A Quencher-Tether-Ligand Probe and Its Application in Biosensor Based on Conjugated Polymer

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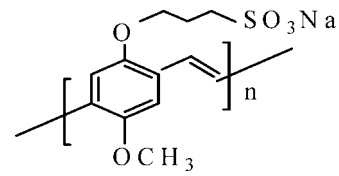
ABSTRACT: Water-soluble conjugated polymers (CPs) are a versatile class of advanced organic materials with excellent photochemistry properties. Using a biotin-modified fluorescence quencher (quencher-tether-ligand, QTL) to specifically sense the presence of avidin based on CP is of great importance. However, the nonspecific interactions between various proteins and CPs greatly baffle the studies of the mechanism and applications of CP-based biosensors. We selected luminescent ruthenium(II) polypyridine biotin complex (Ru-biotin) as a proper probe, which can not only work as a fluorescent quencher but also produces fluorescence resonance energy transfer (FRET) with anionic water-soluble CP (poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenecinylene] (MPS-PPV)). As the emission peak of Ru-biotin produced by FRET cannot be infected by nonspecific proteins, we discussed the MPS-PPV/Ru-biotin/avidin biosensing mechanism based on the fluorescence spectra changes of polymer and Ru-biotin and consequently realized the rapid and specific detection of avidin.

Introduction

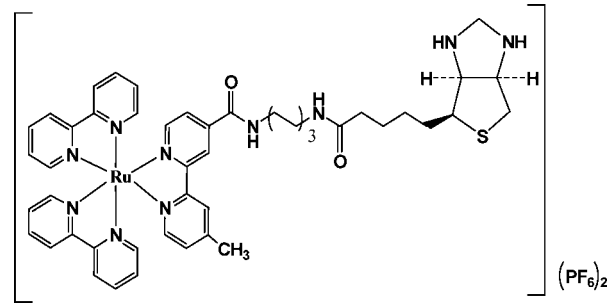
Water-soluble conjugated polymers (CPs) have recently received attention as components in high-performance fluorescence biosensor to improve its sensitivity and selectivity.[1–4] The high sensitivity of CP-based biosensor is attributed to the unique electrical and optical properties of CPs, such as charge transfer, energy transfer, high absorption coefficients, and relatively high fluorescent quantum yields.[5–7] The binding of biotin to avidin is one of the strongest protein−ligand interactions in nature, which is a million times stronger than the antigen−antibody interaction, and this combination is also very stable. Thus, they have been widely used in the biotin−avidin system (BAS) to detect other proteins because of their multilevel amplification effect.[8] Therefore, the detection of avidin has long been the interest of numerous research groups. On the basis of this, Whitten et al. presented a new bioprobe (quencher-tether-ligand, QTL), such as quencher-biotin (Q-biotin), to quench the fluorescence of CP and proposed a simple mechanism whereby avidin's complexation with Q-biotin draws the quencher away from CPs and consequently recovers the CP fluorescence and finally realizes the specific detection of avidin.[9,10] Heeger et al. also pointed out the potential for its application in detecting antibody:antigen pairs or DNA:DNA (DNA:RNA) pairs.[9]

However, some research groups proposed inconsistent ideas on this sensor model. In 2004, Dwight et al. proposed that the addition of avidin did not draw Q-botin away from the CP; instead, it drew Q-botin closer to the polymer, which was not consistent with a simple mechanism of quench/recovery in the avidin/biotin pair.[11] And for the antigen/antibody pair, Heeger et al. demonstrated that because other proteins could also cause a similar phenomenon, the recovery of Q-antibody-quenched fluorescence of CP was not antigen-specific and consequently of no practical value as a biosensor.[12] These researches pointed out that the associations between nonspecific proteins and CPs are very general phenomena, which disturbs the changes of CP fluorescence, perturbs the selectivity of CP-based biosensor, and consequently limits their application. In addition, these different propositions on the sensing mechanism of CPs-based QTL biosensor suggest that the fluorescence change of the polymer alone cannot sufficiently support the study of the sensing mechanism. More evidences are needed to clarify the response mechanism besides the polymer fluorescence spectra.

