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Colorimetric and Fluorometric Assays Based on Conjugated Polydiacetylene Supramolecules for Screening Acetylcholinesterase and Its Inhibitors

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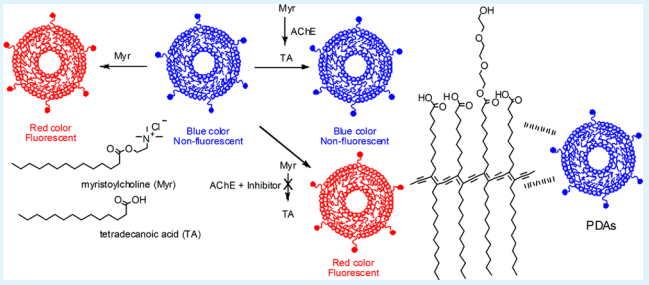
Supporting Information

ABSTRACT: Polydiacetylene supramolecules (PDAs) are unique sensing materials. Upon environmental stimulation, blue PDAs can undergo a colorimetric transition from blue to red accompanied by fluorescence enhancement. In this paper, we report a new PDA system polymerized from a mixed liposome comprising 2-(2-(2-hydroxyethoxy)ethoxy)ethyl pentacosa-10,12-diynoate and pentacosa-10,12-diynoic acid at a 3:7 ratio. The PDA system provided new colorimetric and fluorometric assay methods for screening acetylcholinesterase and its inhibitors through three processes. First, myristoylcholine reacted with PDAs, which then underwent colorimetric and fluorometric transition. Second, acetylcholinesterase catalyzed the hydrolysis of myristoylcholine into tetradecanoic acid, which reduced the myristoylcholine concentration and led to faded color and fluorescence. Third and last, acetylcholinesterase inhibitors retarded the activity of acetylcholinesterase, thereby inducing the recovery of color and fluorescence.



KEYWORDS: *polydiacetylene, conjugated polymer, colorimetric and fluorometric assays, acetylcholinesterase, myristoylcholine, inhibitor*

1. INTRODUCTION

Acetylcholinesterase (AChE) is highly active for the hydrolysis of acetylcholine (ACh) and can regulate the concentration of ACh, which plays a key role in memory and learning as a central neurotransmitter.[1] Elder persons suffering from Alzheimer's disease (AD) have a low ACh level in the hippocampus and cortex, which is the main cause of AD.[2,3] Rational pharmacological treatment of AD is currently based on AChE inhibitors.[4] Therefore, a reliable method for determining the activities of AChE and its inhibitors must be developed for AD treatment.[5] The traditional method of AChE detection is based on the Ellman's reagent. However, this method has the drawbacks of false-positive effects and low sensitivity.[6,7] Fluorometric approaches are preferable because of their high sensitivity.[8,9] Some fluorescent or chemiluminescent sensors based on various platforms including gold nanoparticles, CdS quantum dots, small molecules, etc. for screening AChE inhibitors have been reported.[10−13] However, convenient and rapid methods for screening AChE and its inhibitors are still highly desired.

Conjugated polymers are receiving considerable attention as optical platforms for detecting various species.[14−18] In contrast to small-molecule sensors, probes based on conjugated polymers are more sensitive because the transmission of excitation energy along the conjugated backbone of the polymer can produce amplified optical signal.[14] Polydiacetylenes are conjugated polymers that have been developed as a sensing platform because of their unique optical properties. Upon environmental stimulation, blue polydiacetylene supramolecules (PDAs) can undergo colorimetric transition from blue to red accompanied by fluorescence enhancement. Some systems based on polydiacetylenes have been constructed to detect chemical and biological species.[19−34] Our group has previously reported that PDAs with imidazolium or hydroxybenzaldehyde as head groups undergo colorimetric and fluorogenic transitions upon the addition of anionic and cationic surfactants, respectively.[20,21] In this paper, we report a new PDA system containing optimized polydiacetylene supramolecules polymerized from 2-(2-(2-hydroxyethoxy)-ethoxy)ethyl pentacosa-10,12-diynoate (PCDA-HEP) and pentacosa-10,12-diynoic acid (PCDA). The PDA system showed rapid response to myristoylcholine chloride (Myr) because of the strong ionic interactions between the negatively charged head group of PDAs and the positively charged

