# A Carboxylic Acid-Functionalized Polyfluorene as Fluorescent Probe for Protein Sensing

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**ABSTRACT:** A new water-soluble fluorescent conjugated polyelectrolyte, poly[9,9-bis(3′-butyrate)fluoren-2,7-yl] sodium (BBS-PF), was studied as a fluorescent probe for protein sensing. The conjugated polyelectrolyte BBS-PF shows high fluorescence sensitivity to cytochrome *c*, lysozyme, and bovine serum albumin (BSA). The Stern–Volmer constant ($K_{sv}$) in cytochrome *c* was measured to be as high as $6.1 \times 10^7$ L/mol, approximately twice as that of the other two. Interestingly, it is found that BSA slightly enhances the fluorescence of BBS-PF rather than quenches the fluorescence at its micromolar concentrations. The excitation spectra confirm that the interaction of BBS-PF with the proteins could be different. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 3541–3546, 2011

**Key words:** biosensor; polyelectrolyte; proteins; fluorescence; probe

## INTRODUCTION

Development of new optical biosensors for the detection of nucleic acid and proteins has been an active area of research for the demands of genetic analysis, clinical diagnosis, and environmental analysis.[1–6] In recent years, conjugated polyelectrolytes (CPEs), which can bind nucleic acid or protein surfaces through multivalent electrostatic interactions, have attracted much attention as optical probes in biomacromolecular detection.[7,8] The main advantage of using CPEs compared with small molecules in biosensing is that the transfer of excitation energy along the whole backbone of the CPEs to the reporter results in an amplified fluorescence signal.[9] Three types of signal transduction mechanisms of CPEs for biosensing have been reviewed as Förster resonance energy transfer (FRET) or electron transfer, analyte-induced aggregation of CPEs, and analyte-induced conformational changes of CPEs.[7] Whitten and coworkers pioneered to report the exploration of amplified quenching for biosensing and detected a protein-binding interaction using a biotinylated methyl viologen based on the reversible fluorescence quenching in poly(2-methoxy-5-propyloxy sulfonate phenylene).[10] Heeger and coworkers reported the nonspecific interaction of a sulfonated poly(*p*-phenylenevinylene) (PPV) with cytochrome *c*, myoglobin, and lysozyme, where the fluorescence of the CPE was strongly quenched by the former and to a lesser degree by the later two.[11] Bazan and coworkers reported the interaction between the PPV polyelectrolyte and avidin in combination with a biotinylated quencher.[12] Carboxylated-substituted poly(*p*-phenyleneethynylene)s (PPE) were found to form stable complexes with histone, lysozyme, myoglobin, and hemoglobin and the fluorescence was quenched; yet bovine serum albumin (BSA) did not quench the fluorescence of the PPEs but enhanced it.[13]

Although CPEs such as PPVs and PPEs have been studied extensively as optical probes to detect the interactions with proteins, high fluorescence quantum yields and high fluorescence sensitivity are still challenges to explore new CPE for the probes. Water-soluble polyfluorenes (PFs) have just proven good candidates in this area because of their unique

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properties. Cationic PFs with high fluorescence quantum yields have been successfully used for sequence-specific DNA detection.[14,15] However, anionic PFs are not well-reported to probe proteins. Cao and coworkers synthesized a water-soluble sulfonated-PF and found the fluorescence could be quenched by a biotin-labeled dye in the presence of avidin.[16] Liu and coworkers synthesized some carboxylated-PFs composed of fluorene segments and benzothiadiazole units, which could be used for protein detection and quantification by utilizing their aggregation-induced fluorescence change, and they also investigated the strong fluorescence quenching of the anionic PFs by cationic quenchers and proteins.[17,18]

Here, we report the fluorescence sensing properties of a homemade water-soluble anionic PF, poly[9,9-bis(3′-butyrate)fluoren-2,7-yl] sodium (BBS-PF). The fluorescence quenching of the carboxylic acid-functionalized PF interacted with proteins such as cytochrome *c*, lysozyme, and BSA, and methyl viologen ($MV^{2+}$), $Fe^{3+}$, and $Fe^{2+}$ were investigated. BBS-PF exhibited a high fluorescence quantum yield and a large Stern–Volmer constant value toward different proteins, which makes it a promising probe for protein sensing.