Scheme 1. Chemical Structure of MPS-PPV



Scheme 2. Chemical Structure of Ru-Biotin



In this paper, the anionic water-soluble conjugated polymer we used is poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenecinylene] (MPS-PPV)[7,13] (Scheme 1). We selected a novel biotin-modified QTL probe, luminescent Ru-biotin[14] ($[Ru(bpy)_2L](PF_6)_2$ (bpy = 2,2′-bipyridine; L = 4-(*N*-((6-biotinamido)hexyl)amido)-4′-methyl-2,2′-bipyridine) (Scheme 2), to study the mechanism of the MPS-PPV/Ru-biotin/avidin system in different mediums (water, phosphate buffer (PBS) pH = 7.4, and ammonium carbonate buffer pH = 8.9). The traditional QTL probes, such as methyl viologen-biotin, can merely quench the fluorescence of polymer through electron transfer. Ru-biotin, not only an electron acceptor but also an

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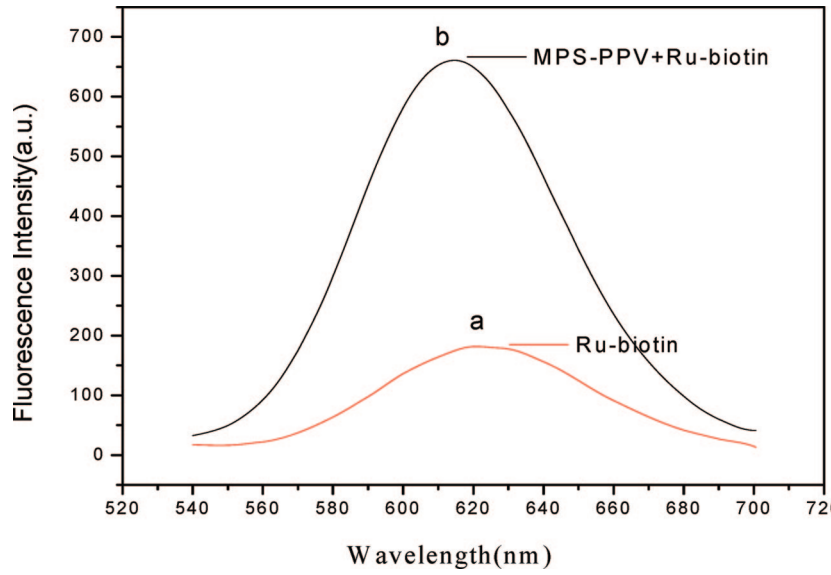


Figure 1. Fluorescent spectra of Ru-biotin and MPS-PPV/Ru-biotin (λ_{ex} = 457 nm) in 0.01 M $(NH_4)_2CO_3$ at pH = 8.9: (a) [Ru-biotin] = 1.0×10^{-5} M; (b) Ru-biotin + MPS-PPV ([RU] = 3.0×10^{-5} M, where RU = repeat unit of polymer).

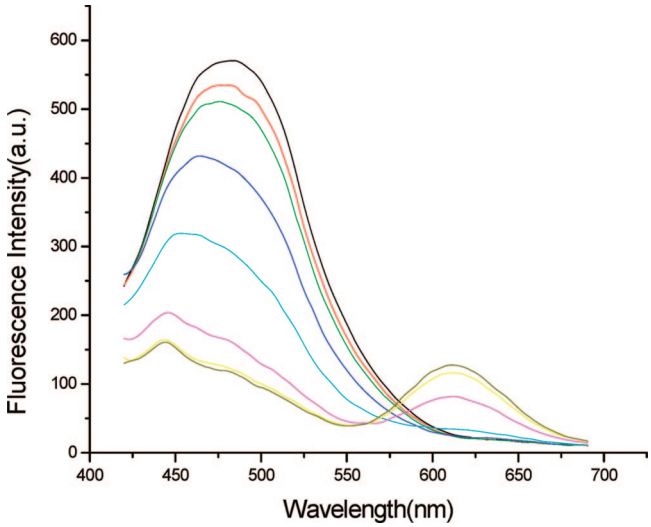


Figure 2. Fluorescent spectra of MPS-PPV quenched by Ru-biotin in pure water (λ_{ex} = 387 nm): MPS-PPV (3.0×10^{-5} M), From top to bottom: [Ru-biotin] = 0.0, 0.003, 0.015, 0.12, 0.45, 1.8, 2.7, and 3.0×10^{-6} M.

