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A Novel Water-Soluble Polythiophene Derivatives Based Fluorescence "Turn-On" Method for Protein Determination

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ABSTRACT: A lable-free, simple, and sensitive fluorescence "turn-on" approach is designed to rapidly detect protein using a conjugated polythiophene derivative (PDPMT-Cl). The fluorescence of PDPMT-Cl solution can be efficiently quenched by $PtCl_4^{2-}$ ions. Upon adding trypsin to the (bovine serum albumin, BSA) PDPMT-Cl–$PtCl_4^{2-}$ solution, the BSA is cleaved into amino acid or peptide fragments, which are stronger $PtCl_4^{2-}$ ions chelators to form more stable complexes with $PtCl_4^{2-}$ ions. Thus, the $PtCl_4^{2-}$ ion is displaced from PDPMT-Cl and the fluorescence of PDPMT-Cl is recovered. By triggering the "turn-on" signal of PDPMT-Cl, it is successful to detect the protein in real time. "Turn-on" response as readout signal is able to effectively reduce background noise and increase detection sensitivity. This method offers good selectivity for detecting protein in the presence of other common amino acids and metal ions. Under optimized conditions, the concentration of BSA in the range of 0.0004–1.75 mg/mL exhibits a linear relationship with the relative fluorescence intensity, and the correlation coefficient is 0.9997. The limit of detection is $4.47 \times 10-4$ mg/mL. The system is successfully applied for detecting protein in milk and egg. Due to the simplicity, sensitivity, and rapid response, this assay shows great potential for protein detection in the future. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2013

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INTRODUCTION

Protein is known as the "building blocks of life" and is one of the most important macromolecules in life science. And sensitive analytical methods for quantification of protein concentration in solution are important in biological laboratories.[1] Bradford,[2] Lowry,[3] and BCA[4] methods are the most commonly used techniques to determine protein concentrations. However, these methods have limitations in sensitivity, protein-to-protein variability, and dynamic range. Sample treatment and measurement procedures can also in some cases be impractical and time-consuming. The methods may require exact or lengthy incubation time with multiple steps at elevated temperatures and hazardous reagents. The methods that rely on the interaction of a dye with proteins,[2–5] detergent-coated proteins,[6,7] or a specific protein functional group[1,8] are dependent on protein surface characteristics and the number of the functional groups. Thus these methods are susceptible to protein-to-protein variability and need to be calibrated for each target separately. Thus, it is essential to develop new methods that could improve the simplicity, selectivity, and sensitivity of protein determination.

Recently, several recent studies have shown that water-soluble, fluorescent conjugated polymers (CPs) provide a useful platform for the development of highly sensitive fluorescence-based sensors for biomolecules[9–12] such as proteins,[9,13–15] DNA,[12,16–18] carbohydrates,[19] and enzymes.[20–24] The high sensitivity of CPs assays takes advantage of the intrinsic fluorescence signal amplification that results from the superquenching behavior of CPs.[9,10,25–30] Such behavior is attributed to a combination of several factors, including delocalization and rapid diffusion of the singlet exciton along the CPs backbone to the quencher "trap site," as well as the electrostatic ion pairing of quenchers and with oppositely charged CPs side chains.[9,25,27,31–33]

As compared with typical fluorescent dyes, these CPs possess a strong light-harvesting ability. And thanks to their delocalized π–π^* electronic structures, the rapid transfer of excitation along the whole backbone to energy/electron receptors is supported, which can remarkably amplify the optical responses by means of either fluorescence superquenching or efficient fluorescence resonance energy transfer (FRET).

Herein, we report a novel cationic polythiophene derivative poly[3-(1,1′-dimethyl-4-piperidinemethylene)thiophene-2,5-diyl chloride] (PDPMT-Cl, see Scheme 1 for chemical structure),[34] and develop a lable-free fluorescence "turn-on" assay for protein

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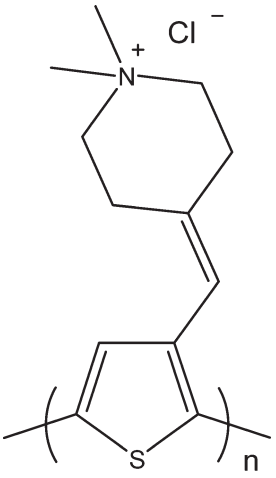

PDPMT-Cl

Scheme 1. Molecular structure of PDPMT-Cl.

in homogeneous aqueous solution. The "turn-on" fluorescence signal response guarantees an easier measurement of low-concentration analyte contrast to the "dark" background, which not only reduces the likelihood of false positive signal but also enhances the detection sensitivity.

