

# Synthesis of water-soluble fluorescent polymeric glycoconjugate for the detection of cholera toxin

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## ABSTRACT

Considering inherence optical properties of adjoint polyfluorenes and special functions of water-soluble conjugated glycopolymers, a triazole chain glycoconjugate via one-pot method were rapidly synthesized to prepare a lactate ligand polyfluorene with a clear fluorescent label by a nickel-catalyzed Yamamoto coupling polymerization. The water solubility and biocompatibility of the glycoconjugated polymer were ameliorated when the lactose group introduced as the side chain of the conjugated polymer. As a fluorescent multivalent system of glycoconjugates containing pyranogalactose groups, the interaction between pyranogalactose group and cholera toxin B subunit was studied by fluorescence spectrophotometric titration. PF-Lac has a broad application prospect in the check of cholera toxin and the study of glycoprotein interaction.

## ARTICLE HISTORY

Received 20 May 2019  
Accepted 1 August 2019

## KEYWORDS

Cholera toxin; fluorescence quenching; lactose; sensors; water-soluble conjugated polymer

## 1. Introduction

Some important bioprocesses, such as cell recognition and divergence, cancer transfer, bacterial and virus infections, are closely related to carbohydrate-mediated biological interactions [1–3]. Multivalent interaction, so-called glycoside cluster effect plays a significant role in binding affinity, particularly in those specific biological interactions [4,5]. Glycopolymers are suitable to be the mimic system of glyco-biomacromolecules considering the polyvalence, molecular weight control and molecular structure control [6–8]. These properties have been shown to influence the biological interactions between carbohydrate-carrying chains and their combined targets [9–11].


Cholera is an acute purging infection caused by ingestion of eatables or water polluted by vibrio cholerae, characterized by sudden purging, followed by vomiting; decreased blood pressure, weak pulse, etc. Without appropriate treatment, it can lead to severe vomiting, massive loss of electrolytes such as sodium and potassium in the blood, systemic electrolyte disturbances and even death [12]. In some developing countries, cholera still represents a major potential threat to the health of both human and animals. Cholera give rise to cholera toxin (CT), which has an AB<sub>5</sub> hexamer structure. Five identical cholera toxin B subunits (CTB) selectively bind to glycolipid ganglioside GM1 [13]. The CTB can attach to intestinal

cells by binding with the pentasaccharide head group of GM1, and this mechanism initiates the disease. Some lactosyl-bearing multivalent systems, such as dendrimers [14,15], nanoparticles [16], and the molecule with aggregation-induced emission feature [17] have been used to study specific combine to CTB. CTB is known to link to lactose by distinguish the end glycolipid section, which is also the end sugar in the GM1 oligosaccharide head [18].

Synthetic fluorescent glycoconjugates have been used as multivalent artificial models of carbohydrate systems and suitable well-defined tools for the study of carbohydrate-based biological events [19–26]. For example, studies on the interaction of carbohydrate-lectin [27] and carbohydrate-bacteria [21] by fluorescent conjugated sugar polymers have been reported. Water-soluble conjugated polymers provide an advantageous platform for the development of new electro-optical devices [28,29], chemical sensors and biosensors [30,31], taking the inherent optoelectronic properties and good water solubility into account. Fluorescent conjugated sugar polymers possessing fluorescent backbones and carbohydrate-binding moieties are used in the investigation of carbohydrate-mediated bio-interaction and biosensor applications because of their water solubility, their unique optical properties and their high sensitivity to external stimuli and good biocompatibility [32,33].

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 Supplemental data for this article can be accessed [here](#).

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It is well known that polyfluorenes exhibit intrinsic optical properties [34] and have been used as various sensors [35]. Herein, a lactosyl-bearing polyfluorene was synthesized and characterized, the latent application of the CT fluorescence sensor is also investigated. The introduction of lactose part can improve the solubility of water and biocompatibility of the polyfluorene system. Moreover, lactose moieties can provide CTB-oriented binding sites via specific biological interactions, thus, enhance the binding affinity via multivalent interactions, which leads to aggregation of lactosyl-bearing polyfluorene and significant fluorescence quenching.