Table 1. K_{sv} of Ru-Biotin to MPS-PPV in Different Solutions

	pure water	0.01 mol/L PBS (pH 7.4)	0.01 mol/L ammonium carbonate (pH 8.9)
K_{sv} (M^{-1})	1.8×10^6	9.33×10^5	1.09×10^6

energy acceptor, can quench the fluorescence of the polymer and produce a new emissive peak at the same time. Different from MPS-PPV, Ru-biotin is a conventional small molecular fluorescent material. The emission peak produced by fluorescence resonance energy transfer (FRET) cannot be easily infected by proteins, but by the distance between the acceptor and donor. Therefore, when we investigated the CPs/Q-biotin/avidin biosensing mechanism, we can get direct proof from the spectra: whether the addition of avidin draws Ru-biotin away from MPS-PPV (the model of Whitten) or draws it closer to MPS-PPV (the model of Dwight). And on the basis of the new mechanism we got, a novel polymer-based QTL probe was applied to specifically determine the presence of avidin.

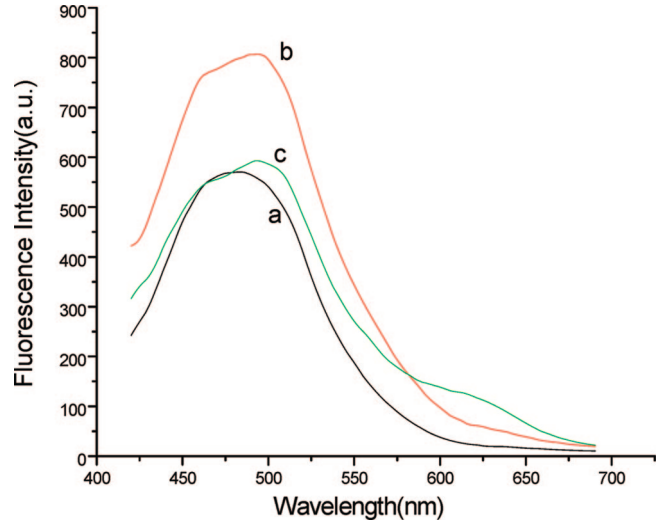


Figure 3. Fluorescent spectra of MPS-PPV (λ_{ex} = 387 nm) quenched by Ru-biotin in pure water with the existence of CTMAB: (a) MPS-PPV (3.0×10^{-5} M); (b) MPS-PPV and [CTMAB] = 4.0×10^{-5} M; (c) MPS-PPV, CTMAB, and [Ru-biotin] = 3.0×10^{-6} M.

Experimental Methods

Apparatus and Reagents. The fluorescence emission spectra were recorded with a LS 55 luminescence spectrometer (Perkin-Elmer Co.). MPS-PPV was prepared according to ref 13, and the molecular weight of MPS-PPV ranges from 150 000 to 290 000. Its molecular weight was estimated by static light scattering measurement.[7] The characterization of MPS-PPV is provided in the Supporting Information (part one). Ru-biotin was offered by Dr. Kenneth Kam-Wing Lo from City University of Hong Kong, and avidin was purchased from Sigma Co. (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Maverick Co., and cationic surfactant cetyltrimethylammonium bromide (CTMAB) was purchased from China Medicine (Group) Shanghai Chemical Reagent Corp. Buffer solutions were prepared with reagent-grade material. The Milli-Q water was used as pure water.

Fluorescence Spectra Measurement. All experiments were carried out at room temperature, and the fluorescence intensity was measured after the sample was allowed to thermally equilibrate. The fluorescence spectra of Ru-biotin were obtained by excited at 457 nm. MPS-PPV solutions (pure water, PBS pH = 7.4, or ammonium carbonate buffer pH = 8.9) were added to a 1 mL quartz

