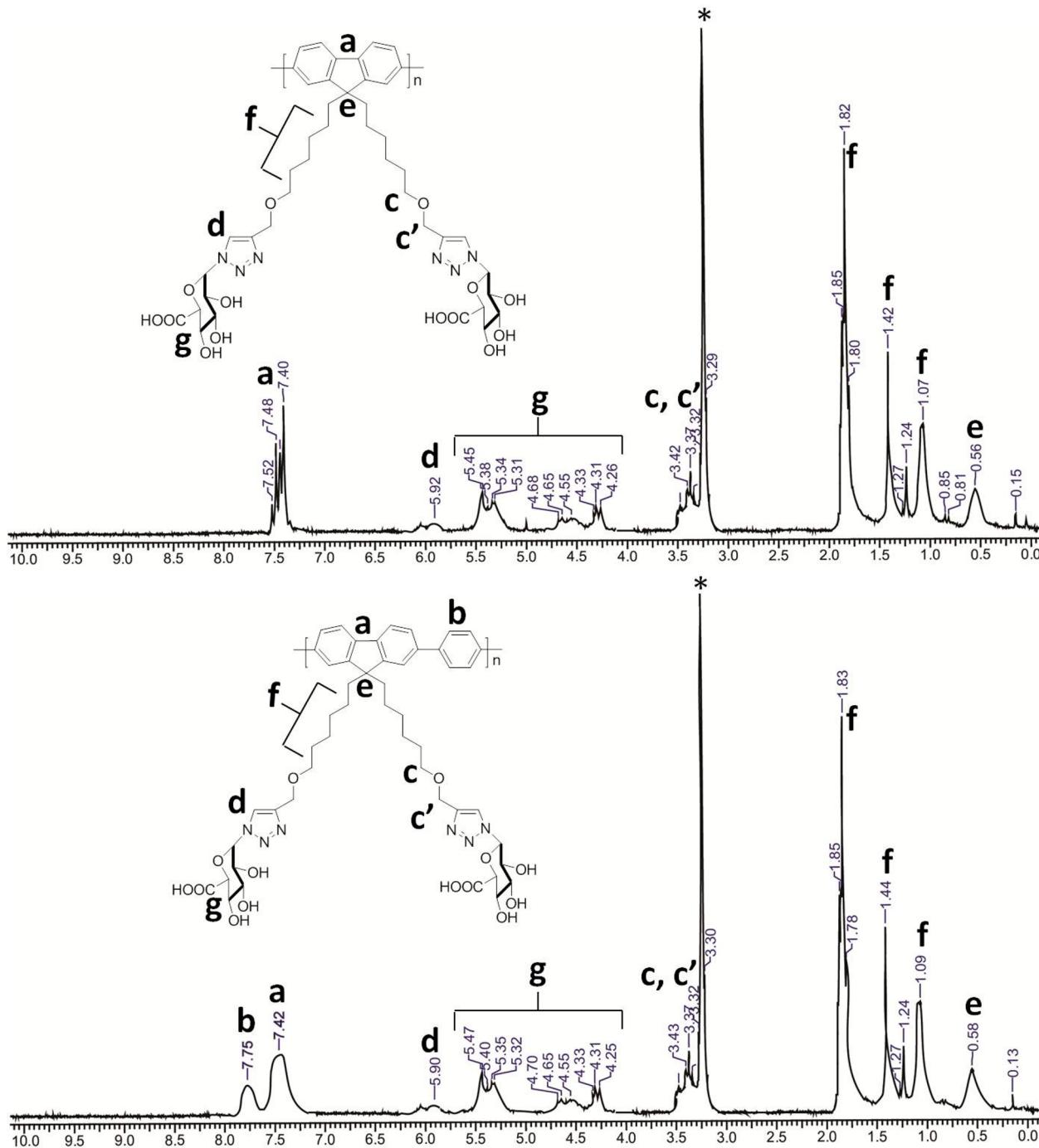


Figure 1. ^1H NMR spectra of PF-Ph-GlcA and PF-GlcA recorded in MeOH-D_4 .

(UDPG1cUA) to achieve final concentrations of 50–500 mg/L (0.86 to 8.6×10 mol/L) bilirubin and 10 to 5×10 mol/L UDPG1cUA. The acceptable pH range for the incubation mixture was 7.0–7.8. For routine incubations, 10–15 g/L Triton X-100 surfactant and 10 mol/L MgCl_2 were also present in the medium. The entire reaction mixture was kept in a nitrogen-rich atmosphere, shielded from strong light, and at 37 °C (with constant, gentle swirling) during the 3–4 h incubation. After incubation the content was extracted with chilled chloroform to remove unconjugated bilirubin from the medium. The contents were then centrifuged to remove the solid materials. The aqueous layer was separated, and the isolation of conjugated bilirubin was done using the Lucassen procedure.³⁶

RESULTS AND DISCUSSION

Synthesis and Structural Characterization. Water-soluble polyfluorenes functionalized with glucuronic acid (GlcA) and glucose (Glu) side chains were synthesized via click chemistry followed by palladium-catalyzed cross-coupling which is shown in Scheme 1a,b. 2,7-Dibromofluorene (**1**) was reacted with 1,6-dibromohexane in the presence of NaH to obtain 2,7-dibromo-9,9-bis(6-bromohexyl)-9*H*-fluorene (**2**). Compound **2** was etherified with propargyl alcohol to obtain 2,7-dibromo-9,9-bis(6-(prop-2-yn-1-yloxy)hexyl)-9*H*-fluorene (**3**). The synthesis of azide-functionalized glucuronic acid and glucose was followed from a literature report, and the scheme