## 2. Experiment

### 2.1 Materials

Peracetylated lactose (1) and HBr (33% solution in acetic acid) were commercially available from Acros Scientific Co. Ltd. Tetrabutylammonium hydrogen sulfate and sodium azide were commercially available from J&K Scientific Co. Ltd. 1,5-cyclooctadiene (1,5-COD), bis(1,5-cyclooctadiene)nickel(0) (1,5-COD-Ni) and 2,2'-bipyridyl (2,2'-Bpy) were bought from Aldrich Chemicals. Sodium methoxide and sodium ascorbate were purchased from Shanghai Chemical Technology Co. Ltd. CTB, bovine serum albumin (BSA) and phosphate buffer solution (PBS, 50 mM, pH 7.3) were bought from Sigma-Aldrich Co. Ltd. N, N-Dimethylformamide (DMF), dichloroethane (DCE), chloroform, methanol and tetrahydrofuran (THF) were dried before use. Unless otherwise stated, Other chemical reagents can be bought commercially and used as they are. The Millipore Purification System (Mily Q Water) was used to purify deionized water. 9,9-bis(4-propargyloxy phenyl)-2,7-dibromo fluorene (2) was prepared according to reported methods [36].

### 2.2 Instrumental characterization and structural analysis

Measurement of Nuclear Magnetic Resonance (NMR) spectra ( $^1\text{H}$  and  $^{13}\text{C}$ ) were on Bruker DMX400 NMR spectrometer (Bruker Daltonics Inc., Germany). VG PLATFORM mass spectrometer was applied to record mass spectra by electrospray ionization (ESI (+)) (VG Elemental, England). The infrared (IR) spectra of small molecules and polymers were recorded with KBr pellets on a Perkin-Elmer Paragon 1000 Fourier transform infrared (FTIR) spectrometer (PerkinElmer Instruments Co. Ltd., USA). Perkin Elmer Lamda 900 UV-vis-NIR spectrophotometer (Perkin Elmer Instruments Co. Ltd.) was used to record UV-vis spectra by a quartz pool with a path length of 1 cm. Measurement of Fluorescence Spectra were used by Perkin Elmer LS55

Luminescence Spectrometer (PerkinElmer Instruments Co. Ltd., USA) at 1 cm Path Length. Screening molecular mass of polymers dissolved in THF was used by Agilent 1100 GPC-SEC Analysis System (Agilent Technologies, USA). The average molecular weight and molecular weight ( $M_n$  and  $M_w$ ) of polystyrene were reckoned by calibration curve of polystyrene reference material.

### 2.3 Synthesis of monomer 3 via one-pot reactions

For the solution of acetylated lactose 1 (1.0 g, 1.5 mmol) in dry chloroform (15 mL), HBr (33% solution in acetic acid, 2.0 mL) was added dropwise in an ice water bath. Stirring the liquor at room temperature was for about 6 h under nitrogen protection until the reaction was accomplished. The volatile compounds are removed by decompression, and chloroform (10 mL), saturated baking soda solution (2.0 M, 5 mL), sodium azide (0.16 g, 4.6 mmol) and tetrabutylammonium bisulfate (0.5 g, 3.0 mmol) were added sequentially. Two hours later, 9,9-bis(4-propargyloxy phenyl)-2,7-dibromo fluorene 2 (292 mg, 1.0 mmol),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (12.5 mg, 0.1 mmol), L-ascorbic acid Sodium Salt (30 mg, 0.30 mmol) and alcohol (5 mL) were added to the solution. The reaction mixture was kept stirring at room temperature for about 16 h. After removing solvent in vacuum, water (20 mL) was added to extract the product with ethyl acetate (170 mL). The merged organic layer was dehydrated with anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated in vacuum. Column chromatography (ethyl acetate-light petroleum, 2:1) was used for purification of the product 3 as foamed solid (0.9 g, 31%).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  19.2, 19.4, 19.5, 19.6, 19.8, 19.9, 20.1, 59.5, 59.5, 60.8, 61.0, 63.5, 65.6, 68.2, 69.6, 70.0, 71.7, 74.6, 75.0, 84.6, 100.2, 113.9, 120.3, 121.0, 128.3, 123.0, 136.2, 136.8, 143.9, 152.5, 156.3, 168.2, 168.5, 169.1, 169.2, 169.3, 169.5.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.77 (s, 6H), 1.95 (s, 6H), 2.01 (s, 6H), 2.03 (s, 6H), 2.05 (s, 6H), 2.06 (s, 6H), 2.14 (s, 6H), 3.95–3.87 (m, 6H), 4.15–4.06 (m, 6H), 4.46 (d,  $J = 10.9$  Hz, 2H), 4.93 (dd,  $J = 2.9, 9.9$  Hz, 2H), 5.08 (d,  $J = 8.2$  Hz, 2H), 5.13 (s, 4H), 5.40–5.34 (m, 6H), 5.83 (d,  $J = 9.8$  Hz, 2H), 6.83 (d,  $J = 9.0$  Hz, 4H), 7.04 (d,  $J = 8.6$  Hz, 4H), 7.45–7.43 (m, 4H), 7.55 (d,  $J = 9.1$  Hz, 2H), 7.76 (s, 2H). ESI(+)-MS: calcd. for  $\text{C}_{83}\text{H}_{90}\text{O}_{36}\text{N}_6\text{Br}_2$ : 1907.45 [M]; found 1946.86 [M + K] $^+$ .