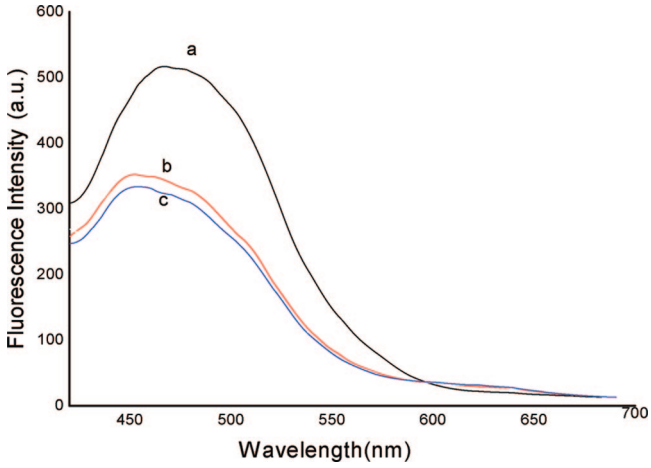


Figure 4. Fluorescence spectra of MPS-PPV (λ_{ex}= 387 nm) quenched by Ru-biotin with the addition of avidin: (a) MPS-PPV (3.0×10^{-5} M); (b) MPS-PPV and [Ru-biotin] = 4.5×10^{-6} M; (c) MPS-PPV, Ru-biotin, and [avidin] = 1.5×10^{-8} M.

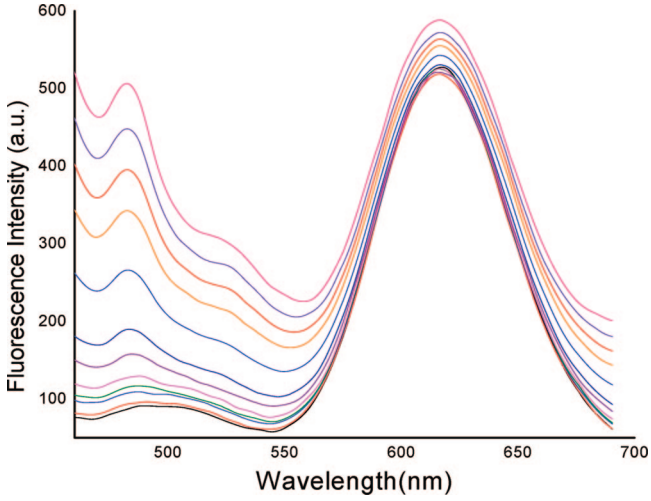


Figure 5. Fluorescent spectra of MPS-PPV (λ_{ex} = 387 nm) quenched by Ru-biotin in PBS (pH = 7.4) with the addition of avidin: [MPS-PPV] = 3.0×10^{-5} M, [Ru-biotin] = 1.5×10^{-5} mol/L, [avidin] = 0, 1.0, 2.0, 3.0, 5.0, 10, 20, 30, 40, 50, 60, and 70×10^{-8} mol/L.

cuvette (sample cell), and the fluorescence spectra were recorded with excitation at 387 nm. Under the same conditions, the fluorescence spectra were recorded while adding varied concentration of different samples to MPS-PPV solutions.

Luminescence Titrations. In a typical procedure, aliquots of the Ru-biotin were added cumulatively to MPS-PPV solutions (pure water, PBS pH = 7.4, or ammonium carbonate buffer pH = 8.9) (1 mL) at 1 min intervals. The emission spectrum of the solution was then measured.

Results and Discussion

Fluorescence Quenching and FRET. In our previous study, we discovered that those transition metal cations which have a d^{6-9} electron-deficient configuration, especially noble metal cations, such as Pd^{2+}, Ru^{3+}, and Pt^{2+}, possess excellent quenching efficiency to MPS-PPV fluorescence.[7] Because the central ion of Ru-biotin is an electron-deficient transition metal ion, Ru-biotin is an excellent MPS-PPV quencher through electron transfer.