Received: January 25, 2013
Accepted: April 1, 2013
Published: April 1, 2013

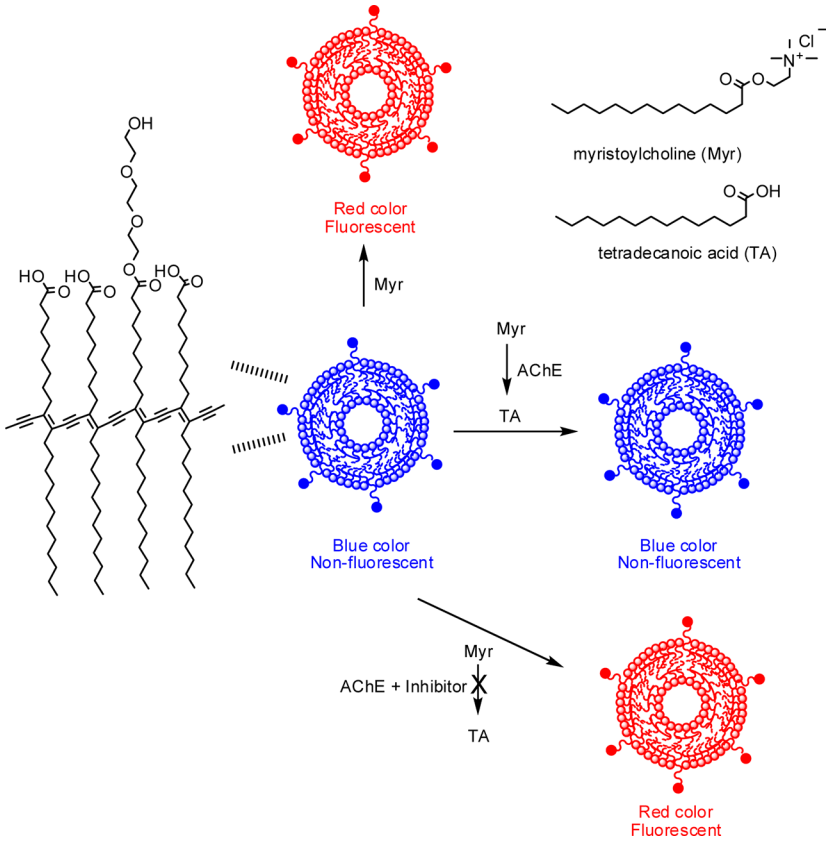


Figure 1. Schematic illustration of AChE activity determination and its inhibitor screening mechanism by the designed PDA liposomes.

ammonium group of Myr (Figure 1). When Myr was pretreated with AChE, Myr was hydrolyzed to generate tetradecanoic acid (TA), which is negatively charged and cannot interact with PDA. Thus, the optical transition from blue to red phase was retarded by AChE. When AChE was treated with inhibitors before incubation with Myr, the hydrolysis activity of AChE to Myr was inhibited. Myr survived and optical changes in PDAs were observed again. The color transitions in above processes were exhibited in Figure S1 (Supporting Information). In the proposed strategy, AChE activity in the presence or absence of inhibitors was monitored using the PDA system.

2. EXPERIMENTAL SECTION

Materials. All materials were obtained from Sigma-Aldrich. Reagents were purchased from Shanghai Lingfeng and used without further purification. Ultrapure water was obtained with a Millipore filtration system.

Measurements. Chromatography was carried out on silica gel 60 (230−400 mesh ASTM). ^{1}H and ^{13}C NMR spectra were recorded on Bruker 500. Mass spectra were obtained using a Waters Micromass Q-Tof micro mass spectrometer. Fluorescence emission spectra were obtained using a RF-5301/PC spectrofluorophotometer. UV absorption spectra were obtained on α-1860A UV-Vis spectrometer. All pH measurements were made with a Sartorius PB-10 meter.

Preparation of liposomes. PDA liposomes in aqueous solution were prepared by initially dissolving a mixture of the PCDA-HEP and PCDA (3:7 mol ratio) in a small amount of DMSO (1 mL). The organic solution was injected into 19 mL of HEPES buffer (20 mM, pH 7.4) with shaking to yield a total monomer concentration of 1 mM. The sample was then sonicated at 80 °C for 25 min. The resulting solution was filtered through a 0.8 μm filter, and the filtrate was cooled at 4 °C for 12 h. Polymerization was carried out at 4 °C by irradiating the solution with 254 nm UV light (1 mW/cm^2) for 15 min.