### 2.4 Synthesis of polymer PF-AcLac by the Yamamoto coupling polymerization

Dry 1,5-cyclooctadiene (0.052 mL, 0.42 mmol) was put in anhydrous DMF (15 mL) solutions of 1,5-COD-Ni (112 mg, 0.41 mmol) and 2,2'-Bpy (64 mg, 0.41 mmol). The mixture was heated and stirred at 80°C for 1 h, and sugar-bearing monomer 3 (286 mg, 0.15 mmol) was added to the purple solution. The mixture was heated and stirred at 80°C under

the nitrogen atmosphere for 24 h. The resulting polymer was precipitated by pouring the reaction mixture into a methanol-water solution. PF-AcLac was isolated as grey powder (200 mg, 78%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.77 (bs, 6H), 1.99 (bs, 6H), 2.05 (bs, 24H), 2.14 (bs, 6H), 3.92 (br, 6H), 4.10 (br, 6H), 4.44–4.53 (m, 4H), 4.95–4.98 (m, 2H), 5.01–5.14 (m, 6H), 5.34–5.39 (m, 6H), 5.81 (br, 2H), 6.82–6.88 (m, 4H), 7.04–7.20 (m, 3H), 7.45–7.51 (m, 3H), 7.56–7.63 (m, 4H), 7.75 (bs, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  19.0, 19.5, 19.7, 19.8, 59.8, 60.6, 60.8, 65.6, 68.0, 69.5, 69.7, 69.9, 71.6, 74.7, 74.8, 76.1, 84.6, 100.1, 113.4, 113.8, 120.1, 126.0, 126.4, 128.4, 130.7, 136.8, 137.7, 143.9, 152.5, 156.4, 168.1, 168.3, 168.5, 169.0, 169.1, 169.2, 169.3. IR:  $\nu$  ( $\text{cm}^{-1}$ ): 2938 (w), 1752 (s), 1593 (m), 1513 (m), 1482 (s), 1372 (m), 1321 (m), 1235 (s), 1111 (s), 809 (m). GPC (THF, polystyrene standard):  $M_n = 28$ , 600 g/mol; polydispersity = 1.74.

## 2.5 Synthesis of polymer PF-Lac

$\text{CH}_3\text{ONa}$  (1.0 M methanol solution) was added to the protected sugar polymer PF-AcLac (150 mg) in  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (v/v = 1:3, 20 mL) solution drop by drop until the solution pH reached 11. The reaction mixture was stirred at room temperature overnight. The solid was gathered by centrifugation at 8000 rpm and washed twice with  $\text{CH}_2\text{Cl}_2$ . After the solvent is removed under diminished pressure, the residue was diluted with 10 mL water. The obtained solution was placed in a cellulose dialysis tube (with a trapped molecular weight of 3,500), dialyzed with water for 3 days, and freeze-dried to obtain the required solid polymer PF-Lac (95.5 mg, 94%). IR:  $\nu$  ( $\text{cm}^{-1}$ ): 3421 (bs), 2934 (w), 1590 (m), 1513 (m), 1479 (s), 1325 (m), 1103 (s), 804 (m).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  3.31–3.44 (m, 6H), 3.51–3.73 (m, 14H), 3.75–3.88 (m, 4H), 4.28 (br, 2H), 5.11 (s, 4H), 5.68 (br, 2H), 6.95–7.04 (m, 4H), 7.15–7.24 (m, 2H), 7.25–7.70 (m, 4H), 7.75–8.10 (m, 4H), 8.44 (bs, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  60.0, 60.9, 68.2, 70.6, 71.9, 73.3, 75.2, 75.6, 77.7, 79.2, 79.7, 86.9, 103.5, 114.7, 123.7, 125.1, 126.5, 127.3, 128.7, 131.7, 134.3, 136.4, 139.2, 142.5, 157.2. GPC (THF, polystyrene standard):  $M_n = 19$ , 900 g/mol; polydispersity = 1.71.

