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Novel Water-Soluble Red-Emitting Poly(*p*-phenylenevinylene) Derivative: Synthesis, Characterization, and Fluorescent Acetylcholinesterase Assays

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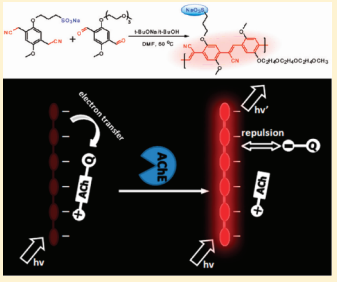
Supporting Information

ABSTRACT: A new cyano-substituted poly(*p*-phenylenevinylene) (PPV) derivative, MEOPS-CNPPV, is synthesized through Knoevenagel condensation of anionic diacetonitrile and neutral dialdehyde and characterized by ^{1}H NMR, IR, elemental analysis, and gel-permeation chromatography (GPC). To our knowledge, the polymer is the first water-soluble red-emitting PPV derivative. The absorption and emission wavelength of this water-soluble conjugated polymer (CP) depend on the solvent. In buffer solution, the fluorescence of MEOPS-CNPPV is quenched by cationic dinitrobenzene derivatives. Further research indicates that dinitrobenzene derivative with a more flexible structure exhibits a larger K_{sv}. Making use of the charge reversal of dinitrobenzene-modified substrate, a "turn-on" method is developed for AChE activity assay with the new polymer as a fluorophore. This convenient and direct fluorometric assay thus provides a platform for novel red-emitting sensory systems.



INTRODUCTION

Conjugated polymers (CPs) have been extensively studied as materials in optical and electronic applications including light-emitting devices,[1,2] photovoltaic cells,[3,4] and thin-film transistors.[5−7] CPs have also attracted interest in sensing areas in recent years. A key advantage of CP-based sensors over devices using small molecule elements is the potential of the CPs to exhibit collective properties that are sensitive to very minor perturbations.[8−10] Recently, water-soluble fluorescent CPs have attracted much attention as novel biosensors for detection of proteins, nucleic acids, and proteases.[11−15] Up to now, most of the fluorescent CPs, such as poly(*p*-phenylenevinylene) (PPV),[1,2] poly(*p*-phenyleneethynylene) (PPE),[16−19] and polyfluorene (PF),[13,20−22] emit blue or green fluorescence upon ultraviolet excitation. However, for many cell and neurobiological applications, red-emitting biosensors would offer distinctive advantages over blue-emitting biosensors. Red emission excited by visible light would permit a better discrimination against cellular autofluorescence, an enhancement of the optical transparency of tissue, and the ability to interrogate thicker specimens.[23] Cyano-substituted poly(*p*-phenylenevinylene) (CN-PPV) has been known as a red-emitting CP and widely used both as electron-transporting material and emissive layer in optoelectronic devices.[2,24−26] Nonetheless, water-soluble CN-PPV has not been reported to date. Furthermore, there is little work involved in the application of CN-PPV as a biosensor.

The hydrolysis of acetylcholine (ACh) by acetylcholinesterase (AChE) is a key process for the regulation of the neural response system, and its breakdown in the brain can accelerate the assembly of amyloid β peptides into amyloid fibrils, which results in Alzheimer's disease (AD).[27,28] AChE activity is traditionally monitored by UV−vis spectroscopy,[29] but such assay methods show low sensitivity. Recently, fluorometric approaches with good sensitivity have been developed to sense the activity of AChE.[30−35] However, most of them adopt indirect and discontinuous methods, which are not suitable or precise enough for enzymological studies. For example, chemiluminescent probes for the AChE activity assay have been achieved by using an enzyme-involved cascade reaction in which AChE assay was converted to the detection of H_2O_2.[30,31] Zhang's group developed two "turn-on" fluorescent probes based on the nucleophilicity of thiocholine.[32,33] However, thio-containing compounds such as cysteine and reduced glutathione will interfere on the assay. Very recently, Wang's group reported two fluorescent probes which used a direct method, but the emission wavelength is in blue-emission region.[35] Therefore, it will be of significance to develop direct methods for AChE activity assay with long wavelength fluorescence.