Synthesis of PCDA-HEP. The diacetylene monomer PCDA-HEP was prepared from commercially available 10,12-pentacosadiynoic acid (Sigma-Aldrich) by coupling the acid. In a typical procedure, 0.375 g (1 mM) of 10,12-pentacosadiynoic acid was dissolved in 20 mL of dry methylene chloride, and then 2 mL of sulfoxide chloride was added dropwise to the solution. The reaction mixture was stirred overnight at room temperature under N_2. The solvent was evaporated in a vacuum, and the crude residue was dissolved in a small amount of methylene chloride. The methylene chloride solution was added dropwise to a tetrahydrofuran solution containing 0.180 g of 2,2′-(ethane-1,2-diylbis(oxy))diethanol, and the reaction mixture was stirred at room temperature overnight under N_2. After reaction completion, the solvent was evaporated under reduced pressure, and the obtained crude product was purified by column chromatography on silica gel (CH_2Cl_2/CH_3OH, 100:2) to yield 2-(2-(2-hydroxyethoxy)ethoxy)-ethyl trideca-4,6-diynoate (90.5 mg, 24.1%). ^{1}H NMR ($CDC1_3$, 500 MHz) δ (ppm): 0.86−0.89 (t, 3H, J = 7.0 Hz), 1.25 (s, 26H), 1.47−1.52 (t, 4H, J = 7.0 Hz), 1.60−1.63 (t, 2H, J = 5.2 Hz), 2.22−2.24 (t, 2H, J = 7.0 Hz), 2.31−2.34 (t, 4H, J = 7.55 Hz), 3.60−3.62 (t, 2H, J = 4.3 Hz), 3.66−3.67 (t, 6H, J = 1.55 Hz), 3.69−3.73 (t, 2H, J = 4.8 Hz), 4.22−4.24 (t, 2H, J = 4.85 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ (ppm): 14.03, 19.12, 19.14, 22.62, 24.79, 28.27, 28.31, 28.70, 28.80, 28.84, 28.99, 29.02, 29.28, 29.42, 29.54, 29.56, 29.58, 29.63, 31.86, 34.09, 61.71, 65.24, 65.32, 66.83, 69.13, 70.31, 70.36, 70.48, 76.75, 77.00, 173.7; HRMS (EI) m/z = 529.3865 [M + Na]$^+$, calc. for $C_{31}H_{54}O_5Na$ = 529.3869.

DLS data. Particle size was measured on Malvern Zetasizer 3000HSA. Each sample was tested twenty times to obtain the average value. The data were obtained directly without filtration after being prepared according to the routine method. The concentration of sample is 100 μM.

Scanning Electron Microscopy (SEM). A SEM image of PDAs was obtained on a Hitachi S-4800 field emission scanning electron microscope. Sample was freshly made and deposited on silicon wafer

with a 20 μL micropipet. Silica wafer deposited with PDAs was dried for at least 5 h in an incubator.

Fluorescent spectroscopy. Stock solutions of PDAs (500 μM) were prepared by mixing 100 μL of polymers (1 mM) and 100 μL of HEPES buffer (10 mM, pH 7.4). In a typical experiment, test solutions were prepared by placing 60 μL of the stock solution into a test tube, diluting the solution to 3 mL with 10 mM HEPES (pH 7.4), and adding an appropriate aliquot of each analysis stock solution. Normally, excitation was at 492 nm, and both excitation and emission slit widths were 5 nm.

Inhibition efficiency. *Method 1.* The inhibition efficiency was estimated as follows:[35] inhibition efficiency = $F_{(\text{inhibitor})} - F_{(\text{no inhibitor})}/F_0 - F_{(\text{no inhibitor})}$. $F_{(\text{inhibitor})}$ and $F_{(\text{no inhibitor})}$ are the fluorescence intensity at 545 nm of the solution containing PDA liposomes [20 μM in HEPES buffer (10 mM, pH 7.4)], myristoylcholine (12 μM), and AChE (0.4 U/mL) in the presence (0, 0.5, 1, 5, and 10 nM) and absence of inhibitors, respectively. F_0 is the fluorescence intensity of the polymer at 545 nm in the absence of AChE and its inhibitors.