of synthesis is shown in Figure S1.³⁷ In short, D-glucuronic acid/D-glucose was initially protected with acetic anhydride in the presence of iodine to give the penta-acetylated product. In the case of D-glucuronic acid an additional step involving conversion of the acetylated ester (COOAc) to its methyl ester derivative (COOMe) was also carried out by refluxing the penta-acetylated product in dry methanol for 24 h. The esters were further reacted with TMS-N_3 and SnCl_4 to obtain the corresponding azido derivatives **4** and **6**, respectively, as the major products which were purified by column chromatography. Click reaction between the azide-functionalized glucuronic acid/glucose and propargyl-functionalized fluorene was carried out using copper-catalyzed azide–alkyne chemistry to get the desired glucuronic acid- and glucose-functionalized fluorene monomers **5** and **7**, respectively. The monomers were copolymerized with 1,4-benzenediboronic bis(pinacol ester) via Suzuki coupling reaction to obtain the glucuronic acid-functionalized fluorene copolymer PF-Ph-GlcA(OAc) and glucose-functionalized fluorene copolymer PF-Ph-Glu(OAc), respectively. A homopolymer of the glucuronic acid-functionalized polyfluorene PF-GlcA(OAc) was also synthesized by using bis(pinacolato)diboron as the coupling reagent. The polymers were deprotected using NaOMe in MeOH:DCM (1:1) and further treated with dilute HCl or TFA (trifluoroacetic acid) to produce PF-Ph-Glu, PF-Ph-GlcA, and PF-GlcA. The polymers were purified by dialyzing the powdered sample against Milli-Q water using 2 kDa molecular weight cut-off dialysis membrane for 2 days. The deprotected glucuronic acid-functionalized polymers were readily soluble in water, DMF, DMSO, and MeOH compared to its precursor protected polymer which was insoluble in water. The glucose-functionalized polyfluorene had poor water solubility compared to the glucuronic acid-functionalized counterpart. The detailed synthetic procedures for the monomers and polymers are given in the Supporting Information (S2). Both protected and deprotected polymers were characterized by NMR spectroscopy for chemical structure analysis and size exclusion chromatography (SEC) (using DMF as solvent) for molecular weight analysis. Broadening of proton NMR peaks and broad distribution in SEC indicated high molecular weight for the polymers. The ^1H and ^{13}C NMR spectra of monomers and all polymers are shown in Supporting Information S3. The proton NMR spectra of deprotected polymers (PF-GlcA and PF-Ph-GlcA) are shown in Figure 1. The spectrum of the glucose-functionalized polymer PF-Ph-Glu is given in the Supporting Information S3n,o,p. The peaks observed at δ 3.7 ppm for methyl ester and δ 2.15–2.0 for acetyl protons in the NMR spectra for the protected polymers were completely absent in the case of deprotected polymers represented in Figure 1. Supporting Information S3-m shows the ^1H – ^1H COSY spectrum of the PF-GlcA(OAc) polymer in CDCl_3 , which was recorded to detect the coupling partners of the various peaks. The molecular weights (M_n and M_w) and polydispersity index (D_M) of the protected and deprotected polymers were determined by size exclusion chromatography (SEC) using DMF as solvent, and the corresponding values are tabulated in Table 1. The molecular weights of the new polymers were comparable with that of polyfluorenes functionalized with mannose reported in the literature.³⁸ Corresponding SEC chromatograms of the polymers are shown in Supporting Information Figure S4. As expected, the observed molecular weights of the protected polymers were larger compared to that

Table 1. Polymer Designation, Molecular Weight, Polydispersity Index, and Yield of Polymers before and after Deprotection

name	M_n^a	M_w^a	polydispersity index (D_M) ^a	yield (%)
PF-GlcA(OAc)	16800	25800	1.54	90
PF-Ph-GlcA(OAc)	20200	38300	1.89	85
PF-Ph-Glu(OAc)	17300	35800	2.07	87
PF-GlcA	15500	24200	1.56	78
PF-Ph-GlcA	17900	35900	1.94	80
PF-Ph-Glu	14200	25600	1.8	85

^aMolecular weights as determined by size exclusion chromatography using DMF as eluent at 30 °C and polystyrene standards for calibration.

of the deprotected polymers due to the elimination of protecting functional groups during deprotection.

Photophysical Properties of the Water-Soluble Polyfluorenes. The normalized absorption and emission spectra of the synthesized polymers were recorded in PBS buffer at pH = 7.4 and 10. Supporting Information Figure S5 compares the normalized emission and excitation spectra at pH = 10 and at the neutral pH = 7.4 for the three polymers. It should be mentioned here that due to poor solubility of the glucose-functionalized reference polymer in water at both the pH studied, its solution remained turbid. Red-shift and broadening of the emission were observed upon changing the pH from 7.4 to 10 for the glucuronic acid appended polyfluorene; however, the absorption spectra remained unaffected. The excitation spectra were comparable with that of the absorption spectra of the polymers. The sensing studies to be discussed later on were all carried out at pH = 10; therefore, the photophysical studies carried out at pH = 10 is discussed in detail here. Figure 2 compares the absorption (top) and emission (bottom) spectra of the three polymers in PBS buffer at pH = 10. PF-Ph-GlcA copolymer and PF-GlcA homopolymer showed absorption centered at 315 and 360 nm, respectively. The absorption spectrum of PF-Ph-Glu was broad (maximum ~360 nm), and the baseline was high due to scattering. The emission maximum of 0.1 OD aqueous solutions of the polymers PF-Ph-Glu excited at 315 nm and PF-Ph-GlcA and PF-GlcA excited at 360 nm was observed around 410–420 nm. The peak appearing at 350 nm in the emission spectra of PF-Ph-GlcA corresponded to the water Raman peak.³⁹ The emission spectrum of the polymers overlapped with the absorption maximum of bilirubin (target analyte) at 455 nm (denoted in dotted line), thereby favoring energy transfer from polymer to bilirubin. The fluorescence quantum yield of the polymers were determined in water at neutral pH using quinine sulfate ($\Phi_f = 0.546$ in 0.1 M H_2SO_4) as reference. The calculated quantum yield for PF-Ph-GlcA polymer was 0.43 and for PF-GlcA was 0.48. The quantum yield of the homopolymer (PF-GlcA) was slightly higher than PF-Ph-GlcA because the solubility of PF-GlcA (5 mg/mL) was better in water compared to that of PF-Ph-GlcA (2 mg/mL). The quantum yield of PF-Ph-Glu was not determined due to the turbid nature of the solution.

Fluorescence Sensing of Free Bilirubin in Water. Sensing of free bilirubin in water was targeted taking the key advantage of spectral overlap between emission of polymers and absorption of bilirubin which is the necessary condition for feasible Förster resonance energy transfer (FRET) to occur.⁴⁰ The glucuronic acid functionalization imparted water solubility

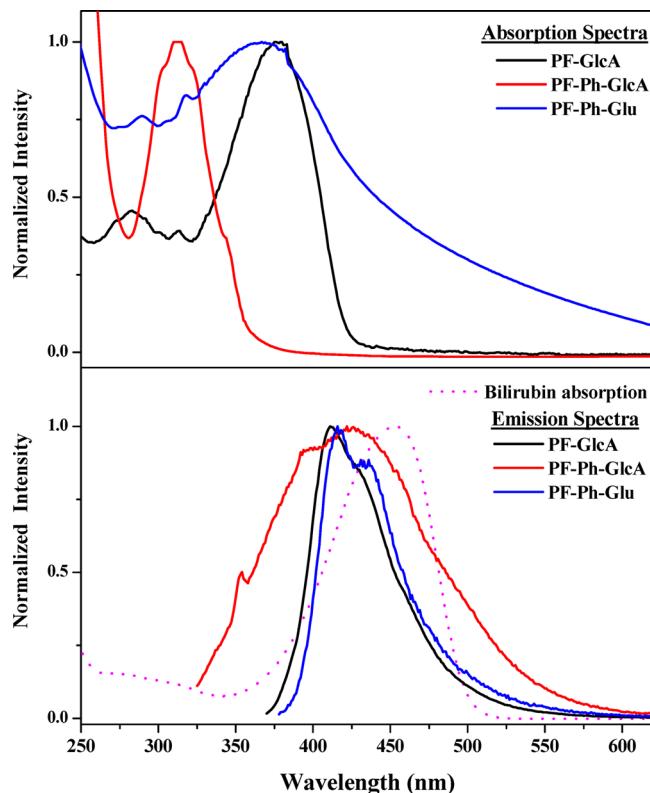
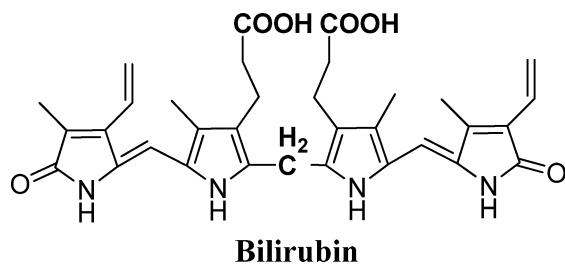