Ru-biotin can also work as an energy acceptor with anionic water-soluble CP (MPS-PPV). The emission wavelength of MPS-PPV λ_{em} = 483 nm is close to the excitation wavelength of Ru-biotin (λ_{ex} = 457 nm). The overlap of the emission of MPS-PPV and the absorption of Ru-Biotin ensures efficient FRET between them.[15–17] In addition, the probe Ru-biotin is positively charged in aqueous solutions, which renders its close proximity to the negatively charged MPS-PPV because of electrostatic interactions and consequently forms a complex with it. As shown in Figure 1, the emission peak of Ru-biotin is at 624 nm; in the presence of the polymer there is a ~10 nm blue shift (from 624 to 614 nm), and a sharp change of fluorescence intensity can be observed, presumably due to the change in polarity in the vicinity of Ru-biotin by the interaction with MPS-PPV.[4]

Thus, when adding Ru-biotin into MPS-PPV aqueous solution, the emission peak of MPS-PPV at 483 nm is quenched, and a new peak appears concomitantly at 614 nm. And with the addition of Ru-biotin, the emission peak of MPS-PPV at 483 nm decreases gradually, with an increase of fluorescence intensity at 614 nm (Figure 2). Similar phenomena can be observed in the buffer system (PBS pH = 7.4 and ammonium carbonate buffer pH = 8.9) (see Supporting Information, part two).

Assuming the quenching of MPS-PPV is a dynamic quenching, we would estimate the dynamic quenching rate constant according to Stern−Volmer equation:

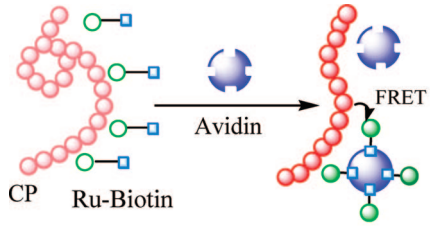
$$F_0/F = 1 + K_q\tau_f[Q] = 1 + K_{sv}[Q]$$

where F_0 and F are the fluorescence intensities of MPS-PPV in the absence and presence of Ru-Biotin, respectively, and the quenching constant (K_{sv}) of Ru-biotin is 1.8×10^6 M^{-1} (see Supporting Information, Figure 3S). The fluorescence lifetime of MPS-PPV is 1.0 ns.[10] The dynamic quenching rate constant was estimated to be $K_q = K_{sv}/\tau_f \approx 1.8 \times 10^{15}$ M^{-1} s^{-1}, which is several orders of magnitude above values for diffusion-controlled quenching (the maximum diffusion colliding quenching constant of various quenchers is 2.0×10^{10} $(mol\ L^{-1})^{-1}$ s).[18] Therefore, we can presume that the quenching procedure is static quenching because a new ionic complex is formed between Ru-biotin and the polymer.

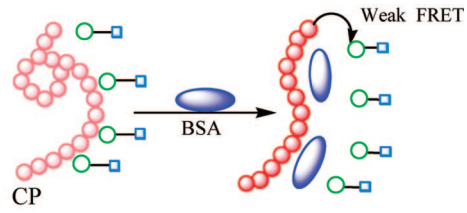
By comparing the quenching constants (K_{sv}, see Supporting Information, part three), we discovered that the quenching efficiency of Ru-biotin to the fluorescence of MPS-PPV varies in different solutions (as shown in Table 1). The quenching efficiencies of Ru-biotin in buffers are lower than that in pure water. The possible reason is that the electrostatic screening in buffer system perturbs the electrostatic interactions between Ru-biotin and MPS-PPV and consequently weakens the quenching ability of Ru-biotin. Since buffer solutions (0.01 M) have the same ionic strength and the pH possesses little influence on the electrostatic effect between Ru-biotin and MPS-PPV, the quenching efficiency differs slightly in PBS and ammonium carbonate buffer solutions.

Efficiency of FRET and Distance. It is well-known that the efficiency of FRET can be greatly influenced by the distance between the acceptor and donor. An experiment was designed to estimate whether there exists a similar effect between Ru-biotin and MPS-PPV. As shown in Figure 3, the cationic surfactant CTMAB forms a stable complex with opposite-charged MPS-PPV, and the fluorescence intensity of MPS-PPV was enhanced.[19–21] The stable complex formed by CTMAB and MPS-PPV prevents interactions between MPS-PPV and Ru-biotin, enlarges the distance between them, and reduces their FRET efficiency. And the ratio of fluorescence at 614 nm to the fluorescence at 483 nm is much lower than that of Figure 2 when the same amount of Ru-biotin (3 μM) was added. So by analyzing the change of the emission peak at 614 nm, we can deduce the distance change between MPS-PPV and Ru-biotin. That is, when using Ru-biotin as a QTL probe, it can help us