EXPERIMENTAL SECTION

Materials and Measurements

PDPMT-Cl was prepared according to the procedure in the literature.[34] Bovine serum albumin (BSA) (0.25 mg/mL aqueous solution) and trypsin (50 μg/mL aqueous solution) were obtained from Shanghai Aladdin Reagent Company (Shanghai, China). K_2PtCl_4 solution (1.0×10^{-4} mol/L); the pH value of NaH_2PO_4-NaOH buffer solution is 8.4. And the other chemical reagents were common commercial level and used as received without further purification. The water was purified using a Millipore filtration system. Fluorescence measurements were carried out on a RF-5301 fluorescence spectrophotometer.

Protein Assay

The quenching experiment was performed by successive addition of $PtCl_4^{2-}$ ion (4.00 mL 1.0×10^{-5}mol/L) to the solution of PDPMT-Cl (1.00 mL 2×10^{-4}mol/L in repeat units (RUs)) at room temperature in 10-mL colorimetric cylinder. And then right amount of BAS and trypsin (1 mL, 50 μg/mL aqueous solution) were added, the fluorescence spectra were recorded with excitation at 463 nm, slit width at 10 and emission at 520 nm. I_0 was used for the fluorescence intensity of PDPMT-Cl + buffer solution (pH = 8.4), I_1 was used for the fluorescence intensity of PDPMT-Cl + $PtCl_4^{2-}$ + buffer solution (pH = 8.4), I_2 was used for the fluorescence intensity of PDPMT-Cl + $PtCl_4^{2-}$ + BSA + trypsin + buffer solution (pH = 8.4), $\Delta I = I_0 - I_1$, was used for fluorescence quenching intensity when $PtCl_4^{2-}$ were added to PDPMT-Cl + buffer solution. $I_R = I_2 - I_1$ was used for fluorescence recovery intensity when BSA + trypsin were added in this system.

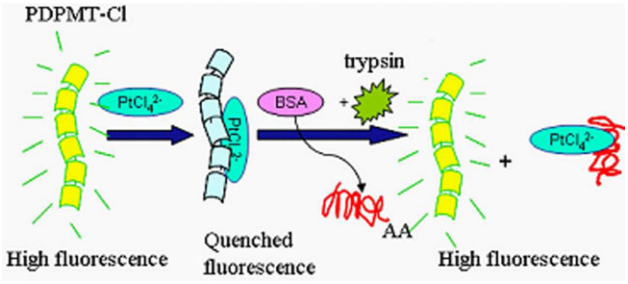

Figure 1. Schematic representations of the detections for protein. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Sample Preparation

About 0.5000 g egg whites were added to a beaker, and they were dissolved by 80 mL water, filtered, and added to 100-mL volumetric flask. Milk sample was prepared by the same procedure. According to the assay method, to assay the BSA concentration of the sample, take a fixed quantity sample solution, and