Figure 2. Absorption and emission spectra of PF-Ph-Glu, PF-Ph-GlcA, and PF-GlcA polymers along with absorption of bilirubin (dotted line, bottom Figure) in water at pH = 10.

to the polymer, facilitating sensing studies to be carried out in water. The bilirubin molecule is a tetrapyrrole moiety, which exists in the ridge tile conformation due to several intramolecular hydrogen bonds. It has been variously reported in the literature as a porphyrin-like structure or linear representation. Single-crystal X-ray structure of bilirubin has confirmed the Z configuration at the C4–C5 and C15–C16 positions.⁴¹ The gross structure of bilirubin is shown in Scheme 2 in its linear representation. Bilirubin is reported to have a very

Scheme 2. Gross Structure of Bilirubin in Its Linear Representation



low solubility of ~ 7 nmol in water at pH = 7.4.⁴² A pH of ~ 10 is required to induce solubility in water by breaking the hydrogen bonding in bilirubin. Therefore, studies involving bilirubin are usually reported at pH = 10 in the literature.⁴² Thus, stock solutions of bilirubin and polyfluorene were prepared at pH = 10. Figure 3a shows the absorption spectrum of the homopolymer PF-GlcA upon addition of various concentration of bilirubin ranging from 1×10^{-6} to 1×10^{-4} M in water at pH = 10. The concentration of the polymer was

kept constant at 1×10^{-6} M ($1 \mu\text{M}$). When the concentration of bilirubin was increased, an increase in the absorption of bilirubin at 455 nm was observed. Figure 3b shows the corresponding emission spectra upon excitation at 360 nm. The notable observation was the quenching of polymer fluorescence with concurrent increase in the emission of bilirubin at 520 nm. Supporting Information Figure S6 shows emission spectra of PF-GlcA, normalized at polymer emission maxima highlighting the FRET-induced bilirubin emission. A clear visual color change from blue fluorescence to light green was observed at higher concentrations of bilirubin, which is shown in the inset image of Figure S6. Bilirubin does not emit in any solvent (including water) due to inherent property of poor quantum yield. However, upon interaction with the polymer, mediated by the glucuronic acid moiety, enhanced green emission of bilirubin was observed. The intensity of the bilirubin emission observed in this PF–bilirubin system was much higher compared to that of bilirubin complexed with HSA protein reported in the literature.⁴³ Direct excitation at the bilirubin absorption maxima at 455 nm showed a steady increase in the bilirubin emission intensity with increase in the concentration of bilirubin as shown in Supporting Information Figure S7. FRET-induced bilirubin emission at 520 nm that was observed upon excitation of the polymer in water highlighted the potential of the polymer as a biosensor.

Similar sensing experiments were conducted for the copolymers (PF-Ph-GlcA and PF-Ph-Glu) in water as shown in Figure 3c–f. Similar conditions of addition of bilirubin to polymer in water were followed, and fluorescence quenching of the polymer was monitored. The changes in the fluorescence intensity of the polymers upon addition of bilirubin were plotted against the concentration of bilirubin, and the result is given in Figure S8a. From the plot it could be seen that the homopolymer PF-GlcA exhibited a sharper drop in the polyfluorene emission upon addition of bilirubin with almost complete quenching of the blue polymer fluorescence at $20 \mu\text{M}$ concentration of bilirubin. The copolymer exhibited complete quenching of the polymer fluorescence at $40 \mu\text{M}$ concentration of bilirubin. On the other hand, the glucose appended polymer PF-Ph-Glu exhibited very poor quenching of polymer fluorescence with only 40% quenching observed even for the highest addition of bilirubin ($1 \times 10^{-4} \text{ M}$). In fact, beyond $10 \mu\text{M}$ of bilirubin, the polymer fluorescence remained unaffected. Figure S8b compares the intensity of FRET-induced bilirubin emission upon excitation of polymers at the polymer absorption maximum, which also showed that the homopolymer exhibited higher FRET-induced bilirubin emission compared to the copolymers at higher bilirubin concentrations in water. The emission from bilirubin was almost non-observable in the case of the glucose appended polymer PF-Ph-Glu. The better sensing efficiency of the homopolymer compared to the copolymers could be attributed to the greater number of glucuronic acid appendage per repeat unit present in the former compared to the latter, which facilitated better polymer–analyte interaction. Isothermal titration calorimetric (ITC) experiments (to be discussed later on) pointed toward a higher binding constant for the homopolymer with bilirubin, which also supported this observation of better sensing efficiency for the homopolymer. Thus, the above experiment stressed the importance of interaction between the polyfluorene and free bilirubin in facilitating the biosensing property of the polymers.

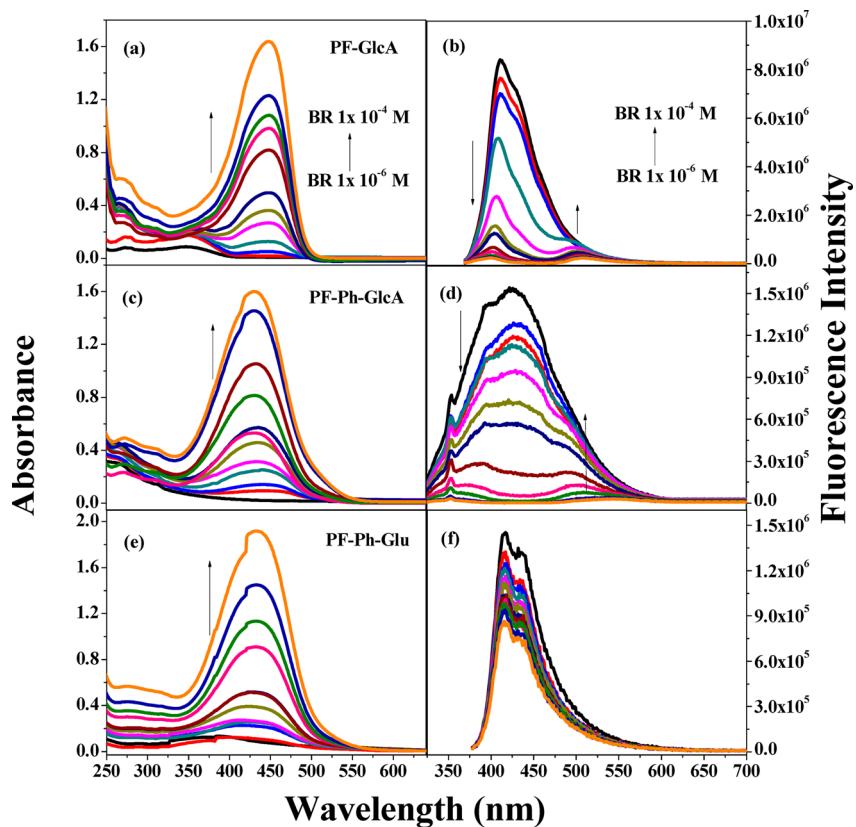


Figure 3. (a, c, e) Absorption and (b, d, f) emission spectra of PF-GlcA, PF-Ph-GlcA, and PF-Ph-Glu ($1 \mu\text{M}$) in water at $\text{pH} = 10$ upon addition of various concentrations of bilirubin from 1×10^{-6} to 1×10^{-4} M.