In this paper, we designed and synthesized a red-emitting water-soluble PPV derivative for the first time. The fluorescence of the red-emitting polymer can be quenched by dinitrobenzene compounds. In view of the fact, we synthesized two substrates containing dinitrobenzene moiety for the AChE detection. The assay for AChE activity was successfully developed through fluorescence "on−off−on" of the red-emitting CP.

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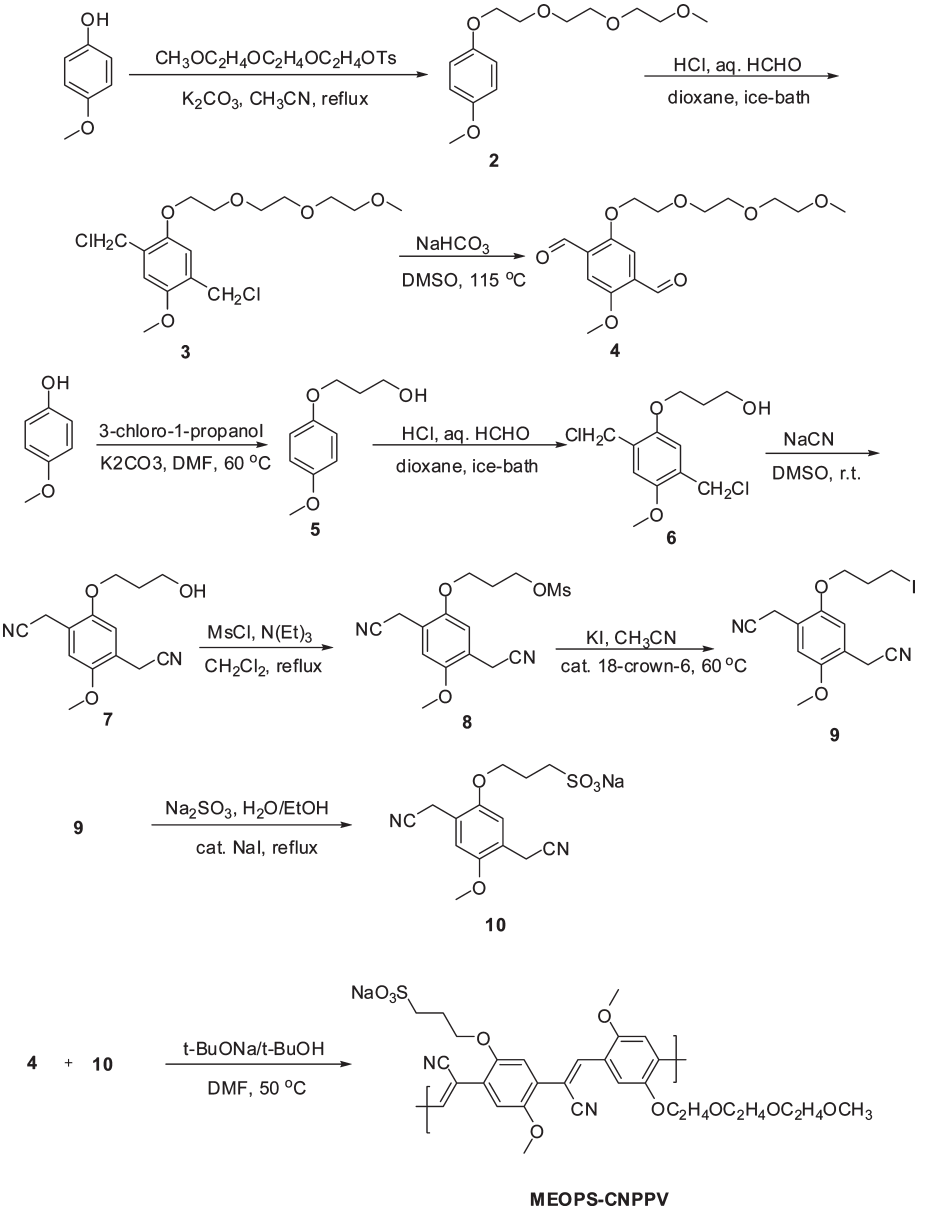
Scheme 1. Synthesis of MEOPS-CNPPV