Scheme 3. Mechanisms for the Fluorescence Change by Addition of Specific Protein (Avidin) (a) and Nonspecific Protein (BSA) (b) in Aqueous Solution



Scheme 3a



Scheme 3b

Table 2. Fluorescence Recovery after Adding 7.0×10^{-7} M Avidin into the MPS-PPV/Ru-Biotin System

	pure water	0.01 mol/L PBS (pH 7.4)	0.01 mol/L ammonium carbonate (pH 8.9)
fluorescence recovery (%)	42.8	95.2	30.5

analyze the mechanism of the system after the addition of avidin: whether avidin draws Ru-biotin away from MPS-PPV or draws them into closer proximity.

Addition of Avidin into Partly Quenched MPS-PPV System. As a charged macromolecule protein, the effect of avidin to the quenched MPS-PPV system is complicated. Dwight[11] observed the addition of avidin further quenched the fluorescence of MPS-PPV, while Whitten[10] observed fluorescence recovery after the addition. On the basis of these seemingly contradictory phenomena, different mechanisms are proposed. We discovered that one of the important diversities in their experiments was that in Dwight's experiment avidin was added when the fluorescence of the polymer was 15% quenched, while in Whitten's experiment that number was 80%. Therefore, in order to systematically examine the effect of avidin on the quenched MPS-PPV system, we carried out our experiments under the condition that the fluorescence of MPS-PPV was partly quenched and completely quenched.

As shown in Figure 4, when the fluorescence of the polymer is partly quenched about 45%, the fluorescence decreases when further adding avidin, similar to the phenomena reported by Dwight et al. 1.5×10^{-8} M avidin quenches an additional 5.8% of the emission intensity, and further increase in the concentration of avidin produces no obvious change on the fluorescence of the polymer. Since the fluorescence of Ru-biotin produced by FRET is very weak as the quantity of Ru-biotin in the system is relatively small, the addition of avidin cannot produce obvious change on the fluorescence. We still lacked the direct proof to study MPS-PPV/Ru-biotin/avidin system sensing mechanism. So we transferred our focus to the situation that the fluorescence of the polymer is completely quenched.

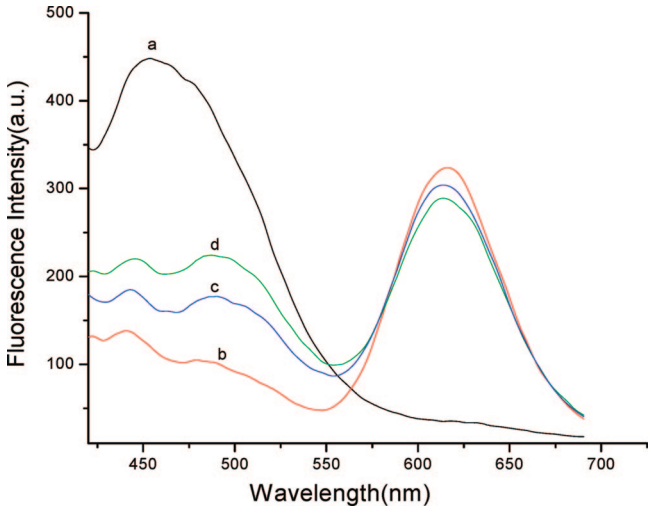


Figure 6. Fluorescence spectra of MPS-PPV ($\lambda_{ex} = 387$ nm) in 0.1 M $(NH_4)_2CO_3$ at pH = 8.9: (a) MPS-PPV (3.0×10^{-5} M); (b) MPS-PPV and [Ru-biotin] = 1.2×10^{-5} M; (c) MPS-PPV, Ru-biotin, and [BSA] = 3×10^{-6} M; (d) MPS-PPV, Ru-biotin, and [BSA] = 5×10^{-6} M.