Sensing of Bilirubin in Human Serum Samples. To understand the reliability of the new polymers for biosensing applications, sensing studies were proposed to be carried out in human serum containing interferences such as hemoglobin, proteins, triglyceride, cholesterol, glucose, and metal ions. Although the homopolymer (PF-GlcA) showed better sensing activity in water, it could not be used for sensing studies in human serum due to its tendency to aggregate (discussed in detail later on). The bilirubin sensing studies in human serum was conducted with the copolymers PF-Ph-GlcA and PF-Ph-Glu. 0.1 mL of human serum was mixed with free bilirubin in the concentration range of 1×10^{-6} to 1×10^{-4} M, and this mixture was added to $1 \mu\text{M}$ solution of the polymers. A blank experiment was performed by recording the emission of bilirubin in water as well as in serum at $\text{pH} = 10$ in the absence of polymer. Supporting Information Figure S9 compares the emission intensity from bilirubin upon excitation at 325 nm (the polymer absorption wavelength which was used to excite the polymer in the actual bilirubin sensing experiments) as well as at 455 nm (the bilirubin absorption maximum). Bilirubin in water at $\text{pH} = 10$ did not exhibit any fluorescence upon excitation at 325 or 455 nm . In serum at $\text{pH} = 10$, bilirubin exhibited a weak emission centered at 520 nm , indicating that selective excitation of polymer at 325 nm was not possible due to the not so negligible absorption of bilirubin at 325 nm . Direct excitation at 455 nm resulted in sharp emission from bilirubin at 520 nm . Figure 4a–d demonstrates the changes in the photophysical properties of the probe for various concentrations of the analyte. In the absorption spectrum, human serum exhibited strong absorption of light below 300 nm , indicating the presence of other analytes. In the

emission spectrum of PF-Ph-GlcA (Figure 4b), blue fluorescence of the polymer quenched upon addition of bilirubin with concurrent appearance of light green emission from bilirubin. The photograph of the visual color change from blue to green observed in the serum is given as an inset in Figure 4b, highlighting the sensing ability of PF-Ph-GlcA in human serum. The emission plot also shows the emission from bilirubin in serum at $\text{pH} = 10$ which indicated that its intensity was negligible compared to the FRET-induced emission from bilirubin in the presence of polymer. From Figure 4d it could be seen that the quenching of fluorescence of polymer upon addition of bilirubin was much less for the reference glucose appended polymer PF-Ph-Glu, and also the FRET-induced bilirubin emission was almost negligible. Figure 5 compares the efficiency of FRET-induced bilirubin emission for both polymers for increasing additions of bilirubin. The plot gives the ratio of the integrated areas of bilirubin emission (486 – 600 nm) to that of polymer emission (335 – 486 nm) as a function of increasing concentration of bilirubin. PF-Ph-GlcA exhibited more than 5-fold increase in bilirubin emission compared to PF-Ph-Glu at the highest added bilirubin concentration of 10^{-4} mol. Supporting Information Figure S10 compares the reduction in fluorescence emission of the polymers (area under the emission plot) as a function of increasing bilirubin concentration. The drop was much steeper for the glucuronic acid-functionalized polymer, thereby once again establishing its superiority as a bilirubin sensor. Although the photophysical criteria for FRET-based energy transfer from polymer to bilirubin was favorable for both polymers PF-Ph-GlcA and PF-Ph-Glu, the higher tendency of the glucuronic acid–bilirubin interaction in the former compared to the low or non-

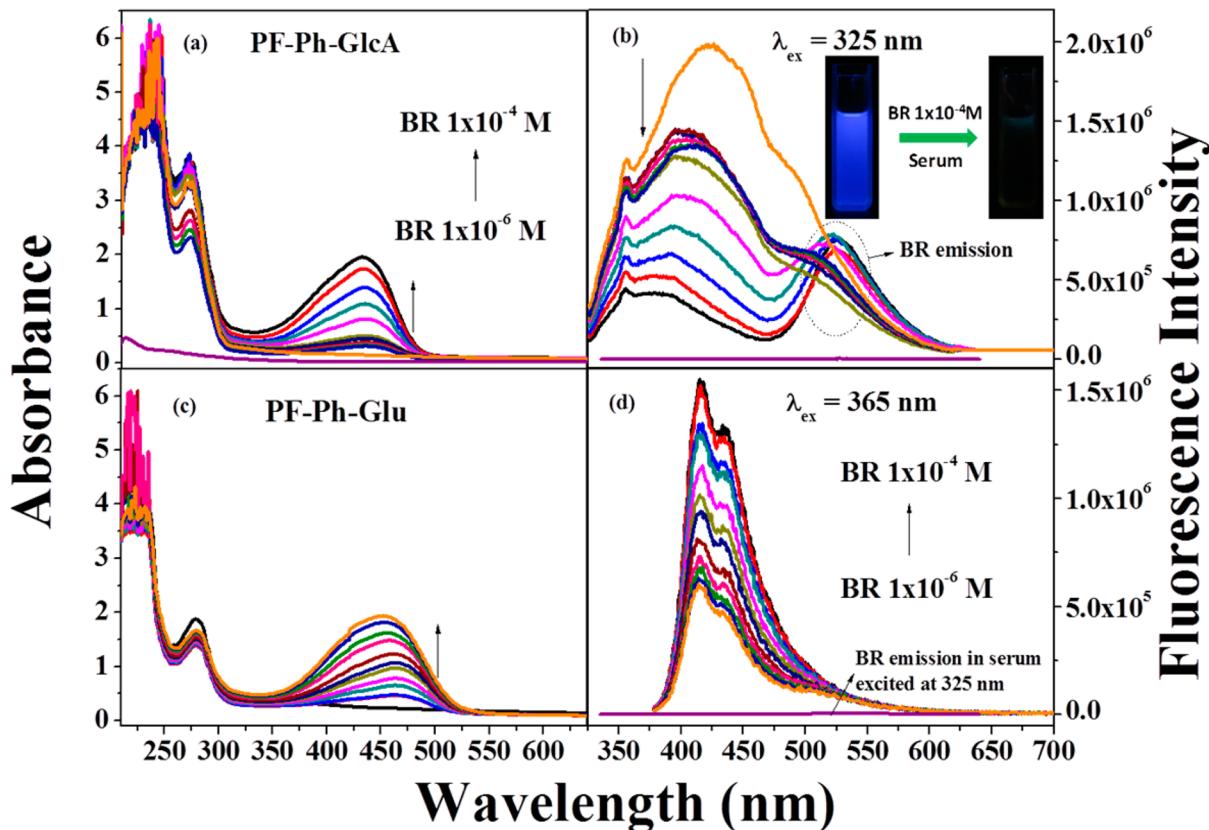


Figure 4. (a, c) Absorption and (b, d) emission spectra of PF-Ph-GlcA and PF-Ph-Glu, respectively, in human serum upon addition of various concentrations of bilirubin from 1×10^{-6} to 1×10^{-4} M in phosphate buffered saline at pH = 10. The emission from 1×10^{-4} M bilirubin in serum upon excitation at the polymer absorption wavelength is also included in the emission spectra.