## 2.6 Studies of the interactions between PF-Lac and CTB by fluorometric titration analysis

PF-Lac stock solution was prepared in PBS aqueous solution (50 mM, pH 7.3). Equivalent CTB (2  $\mu\text{L}$ ) was added to the same PBS buffer and PF-Lac solution ( $1.5 \times 10^{-6}$  M, 2.0 mL). The final concentration of CTB was ranged from  $1.0 \times 10^{-8}$  to  $8.0 \times 10^{-8}$  M. After each addition, the samples were balanced overnight and the spectra were recorded. The excitation wavelength was 320 nm and the emission scan ranged from 310 to 630 nm.

## 2.7 Studies on the selectivity of fluorometric detection for CTB

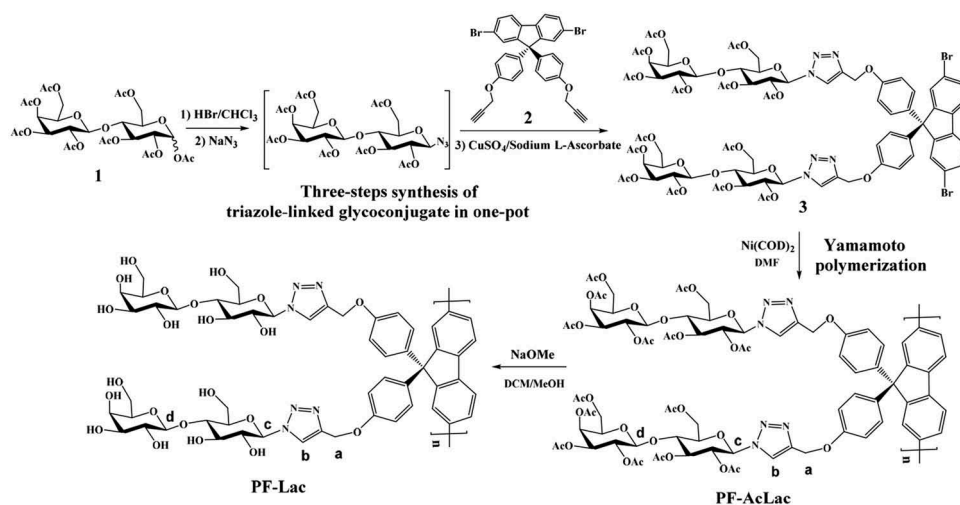
Preparation of PF-Lac stock solution was in PBS (50 mM, pH 7.3). BSA (2  $\mu\text{L}$ ) and CTB (2  $\mu\text{L}$ ) fractions in alike PBS buffer were injected in PF-Lac solution ( $1.5 \times 10^{-6}$  M, 2.0 mL) subsequently. After each addition, the sample was balanced overnight and the spectra were recorded. The final concentrations of BSA and CTB were  $8.0 \times 10^{-8}$  M, respectively. The excitation wavelength is 317 nm and the radiate scanning range is 330–630 nm.

## 3. Results and discussion

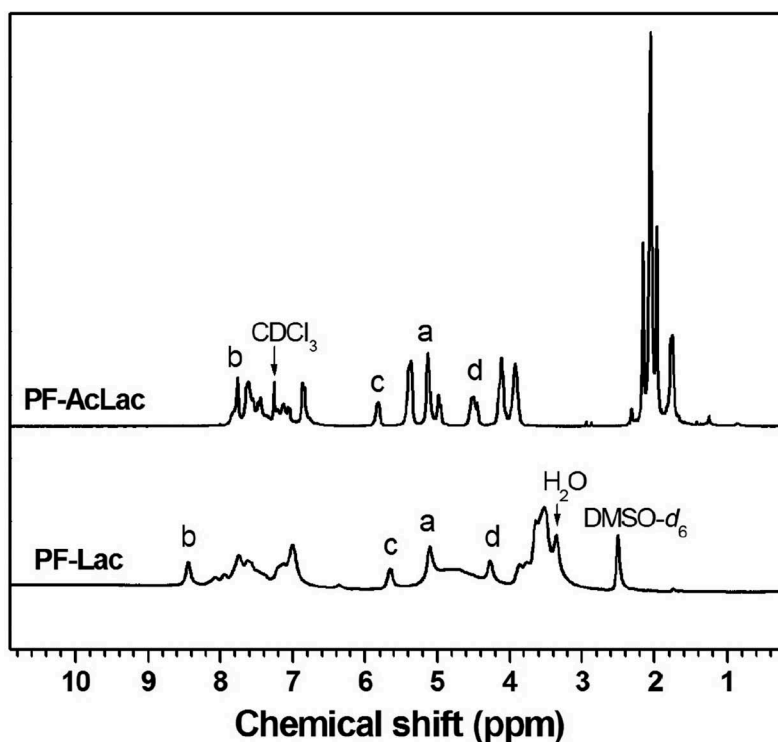
### 3.1 Synthesis and characterization of monomer and polymers

