ACS APPLIED MATERIALS & INTERFACES

RESEARCH ARTICLE

www.acsami.org

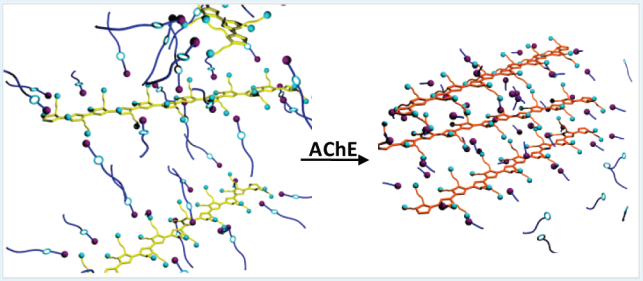
Colorimetric Assays for Acetylcholinesterase Activity and Inhibitor Screening Based on the Disassembly−Assembly of a Water-Soluble Polythiophene Derivative

Yugang Li, Hua Bai, Chun Li,* and Gaoquan Shi

Department of Chemistry, Tsinghua University, Beijing 100084, China

Supporting Information

ABSTRACT: A complex between an anionic polythiophene derivative (PT−COO^-) and a cationic surfactant, myristoylcholine, has been prepared and applied to be colorimetric probe for acetylcholinesterase (AChE) assays. The complex formation process, AChE activity assay and inhibitor screening has been studied by absorption spectroscopy. It was confirmed that the introduction of myristoylcholine into PT−COO^- phosphate buffer solution resulted in the disassembly of PT−COO^- aggregates, and further addition of AChE into the above solution led to the reassembly of PT−COO^- due to the catalyzed hydrolysis of myristoylcholine and the collapse of the complex. The colorimetric assay for AChE can be readily realized with the concentration of AChE as low as 0.2 U/mL. The results also demonstrate that the colorimetric approach can be applied for screening inhibitors of AChE.



KEYWORDS: acetylcholinesterase, myristoylcholine, polythiophene, biosensor, aggregation

INTRODUCTION

Acetylcholinesterase (AChE) is an enzyme that degrades the neurotransmitter acetylcholine, playing important roles in human and animal function and health.[1] AChE is the target of many Alzheimer's dementia drugs, nerve gases, particularly the organophosphates and insecticides. All these agents are known as the inhibitors of AChE, blocking the function of AChE and thus causing excessive acetylcholine to accumulate in the synaptic cleft. The excess acetylcholine causes neuromuscular paralysis throughout the entire body, leading to death by asphyxiation.[2] Therefore, it is important to be able to monitor easily the AChE activity and screening the inhibitors of AChE.[3] Although many well-known techniques, including Ellman method,[4] colorimetric assay based on gold nanoparticles,[5] fluorescent approaches based on synthetic probes[6−9] and semiconductor nanoparticles,[10] and various electrochemical methods,[3] have been developed to monitor AChE activity and screen the inhibitors of AChE, it is still attractive to find convenient and simple analytical methods for AChE assays.