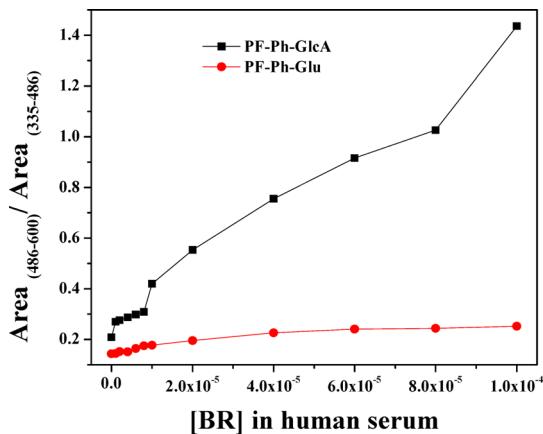


Figure 5. Ratio of integrated area of FRET induced bilirubin emission (486–600 nm) to that of the polymer emission (335–486 nm) for PF-Ph-GlcA and PF-Ph-Glu in serum as a function of bilirubin concentration.

interacting glucose–bilirubin combination in the latter acted in favor of PF-Ph-GlcA to behave as a better sensor for bilirubin in human serum. Additionally, the low solubility of PF-Ph-Glu also could have resulted in the poor performance of PF-Ph-Glu as an efficient bilirubin sensor. Thus, among the three polymers examined for sensing bilirubin, only the glucuronic acid appended copolymer, i.e. PF-Ph-GlcA, was found to be effective for sensing bilirubin in human serum. Therefore, only PF-Ph-GlcA was taken up for further detailed studies to better understand its biosensing characteristics.

An experiment was conducted to study the sensing efficiency of PF-Ph-GlcA in the presence of fixed concentration of bilirubin (100 μM) in serum at various pH. Figure 6 compares

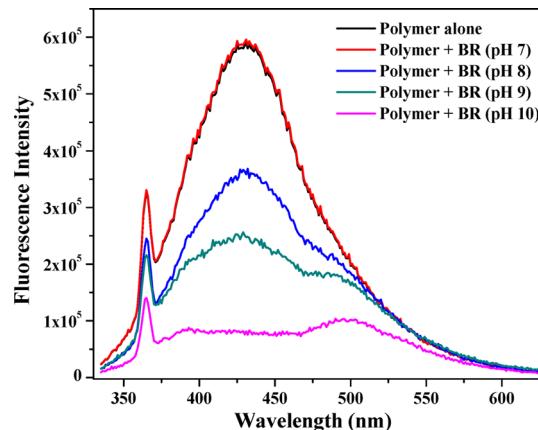


Figure 6. Effect of varying pH (7–10) on the fluorescence emission spectra of 0.1 OD solution ($\sim 1.25 \times 10^{-6}$ M) of PF-Ph-GlcA in the presence of 100 μM of bilirubin in human serum.

the intensity of emission from a 0.1 OD solution (1.3 μM) of PF-Ph-GlcA in serum at pH varying from 7.0 to 10. No quenching of polymer fluorescence was observed at pH = 7, since the bilirubin was not soluble at this pH. As the pH was slowly increased, one could visually observe the bilirubin solution turning yellow due to its dissolution. Complete dissolution of bilirubin with absence of any undissolved

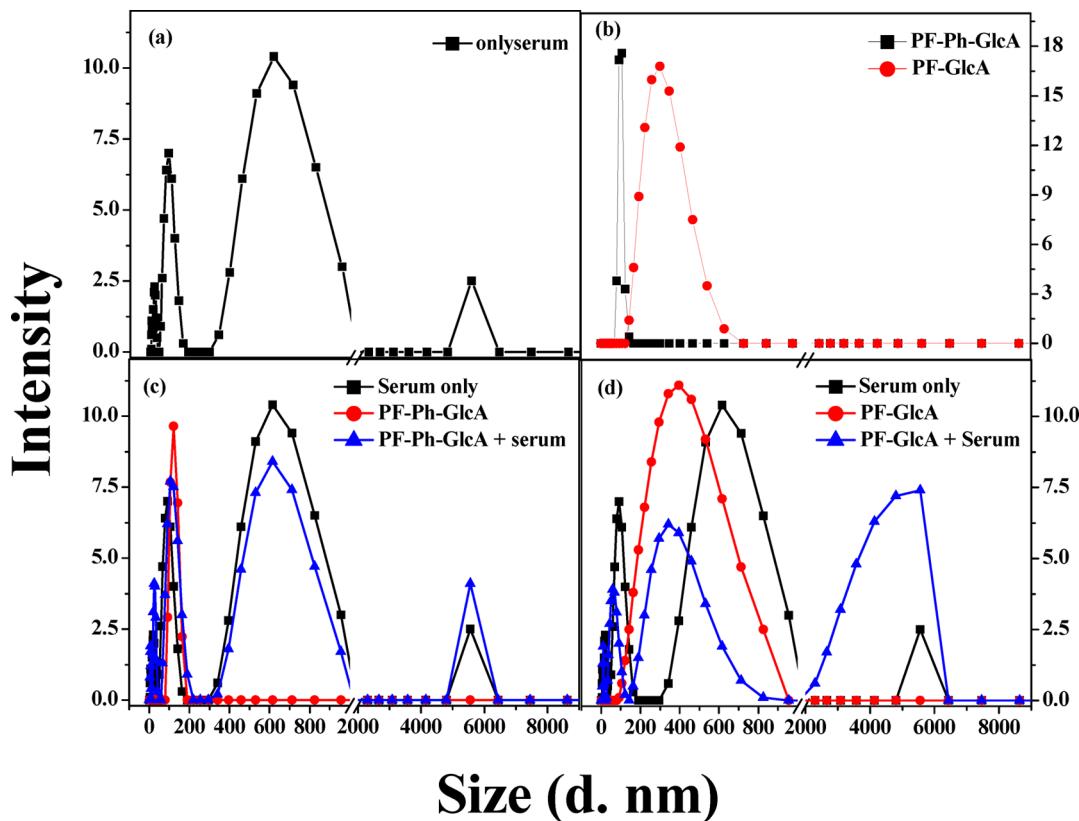


Figure 7. DLS plot of (a) serum, (b) polymers in water, (c) PF-Ph-GlcA, and (d) PF-GlcA in PBS buffer at pH = 10 upon addition of serum. The concentration of polymers was 1 μM , and serum of 100 μL was added to the polymer solution.