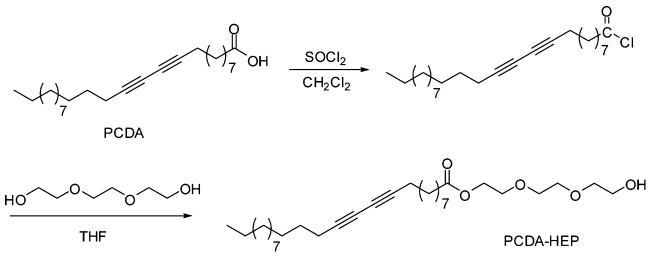
Method 2. Another method for inhibition efficiency estimation was as follows: inhibition efficiency = $R_{(\text{inhibitor})} - R_{(\text{no inhibitor})}/R_0 - R_{(\text{no inhibitor})}$. $R_{(\text{inhibitor})}$ and $R_{(\text{no inhibitor})}$ are the $A_{\text{red}}/A_{\text{blue}}$ ratio of the solution containing PDA liposomes [20 μM in HEPES buffer (10 mM, pH 7.4)], myristoylcholine (12 μM), and AChE (0.4 U/mL) in the presence (0, 0.5, 1, 5, and 10 nM) and absence of inhibitors, respectively. R_0 is the $A_{\text{red}}/A_{\text{blue}}$ ratio of the polymer in the absence of AChE and its inhibitors.

IC_{50} value. The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or inhibitor is needed to inhibit a given biological process by half. On the basis of the inhibition efficiency curve, we can obtain the value of the abscissa when the inhibition efficiency is 50%.

3. RESULTS AND DISCUSSION

Preparation of PDAs. PCDA-HEP was synthesized using 10,12-pentacosadiynoic acid and 2,2′-(ethane-1,2-diylbis(oxy))-diethanol as the starting materials according to the route shown in Scheme 1. The experimental procedures and characterization

Scheme 1. Synthesis of PCDA-HEP



data are detailed in the Experimental Section. The liposome comprising PCDA-HEP and PCDA at a ratio of 3:7 was then converted to PDAs by a routine procedure. UV irradiation of the suspensions derived from self-assembled mixed monomers for 15 min resulted in deep blue PDAs. The mean particle sizes of the PDAs were measured to be about 250 nm by dynamic light scattering experiments (see Figure S2 in the Supporting Information). The SEM analysis showed the PDAs were nearly spherical with sub 100 nm diameters, and multilamellar liposomes as well as aggregates formed (see Figure S3 in the Supporting Information).

Color and spectral changes of PDAs after adding myristoylcholine. The concentration-dependent colorimetric changes of polymer (500 μM) in the presence of various amounts of Myr were examined (Figure 2a). With increased Myr concentration from 0 to 200 μM, the PDAs exhibited clear