Sensory technology based on conjugated polyelectrolytes (CPEs) has attracted increasing interest during the past decade because of its signal amplification effect and potential for applications in sensing bioanalytes.[11−15] Among various CPEs, water-soluble poly(3-alkoxy-4-methylthiophene)s (P3RO-4MeTs) have attracted critical attention in recent years because of their sensitive chain conformation to external stimuli. This unique structure characteristic has been applied to design optical sensors based on both colorimetric and fluorometric modes, providing advantages over the other CPEs since the colorimetric protocol possesses inherent merits including visual detection and real-time in situ responses.[13] Up-to-date, several kinds of cationic P3RO-4MeTs have been synthesized and applied to be colorimetric and fluorescent probes for the detection of DNA and proteins,[13,16−18] monitoring the helicity and conformational transition in polysaccharides,[19,20] and sensing small bioanions such as nucleotides,[21,22] folic acid,[23] glutathione,[24] and taurine.[25] For sensing small anions, analyte-induced aggregation of cationic P3RO-4MeT through electrostatic, hydrophobic, and aromatic stacking cooperative interactions has been proposed to account for the remarkable solution color change.[21−25] More recently, we have developed a colorimetric strategy for the selective detection of surfactants based on the disassembly of P3RO-4MeT aggregates.[26] As part of our interest in the development of colorimetric sensing systems based on P3RO-4MeT, herein, we report a simple and convenient colorimetric method for AChE activity assay and inhibitor screening based on an anionic P3RO-4MeT derivative (PT−COO^-, Figure 1). Although CPE-based assays for enzyme activity and inhibitor screening have been widely studied during the past decade, most of these examples are based on the fluorometric mode,[27−42] and few based on colorimetric CPE-based assays have been reported.[43] The design rationale for colorimetric AChE assay is illustrated in Figure 1. Commercially available myristoylcholine was used as a substrate for AChE. The cationic surfactant, myristoylcholine,

Received: January 25, 2011
Accepted: March 26, 2011
Published: March 26, 2011

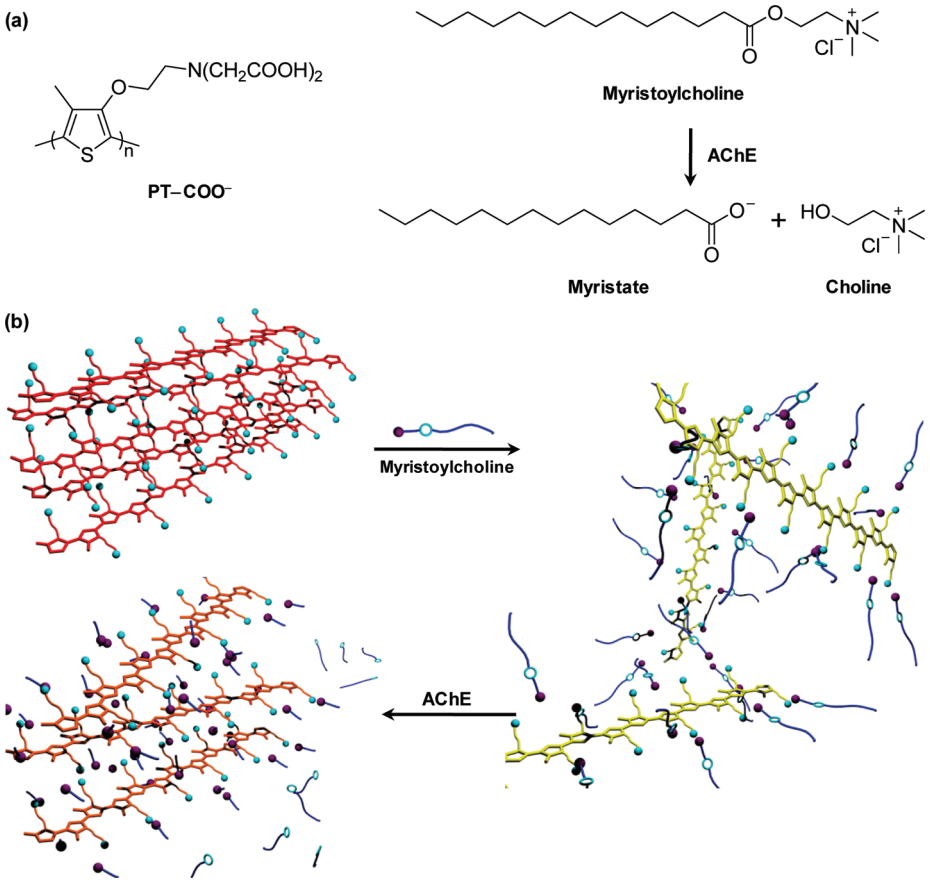


Figure 1. (a) Chemical structures of PT−COO^- and myristoylcholine, and reaction scheme for the hydrolysis of myristoylcholine catalyzed by AChE. (b) The disassembly reassembly process of PT−COO^- in the presence of myristoylcholine and AChE.