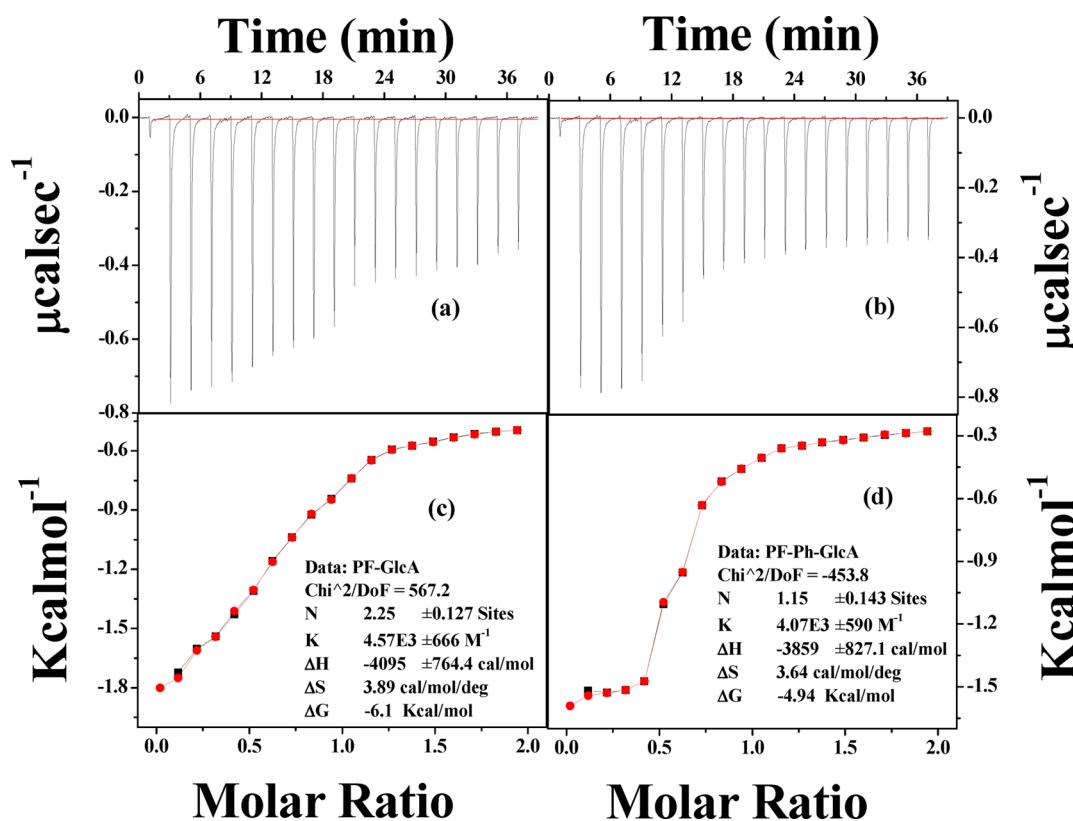


Figure 8. Isothermal calorimetric thermograms (a, c) for the injection of 2 μL of 20 mM bilirubin into 2 mM PF-GlcA and PF-Ph-GlcA at an interval of 3 min at pH = 12 and (b, d) the respective sigmoidal fit.

particles was observed between pH = 9 and 10 (Supporting Information photograph S11). The observation from the fluorescence measurements also correlated by exhibiting the highest quenching of polymer emission at pH = 10. This study also validated the conduction of the fluorescence based bilirubin sensing experiments at pH = 10.

Although the homopolymer PF-GlcA had exhibited better sensing efficiency for bilirubin in water compared to the copolymer, its employment for sensing studies in serum could not be carried out. This was due to the considerable quenching of fluorescence as well as aggregate emission at 550 nm, which hampered observation of the bilirubin emission beyond 520 nm in serum. Supporting Information Figure S12 shows the reduction in fluorescence intensity \sim 410 nm and aggregate emission at 550 nm exhibited by the homopolymer (PF-GlcA) in serum.

Dynamic light scattering (DLS) studies conducted both in water and in serum also supported the tendency of the homopolymer to form large size aggregates in serum. The DLS spectrum of human serum is shown in Figure 7a. In water, PF-GlcA exhibited particle size of 330 nm whereas PF-Ph-GlcA formed smaller particles with size of 110 nm (Figure 7b). Figure 7a–d compares the size versus intensity plot from the DLS studies for the three polymers in water and serum. Figure 7a shows that the serum itself exhibited multimodal distribution of particle size due to presence of proteins and other ingredients. From Figure 7c,d it could be seen that while the copolymer did not exhibit any changes in the inherent size of the particles upon addition of serum (Figure 7c), the homopolymer exhibited both broadening of its inherent particle size and the appearance of new broad peak at higher particle size (Figure 7d). It could be inferred from the above results that the homopolymer PF-GlcA with higher volume ratio of glucuronic acid tend to adhere to the hydrophilic patches of proteins⁴⁴ in serum, resulting in polymer aggregation in serum. Therefore, it is essential to design a polymer with optimum content of glucuronic acid appendage so as to avoid adherence to proteins.

Identification of the Interactions between Polymer and Bilirubin. The nature of interaction existing between the new polymers appended with D-glucuronic acid and bilirubin in water medium was identified using isothermal titration calorimeter (ITC). ITC measures the heat changes produced in the course of the addition of bilirubin acting as ligand to the polymer acting as the macromolecule. High concentration of bilirubin (20 mM) was required to observe saturation of polymer–bilirubin interaction in the ITC experiment. Such high concentrations of bilirubin could be fully solubilized only in PBS buffer at pH = 12. Thus, 20 mM of bilirubin solution in PBS buffer at pH = 12 was added in injections of 2 μ L volume to 200 μ L polymer solution at a concentration of 2 mM. Corresponding results of heat changes during the course of addition for both the polymers PF-GlcA and PF-Ph-GlcA are given in Figures 8a,b and 8c,d. The outcome of the results revealed the exothermic nature of the interaction between polymer and bilirubin. To eliminate the solvent–solvent and solvent–bilirubin interactions, blank reactions without polymer was also carried out. The blank heat change value was subtracted from the heat change value for the polymer–bilirubin complex. The fitting in Figure 8c,d and calculation methods are given in the Materials and Methods section. The free energy change for the reactions obtained in the negative scale indicated the spontaneity of the binding of bilirubin with

the polymer. The free energy change ΔG for the binding of bilirubin with PF-GlcA was -6.1 kcal/mol while that for binding with PF-Ph-GlcA polymer was -4.94 kcal/mol. Generally, the Gibbs free energy change for electrostatic type of interactions and hydrogen bonding interactions falls in the range of $-(15-30)$ and $-(2-10)$ kcal/mol, respectively.⁴⁵ From the values of ΔG of the polymer–bilirubin complex, the type of interaction between the polymer and bilirubin was identified to be that of hydrogen-bonding interactions. The OH and COOH groups of glucuronic acid appendage on the polyfluorene could be expected to play a key role in making hydrogen bond interactions with COOH and NH moieties present in the pyrrole ring of the bilirubin.

The binding constants for the interaction between bilirubin and polyfluorenes were 4.57×10^3 M⁻¹ for PF-GlcA polymer and 4.07×10^3 M⁻¹ for PF-Ph-GlcA. The stoichiometry of the interaction was obtained as 2.25 for homopolymer (PF-GlcA) and 1.13 for copolymer (PF-Ph-GlcA). As the homopolymer PF-GlcA contained relatively more glucuronic acid side chains per mole than the copolymer PF-Ph-GlcA, the PF-GlcA has higher possibility for noncovalent interactions, resulting in a higher binding constant. Therefore, the homopolymer showed relatively higher sensing activity in water compared to the copolymer. Unfortunately, the sensing activity of homopolymer in serum was hindered due to its severe aggregation in serum.