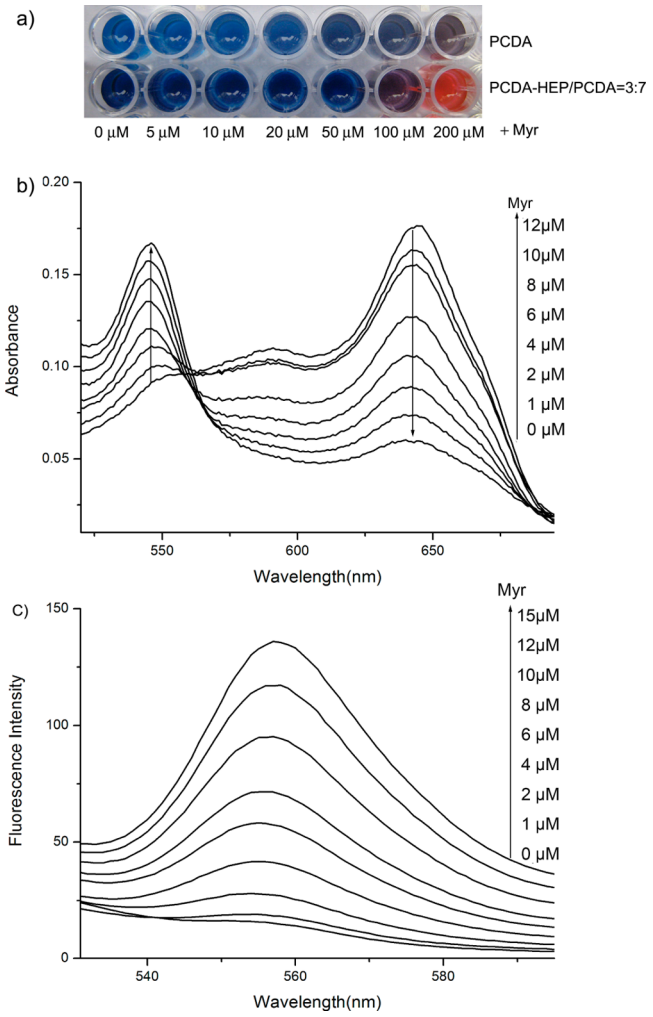


Figure 2. (a) Colorimetric responses of PDA liposomes solution (500 μM) upon incubation with various concentrations of Myr (0, 5, 10, 20, 50, 100, and 200 μM) after 5 min incubation in HEPES buffer (10 mM, pH = 7.4). Top row: PDAs prepared from pure PCDA. Bottom row: PDAs prepared from PCDA-HEP and PCDA at a 3:7 ratio. (b) Vis absorption spectra of PDAs (20 μM) in HEPES buffer (10 mM, pH = 7.4) upon incubation with various Myr concentrations (0, 1, 2, 4, 6, 8, 10, and 12 μM). (c) Fluorescence spectra of PDAs (20 μM) in the presence of various Myr concentrations (0, 1, 2, 4, 6, 8, 10, 12, and 15 μM) in HEPES buffer (10 mM, pH = 7.4) (λ_{ex} = 492 nm). Each spectrum is recorded at 5 min after the addition of Myr to PDA solution.

phase transition from blue to red. In contrast, the colorimetric change of the PDAs prepared from pure 10,12-pentacosadiynoic acid was much weaker even if as much as 200 μM of Myr was added. When other cationic analytes including cetyl trimethylammonium bromide (CTAB), Hg^{2+}, Ag^{+}, Mg^{2+}, Na^{+}, Fe^{3+}, and Ni^{2+} were added to the PDA solution prepared from PCDA-HEP and PCDA (3:7 mol ratio), only CTAB gave a good response (see Figure S4 in the Supporting Information), indicating that the PDAs reacted only with cationic surfactants. Our previous reports have shown that the optical changes of PDAs induced by surfactants are connected with long alkyl chains and charged head groups.[20,21] Especially, when the electrostatic interaction is not enough to have an effect on the backbone chain of PDAs, the long alkyl chain played a key role for penetration of the surfactant into the polymer, leading to

dx.doi.org/10.1021/am400260y | ACS Appl. Mater. Interfaces 2013, 5, 3275−3280

the conformational transitions in the conjugated (ene−yne) PDA backbone.

The Myr-induced phase transition of the PDAs was further monitored by Vis absorption spectroscopy at room temperature. Figure 2b shows that the addition of Myr caused a gradual decrease in absorption at 644 nm with a concomitant increase at 545 nm. Given that the blue-to-red transition of the PDAs was accompanied by fluorescence enhancement, the Myr-promoted reaction was also monitored by fluorescence spectroscopy. As shown in Figure 2c, the polymer had a large fluorescent enhancement with the addition of Myr. The overall emission change upon the addition of Myr was about 9-fold even when as little as 15 μM of Myr was added.

Spectral changes of PDAs after the Myr was pretreated with AChE. After AChE pretreatment of the Myr-containing solution, it was added to the polymer solution. The resulting fluorescence intensity of the polymer was found to depend on the incubation time between Myr and AChE (see Figure S5 in the Supporting Information). The hydrolysis reaction that occurred upon AChE treatment caused Myr to be hydrolyzed to TA, which cannot induce optical changes in the polymer. Longer incubation times resulted in lower emission intensities. When the incubation was carried out for 10 min, the emission intensity of the polymer added with the reaction mixture of Myr and AChE decreased to nearly that of the polymer without added Myr. On the other hand, the degree of fluorescence enhancement of the polymer decreased with increased AChE concentration. After Myr was hydrolyzed by AChE (0.4 U/mL) for 10 min, the resulting mixture induced a negligible change in the polymer fluorescence (Figure 3a). Similarly, the Vis spectra showed that the degree of absorption change of the polymer decreased with increased AChE concentration (Figure 3b).