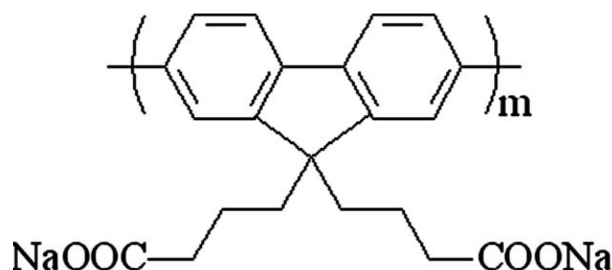
## EXPERIMENTAL

### Materials

All materials and reagents used in this work are commercial products of high grade employed as received without further purification unless otherwise stated. Cytochrome *c*, lysozyme, BSA, and the sodium phosphate buffer (pH = 7.5) were purchased from Shanghai Sangon Biological Engineering Technology and Services, Co., Ltd. (Shanghai, China). Methyl viologen ($MV^{2+}$) was purchased from Aldrich (St. Louis, MO).

### Characterizations

UV–vis absorption and fluorescence spectra were measured using a Shimadzu UV-3150 spectrophotometer and a Shimadzu RF-6301PC spectrophotometer, respectively. All optical measurements of BBS-PF ($1.0 \times 10^{-7}$ M) were performed in 10 m*M* sodium phosphate buffer at pH 7.5 at room temperature (around 25°C). Fluorescence emission spectra were acquired upon excitation at 375 nm and fluorescence excitation spectra were acquired for 416 nm emission. The fluorescence quantum yield, Φ, of the polymer was determined in phosphate buffer at pH 7.5 by using quinine sulfate in sulfuric acid (1.0*N*) as a standard with a known Φ of 0.55.[19] In this work, sodium phosphate buffer (pH 7.5) was used as media to study the interaction of BBS-PF and proteins.



**Scheme 1** Chemical structure of BBS-PF.

## RESULTS AND DISCUSSION

### Chemical structure of BBS-PF

BBS-PF studied here was synthesized by Suzuki coupling reaction, followed by hydrolysis.[20] Scheme 1 shows the chemical structure of BBS-PF, the number-average molecular weight ($M_n$) and distribution of which are $1.38 \times 10^4$ and 3.2, respectively, measured by gel permeation chromatography using polystyrene as a standard and tetrahydrofuran as the eluant. The UV–vis absorption spectrum of BBS-PF in phosphate buffer shows a strong band with a peak at around 375 nm, which can be attributed to the $\pi$–$\pi^*$ transition of the polymer backbone. The fluorescence emission spectrum shows a main peak at around 415 nm and a shoulder at about 435 nm. The fluorescence quantum yield Φ was measured to be 0.41 in phosphate buffer with quinine sulfate as a reference. The Φ value is much higher than that of carboxylated-PPEs (only 0.08),[13] and also higher than that of the reported propanoate-PF (0.25).[17]

### Protein sensing of the fluorescent BBS-PF

The fluorescence quenching process that is at the core of sensing principle for conjugated polymers can be described by the Stern–Volmer equation.[21]

$$F_0/F = 1 + K_{SV}[Q]$$

where $F_0$ and $F$ are the fluorescence intensity of the conjugated polymer before and after addition of a given molar concentration of quencher [Q], respectively. $K_{SV}$ denotes the Stern–Volmer constant and can be extracted as the slope from a graph that plots quencher concentration versus $F_0/F$. Under conditions where all other variables are held constant, the higher the $K_{SV}$, the lower the concentration of quencher required to achieve fluorescence quenching, and the higher sensitivity the conjugated polymer used as optical probe to detect biomacromolecules. To investigate the sensitivity of the PF BSS-PF as

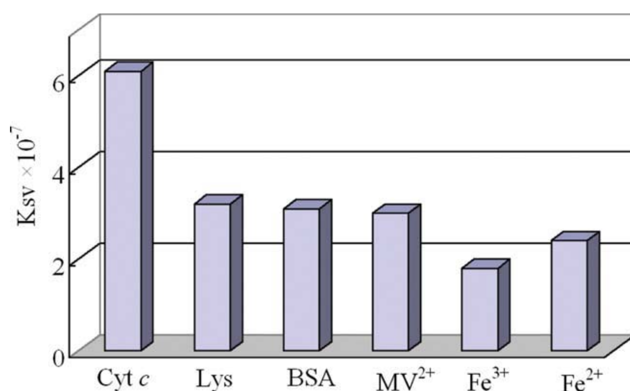



**Figure 1** Bar graph of the Stern–Volmer constants for the quenching of BBS-PF by cytochrome *c* (Cyt *c*), lysozyme (Lys), BSA, methyl viologen ($MV^{2+}$), $Fe^{3+}$, and $Fe^{2+}$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