Addition of Avidin into Completely Quenched MPS-PPV System. When the fluorescence of MPS-PPV is quenched about 85% by Ru-biotin, further addition of Ru-biotin cannot decrease the fluorescence intensity of MPS-PPV, and this can be regarded as complete quenching. In this case, additions of avidin in three different mediums (pure water, PBS buffer pH = 7.4, and ammonium carbonate buffer pH = 8.9) produce similar results. As shown in Figure 5, with the increase of avidin concentration, the fluorescence intensities at 483 and 614 nm increase respectively. The fluorescence of the polymer at 483 nm recovered with the addition of the avidin. However, we do not think this phenomenon means the addition of avidin draws Ru-biotin away from MPS-PPV. From the fluorescence increase of Ru-biotin at 614 nm we can directly infer that the complex formed by the complexation between Ru-biotin and polymer was not destroyed. Instead, it shortens the distance between Ru-biotin and MPS-PPV, enhances their FRET efficiency, and thus increases Ru-biotin fluorescence intensity.

On the basis of the results of our experiments, the MPS-PPV/Ru-biotin/avidin biosensing mechanism is deduced (Scheme 3a). Ru-biotin combines with the polymer MPS-PPV through electrostatic attraction and thus quenches the fluorescence of MPS-PPV because of electron transfer. The addition of avidin, which selectively binds onto the biotin substructure, is described to encapsulate Ru-biotin. However, avidin, a positively charged macromolecule which possessed a pI of 10, moves toward the polymer by electrostatic attraction and hydrophobic interactions.[22] Thus, the addition of avidin does not draw Ru-biotin away from MPS-PPV. The possible reason for fluorescence increase of the polymer is that, on one hand, the proximity of polymer and macromolecule avdin changes the conformation of the polymer, and avidin competes with polymer for the quencher Ru-biotin because of the specific binding between avidin and biotin. On the other hand, because Ru-biotin−avidin possesses larger and positively charged structure and approaches MPS-PPV, it elongates and separates polymer chains, breaks up aggregation, and increases the average interchromophore distance, rendering a decrease in self-quenching. Therefore, an increase of polymer fluorescence at 483 nm is observed.

Meanwhile, we also discussed the influence of system environment on fluorescence recovery of MPS-PPV. As shown in Table 2, the percentages of MPS-PPV fluorescence recovery in pure water, phosphate buffer, and ammonium carbonate buffer differ greatly. In phosphate buffer system (pH = 7.4), the addition of avidin can realize almost complete recovery, obviously better than the enhanced phenomenon observed in

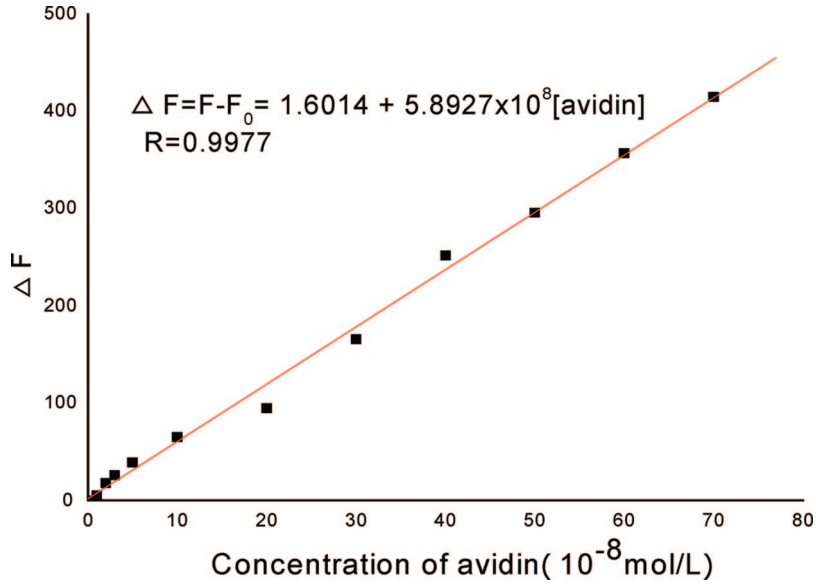


Figure 7. Linear relationship between the concentration of avidin and the changed fluorescence intensity at an emission wavelength of 483 nm in PBS (pH = 7.4). [MPS-PPV]= 3.0×10^{-5} M, [Ru-biotin] = 1.5×10^{-5} M; [avidin] = 1.0, 2.0, 3.0, 5.0, 10, 20, 30, 40, 50, 60, and 70×10^{-8} M; λ_{ex} = 387 nm.