Fluorescence Lifetime. For a better understanding of the energy transfer processes taking place between the polymer and bilirubin, time-correlated single photon counting (TCSPC) analysis of the fluorescence lifetime was utilized. Time-resolved fluorescence decays were collected for the polymers PF-Ph-GlcA and PF-GlcA at 420 and 410 nm with excitation from nano LED at 320 and 375 nm in PBS buffer at pH = 10 at 25 °C. The decay curve for the polymer PF-Ph-GlcA is shown in Figure S13a. The fluorescence decays were fitted to a biexponential fit, and the lifetime values are given in Supporting Information Table 1. PF-Ph-GlcA exhibited a lifetime of $\tau_1 = 457$ ps and $\tau_2 = 2.42$ ns with $\alpha_1 = 0.87$ and $\alpha_2 = 0.13$. Upon addition of various concentrations of bilirubin, the lifetime of the polymer decreased drastically to a minimum value of $\tau_1 = 21$ ps and $\tau_2 = 2.56$ ns with $\alpha_1 = 1$ and $\alpha_2 = 0$. Time-resolved fluorescence decays were also collected at 520 nm (bilirubin emission) for 320 nm excitation. Figure S13c reveals the decay curve for excitation at 320 nm and collection at 520 nm upon various additions of bilirubin to PF-Ph-GlcA polymer. This graph confirmed the increase in the lifetime of bilirubin in the polymer–bilirubin complex, giving evidence for the FRET process. The increase in the lifetime values of bilirubin are given in the Supporting Information (Table S2). Similar behavior was observed for PF-GlcA homopolymer that showed lifetime of $\tau_1 = 470$ ps and $\tau_2 = 3.54$ ns with $\alpha_1 = 0.89$ and $\alpha_2 = 0.11$. The lifetime value of the polymer decreased continuously with the addition of bilirubin, and on final addition of analyte the value reached $\tau_1 = 52$ ps and $\tau_2 = 1.54$ ns with $\alpha_1 = 0.97$ and $\alpha_2 = 0.03$. The corresponding decay curve for polymer (PF-GlcA) is shown in Figure S13b,d. The decay curve for excitation at 375 nm and observation at 520 nm is given in Figure S13d. The rise in the lifetime of the bilirubin values is tabulated in Supporting Information (Table S2). Simultaneous decrement in fluorescence lifetime of the polymer and increase in the lifetime of the bilirubin strongly supported the occurrence of FRET from polymer to bilirubin.

Selectivity and Sensitivity of Bilirubin Sensing in Human Serum. Sensing of analyte in the presence of crucial

interferences is important in order to establish the potential of the sensor for real world biosensing applications. Human serum contains components such as hemoglobin, proteins, iron, cholesterol, glucose, sodium, etc. The D-glucuronic acid appended PF-Ph-GlcA polymer could selectively sense bilirubin in the presence of these components of blood. The porphyrin unit of hemoglobin has structural similarity to bilirubin, but as demonstrated by a control experiment, where the polymer was added to human serum sample in the absence of added bilirubin (Figure S14), the emission of polymer (PF-Ph-GlcA) did not quench in the serum. Despite the similarity in chemical structure, the closed ring structure of hemoglobin prevented hydrogen-bonding interaction with the polymer. In fact, human serum albumin (HSA) is known to bind to free bilirubin with high affinity;³² however, the polymer exhibited competitive binding with free bilirubin in human serum sample via the hydrogen-bonding interactions of its D-glucuronic acid appendage. This was evident from the quenching of polymer emission in human serum samples containing bilirubin.

Other notable interferences such as biliverdin which is the structural analogue of bilirubin, rhodamine, cholesterol, glucose, and sucrose were supplemented in fixed concentration (1×10^{-4} M) to 100 μ L serum in separate experiments. The polymer PF-Ph-GlcA concentration was fixed at 1 μ M, and the total volume of the experimental solution was made up to 2.5 mL with PBS buffer. The absorption spectrum of biliverdin and rhodamine along with emission of PF-Ph-GlcA polymer is given in Figure S15. Each sensing experiment was performed four times, and the average fluorescence response of the polymer was recorded and plotted against respective analytes in Figure 9a as a bar graph representation with error bars (the corresponding emission spectrum is given in Figure S16). A huge decrement in fluorescence intensity of polymer was observed only with the addition of bilirubin, highlighting the high selectivity of new polymer toward bilirubin in human blood serum samples. To differentiate the type of bilirubin, the fluorescence experiment under same conditions were conducted keeping conjugated bilirubin as the analyte. Conjugated bilirubin (con.BR) was prepared following the reported literature procedure, and the details are given in the Experimental Section.³⁶ There was not much change observed for the emission intensity of the polymer in the presence of conjugated bilirubin, indicating its poor interaction with the polymer (Figure S7). Conjugated bilirubin was incapable of hydrogen-bonding interactions with the glucuronic acid appendage, thereby disabling the polymer from binding the conjugated bilirubin. From Figure S15, it could be seen that both biliverdin and rhodamine exhibited reasonable spectral overlap with polymer emission; however, a very low (for biliverdin) or no quenching of polymer emission was observed when these were added to the polymer in serum. Biliverdin is a structural analogue of bilirubin, with an extra double bond in the C10 position, which makes the two dipyrromethene units conjugated.⁴⁶ Although this structural change is very small, it makes biliverdin more rigid compared to bilirubin. The probability of hydrogen bond interaction between biliverdin and glucuronic acid is good; however, a probable nonfavorable conformation of the former could be the reason for the observed poor quenching of polymer fluorescence.

Besides selective sensing, sensitivity of detecting nanomolar amounts of analyte is also an important requirement for an efficient sensor. Therefore, nanomolar amounts of bilirubin (1–150 nM) were added to a 2×10^{-5} M solution of PF-Ph-GlcA

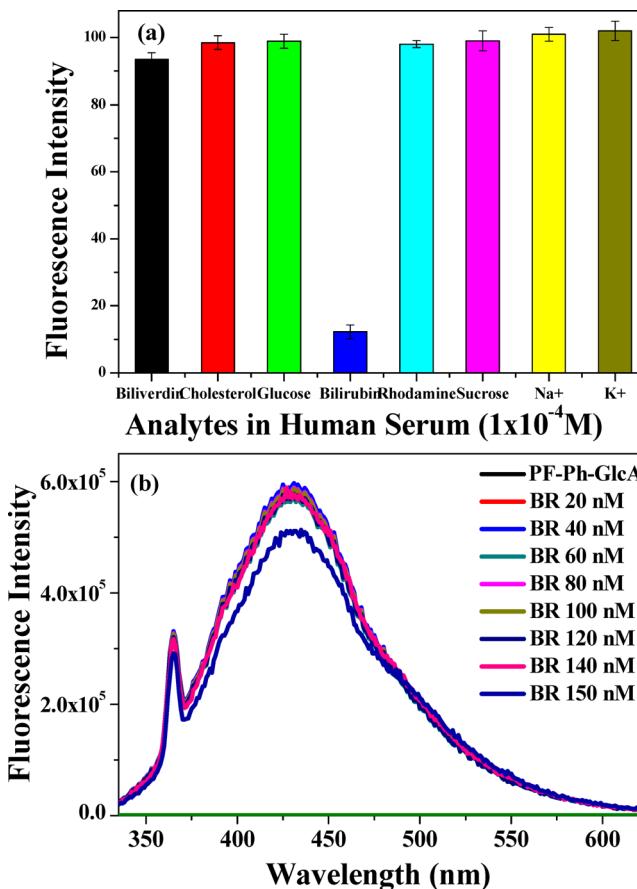


Figure 9. (a) Bar diagram depicting the effect of various interfering analytes on the fluorescence intensity of PF-Ph-GlcA in human serum. The analyte concentration was kept constant at 1×10^{-4} M. (b) Nanomolar sensing by PF-Ph-GlcA in human serum; [polymer] = 1×10^{-5} M.

in human serum at pH = 10. Figure 9b depicts the change in fluorescence emission from polymer upon addition of various nanomolar concentration of bilirubin. A 10% reduction in total area of fluorescence emission was observed upon addition of 150 nm of bilirubin. This was well below the clinically relevant range of <25 to >50 μ mol/L bilirubin observed in the human blood serum. Thus, the selectivity and sensitivity experiments demonstrated the superior performance of the glucuronic acid appended polyfluorene PF-Ph-GlcA as a biosensor for sensing bilirubin in human serum.