optical probe for protein detection, we exposed it to a series of commercially available proteins such as cytochrome *c*, lysozyme, and BSA and methyl viologen in phosphate buffer. Methyl viologen ($MV^{2+}$), which is a highly efficient electron transfer quencher, a single of which can quench the entire conjugated polymer,[10] is used to compare the quenching efficiency of the proteins.

Figure 1 shows the obtained large Stern–Volmer constants ($K_{SV}$) for the quenching BSS-PF by proteins, $MV^{2+}$, and iron ions at their low concentrations below $8 \times 10^{-9}$ M. Cytochrome *c* shows the largest Stern–Volmer constant, $K_{SV} = 6.1 \times 10^7$ L/mol, approximately twice as that of others. The Stern–Volmer constants for lysozyme, BSA, and $MV^{2+}$ are $3.2 \times 10^7$, $3.1 \times 10^7$, and $3.0 \times 10^7$ L/mol, respectively. These $K_{SV}$ values are comparable with those for the quenching of PPV polyelectrolytes, where $K_{SV}$ for cytochrome *c* was measured to be $3.2 \times 10^8$ L/mol, and lysozyme could also reduce the fluorescence by 10% at a concentration of $10^{-10}$ M as that of cytochrome *c*.[11] Comparing with PPE polyelectrolytes, the quenching constants are much higher since the $K_{SV}$ for the quenching of PPEs by cytochrome *c* and lysozyme is only $\sim 10^5$ L/mol.[13] Liu and coworkers reported that cytochrome *c* and $MV^{2+}$ quenched the fluorescence of propanoate-PF with $K_{SV}$ values of $7.0 \times 10^7$ and $3.2 \times 10^7$ L/mol, respectively.[17] For the phosphatepropyl-PF, the $K_{SV}$ values were reported to be $3.0 \times 10^7$ and $2.8 \times 10^6$ L/mol, respectively; for the phosphatehexyl-PF, the values were $2.1 \times 10^8$ and $1.6 \times 10^6$ L/mol, respectively.[22] The fluorescence quenching by proteins can be explained by multivalency nonspecific interactions between the CPEs and the quenchers.[23]

Cytochrome *c*, a heme-containing protein, is a cationic polyelectrolyte at neutral pH and can form complexes with anionic polyelectrolytes.[24] Lysozyme, a nonmetalloprotein, is also a highly positively charged polyelectrolyte, yet BSA, a nonmetalloprotein, is a negatively charged polyelectrolyte. The metalloprotein cytochrome *c* formed complex with BBS-PF through electrostatic interaction, where the FRET could occur from BBS-PF to cytochrome *c* since the UV–vis absorption spectrum of which overlaps well with the emission spectrum of BBS-PF shown in Figure 2. Besides the FRET, the electron transfer, resulting from the redox of metalloporphyrin in cytochrome *c*, could be another mechanism of fluorescence quenching of BBS-PF. To confirm the electron transfer quenching of cytochrome *c*, we exposed the BBS-PF in ferric and ferrous solutions. The $K_{SV}$ for $Fe^{3+}$ and $Fe^{2+}$ were found to be $1.7 \times 10^7$ and $2.4 \times 10^7$ L/mol, respectively, as shown in Figure 1. The fluorescence quenching induced by $Fe^{3+}$ and $Fe^{2+}$ results from the static electron transfer quenching,[9,25] since the extinction coefficient of BBS-PF decreased obviously upon addition of the ions and the absorption onset was red-shifted by about 3 nm. So cytochrome *c* could quench the fluorescence by the electron transfer from BBS-PF to the metal iron in the protein, besides the FRET.