Spectral changes of PDAs after adding the inhibitors. The hydrolysis rate of Myr induced by AChE was slowed down upon the addition of AChE inhibitors. When the reaction mixture of Myr, AChE, and AChE inhibitors was added to the polymer-containing solution, the optical changes are similar with that of polymer only with Myr. Accordingly, we evaluated the inhibitory activity of the inhibitors using the polymer, Myr, and AChE. Neostigmine, as a typical AChE inhibitor, was selected to carry out the inhibitory activity assay. After Myr (12 μM) and AChE (0.4 U/mL) with different concentrations of neostigmine (0, 0.5, 1, 5, and 10 nM) were incubated for 10 min at room temperature, the mixture was added to the solution containing the polymer. As expected, in the presence of neostigmine, the fluorescence intensity of the polymer gradually increased with increased neostigmine concentration (Figure 4a). On the basis of the relationship between the inhibition efficiency and neostigmine concentration, the corresponding IC_{50} value of neostigmine toward AChE activity was estimated to be 4.2 nM (Figure 4b). Furthermore, by monitoring the changes in the Vis spectra in the presence of various neostigmine concentrations, the IC_{50} value of neostigmine toward AChE activity was estimated to be 6.9 nM (see Figure S6 in the Supporting Information). Similarly, using the fluorometric and colorimetric methods, the IC_{50} values of tacrine toward AChE activity were estimated to be 150 and 112 nM (see Figures S7 and S8 in the Supporting Information), respectively, which are similar to previous results.[12,36] The IC_{50} values of dimethyl methylphosphonate (DMMP) toward AChE activity were estimated to be 201 μM by the fluorometric method and 384 μM by the colorimetric method (see Figures S9 and S10 in the Supporting Information). The inhibiting abilities of different inhibitors for the activity of AChE are different. For the three inhibitors mentioned above, the inhibiting efficiencies toward AChE are exhibited according to the following order: neostigmine > tacrine ≫ DMMP. There are some differences in the IC_{50} values obtained for one inhibitor using the different methods. It might be attributed to the different instruments used and the different quantification means by method 1 and method 2 to process the data from fluorescence and UV-Vis spectrometers, respectively.

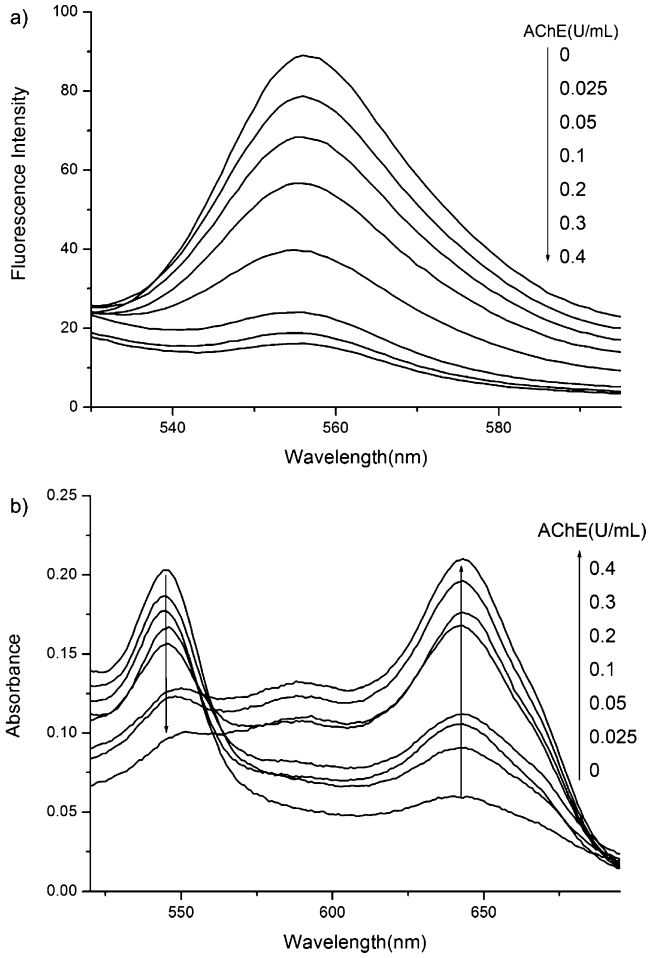


Figure 3. (a) Fluorescence spectra of PDAs (20 μM) containing Myr (12 μM) and different amounts of AChE (0, 0.025, 0.05, 0.1, 0.2, 0.3, and 0.4 U/mL); the solution containing Myr was pretreated with AChE for 10 min and added PDA liposomes. Then, each spectrum was recorded after another 5 min incubation (λ_{ex} = 492 nm). (b) Vis spectra of the above process. All experiments were carried out in HEPES buffer (10 mM, pH = 7.4) at room temperature.