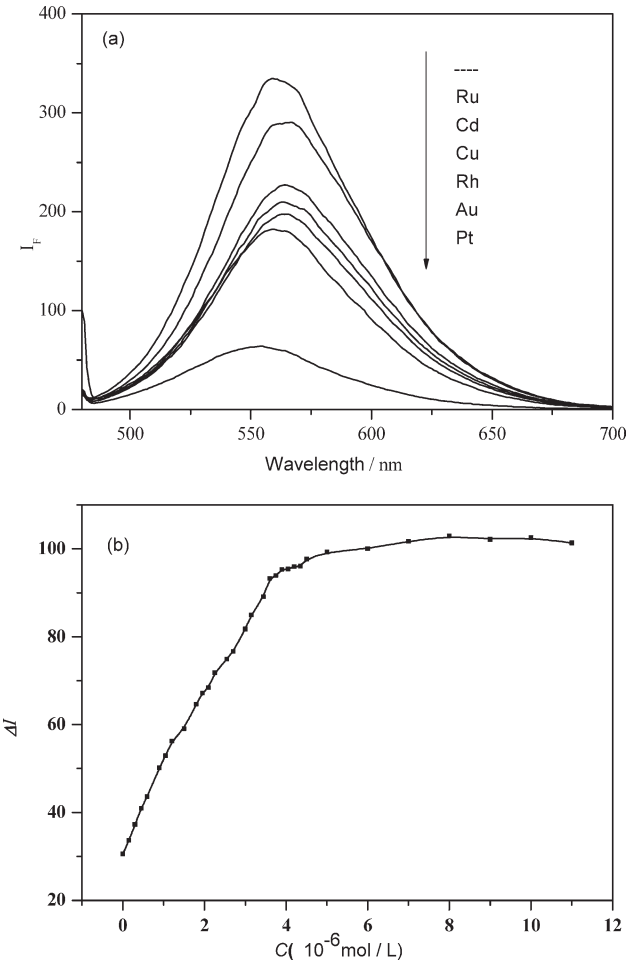

Figure 2. (a) Fluorescence spectra of PDPMT-Cl in the presence of various metal ions. [PDPMT-Cl] = 2.0×10^{-5} mol/L; [$PtCl_4^{2-}$] = 2.0×10^{-5} mol/L; NaH_2PO_4-NaOH buffer solution pH = 8.4, (b) ΔI plot of PDPMT-Cl in the presence of various $PtCl_4^{2-}$ concentration. [PDPMT-Cl] = 6.0×10^{-6} mol/L; NaH_2PO_4-NaOH buffer solution pH = 8.4.

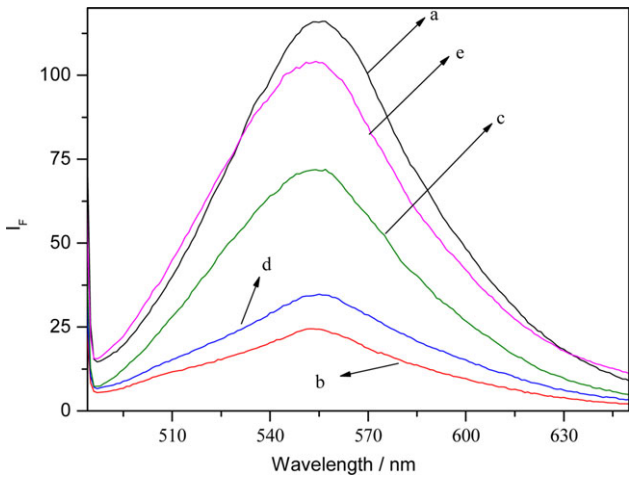

Figure 3. Emission spectra of PDPMT-Cl (a), PDPMT-Cl+ $PtCl_4^{2-}$ (b), PDPMT-Cl+ $PtCl_4^{2-}$+BSA (c), PDPMT-Cl+ $PtCl_4^{2-}$+trypsin (d), PDPMT-Cl+ $PtCl_4^{2-}$+BSA+trypsin (e). [PDPMT-Cl] = 8.0×10^{-6} mol/L; [$PtCl_4^{2-}$] = 4.0×10^{-6} mol/L; [BSA] = 1.0 mg/mL; [trypsin] = 5.0 μg/mL; NaH_2PO_4-NaOH buffer solution pH = 8.4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

add a fixed quantity BSA standard solution, according to the assay method to do the recovery test.

RESULTS AND DISCUSSION

Our new assay strategy for protein is illustrated in Figure 1. Conjugated polythiophene derivative (PDPMT-Cl) is selected as the optical probe. $PtCl_4^{2-}$ ion is a good fluorescence quencher of conjugated polymer, thus, can highly efficiently quench the fluorescence of PDPMT-Cl. Upon adding protease to the solution, the substrate protein is cleaved into amino acid or peptide fragments, which are stronger $PtCl_4^{2-}$ chelators and form more stable complexes with $PtCl_4^{2-}$ ions.[35,36] Thus, the $PtCl_4^{2-}$ ion is displaced from PDPMT-Cl and the fluorescence of PDPMT-Cl is recovered. By triggering the "turn-on" signal of PDPMT-Cl, it is possible to detect the protein in real time.

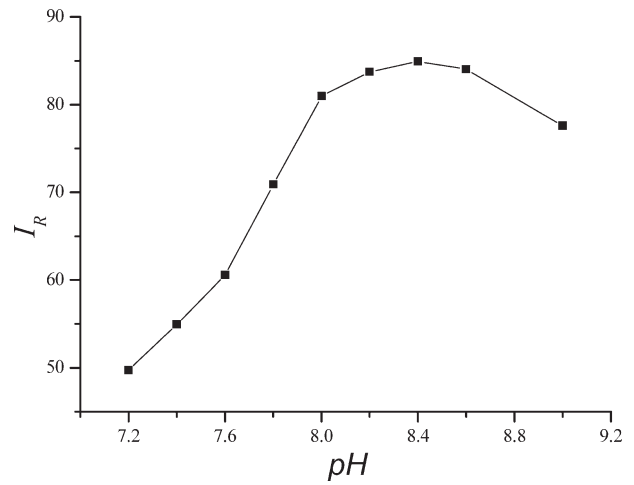

Figure 4. The effect of the pH of buffer solution on the fluorescence intensity. [PDPMT-Cl] = 2.0×10^{-5} mol/L; [$PtCl_4^{2-}$] = 4.0×10^{-6} mol/L; [trypsin] = 5.0 μg/mL.