For the nonmetalloproteins such as lysozyme and BSA, no FRET and electron transfer exist in the complex, since they are lack of chromophore, which can accept the excited state energy from the conjugated polymer and lack of functional center, which can accept the electrons. Thayumanavan and coworkers studied the quenching properties of a fluorogenic carboxylated polymer by metalloproteins and showed that the metalloproteins could quench the fluorescence by FRET and electron transfer but the nonmetalloproteins not.[26] In this study, the increased quenching constant, $K_{SV}$, for cytochrome *c*,

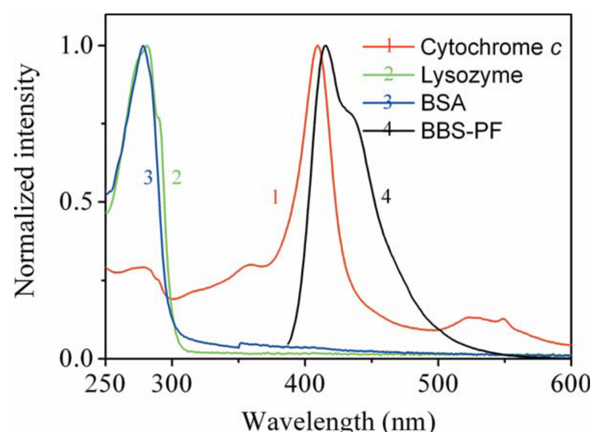



**Figure 2** UV–vis absorption spectra of cytochrome *c* (curve 1), lysozyme (curve 2), and BSA (curve 3), and emission spectrum of BBS-PF in phosphate buffer at pH 7.5 (curve 4). [Color figure can be viewed in the online

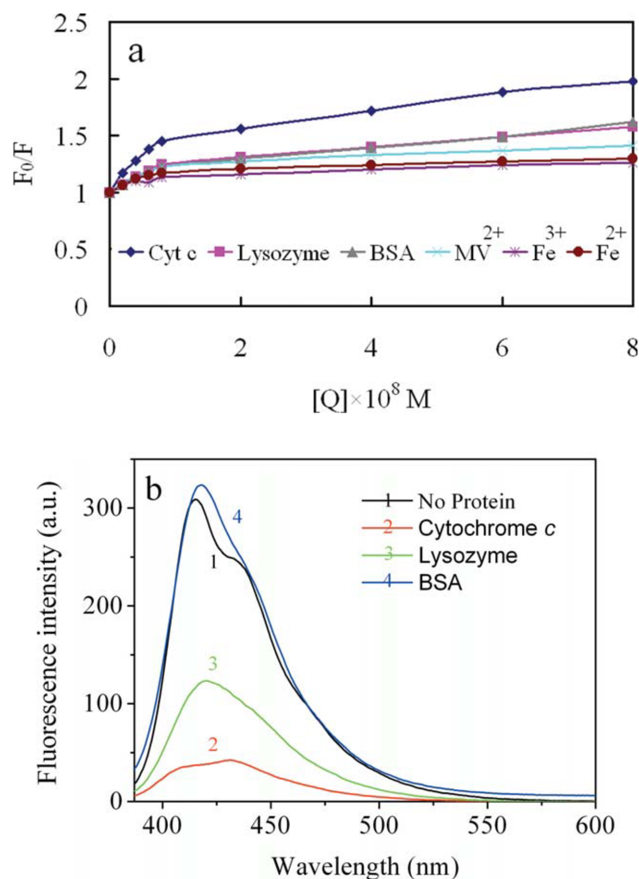



**Figure 3** (a) $F_0/F$ plots for BBS-PF with cytochrome *c*, lysozyme, BSA, methyl viologen ($MV^{2+}$), $Fe^{3+}$, and $Fe^{2+}$. (b) Fluorescence spectra of BBS-PF in the absence and presence of the proteins at the concentration of 4 μ*M*, excitation at 375 nm: no protein (curve 1), cytochrome *c* (curve 2), lysozyme (curve 3), and BSA (curve 4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

comparing with that for lysozyme and BSA, should be ascribed to the FRET and electron transfer from BBS-PF to the metalloprotein. Another contribution to the fluorescence quenching for cytochrome *c* should be ascribed to the protein-induced aggregation of the CPE.[7,11] Lysozyme, a polycation, could quench the fluorescence by the protein-induce aggregation at low concentrations with a $K_{SV}$ of 3.2 × $10^7$ L/mol. However, the lysozyme quenching saturated at around 60% of its original fluorescence, yet the fluorescence could be almost fully quenched by cytochrome *c*. BSA, a polyanion, could also quench the fluorescence at low concentrations ($K_{SV}$ = 3.1 × $10^7$ L/mol), however, it increased the fluorescence a little at high concentrations, as has not been reported before and will be discussed later.[11,13,17]