EXPERIMENTAL SECTION

Materials. 2-(2-(2-Methoxyethoxy)ethoxy)ethyl-4-methylbenzenesulfonate, 1-methoxy-4-(2-(2-(2-methoxyethoxy)ethoxy)-ethoxy)benzene (**2**), 1,4-bis(chloromethyl)-2-methoxy-5-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (**3**), 3-(4-methoxyphenoxy)propan-1-ol (**5**), and 3-(2,5-bis(chloromethyl)-4-methoxyphenoxy)propan-1-ol (**6**) were prepared according to literature procedure.[36,38] AChE was purchased from Sigma, and the solution of the enzyme was cooled in ice before use. Other chemicals were purchased from commercial sources and were used without further purification unless otherwise noted.

Instruments. ^{1}H NMR and ^{13}C NMR spectra were recorded on a MECUYRVX300 spectrometer in $CDCl_3$, D_2O, and dimethyl sulfoxide (DMSO)-d_6 using tetramethylsilane as an internal reference, respectively. Mass spectra were measured on a ZAB 3F-HF mass spectrophotometer. Liquid chromatography mass spectra were measured on a Waters ZQ-Mass electrospray ionizer (ESI). Elemental analyses of carbon, hydrogen, and nitrogen were performed on a Carlorerba-1106 microanalyzer. Gel permeation chromatography (GPC) analyses were conducted on an Waters 2690D separations module equipped with Waters 2410 refractive index detector, using polystyrene as standard and dimethylformamide (DMF) as eluent. Fourier transform infrared (FT-IR) experiments were carried out on a Perkin-Elmer spectrometer with KBr pellets. UV−vis absorption spectra were recorded on Shimadzu UV-2550 recording spectrophotometer. PL spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer at 37 °C.

Synthesis of the Polymer (MEOPS-CNPPV). Samples of **4** (163 mg, 0.5 mmol) and **10** (173 mg, 0.5 mmol) were dissolved in DMF (10 mL). Then a solution of *t*-BuONa (12 mg, 0.1 mmol)

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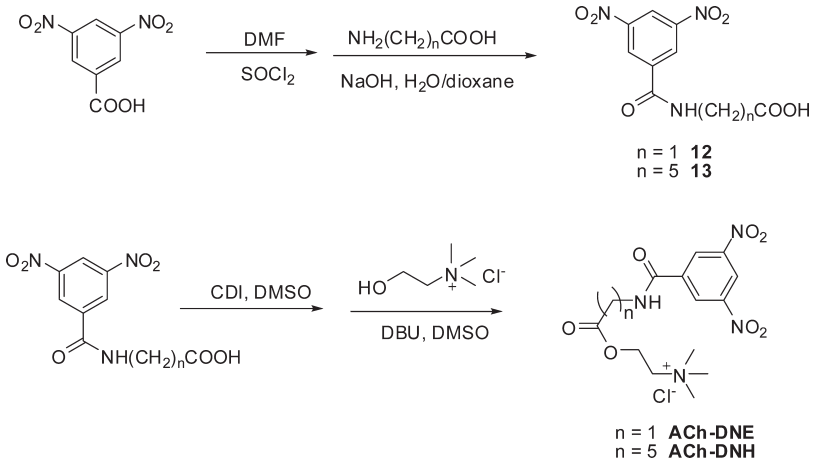
Scheme 2. Synthesis of AChE Substrates



in *t*-BuOH (1 mL) was added to the mixture. The reaction mixture was heated at 50 °C for 48 h. After cooling to room temperature, the mixture was poured into acetone and filtrated. The residue was dissolved in DMF and reprecipitated in vigorously stirred acetone. The collected solid was dried in vacuum to afford MEOPS-CNPPV as a brown powder (100 mg, yield: 31.4%). ^{1}H NMR (DMSO-d_6, 300 MHz), δ (ppm): 8.1−7.8 (m, 4H), 7.25 (m, 2H), 4.2 (m, 4H), 3.9−3.8 (m, 7H), 3.6−3.4 (m, 9H), 3.16 (s, 3H), 2.6−2.4 (m, 2H), 2.1 (m, 2H). Anal. Calcd. for $C_{30}H_{33}N_2O_{10}S$: C, 56.60; H, 5.22; N, 4.40. Found: C, 54.84; H, 5.69; N, 4.23.