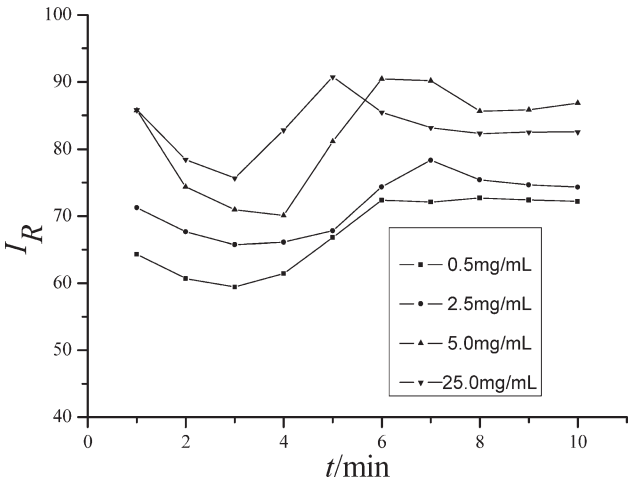

Figure 5. The effect of different concentration of trypsin on the fluorescence intensity. [PDPMT-Cl] = 2.0×10^{-5} mol/L; [$PtCl_4^{2-}$] = 4.0×10^{-6} mol/L; [BSA] = 1.0 mg/mL; NaH_2PO_4-NaOH buffer solution pH = 8.4.

The fluorescence quenching of PDPMT-Cl was examined in the presence of different metal ions. From Figure 2(a), it can be seen that the fluorescence of PDPMT-Cl was efficiently quenched by $PtCl_4^{2-}$ ion. The quenching efficiency could reach the highest in 3 min, and it remained unchanged in 3 h. It can be clearly seen from Figure 2(b) that upon the addition of increasing amounts $PtCl_4^{2-}$ ions caused a decrease in fluorescence intensity of PDPMT-Cl, when the concentration of $PtCl_4^{2-}$ ion is less than 4.0×10^{-6}mol/L, ΔI and Pt has a good linear relationship. When the concentration of $PtCl_4^{2-}$ ion is above 4.0×10^{-6}mol/L, ΔI tends to be stable, so when the concentration of $PtCl_4^{2-}$ ion and PDPMT-Cl are all at 4.0×10^{-6}mol/L, they can form 1: 1 complex, and at this time, the fluorescence

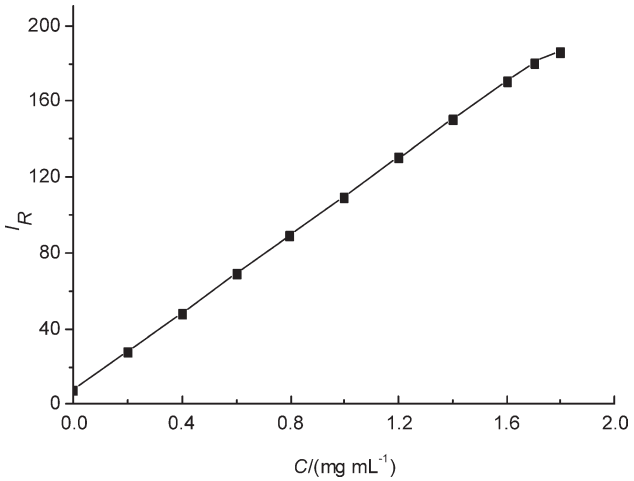

Figure 6. The emission intensities of the system in the presence of different BSA concentration. [PDPMT-Cl] = 2.0×10^{-5} mol/L; [$PtCl_4^{2-}$] = 4.0×10^{-6} mol/L; [trypsin] = 5.0 μg/mL; NaH_2PO_4-NaOH buffer solution pH = 8.4.