Figure 3(a) displays the Stern–Volmer plots for BBS-PF with the proteins and cationic quenchers, in which effective quenching is observed at low concentrations but less effective quenching occurs at higher concentrations. The above quenching constants $K_{SV}$ were obtained from the linear part of the graph at low concentrations. When the concentration increased to micromolar, cytochrome *c* and lysozyme quenched the fluorescence of BBS-PF further, but BSA increased the fluorescence a little. Figure 3(b) shows the fluorescence spectra of BBS-PF in the absence and presence of the proteins at the concentration of 4 × $10^{-6}$ M. BSA is a fatty acid transporter and has surfactant qualities.[27] The increased fluorescence of BBS-PF upon addition of BSA to the concentration of micromolar should be ascribed to that the BBS-PF's backbone is efficiently complexed by the hydrophobic patches on the surface of BSA and its negative charge does not play a significant role under this condition.[13]

To obtain insight into the aggregation of the CPE induced by protein, excitation spectra of BBS-PF were measured in the proteins. For cytochrome *c* and lysozyme, the relative intensity of the excitation bands of BBS-PF decreased and the bands blue-shifted as the amount of the proteins increased both at their nanomolar and micromolar concentrations; for BSA at nanomolar concentrations, the intensity decreased and the bands blue-shifted a little as the amount increased, however, the intensity increased and the bands red-shifted when the amount increased to micromolar concentration. Figure 4(a–c) depicts the spectral blue-shift of BBS-PF under the nanomolar of cytochrome *c*, lysozyme, and BSA, respectively; Figure 4(d,e) depicts the blue-shift of BBS-PF under the micromolar of cytochrome *c* and lysozyme, respectively; Figure 4(f) depicts the red-shift of BBS-PF under the micromolar of BSA. For cytochrome *c*, the band peak blue-shifted gradually from 382 to 365 nm when the protein increased from 0 to 4 μ*M*; yet for BSA, the band peak red-shifted from 382 to 385 nm. The blue-shift indicated a strong π-stacking and interchain interaction of BBS-PF by the addition of proteins, as resembles the aggregation of MEH-PPV in solvent at high concentrations.[28] The aggregation induced by the proteins, not only the positively charged proteins such as cytochrome *c* and lysozyme but also the negatively charged BSA, results in the interchain interaction between the conjugated polymers, which in turn results in fluorescence quenching due to π-stacking of the backbones of the polymers.[29] For BSA at micromolar concentrations, the red-shift indicted that the aggregation of BBS-PF in the solvent became weak, resulted from the formed complex with the BSA surfactant, where the negative charge does not play a significant role.[13] The different fluorescence response of BBS-PF to proteins might find its great potential applications as optical probe for biomacromolecule detection.