Synthesis of 2-(3,5-Dinitrobenzamido)acetic Acid (12). A solution of 3,5-dinitrobenzoic acid (5.30 g, 25 mmol) in thionyl chloride (30 mL) was added to a flask and refluxed for 4 h. After thionyl chloride was removed by evaporation, anhydrous dioxane (20 mL) was added. To a solution of glycine (1.41 g, 18.8 mmol) in a water/dioxane mixture (1:1, 60 mL) sodium hydroxide (2.00 g, 50.0 mmol) in H_2O (5 mL) and dioxane solution of 3,5-dinitrobenzoyl chloride were successively added. The mixture was stirred for 6 h at room temperature and diluted with brine. The mixture was washed with diethyl ether, acidified with hydrochloric acid to pH 4, and extracted with ethyl acetate. The organic solution was washed with brine, dried (sodium sulfate), and evaporated. The residue was purified by silica gel column chromatography (1:1 chloroform−ethyl acetate) to give **12** as a white solid (4.29 g, yield: 85%). ^{1}H NMR (DMSO-d_6, 300 MHz), δ (ppm): 9.66 (br, 1H), 9.07 (s, 2H), 8.98 (s, 1H), 4.03 (s, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz), δ (ppm): 175.2, 162.3, 148.9, 137.7, 128.0, 121.3, 42.3. ESI-MS: m/z 224 (M^+-45 (COOH)). Anal. Calcd. for $C_9H_7N_3O_7$: C, 40.16; H, 2.62; N, 15.61. Found: C, 40.36; H, 2.95; N, 15.37.

Synthesis of 6-(3,5-Dinitrobenzamido)hexanoic Acid (13). Compound **13** was obtained from 6-aminocaproic acid by a similar protocol to **12**, yield 87%. ^{1}H NMR (DMSO-d_6, 300 MHz), δ (ppm): 12.09 (br, 1H), 9.18 (br, 1H), 9.04 (s, 2H), 8.94 (s, 1H), 3.31 (t, J = 7.5 Hz, 2H), 2.21 (t, J = 7.5 Hz, 3H), 1.58−1.48 (m, 4H), 1.37−1.32 (m, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz), δ (ppm): 175.1, 162.5, 148.8, 137.7, 128.1, 121.3, 40.0, 34.2, 29.2, 26.7, 24.9. ESI-MS: m/z 325 (M^+). Anal. Calcd. for $C_{13}H_{15}N_3O_7$: C, 48.00; H, 4.65; N, 12.92. Found: C, 48.19; H, 4.96; N, 13.03.

Synthesis of 2-(2-(3,5-Dinitrobenzamido)acetoxy)-*N*,*N*,*N*-trimethylethanaminium Chloride (ACh-DNE). A solution of **12** (1.62 g, 6.0 mmol) and carbonyldiimidazole (1.07 g, 6.6 mmol) in DMSO (30 mL) was stirred under Ar for 30 min. The reaction mixture was then transferred with a syringe to a flask containing a solution of choline chloride (0.92 g, 6.6 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (0.9 mL, 6 mmol) in DMSO (15 mL). The resulting mixture was stirred at 40 °C for 24 h, and then the solvent was removed under vacuum. The residue was purified by silica gel column chromatography (65:25:4 CH_2Cl_2-MeOH-H_2O) to give ACh-DNE (0.49 g, yield: 21%). ^{1}H NMR (D_2O, 300 MHz), δ (ppm): 9.07 (s, 1H), 8.84 (s, 2H), 4.48 (br, 2H), 4.13 (s, 2H), 3.59 (t, J = 4.0 Hz, 2H), 3.01 (s, 9H). ^{13}C NMR (D_2O, 75 MHz), δ (ppm): 174.9, 162.4, 148.6, 137.5, 128.0, 121.1, 64.10, 57.7, 53.2, 39.5. ESI-MS: m/z 355 (M^+-Cl). Anal. Calcd. for $C_{14}H_{19}ClN_4O_7$: C, 43.03; H, 4.90; N, 14.34. Found: C, 43.22; H, 5.03; N, 14.23.