Table I. The Interference of the Other Substances on the Fluorescence Intensity

The interference material	Concentration (mol/L)	Interference (%)
Amylum	1.0×10^{-4}	0.89
ATP	1.0×10^{-4}	0.60
L-Histidine	1.0×10^{-4}	−1.12
Glucose	1.0×10^{-4}	−3.64
DL-Alanine	1.0×10^{-4}	−4.43
L-Proline	1.0×10^{-4}	3.23
L-Phenylalanine	1.0×10^{-4}	−0.02
DL-Cysteine	1.0×10^{-4}	0.74
L-Tryptophan	1.0×10^{-4}	−3.43
L-Arginine	1.0×10^{-4}	4.22
L-Serine	1.0×10^{-4}	2.09
Na^+	1.0×10^{-3}	0.66
Mg^{2+}	1.0×10^{-3}	0.65
Ca^{2+}	1.0×10^{-3}	0.78
Fe^{3+}	1.0×10^{-3}	−3.51
K^+	1.0×10^{-3}	−0.44
Al^{3+}	1.0×10^{-3}	1.26
L-Cysteine	1.0×10^{-4}	4.19

quenching degree was the highest, so the concentration of $PtCl_4^{2-}$ ion is chosen as 4.0×10^{-6} mol/L.

Figure 3 compares the fluorescence spectra observed before and after the addition of BSA to the solution of PDPMT-Cl/ $PtCl_4^{2-}$ with trypsin. As shown in Figure 3, the emission spectra of PDPMT-Cl is curve a, and upon adding $PtCl_4^{2-}$ ions to the solution of PDPMT-Cl, the fluorescence of PDPMT-Cl solution can be efficiently quenched by $PtCl_4^{2-}$ ions (curve b). When added the BSA and trypsin to the PDPMT-Cl/ $PtCl_4^{2-}$ solution, there was no significant change (curve c, d), but upon addition of BSA and trypsin together, the fluorescence had obvious recovery (curve e). Trypsin, a member of the serine protease family, will cleave peptides on the C-terminal side of lysine and arginine amino acid residues to expose more binding sites hiding inside the BSA to $PtCl_4^{2-}$ ions and produce amino acid or peptide fragments with stronger chelating ability to $PtCl_4^{2-}$ than that of serine,[35,36] which result in the recovery of PDPMT-Cl fluorescence.

The effect of different pH values of NaH_2PO_4-NaOH buffer solution on the fluorescence intensity were investigated (Figure 4). It turned out that with the increase of pH value, the fluorescence intensity of the system first increased, then decreased. What's more, when the pH value is in the range of 8.2–8.6, the fluorescence intensity is the highest and stable. Thus, the optimum pH value of the buffer solution is 8.4.

Moreover, the effect of different concentrations of trypsin on the fluorescence intensity were also investigated (Figure 5). The experimental results show that the system became stable after adding trypsin in 8 min, and with the increase of trypsin, the fluorescence recovery efficiency was gradually increased. And when the concentration of trypsin was 5.0 μg/mL, the fluorescence recovery efficiency of the system was the highest and unchanged. So the system can choose 5.0 μg/mL of the amount of trypsin for experimental concentration.

According to the experimental method, the fluorescence intensities of the system were investigated in the presence of different amount of BSA. As shown in Figure 6, I_R and the concentration of BSA have a good linear relationship in the range of 0.0004–1.75 mg/mL. The regression equation is $I_R = 100.85c + 8.468$, and the related coefficient is 0.9997. The detection limit is 4.47×10^{-4} mg/mL.

Table I shows the effect of common ions, some kinds of amino acids, amylum, and cane sugar, etc. on the detection of BSA, and the interference level is ±5%.

According to the sample treatment method to deal with the egg white and milk samples, and then determined the concentration of BSA. Moreover, we compared the the results with the biuret method determination results, and the labeled recovery test was also investigated, they were all shown in Table II. From Table II, it can be seen that the relative standard deviation is less than 3.21%, and recovery rate is between 96.33% and 103.88%.

CONCLUSION

In summary, the amplified fluorescence quenching of PDPMT-Cl by $PtCl_4^{2-}$ through coordinate interaction has been employed to develop a label-free, simple, homogeneous, and real-time protein assay through competition bonding mechanism. "Turn-on" response as readout signal is able to effectively reduce background noise and increase detection sensitivity. These tests do not require sophisticated instrumentation and should be applicable to standard fluorescence assays. We expect that the assays of other proteins will be generated in a similar fashion. What's

Table II. Results for the Determination of Protein in the Samples ($n = 6$)

Sample	Biuret method Found (mg/g)	This method Found (mg/g)	Added (mg)	Recovered (mg)	Recovery (%)	RSD (%)
Egg white	142.21	141.82	50.0	193.76	103.88	2.96
			100.0	238.12	96.30	3.21
Fresh milk	37.62	38.12	50.0	87.26	98.28	1.19
			100.0	136.89	98.77	2.56

more, this method is successfully applied to determine the protein in egg white and milk.

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