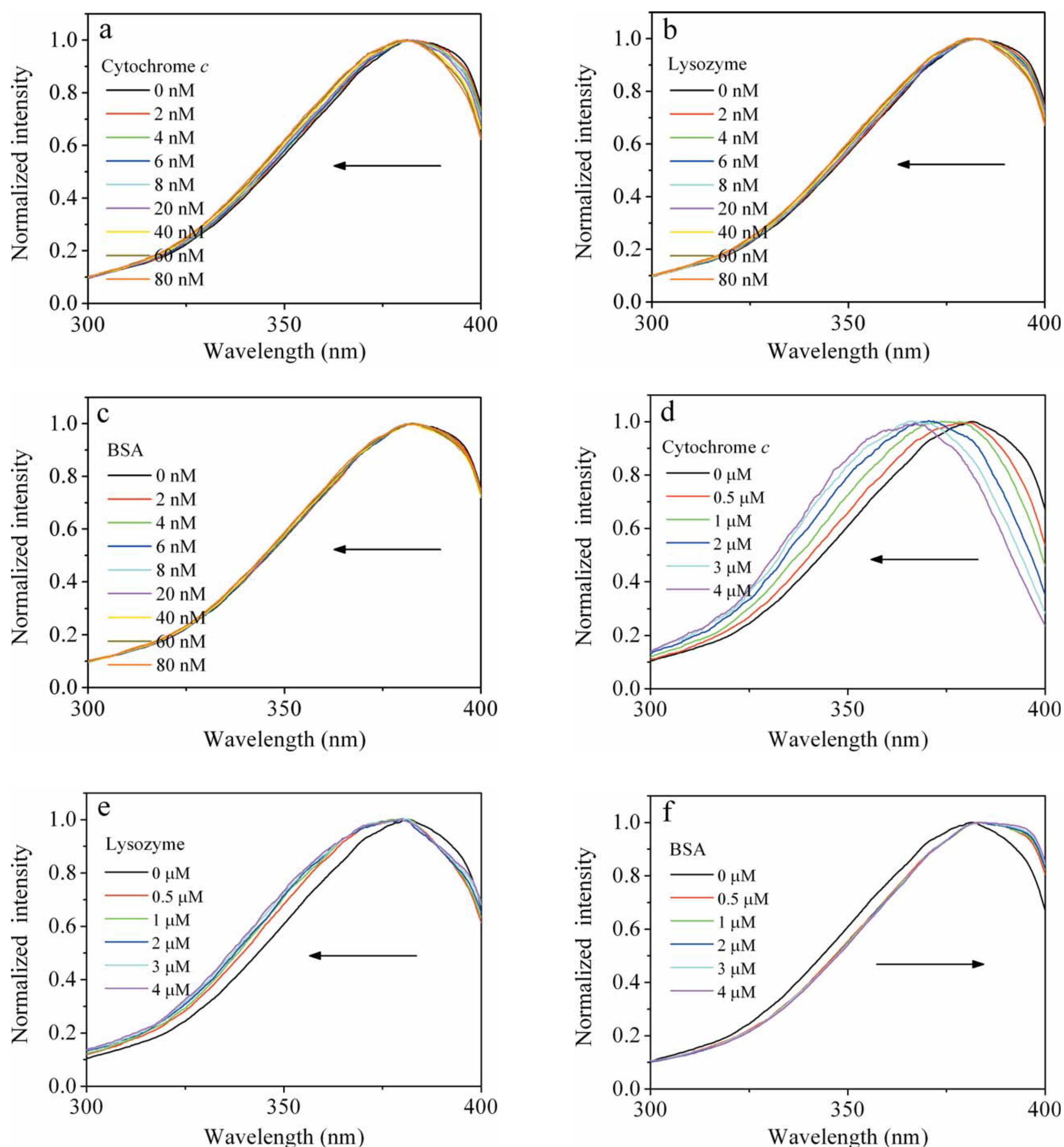



**Figure 4** Excitation spectra of BBS-PF for 416 nm emission with different concentrations of 0, 2, 4, 6, 8, 20, 40, 60, and 80 n*M* of cytochrome *c* (a), lysozyme (b), and BSA (c); excitation spectra of BBS-PF for 416 nm emission with different concentrations of 0, 0.5, 1, 2, 3, and 4 μ*M* of cytochrome *c* (d), lysozyme (e), and BSA (f). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## CONCLUSIONS

Here, we present the protein sensing behavior of a highly fluorescent water-soluble CPE, poly[9,9-bis (3′-butyrate)fluoren-2,7-yl] sodium (BBS-PF). Cytochrome *c*, lysozyme, and BSA can efficiently quench the fluorescence of BBS-PF under their low concentrations at neutral pH. The quenching constant $K_{SV}$ ($6.1 \times 10^7$ L/mol) for cytochrome *c* is twice those for lysozyme and BSA. The high quenching efficiency of the BBS-PF/cytochrome *c* system could be attributed to FRET, electron transfer effect, and the protein-induced aggregation through the electrostatic interactions. For the nonmetalloproteins including positively charged lysozyme and negatively charged BSA, the fluorescence quenching could be ascribed to the

protein-induced aggregation. Interestingly, it should be noted that the negatively charged BSA quenches the fluorescence at its low concentration, but it increases the fluorescence a little at its micromolar concentration. The excitation spectra confirm that the BBS-PF aggregation induced by cytochrome *c* and lysozyme should be different from that induced by BSA. Based on the high selectivity of fluorescence response, the high fluorescence yield, and the good water-soluble properties, this carboxylic acid-functionalized PF can be used as a suitable fluorescent probe for protein detection.

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