The synthetic routes of monomers and sugar polymers are outlined in Scheme 1. For monomer preparation, a rapid one-pot synthetic method to triazole-linked glycoconjugate from commercially available sugar acetate was used [37,38], which includes bromination, azidation and Cu(I)-catalyzed Huisgen 1,3-dipole cyclic addition reaction. As shown in Scheme 1, peracetylated lactose was used as the starting material for this method. After bromination with HBr in acetic acid and removal of all volatiles of solvents and acids, subsequent azide conversion can furnish intermediate acetylated lactosyl azide. 1,3-Dipolar selective ligation between propargyl fluorene derivative 2 and sugar azide intermediate catalyzed by in situ copper (I) are easy to produce lactose-containing monomer 3. A signal peak at 7.76 ppm (2H) in the  $^1\text{H}$  NMR spectrum as well as two peaks at 120.6 ppm and 144.0 ppm in the  $^{13}\text{C}$  NMR spectrum confirmed the formation of the triazole ring.

The Ni-catalyzed Yamamoto coupling polymerization of monomer 3 can afford a well-defined and protected glycopolymer, PF-AcLac, which is a poly(fluorene) containing the side-chain lactosyl derivatives. The PF-AcLac was treated with  $\text{CH}_3\text{ONa}/\text{CH}_3\text{OH}$  to deprotect all acetyl groups. The desired water-soluble conjugated glycopolymers (PF-Lac) were obtained in excellent yield (94%). The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of polymers PF-AcLac and PF-Lac are shown in Figure 1 and the supporting information, which can prove the successful preparation of the desired polymers. There are two reasons for HNMR signal of proton b down-field shifted significantly from PF-AcLac to PF-Lac. One is that the chemical environment of proton b is changed due to fully de-acetylation from PF-AcLac to PF-Lac. The more important is that the solvent for HNMR study is different from PF-AcLac to PF-Lac. For PF-AcLac, solvent is  $\text{CDCl}_3$ . While for PF-Lac, solvent is  $\text{DMSO}-d_6$ . The potential hydrogen bond acceptor would lead to shift the proton signal downfield [39]. Selection of 9,9-bis(4-hydroxyphenyl) fluorene as polymeric monomers is beneficial to provide more effective



**Scheme 1.** Synthetic routes to the monomer 3 and polymer PF-Lac.

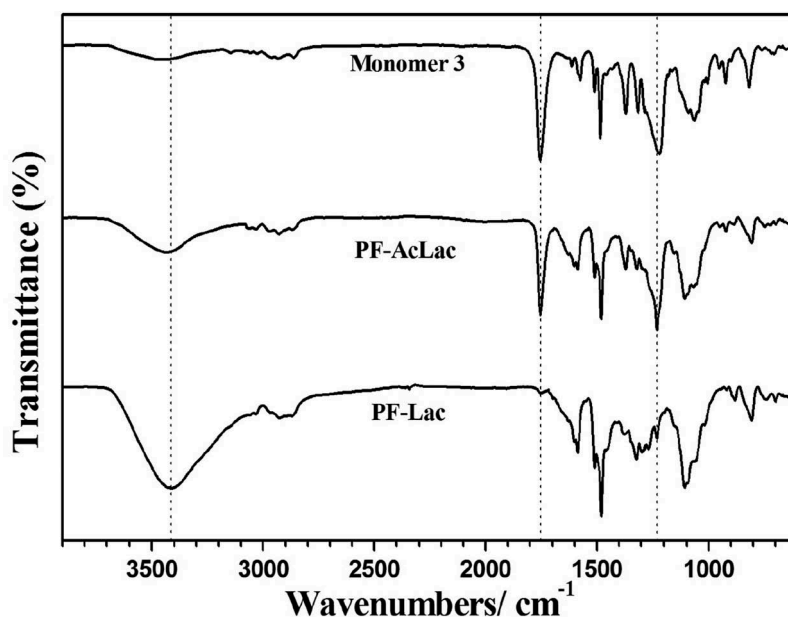


**Figure 1.**  $^1\text{H}$  NMR spectra of polymer PF-AcLac (in  $\text{CDCl}_3$ ) and polymer PF-Lac (in  $\text{DMSO}-d_6$ ).

shielding effect on the polyfluorenyl skeleton, because there is a non-flexible phenylene spacer between the main chain of the polymer and the side chain of the sugar, which can inhibit the formation of aggregates or receptors [40].