can form a complex with the anionic CPE PT−COO^- through electrostatic interaction, and the synergistic effect that arises from the hydrophobic interaction between hydrophobic alkyl chains of surfactants promotes the disassembly of PT−COO^- aggregates.[26] Upon addition of AChE, myristoylcholine can easily be catalytically hydrolyzed into myristate and choline. Accordingly, the complex of PT−COO^- and cationic surfactant formed in aqueous solution would be collapsed due to the electrostatic repulsion between PT−COO^- and myristate, a hydrolyzed product of myristoylcholine. Therefore, PT−COO^- reaggregates, and the AChE-catalyzed hydrolysis can be monitored easily by recording the absorption spectral changes of PT−COO^-. Furthermore, in the presence of inhibitors, the AChE-catalyzed hydrolysis of myristoylcholine would be retarded, and thus the assay can also be applied for screening the inhibitors of AChE.

EXPERIMENTAL SECTION

Materials and Measurements. Anionic water-soluble polythiophene derivative, PT−COO^-, was synthesized and purified as reported previously.[26] Myristoylcholine, AChE (from *Electrophorus electricus*), and neostigmine bromide were purchased from Sigma-Aldrich. All other chemicals were purchased from Beijing Chem. Reagents Co. (Beijing, China) and were used as received. Ultrapure water (18.2 MΩ cm at 25 °C) was obtained with a Millipore filtration system. Stock solutions of PT−COO^-, myristoylcholine, and AChE in 10 mM phosphate buffer (76.9 mM NaCl, pH 8.0) were prepared immediately before use and maintained below 4 °C. All absorption spectra for AChE assays were collected at 37 °C by using a Hitachi 3010 UV−visible spectrometer.

Assay for AChE Activity. A mixture of PT−COO^- (0.05 mM) and myristoylcholine (0.05 mM) in 10 mM phosphate buffer (76.9 mM NaCl, pH 8.0) was incubated for 10 min at 37 °C. AChE with the given concentration (0.2, 0.5, 1.0, and 1.5 U/mL) was then added into the mixture, and its time course of the absorbance at 500 nm was monitored immediately at 37 °C by UV−visible spectrometer.

Assay for AChE Inhibition. A solution (0.8 mL) of PT−COO^- and myristoylcholine was preincubated in 10 mM phosphate buffer (76.9 mM NaCl, pH 8.0) at 37 °C for 10 min. Neostigmine bromide with different concentrations (10−100 nM) were incubated with AChE in 10 mM phosphate buffer (0.2 mL, 76.9 mM NaCl, pH 8.0) at 37 °C for 15 min, then mixed with the preincubated solution to give a solution containing 1.5 U/mL of AChE, 0.05 mM of PT−COO^- and 0.05 mM of myristoylcholine. After mixing, the absorbance of the resulting solutions at 500 nm was monitored immediately at 37 °C. Inhibition efficiency (*IE*) was determined by monitoring absorbance changes of PT−COO^- by using the following equation: $IE = A_{t(\text{inhibitor})} - A_{t(\text{no inhibitor})}/A_0 - A_{t(\text{no inhibitor})}$, in which A_0 refers to the absorbance of PT−COO^- at 500 nm in the absence of AChE and inhibitor, and $A_{t(\text{inhibitor})}$ and $A_{t(\text{no inhibitor})}$ are the absorbance of PT−COO^- during the hydrolysis reaction with AChE in the presence and the absence of inhibitor, respectively.