Synthesis of 2-(6-(3,5-Dinitrobenzamido)hexanoyloxy)-*N*,*N*,*N*-trimethylethanaminium Chloride (ACh-DNH). ACh-DNH was obtained from **13** by a similar protocol to ACh-DNE with DBU 5% molar equiv, yield 48%. ^{1}H NMR (D_2O, 300 MHz), δ (ppm): 9.06 (s, 1H), 8.79 (s, 2H), 4.39 (br, 2H), 3.57 (t, J = 4.0 Hz, 2H), 3.28 (t, J = 6.6 Hz, 2H), 3.05 (s, 9H), 2.31 (t, J = 7.5 Hz, 2H), 1.50−1.47 (m, 4H), 1.27−1.25 (m, 2H). ^{13}C NMR (D_2O, 75 MHz), δ (ppm): 174.2, 163.0, 147.4, 136.6, 126.9, 120.1, 64.1, 57.8, 53.4, 39.6, 33.0, 27.7, 25.3, 23.3. ESI-MS: m/z 411 (M^+-Cl). Anal. Calcd. for $C_{18}H_{27}ClN_4O_7$: 48.38; H, 6.09; N, 12.54. Found: C, 48.04; H, 6.08; N, 12.56.

RESULTS AND DISCUSSION

Synthesis and Characterization. The synthesis of the polymer MEOPS-CNPPV and substrates for AChE are outlined in Schemes 1 and 2, respectively. 4-Methoxyphenol was first alkylated by 2-(2-(2-methoxyethoxy)ethoxy)ethyl-4-methylbenzenesulfonate or 3-chloropropanol to give **2** and **5**, which then underwent direct chloromethylation to afford **3** and **6**, respectively. Oxidation of **3** by dimethylsulfoxide produced the terephthalaldehyde monomer **4**. After reacting with sodium cyanide, **6** was transformed into **7**. Then **7** was converted to sulfonate monomer **10** in three steps according to the literature procedure.[36] Finally, the polymer was synthesized by the Knoevenagel condensation reaction of the neutral dialdehyde **4** with the anionic diacetonitrile **10**. The substrates for AChE were synthesized by amidation of 3,5-dinitrobenzoic acid with ω-amino acid,

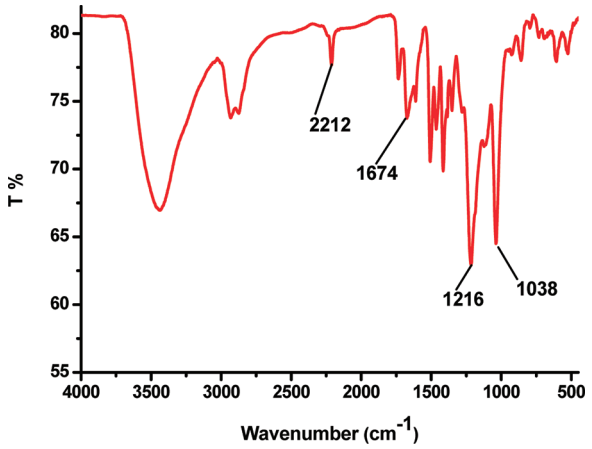


Figure 1. FT-IR spectrum of MEOPS-CNPPV.