FT-IR spectra demonstrated the successful polymerization and deacetylation of conjugated glycopolymers. As shown in Figure 2, absorption bands at 2850–2950  $\text{cm}^{-1}$  in the FT-IR spectra of the monomer, polymers PF-AcLac and PF-Lac represent CH stretching from alkyl chains of

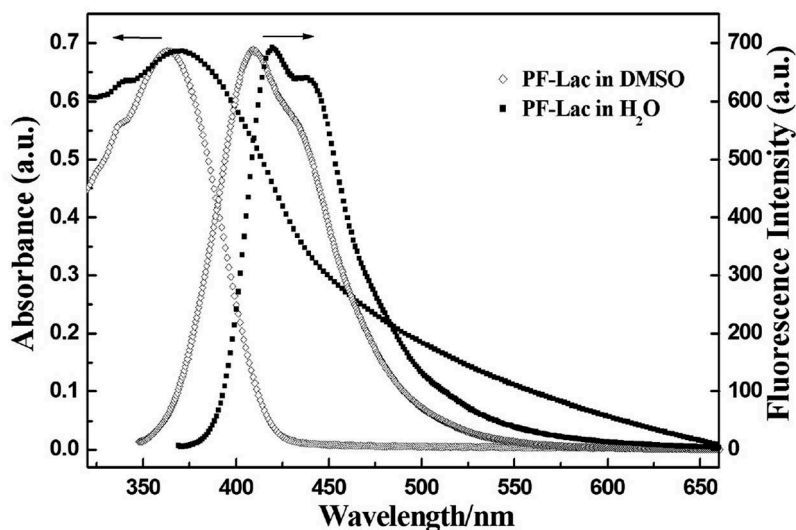
monomers. After treating PF-AcLac with  $\text{CH}_3\text{ONa}/\text{CH}_3\text{OH}$ , the bands at 1750  $\text{cm}^{-1}$  ( $\text{C}=\text{O}$ ) and 1200  $\text{cm}^{-1}$  ( $\text{C}-\text{O}$  stretching in ester) of PF-AcLac disappeared and confirmed perdeacetylation. Moreover, in the FT-IR spectra, there is a wide absorption band at 3400  $\text{cm}^{-1}$  of polymer PF-Lac showed the extension of  $-\text{OH}$ , which are the free hydroxyl groups on the sugar part. This information also proves clearly that the polymerization of sugar-containing aromatic monomer is a good way to prepare well-defined conjugated glycopolymers.



**Figure 2.** IR spectra of monomer 3, polymer PF-AcLac, and polymer PF-Lac.

PF-AcLac is soluble in general solvents, such as  $\text{CHCl}_3$ ,  $\text{CH}_2\text{Cl}_2$ , acetyl acetate and THF, but not soluble in methanol and water. Gel permeation chromatography (GPC) analysis of polystyrene standard materials indicate that the number average molecular weight ( $M_n$ ) of PF-AcLac is 28,600 g/mol with polydispersity of 1.74. After deprotection, the solubility of glycopolymer PF-Lac was different from its precursor. PF-Lac is soluble in water medium and even better in DMF and DMSO. Moreover, it exhibits intrinsic optical properties. For the PF-Lac in DMSO solution, the maximum absorption and emission peaks were

found at 366 nm and 415 nm, and the vibration peak at 433 nm. These peaks are due to the  $\pi$ - $\pi^*$  transition of the main chain of polyfluorene. In aqueous solution, the maximum absorption peak of PF-Lac is at 378 nm, the maximum emission peak is at 424 nm and the vibration peak is at 442 nm. As shown in Figure 3, we can see that the absorption and emission spectra of conjugated glycopolymer PF-Lac in water exhibit a significant red shift compared with the polymer in DMSO solution, because the planar conformation or aggregation of the polymer backbone is enhanced due to increased solvent polarity [41].