4. CONCLUSIONS

We developed a PDA system polymerized from a mixed liposome comprising PCDA-HEP and PCDA at a 3:7 ratio. The sensing system provided dual assays for the screening AChE activity in the presence or absence of AChE inhibitors. This new assay approach was based on three processes. First, Myr reacted with PDAs, which then underwent colorimetric and fluorometric transitions. Second, AChE catalyzed the hydrolysis of Myr to TA, which reduced the Myr concentration and led to faded color and fluorescence. Third and last, the inhibitors retarded the activity of AChE, thereby inducing the

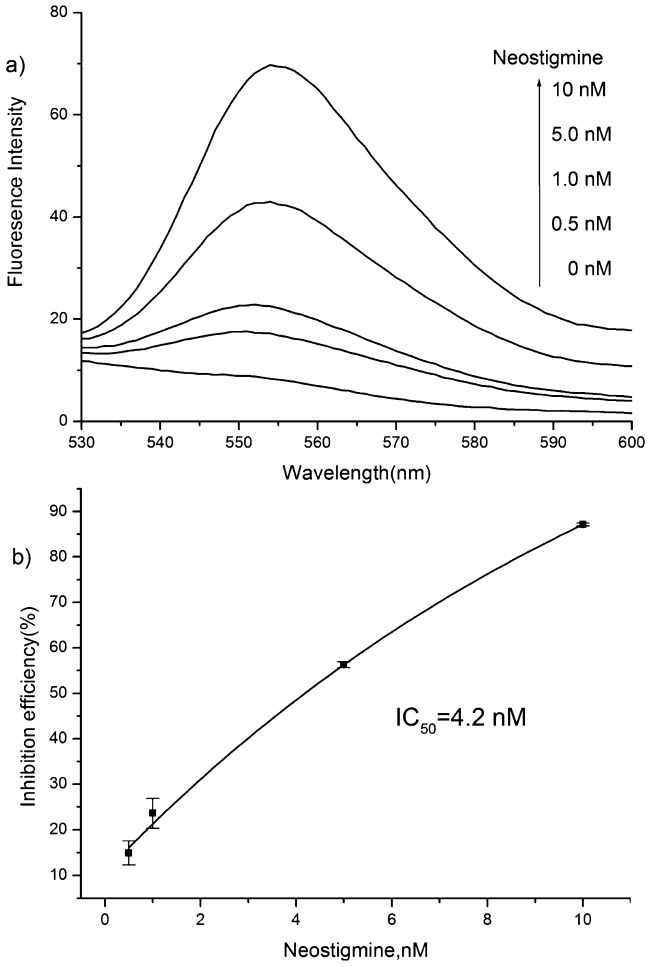


Figure 4. (a) Fluorescence spectra of PDAs (20 μM) added the resulting solutions after the reaction between Myr (12 μM) and AChE (0.4 U/mL) in the presence of different concentrations of neostigmine (0, 0.5, 1, 5, and 10 nM). (b) Inhibition efficiency of neostigmine toward AChE vs the concentration of neostigmine.

recovery of color and fluorescence. The process above mentioned can also be observed conveniently by the naked eye. Furthermore, fluorescent and colorimetric assays provided a simple, rapid, and easy operation for screening acetylcholinesterase and its inhibitors. The presented PDA system will be a potential platform for high-throughput screening of AChE inhibitors and relevant drug discovery.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The photograph of color changes of polymer in the presence of Myr and the resulting solution upon incubation of Myr and AChE; the DLS and SEM data of PDAs; the colorimetric responses of PDAs with various cationic analytes; the emission intensity of polymer upon the addition of the resulting solution of Myr and AChE after reacting for various times; the evaluation of inhibition efficiency of neostigmine, tacrine, and DMMP by the colorimetric and fluorometric assay method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants from NSFY of China (21002049), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (Grant No. 10KJB150005), the Opening Project of State Key Laboratory of Chemo/Biosensing and Chemometrics of Hunan University, and the Scientific Research Foundation for the Returned Overseas Chinese Scholars of State Education Ministry. This work was also supported by a Korean National Creative Research Initiative (2012R1A3A2048814).

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