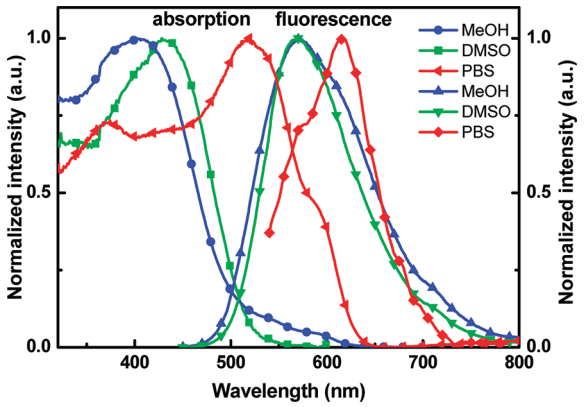


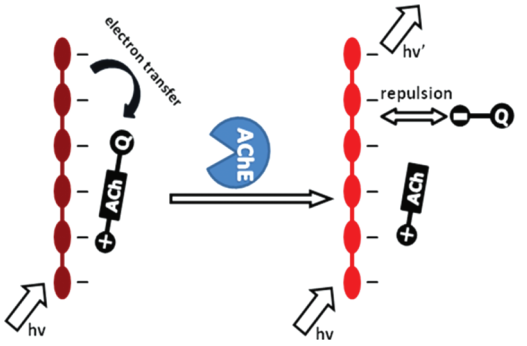
Figure 2. Absorption and fluorescence spectra of MEOPS-CNPPV in different solvents (PBS: 5 mM potassium phosphate buffer with pH of 8.0). Excitation wavelength: MeOH, 402 nm; DMSO, 428 nm; PBS, 520 nm.

followed by esterification with choline chloride. All intermediates and final products were characterized by ^{1}H NMR, ^{13}C NMR, mass spectra, and elemental analyses (see the Experimental Section).

The structure of the polymer was characterized by ^{1}H NMR, IR, elemental analysis, and GPC. Figure 1 shows the FT-IR spectrum of the polymer. The symmetric and asymmetric S═O stretching vibrations of sulfonate groups were observed at 1038 cm^{-1} and 1216 cm^{-1}, respectively; the characteristic C═C and C≡N stretching vibrations appeared at 1677 cm^{-1} and 2212 cm^{-1}, respectively. On the basis of GPC, the number-average molecular weight of the polymer is 7.0×10^4 g/mol by using a calibration curve of polystyrene standards in DMF, with a low polydispersity index (PDI; M_w/M_n) value of 1.13. The narrow distribution can be attributed to the Knoevenagel condensation reaction.[37] MEOPS-CNPPV is soluble in water and polar solvents with a water solubility of 2 mg/mL.

Photophysical Properties. The normalized UV−vis absorption and photoluminescence (PL) spectra of MEOPS-CNPPV in different solvents are shown in Figure 2. In methanol and DMSO, the polymer shows the absorption peaks at 402 nm and 428 nm, as well as the emission peaks at 571 nm and 570 nm, respectively. In contrast, a significant red shift is observed both in the absorption and fluorescence maxima (λ_{abs} = 520 nm and λ_{em} = 617 nm) in phosphate buffer saline (PBS). The significant red