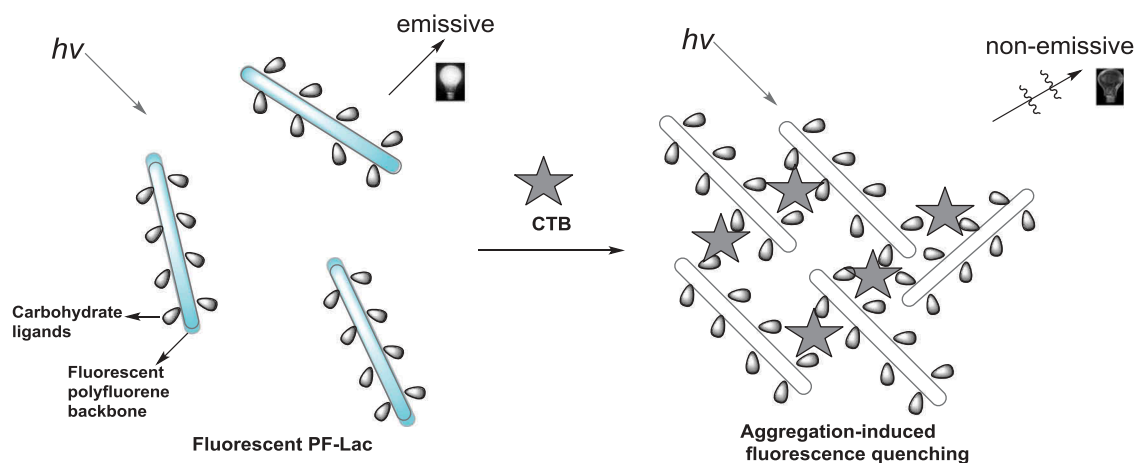
**Figure 3.** UV-vis absorption and fluorescence spectra of polymer PF-Lac in DMSO and water.

### 3.2 Fluorescence 'turn-off' detection of CT

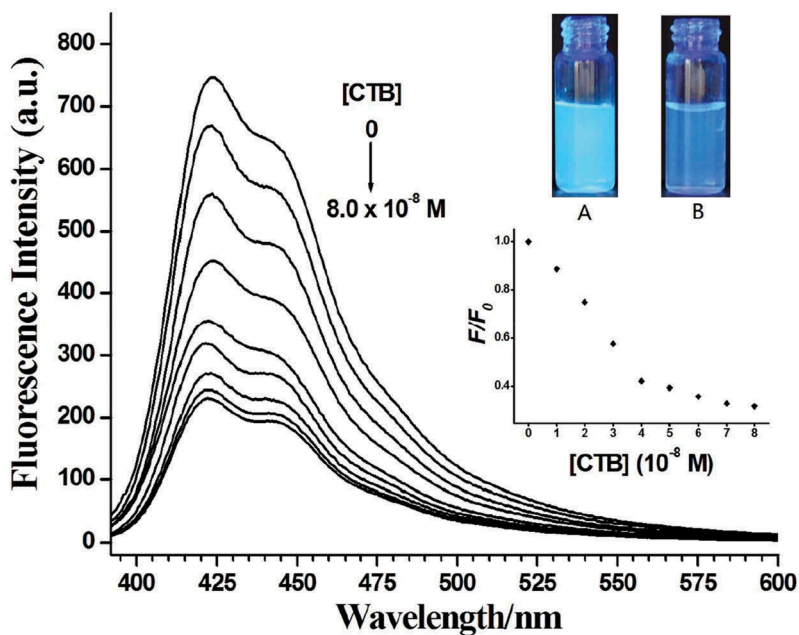
As mentioned above, CT consists of the five same monomers with a galactose binding site of GM1 ganglioside. Therefore, various types of molecules containing identical terminal galactose groups can be designed to bind to CTB. Lactose composition of amylose and a terminus-galatosum unit are usually used to design CT transducer. Water-soluble PF-Lac, possessing both a polyfluorene backbone and many lactosyl units, shows high photoluminescence in water. We can assume that when CTB was

added into the dilute water solution of PF-Lac, the binding of CTB with poly lactose-attached polyfluorene occurred, leading to the aggregation of PF-Lac. Therefore, the fluorescence of PF-Lac is quenched significantly due to aggregation, which can be used for CTB sensing. Schematic representation of fluorescence 'turn-off' assay for CTB by PF-Lac was shown in Scheme 2.