pure water and ammonium carbonate buffer (see Supporting Information, part four). We regard the fluorescence recovery is greatly influenced by two factors: pH value and ion strength of the medium. Since avidin possesses a pI of 10, it is positively charged in these mediums. However, the charge quantity of avidin depends on the pH value of the medium. Because the charge on the surface of avidin is less in ammonium carbonate buffer (pH = 8.9) than that in water and in PBS buffer (pH = 7.4), the electrostatic force between avidin and the polymer is weaker, which makes avidin less effective to approach the polymer and more difficult to recover the fluorescence of the polymer. The reason why the percentage of fluorescence recovery is higher in PBS than in pure water is that from the quenching constant we can know that the interaction between polymer and Ru-biotin is strongest in water, which means it is more difficult for avidin to compete with polymer for Ru-biotin. In addition, the ion strength in buffer is stronger than that in pure water, which is beneficial for the extension of polymer conformation and the separation of chains. Therefore, the percentage of fluorescence recovery of MPS-PPV is the highest in PBS buffer.

Effects of Nonspecific Proteins on the Fluorescence Quenching of MPS-PPV by Ru-Biotin. When adding nonspecific proteins, such as BSA, into highly quenched (85% quenched) MPS-PPV/Ru-biotin system (pure water, PBS buffer pH = 7.4, or ammonium carbonate buffer pH = 8.9), the fluorescence intensity at 483 nm increases, while the fluorescence at 614 nm decreases (Figure 6). BSA is known to be a fatty acid transporter and has surfactant qualities.[23] The backbone of MPS-PPV is efficiently complexed by the hydrophobic patches on the surface of BSA, and the quencher/polymer ground-state complex is broken up by it. The interaction between macromolecules BSA and MPS-PPV enhances the fluorescence of MPS-PPV (at 483 nm). However, since BSA enlarges the distance between MPS-PPV and Ru-biotin, it results in a decrease of FRET efficiency, and the decrease of the fluorescence intensity at 614 nm is observed. Although nonspecific protein BSA (Scheme 3b) can also enhance the fluorescence of MPS-PPV, we can still distinguish avidin from other nonspecific proteins by analyzing the spectra's change of Ru-biotin at 614 nm.

Specific and Sensitive Detection of Avidin. The addition of avidin can increase the emission peak of Ru-biotin at 614 nm, which is immune from the nonspecific interaction between nonspecific proteins and the polymer. Therefore, the application of this novel QTL probe Ru-biotin ensures the selectivity of this system. Meanwhile, on the basis of the change of the high sensitive fluorescence material MPS-PPV, we realized the sensitive detection of avidin. We discovered that in PBS buffer system the addition of 7×10^{-7} M avidin recovers the fluorescence of the probe at 483 nm to 95.2% (80.2% increase, ΔF = 417). Under the same experimental conditions, the addition of BSA (5×10^{-7} M) can only enhance the fluorescence of MPS-PPV for 1.9% (ΔF = 11) and a large amount of BSA (5×10^{-6} M) can realize 16.2% of fluorescence change (see Supporting Information, Figure 11S). Therefore, we can regard that the existence of BSA will not perturb the quantitative detection of avidin. As shown in Figure 7, there exists a linear relationship between the concentration of avidin and the changed fluorescence intensity at an emission wavelength of 483 nm. We realized the specific detection of avidin with the detection limit 5 nM (3 times of the system noise ($\Delta F'$) is 4.12).

$$\Delta F = F - F_0 = 1.60 + 5.89 \times 10^8[\text{avidin}] \quad (1)$$

Conclusion

In this paper, we selected a new QTL probe Ru-biotin to study the mechanism of anionic fluorescent polymer/Q-biotin/avidin biosensor in aqueous solutions (pure water, ammonium carbonate buffer (pH = 8.9)). It works not only as a quencher with high quenching efficiency but also as an energy acceptor producing FRET with anionic water-soluble CP (MPS-PPV). On the basis of this, we realized the rapid, sensitive, and specific detection of avidin. In principle, our technique can be extended to use proper quenchers, such as Ru-antibody and Ru-DNA (RNA), to detect their specific counterports, such as antibody and DNA (RNA).

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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