Selectivity of the AChE Assay. To evaluate the specificity of the AChE assay, enzymes and proteins including hemoglobin (Hb), glucose oxidase (GOx), and bovine serum albumin (BSA) were used in place of AChE in control experiments. The procedure and parameters for control

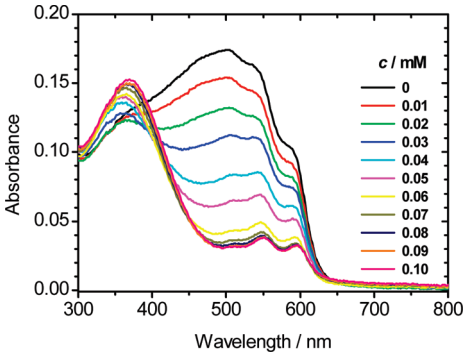


Figure 2. Variation in the absorption spectra of PT−COO^- (0.05 mM) in 10 mM phosphate buffer (76.9 mM NaCl, pH 8.0) with increasing concentrations of myristoylcholine as indicated.

experiments are the same as those for AChE activity assay except for setting the concentrations of enzymes and proteins as 8 nM.

RESULTS AND DISCUSSION

Myristoylcholine-Induced Disassembly of PT−COO^- Aggregates. Figure 2 compares absorption spectra of PT−COO^- and its mixtures with different amounts of myristoylcholine in 10 mM phosphate buffer (pH 8.0) at 25 °C. As reported previously,[26] PT−COO^- shows satisfied solubility in buffer solution and exhibits an absorption maximum at 500 nm together with two broad shoulders around 535 and 588 nm. These results indicate that PT−COO^- adopted a planar conformation and partially aggregated in aqueous solution.[26] Upon the introduction of increasing amount of myristoylcholine into PT−COO^- solution, the magnitudes of the absorption bands at the longer wavelengths associated with the aggregated state decreases gradually. Simultaneously, the absorption maximum is blue-shifted to 368 nm along with a solution color change from pink-red to hermosa pink, signifying the dissociation of PT−COO^- aggregates.[44]

To elucidate the molecular mechanism behind this observation, some preliminary considerations are discussed as follows. It is reported that pK_a values of *N*-methyliminodiacetic acid are pK_{a1} = 2.5 and pK_{a2} = 9.6, respectively,[45] thus, the pK_{a2} value of PT−COO^- was estimated to be around 9−10 due to the similarity between *N*-methyliminodiacetic acid and the side chains of PT−COO^-. In phosphate buffer (pH 8.0) some terminal groups of PT−COO^- side chains are existed as −COOH, and the intermolecular hydrogen binding between carboxylic acid moieties may be the primary driving force for the formation of PT−COO^- aggregates (see Figure S1 in the Supporting Information). Upon addition of myristoylcholine into PT−COO^- solution, the electrostatic interaction between the cationic charge in the ammonium group of surfactant and the negative charge on the *N*-alkyliminodiacetic acid group in PT−COO^- along with the hydrophobic interaction between hydrophobic alkyl chains of surfactants promotes the disassembly of PT−COO^- aggregates. These interactions shift the $\pi-\pi^*$ transition to shorter wavelengths and lead to a color change from pink-red to hermosa pink. Moreover, the concentration of myristoylcholine in the present system is much lower than its cmc of 2.5 mM.[8] Therefore, one can inculde that the absorption spectral change would be due to the formation of PT−COO^-/ myristoylcholine complex.

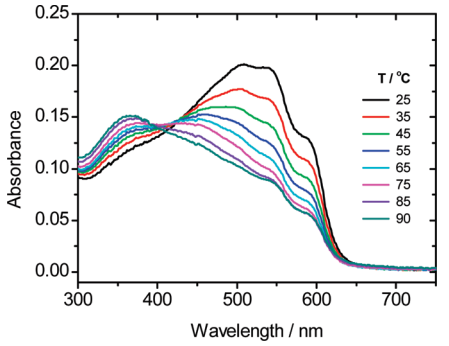


Figure 3. Temperature-dependent absorption spectra of PT−COO^- (0.05 mM) in phosphate buffer (10 mM, pH 8.0).