Mechanism of Sensing. The aforementioned experiments displayed the ability of the glucuronic acid appended polyfluorene to function as a highly selective and sensitive visual sensor for sensing bilirubin. The appendage of D-glucuronic acid performed the dual role of imparting water solubility to the polymer and interacting with the analyte through hydrogen bonding. The polymer exhibited good extent of spectral overlap with bilirubin in water as well as in human serum, which was the necessary condition to facilitate energy transfer from polymer to bilirubin. When bilirubin was added to the polymer in aqueous medium or in serum, the appendage of D-glucuronic acid interacted with bilirubin enabling the binding of bilirubin with the polymer (highlighted schematically in Figure 10). The binding of bilirubin with polymer, in turn, facilitated energy transfer from polymer to bilirubin, which was manifested as a visual fluorescence color change from blue to

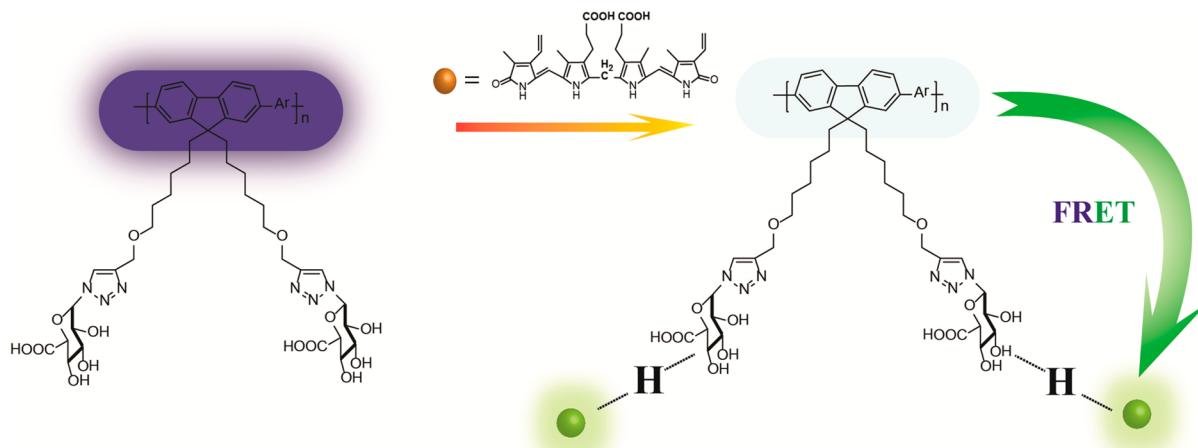


Figure 10. Schematic diagram showing mechanism of FRET-induced sensing of bilirubin in human serum using PF-Ph-GlcA.

light green. In contrast, a reference polymer designed with D-glucose as the appendage in place of D-glucuronic acid (PF-Ph-Glu) could not perform as effectively as PF-Ph-GlcA even though its photophysical properties were favorable for FRET-based energy transfer. The glucose appendage was not successful in affording the high water solubility that was made feasible by the glucuronic acid appendage. PF-Ph-GlcA was highly selective toward free bilirubin compared to other interferences and biological substances present in the serum due to the feasible hydrogen-bonded interactions and favorable spectral overlap existing only for the bilirubin-polymer pair. Though competition for binding sites could be expected to be present in the pool of interferences, binding of bilirubin in minimum sites was sufficient for FRET-based energy transfer to occur due to the well-known molecular wire effect⁴⁷ that is inherent for the conjugated polymer backbone. It also successfully demonstrated its efficiency in sensing nanomolar amounts (~ 150 nm) of bilirubin in human serum. Consolidating all the observations, the combined role of spectral overlap and noncovalent interactions afforded high selectivity and nanomolar sensitivity for the sensing of free bilirubin in water as well as in human blood serum.

CONCLUSION

Novel water-soluble polyfluorenes were designed and successfully synthesized by using click chemistry followed by Suzuki polymerization. The polymers were structurally characterized by NMR spectroscopy and size exclusion chromatography. The appendage of D-glucuronic acid to polyfluorene imparted water solubility through hydrogen-bonded interactions of polar $-\text{OH}$ and $-\text{COOH}$ groups with the aqueous medium. In analogy to the key role of enabling easy excretion of free bilirubin from the body played by D-glucuronic acid in the body metabolism, the appendage of D-glucuronic acid to polyfluorene afforded the added advantage of interactive site for the analyte bilirubin. Taking advantage of the water solubility and spectral overlap of the polymer emission with bilirubin absorption, the sensing of free bilirubin in water was targeted. A complete quenching of the blue polymer fluorescence was observed at $20\ \mu\text{M}$ concentration of bilirubin addition for PF-GlcA and $40\ \mu\text{M}$ for PF-Ph-GlcA. Energy transfer from polymer to bilirubin was proved by the decrease in polymer fluorescence lifetime with concurrent increase in the emission as well as the lifetime of

bilirubin. Isothermal titration calorimetric (ITC) experiments revealed hydrogen-bonding interaction between the polymers and bilirubin with a strong binding constant of $\sim 4.5 \times 10^3\ \text{M}^{-1}$. Although both polymers exhibited similar sensing activity toward bilirubin in water, their sensing activity in human serum was entirely different. The homopolymer PF-GlcA aggregated in serum with aggregate emission observed at 550 nm, which was also supported by DLS experiments conducted in both water and serum media. DLS experiments showed that PF-GlcA formed particles with size of 330 nm in water, which increased considerably in serum. The copolymer PF-Ph-GlcA was successful as an efficient sensor for bilirubin in human serum with visual change of emission color from blue to green for FRET-induced bilirubin emission at 520 nm. The sensing of bilirubin in human blood serum was found to be sensitive even in the presence of crucial interferences such as hemoglobin, biliverdin, proteins, triglyceride, cholesterol, metal ions, and sugars. Nanomolar sensing of bilirubin could also be demonstrated successfully in human serum using PF-Ph-GlcA as the conjugated polymer sensor. Thus, we have demonstrated for the first time the successful application of water-soluble conjugated polymers based on polyfluorenes in the selective and sensitive (sensing ~ 150 nm bilirubin in human serum) sensing of free bilirubin in the clinically relevant range of <25 to $>50\ \mu\text{mol/L}$ in the human blood serum. These novel polyfluorenes appended with glucuronic acid could thus function as a highly selective and sensitive material for the visual sensing of free bilirubin in human blood serum via the combined operation of energy transfer and noncovalent interactions.

ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures and characterizations for all monomers and polymers. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macromol.5b00043.

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Notes

The authors declare no competing financial interest.

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