Scheme 3. Assay of AChE Activity



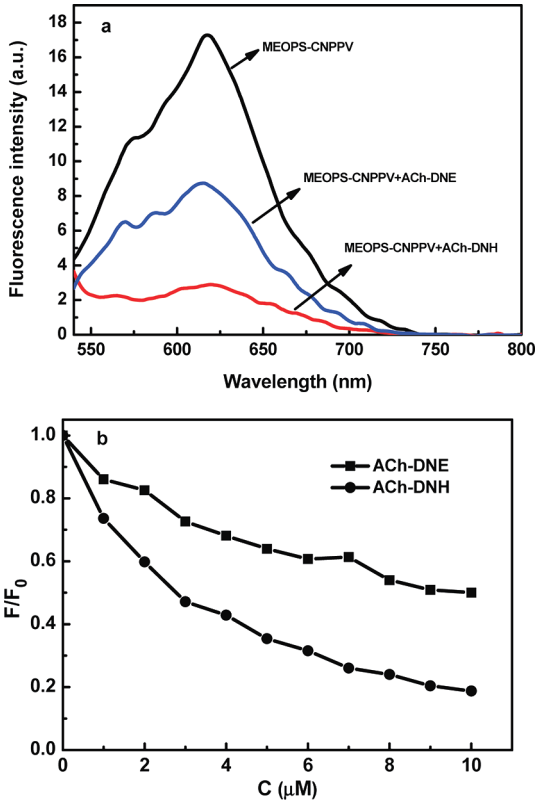


Figure 3. (a) Fluorescence emission changes of MEOPS-CNPPV upon addition of ACh-DNE (0.1 mM) or ACh-DNH (0.1 mM). (b) The dependence of the fluorescence intensity of MEOPS-CNPPV at 617 nm on the concentrations of ACh-DNE and ACh-DNH. All of the experiments were carried out in PBS buffer (pH 8.0, 5 mM) with 1 μM MEOPS-CNPPV (in monomer repeat units). The excitation wavelength is 520 nm.

shift of absorption could be attributed to the aggregation of the polymer in aqueous solution,[38] while the red shift of emission could be attributed to the synergy of the aggregation and intramolecular charge transfer (ICT) of the polymer in very polar aqueous solution.[39] The fluorescence quantum yield of MEOPS-CNPPV in PBS was found to be 6.5%. The low quantum yield may be attributed to the red-emission and aggregation of MEOPS-CNPPV in PBS.

Fluorescent AChE Assays. The fluorescence assay for AChE activity is illustrated in Scheme 3. The cationic AChE substrate

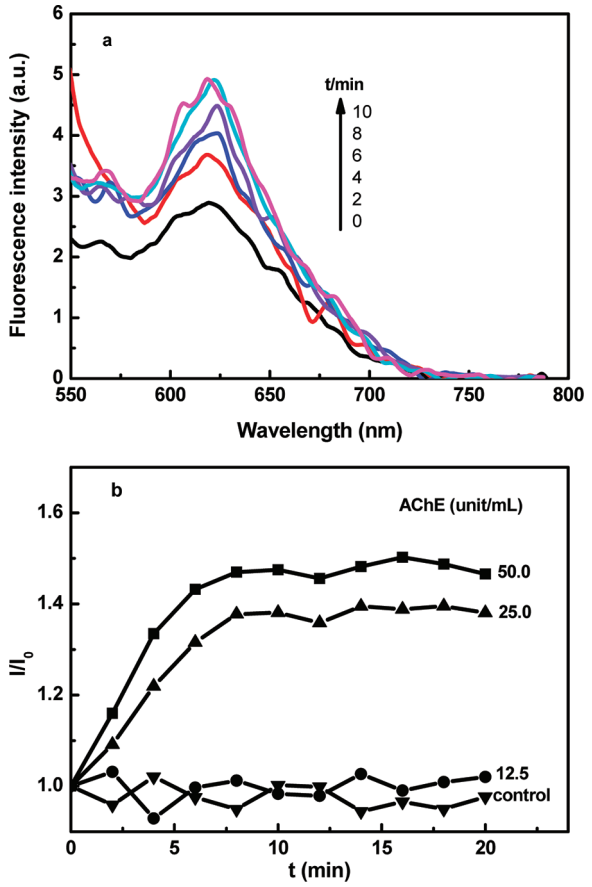


Figure 4. (a) Fluorescence spectra of MEOPS-CNPPV/ACh-DNH as a function of reaction time for AChE-catalyzed hydrolysis; [MEOPS-CNPPV] = 1 μM, [ACh-DNH] = 0.1 mM, [AChE] = 25 units/mL. The experiment was carried out with a fixed concentration of AChE. (b) I/I_0 of MEOPS-CNPPV at 617 nm versus reaction time in the hydrolysis of ACh-DNH with varying concentrations of AChE; [MEOPS-CNPPV] = 1 μM, [ACh-DNH] = 0.05 mM, [AChE] = 12.5 − 50 units/mL. Fluorescence measurements were made in PBS buffer (pH 8.0, 5 mM) with excitation at 520 nm.

can form a complex with the anionic polymer MEOPS-CNPPV through electrostatic interactions. The fluorescence of MEOPS-CNPPV is quenched by the nitrobenzene moiety by an electron-transfer process.[40] Upon the addition of AChE, the AChE substrate will be hydrolyzed by the catalysis of AChE, in which process the substrate is decomposed into choline and a negatively charged residue that contains the dinitrobenzene moiety. The quencher moiety is repelled away from MEOPS-CNPPV, and the fluorescence of the polymer is recovered. Consequently, the hydrolysis catalyzed by AChE can be monitored by the fluorescence recovery of MEOPS-CNPPV.