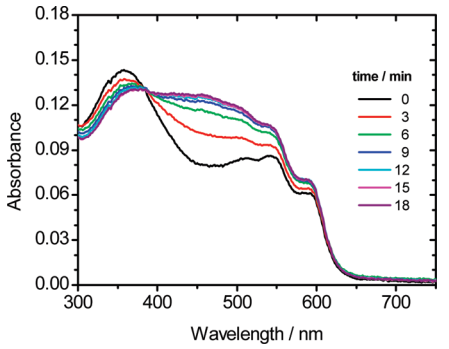


Figure 4. Absorption spectra of PT−COO^-/myristoylcholine (0.05 mM) in phosphate buffer (10 mM; NaCl, 76.9 mM; pH 8.0) in the presence of AChE (1.5 U/mL) incubated at 37 °C for different periods.

To further confirm that the spectral change is due to the disassembly of PT−COO^- aggregates, temperature-dependent absorption spectra of PT−COO^- were collected (Figure 3). It can be seen that with increasing temperature, the magnitudes of the absorption bands which originate from PT−COO^- aggregates, are decreased gradually, and a broad peak around 384 nm attributed to nonaggregated PT−COO^- chains is developed, signifying that the aggregates formed at room temperature are dissociated gradually. The spectral characteristic at higher temperature is similar with that observed for PT−COO^- in the presence of myristoylcholine, supporting the viewpoint that the addition of cationic surfactants promotes the disassembly of PT−COO^- aggregates.

Colorimetric Assay for AChE Activity. Figure 4 shows the absorption spectra of PT−COO^-/myristoylcholine complex (0.05 mM) in 10 mM phosphate buffer (pH 8.0) in the presence of AChE (1.5 U/mL) after different reaction periods at 37 °C. It can be seen that the absorbance at the longer wavelengths originated from the aggregated structures was gradually increased with the incubating time from 0 to 18 min. The control experiments demonstrate that AChE has little effect on the absorption spectrum of PT−COO^- (see Figure S2 in the Supporting Information). These observations indicate that the spectral changes were due to the collapse of PT−COO^-/ myristoylcholine complex by the AChE-catalyzed hydrolysis of myristoylcholine.[46] Moreover, the absorbance ratio of 384 to 500 nm did not change any more after 18 min, indicating that the hydrolysis of myristoylcholine was nearly completed. Figure 5

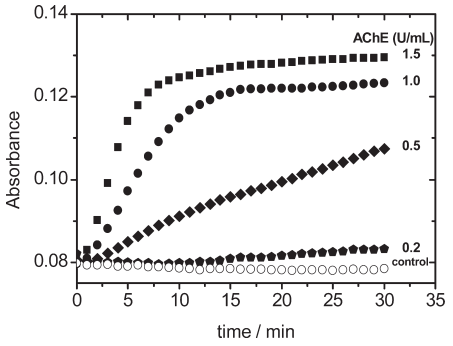


Figure 5. Absorbance of PT−COO^- (0.05 mM) at 500 nm vs incubating time in the hydrolysis of myristoylcholine (0.05 mM) with various concentrations of AChE in 10 mM phosphate buffer (pH 8.0).