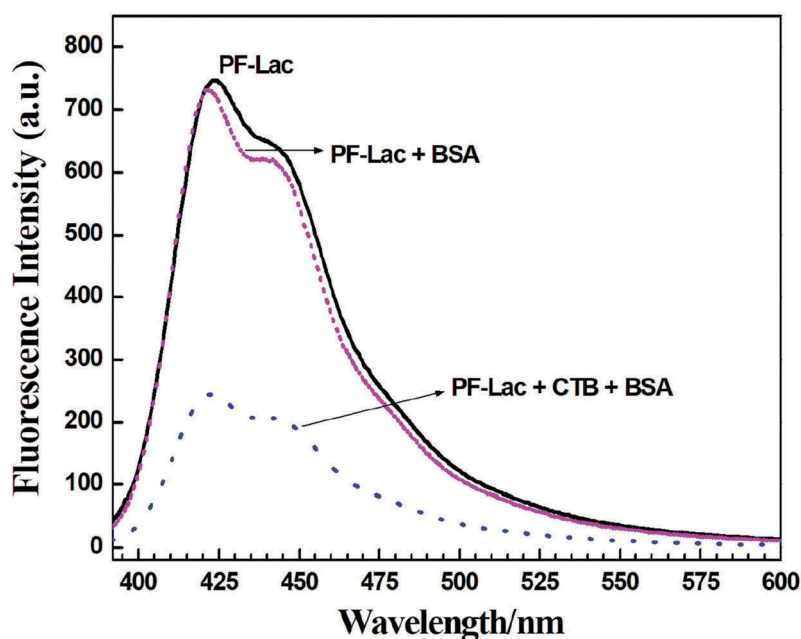
The interaction between PF-Lac and CTB was studied by detecting the fluorescence intensity of PF-Lac in the presence of CTB in PBS. As shown in Figure 4, when CTB ( $0\text{--}8.0 \times 10^{-8}$  M) was gradually



**Scheme 2.** Schematic representation of fluorescence 'turn-off' assay for CTB by PF-Lac.



**Figure 4.** Fluorescence spectra of polymer PF-Lac ( $1.5 \times 10^{-6}$  M) in the absence and presence of various concentrations of CTB in PBS solution at room temperature. CTB concentrations from top to bottom are 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and  $8.0 \times 10^{-8}$  M (Inset displays the photos of the corresponding solutions of PF-Lac in the absence (A) and presence (B) of CTB ( $8.0 \times 10^{-8}$  M) under UV light (365 nm) illumination).



**Figure 5.** Fluorescence spectra of polymer PF-Lac, polymer PF-Lac with the addition of BSA, and polymer PF-Lac following the addition of BSA and CTB at room temperature.

added to the solution of PF-Lac ( $1.5 \times 10^{-6}$  M) in the PBS (50 mM, pH = 7.3), most of fluorescence was quenched. The possible mechanism of fluorescence quenching of PF-Lac is attributed to the aggregation of PF-Lac/CTB binding, resulting in self-quenching. In static quenching, the quencher forms a ground-state complex with the fluorophore, and a linear Stern–Volmer relationship ( $F_0/F = 1 + K_{SV}[Q]$ ) occurs [25,42].  $K_{SV}$ , as the Stern–Volmer constant, indicates the efficiency of quenching. The Stern–Volmer quenching constant of polymer PF-Lac by CTB was calculated as  $1.6 \times 10^7 \text{ M}^{-1}$  based on the linear part of the curve (Figure S1).

To study the selectivity of fluorescence check for CTB and the specificity of carbohydrate–protein interaction, the PF-Lac assay was conducted with other biomacromolecules. We can see that the photoluminescence intensity of PF-Lac did not change significantly at presence of the BSA protein under the same conditions (Figure 5). In addition, when PF-Lac was presented with the same amount of CTB and BSA in PBS solution, the intensity of fluorescence quenching was almost identical as that observed in the solution of CTB. These results revealed that PF-Lac shows great potential use in the selective detection of biomacromolecules.

#### 4. Conclusion

Fluorescent conjugated polyfluorene with side-chain lactopyranosyl ligand (PF-Lac) was synthesized by one-

pot formation of triazole-linked glycoconjugates and Yamamoto C–C coupling polymerization. Using fluorescent fluorene scaffolds and sugar-binding ligands, this well-defined fluorescent glycoconjugate polymer is used to study the interaction of CTB subunits by fluorescence spectrophotometric titration. When CTB is presented in deliquate PF-Lac aqueous solution, the binding of CTB with polyfluorene containing multilactose groups occurred, which cause the accumulation of PF-Lac. Therefore, the fluorescence of PF-Lac is quenched due to self-aggregation. We believe that these methods for insight into carbohydrate-mediated biological interactions have shown potential applications in the sensing of biomacromolecules and the glycobiology study.

#### Disclosure statement

No potential conflict of interest was reported by the authors.

#### Funding

This work was supported by the Science and Technology Cooperation Project of Guizhou Province (Grant QKHLHZ-2015–7564) and the PhD Start-up Foundation of Zunyi Medical College (Zhuhai Compus) (Grant F-710)

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