To choose a better substrate and determine the sensitivity of the protocol, we initially investigated the fluorescence response of the new polymer MEOPS-CNPPV to the two different substrates ACh-DNE and ACh-DNH. As shown in Figure 3, after the addition of ACh-DNE or ACh-DNH, the fluorescence of MEOPS-CNPPV was quenched more efficiently by ACh-DNH. With a concentration of 0.1 mM, the fluorescence of MEOPS-CNPPV was almost completely quenched in the presence of ACh-DNH, whereas it is slightly quenched by ACh-DNE (Figure 3a). The quenching efficiency is related to the Stern−Volmer constant (K_{sv}) that can be determined by the Stern−Volmer equation. From the titration of fluorescence (Figure 3b), the K_{sv} for ACh-DNE and ACh-DNH is determined to be 1.04×10^5 M^{-1} and 4.05×10^5 M^{-1}, respectively. Presumably, the flexible structure of ACh-DNH endowed by the longer chain of ACh-DNH, compared with ACh-DNE, makes it easier for the dinitrobenzene moiety of ACh-DNH to close to the backbone of the polymer. Because of the better quenching efficiency of ACh-DNH, it was used to the subsequent test of AChE activity.

The results of the fluorescence-monitored hydrolysis for ACh-DNH by AChE are shown in Figure 4. Upon the addition of AChE to the MEOPS-CNPPV/ACh-DNH complex ([MEOPS-CNPPV] = 1 μM, [ACh-DNH] = 0.1 mM) in PBS (pH 8.0, 5 mM) at 37 °C, the fluorescence of MEOPS-CNPPV was observed to rise gradually in 10 min (final [AChE] = 25 units/mL) (Figure 4a). This result indicates that AChE catalyzed the hydrolysis of ACh-DNH, consequently leading to a "turn-on" response of MEOPS-CNPPV fluorescence. The fluorescence recovery of MEOPS-CNPPV depends on the concentration of AChE (Figure 4b). An increase in the concentration of AChE leads to a fast cleavage reaction rate and a high fluorescence recovery. AChE with concentrations of ca. 12.5 units/mL can be detected. Though the assay is not as sensitive as the chemiluminesence technique, it has two advantages: (i) long wavelength excitation and emission cause less damage and exhibit less autofluorescence background; (ii) this continuous and direct fluorometric assay is convenient and suitable for enzymological studies. We believe that the sensitivity can be improved by designing more substrate structures with a high binding constant to MEOPS-CNPPV.

CONCLUSION

We have developed a new water-soluble red-emitting polymer MEOPS-CNPPV. The structural design of the polymer combines the red emission of the CNPPV backbone and the water-solubility of sulfonic side chain. The polymer is the first example of water-soluble red-emitting PPV derivative. By taking advantage of charge reversal of a quencher-modified dinitrobenzene moiety (substrate of AChE), the new red-emitting water-soluble polymer has been successfully applied to monitor AChE activity. The red emission of MEOPS-CNPV permits a better discrimination against cellular autofluorescence and can have a perspective for polymer-based biosensor. We believe the red-emitting water-soluble CP will find more application in sensor areas.

ASSOCIATED CONTENT

Supporting Information. Synthesis of the intermediates **4**, **7**, **8**, **9**, and **10**; ^{1}H NMR and ^{13}C NMR spectra of the intermediates, the substrates ACh-DNE and ACh-DNH, and the polymer MEOPS-CNPPV. This material is available free of charge via the Internet at http://pubs.acs.org.

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