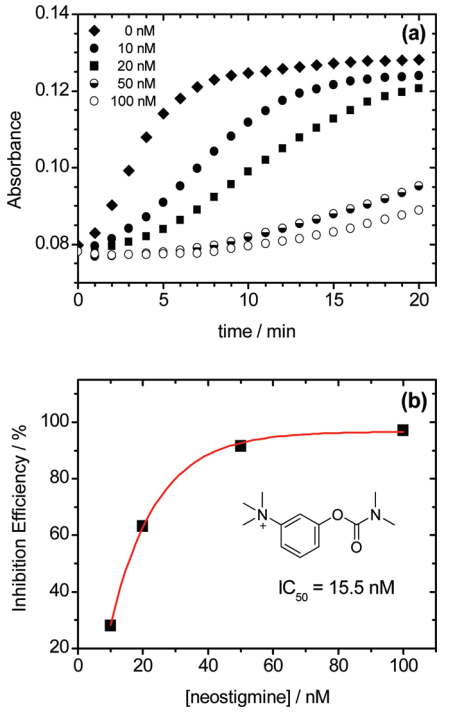


Figure 6. (a) Absorbance of PT−COO^-/myristoylcholine (0.05 mM) at 500 nm versus reaction time in the presence of AChE (1.5 U/mL) with various concentration of neostigmine as indicated in 10 mM phosphate buffer (NaCl 76.9 mM, pH 8.0). (b) Plot of the inhibition efficiency of neostigmine for AChE versus the concentration of neostigmine.

compares the time-dependent absorbance changes of PT−COO^- at 500 nm on the concentration of AChE. It is clearly that the absorbance change depends on the concentration of AChE. An increase in the concentration of AChE leads to a fast initial hydrolysis reaction rate and an enhanced spectral change. The limit of detection of this assay is evaluated to be 0.2 U/mL, which is comparable to those of the reported optical methods.[7,8]

Assay for AChE Inhibition. Inhibitor screening for AChE has been attracted increasing attention in recent years since it can be applied to the discovery of new drugs and detection of nerve agents and pesticides. To check the possibility that the system described herein is used to screen AChE inhibitors, neostigmine, a well-known inhibitor for AChE, was selected as an example for our study. Figure 6a shows the absorbance of PT−COO^- at 500 nm versus the reaction time for the solutions containing PT−COO^- (0.05 mM), myristoylcholine (0.05 mM), AChE (1.5 U/mL), and different amounts of neostigmine (0, 10, 20, 50, 100 nM). It is clearly seen that the introduction of neostigmine to the solution leads to small absorbance (500 nm) enhancement, and the absorbance change becomes smaller after addition of more neostigmine. The inhibition ability of neostigmine is evaluated by an IC_{50} value, which is the inhibitor concentration required for 50% inhibition of AChE activity. The IC_{50} value of neostigmine toward AChE was calculated to be 15.5 nM from the plot of inhibition efficiency versus inhibitor concentration (Figure 6b), being comparable to that reported previously.[47] This result indicates that the strategy presented here provides a sensitive, rapid, and simple method for screening AChE inhibitors.

To evaluate the specificity of the colorimetric assay toward AChE, three proteins including Hb, GOx, and BSA were selected for control experiments (see Figure S4 in the Supporting Information). It was demonstrated that the absorbance of PT−COO^- at 500 nm was increased distinctly only for the addition of AChE, whereas upon the introduction of Hb, GOx, and BSA, the absorbance was changed slightly. These results indicate that the colorimetric PT−COO^-/myristoylcholine-based assay exhibits satisfied selectivity toward AChE.

CONCLUSIONS

In conclusion, we have developed an efficient assay for AChE activity and inhibitor screening based on the disassembly reassembly of an anionic CPE, PT−COO^-. The preliminary electrostatic interaction between myristoylcholine and anionic polythiophene derivative produces the simple salt association initially, and then the synergistic effect of hydrophobic interaction of the long alkyl chains would be responsible for promoting the dissociation of PT−COO^- aggregate. The introduction of AChE into the solution can catalyze the hydrolysis of myristoylcholine, and thus the reassembly of PT−COO^- occurs, and the absorption spectral changes can be applied to AChE assays. The present work will not only extend the application scopes of the colorimetric sensing systems based on P3RO-4MeT but also provide a fast, simple, and convenient approach for monitoring AChE activity and its inhibitor screening. We expect that the present method can be applied to high-throughput screening of AChE inhibitors and sensitive detection of pesticides.

ASSOCIATED CONTENT

Supporting Information. Absorption spectra of PT−COO^- in aqueous solutions with different pH values; absorption spectra of PT−COO^- in the presence of AChE; fluorescence quenching of PT−COO^-/myristoylcholine solution incubated different time with AChE; variation in the absorbance at 500 nm following 15-min incubation with Hb, GOx, and BSA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: chunli@mail.tsinghua.edu.cn. Tel: +86-10-6279-8909. Fax: +86-10-6277-1149.

■ ACKNOWLEDGMENT

The authors gratefully thank the National Natural Science Foundation of China (50973054, 50873092) for financial support of this work.

■ REFERENCES

(1) Soreq, H.; Seidman, S. *Nat. Rev. Neurosci.* **2001**, *2*, 294–302.
(2) Purves, D.; Augustine, G. J.; Fitzpatrick, D.; Hall, W. C.; LaMantia, A.-S.; McNamara, J. O.; White, L. E., Eds. *Neuroscience*, 4th ed.; Sinauer-Associates: Sunderland, U.K., 2008; pp 121−2.
(3) Miao, Y.; He, N.; Zhu, J. *Chem. Rev.* **2010**, *110*, 5216–5234.
(4) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88–90.
(5) Pavlov, V.; Xiao, Y.; Willner, I. *Nano Lett.* **2005**, *5*, 649–653.
(6) Maeda, H.; Mastuno, H.; Ushida, M.; Katayama, K.; Saeki, K.; Itoh, N. *Angew. Chem., Int. Ed.* **2005**, *44*, 2922–2925.
(7) Feng, F.; Tang, Y.; Wang, S.; Li, Y.; Zhu, D. *Angew. Chem., Int. Ed.* **2007**, *46*, 7882–7886.
(8) Wang, M.; Gu, X.; Zhang, G.; Zhang, D.; Zhu, D. *Anal. Chem.* **2009**, *81*, 4444–4449.
(9) Peng, L.; Zhang, G.; Zhang, D.; Xiang, J.; Zhao, R.; Wang, Y.; Zhu, D. *Org. Lett.* **2009**, *11*, 4014–4017.
(10) Gill, R.; Bahshi, L.; Freeman, B.; Willmer, I. *Angew. Chem., Int. Ed.* **2008**, *47*, 1676–1679.
(11) Chen, L.; McBranch, D. W.; Wang, H.-L.; Helgeson, R.; Wudl, F.; Whitten, D. G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12287–12292.
(12) Thomas, S. W.; Joly, G. D.; Swager, T. M. *Chem. Rev.* **2007**, *107*, 1339–1386.
(13) Ho, H.-A.; Najari, A.; Leclerc, M. *Acc. Chem. Res.* **2008**, *41*, 168–178.
(14) Feng, F.; He, F.; An, L.; Wang, S.; Li, Y.; Zhu, D. *Adv. Mater.* **2008**, *20*, 2959–2964.
(15) Liu, Y.; Ogawa, K.; Schanze, K. S. *J. Photochem. Photobiol. C* **2009**, *10*, 173–190.
(16) Ho, H.-A.; Bera-Aberem, M.; Leclerc, M. *Chem.—Eur. J.* **2005**, *11*, 1718–1724.
(17) Ho, H.-A.; Leclerc, M. *J. Am. Chem. Soc.* **2004**, *126*, 1384–1387.
(18) Le Floch, F.; Ho, H.-A.; Leclerc, M. *Anal. Chem.* **2006**, *78*, 4727–4731.
(19) Li, C.; Numata, M.; Bae, A.-H.; Sakurai, K.; Shinkai, S. *J. Am. Chem. Soc.* **2005**, *127*, 4548–4549.
(20) Li, C.; Numata, M.; Hasegawa, T.; Sakurai, K.; Shinkai, S. *Chem. Lett.* **2005**, *34*, 1354–1355.
(21) Li, C.; Numata, M.; Takeuchi, M.; Shinkai, S. *Angew. Chem., Int. Ed.* **2005**, *44*, 6371–6374.
(22) Yao, Z.; Feng, X.; Hong, W.; Li, C.; Shi, G. *Chem. Commun.* **2009**, 4696–4698.
(23) Yao, Z.; Li, C.; Shi, G. *Langmuir* **2008**, *24*, 12829–12835.
(24) Yao, Z.; Feng, X.; Li, C.; Shi, G. *Chem. Commun.* **2009**, 5886–5888.
(25) Yao, Z.; Bai, H.; Li, C.; Shi, G. *Chem. Commun.* **2010**, *46*, 5094–5096.
(26) Yao, Z.; Li, Y.; Li, C.; Shi, G. *Chem. Commun.* **2010**, *46*, 8639–8641.
(27) Kumaraswamy, S.; Bergstedt, T.; Shi, X.; Rininsland, F.; Kushon, S.; Xia, W.; Ley, K.; Achyuthan, K.; McBranch, D.; Whitten, D. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 7511–7515.
(28) Rininsland, F.; Xia, W.; Wittenburg, S.; Shi, X.; Stankewicz, C.; Achyuthan, K.; McBranch, D.; Witten, D. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15295–15300.
(29) Wosnick, J. H.; Mello, C. M.; Swager, T. M. *J. Am. Chem. Soc.* **2005**, *127*, 3400–3405.
(30) Pinto, M. R.; Schanze, K. S. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 7505–7510.
(31) Liu, Y.; Ogawa, K.; Schanze, K. S. *Anal. Chem.* **2008**, *80*, 150–158.
(32) Liu, Y.; Schanze, K. S. *Anal. Chem.* **2008**, *80*, 8605–8612.
(33) Chemburu, S.; Ji, E.; Casana, Y.; Wu, Y.; Buranda, T.; Schanze, K. S.; Lopez, G. P.; Whitten, D. G. *J. Phys. Chem. B* **2008**, *112*, 14492–14499.
(34) Liu, Y.; Schanze, K. S. *Anal. Chem.* **2009**, *81*, 231–239.
(35) An, L.; Tang, Y.; Feng, F.; He, F.; Wang, S. *J. Mater. Chem.* **2007**, *17*, 4147–4152.
(36) Feng, F.; Tang, Y.; He, F.; Yu, M.; Duan, X.; Wang, S.; Li, Y.; Zhu, D. *Adv. Mater.* **2007**, *19*, 3490–3495.
(37) An, L.; Wang, S.; Zhu, D. *Chem. Asian J.* **2008**, *3*, 1601–1606.
(38) An, L.; Liu, B.; Wang, S. *Biomacromolecules* **2009**, *10*, 454–457.
(39) Feng, X.; Duan, X.; Liu, L.; Feng, F.; Wang, S.; Li, Y.; Zhu, D. *Angew. Chem., Int. Ed.* **2009**, *48*, 5316–5321.
(40) Feng, F.; Liu, L.; Yang, Q.; Wang, S. *Macromol. Rapid Commun.* **2010**, *31*, 1405–1421.
(41) Zhang, Y.; Wang, Y.; Liu, B. *Anal. Chem.* **2009**, *81*, 3731–3737.
(42) Wang, Y.; Zhang, Y.; Liu, B. *Anal. Chem.* **2010**, *82*, 8604–8610.
(43) Tang, Y.; Feng, F.; He, F.; Wang, S.; Li, Y.; Zhu, D. *J. Am. Chem. Soc.* **2006**, *128*, 14972–14976.
(44) Li, C.; Numata, M.; Takeuchi, M.; Shinkai, S. *Chem.— Asian J.* **2006**, *1*, 95–101.
(45) Hoeschele, J. D.; Farrell, N.; Turner, W. R.; Rithner, C. D. *Inorg. Chem.* **1988**, *27*, 4106–4113.
(46) The fluorescence method can also be applied to monitoring the hydrolysis of myristoylcholine and AChE activity (see Figure S3 in the Supporting Information).
(47) Kishibayashi, N.; Ishii, A.; Karasawa, A. *Jpn. J. Pharmacol.* **1994**, *66*, 397–403.