Continuous Fluorometric Assays for Acetylcholinesterase Activity and Inhibition with Conjugated Polyelectrolytes**
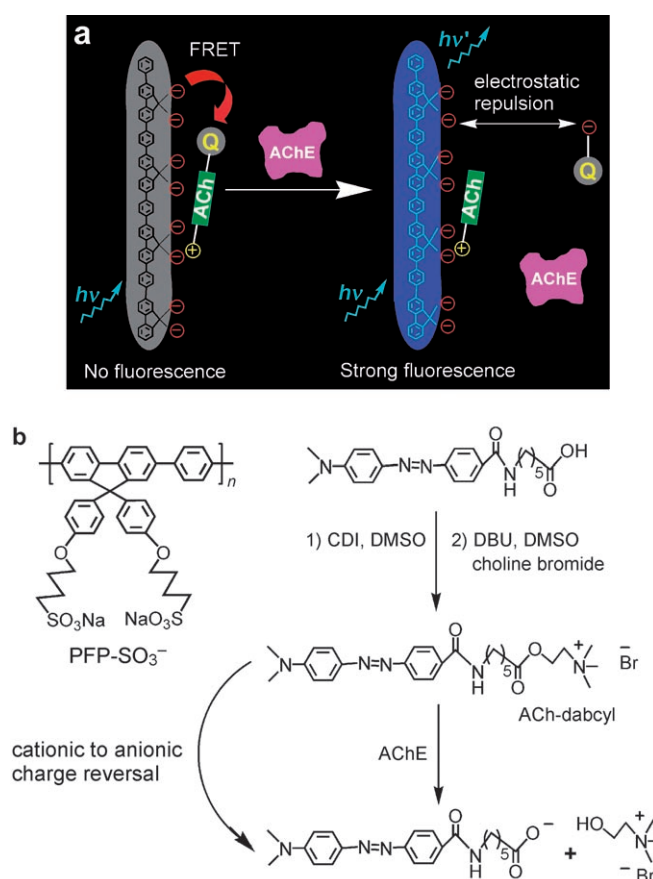
Fude Feng, Yanli Tang, Shu Wang, Yuliang Li, and Daoben Zhu*

Alzheimer's disease (AD) is a chronic, progressive neurodegenerative disorder that affects a significant proportion of the population worldwide.[1] The hydrolytic breakdown of acetylcholine (ACh) in the brain by acetylcholinesterase (AChE)[2] can accelerate the assembly of amyloid β peptides into amyloid fibrils, a process that results in AD.[3] Currently, the inhibition of AChE with the aim of increasing the levels of ACh is proving to be an effective strategy for AD therapies.[4] AChE activity and inhibition are monitored traditionally by UV/Vis spectroscopy.[5] However, a disadvantage of this method is its low sensitivity. The most common assays for AChE involve an enzyme cascade in which AChE converts ACh into choline, which is then oxidized by choline oxidase with the production of H_2O_2. In the presence of horseradish peroxidase (HRP), the H_2O_2 produced is detected by fluorescence, chemiluminescence, electroanalysis, or nanotechnology.[6–9] With these methods, two or more chemical reactions and several additional enzymes are involved in the detection process, which makes the assays complex, expensive, and time-consuming. Furthermore, the assays are not very suitable or precise enough for enzymological studies because of the indirect and discontinuous methods used for the measurement of reaction kinetics. Therefore, a new, sensitive, simple, and continuous pathway for AChE assays is urgently needed.

Conjugated polymers have attracted attention as optical platforms in highly sensitive bioassays for proteins and nucleic acids,[10–18] as well as nucleases, proteases, and kinases.[19–21] In contrast to bioassays with small-molecule counterparts, the transfer of the excitation energy along the whole backbone of the conjugated polymer to the reporter results in the amplified fluorescence signal.[10] Herein we describe a new fluorescence turn-on assay for AChE activity and inhibition based on the reversible fluorescence quenching of an anionic conjugated polymer. The fluorescence quenching is mediated by the charge reversal of modified ACh upon hydrolysis.

Our fluorescence assay for AChE is illustrated in Scheme 1a. An ACh derivative labeled with the widely used quencher dabcyl was synthesized as a substrate for AChE. The resulting cationic compound ACh–dabcyl (see Scheme 1b for its chemical structure and synthesis) can form a complex with the anionic polymer poly{1,4-phenylene-[9,9-bis(4-phenoxybutylsulfonate)]fluorene-2,7-diyl} (PFP-SO_3^-)[22] through electrostatic interactions. The fluorescence of PFP-SO_3^- is quenched efficiently by the dabcyl moiety through the transfer of fluorescence resonance energy.[23] Upon the addition of AChE, ACh–dabcyl undergoes catalytic hydrolysis to produce choline and a negatively charged residue that contains the dabcyl moiety. As a result of the

[*] F. Feng, Y. Tang, Prof. S. Wang, Prof. Y. Li, Prof. D. Zhu
National Laboratory for Molecular Sciences
Key Laboratory of Organic Solids
Institute of Chemistry
Chinese Academy of Sciences
Beijing, 100080 (P.R. China)
Fax: (+86) 10-6263-6680
E-mail: wangshu@iccas.ac.cn

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Scheme 1. a) Assays of AChE activity. b) Chemical structure of the anionic conjugated polymer PFP-SO_3^-, and the synthesis of cationic ACh–dabcyl. CDI = 1,1′-carbonyldiimidazole, DBU = 1,8-diazabicyclo-[5.4.0]undec-7-ene, DMSO = dimethyl sulfoxide, FRET = fluorescence resonant energy transfer.

charge reversal, the dabcyl moiety is repulsed by PFP-SO$_3^-$ and moves away from the electrostatic complex. Therefore, the fluorescence intensity of PFP-SO$_3^-$ is recovered, and the AChE-catalyzed hydrolysis can be monitored in a continuous and real-time manner. Furthermore, the inhibition of AChE can also be analyzed with the PFP-SO$_3^-$/ACh–dabcyl complex.

To determine the sensitivity of the protocol, the fluorescence quenching of PFP-SO$_3^-$ (2.0 µM in repeat units (RUs)) was examined with ACh–dabcyl in phosphate buffer solution (25 mM, pH 8.0; see the Supporting Information). The quenching efficiency is related to the Stern–Volmer constant (K_{sv}) and is determined by monitoring measurable fluorescence changes by using the Stern–Volmer equation [Eq. (1)][24] in which F_0 is the fluorescence-emission intensity in the absence of the quencher, F is the fluorescence-emission intensity in the presence of the quencher, and [Q] is the concentration of the quencher.

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

At low concentrations, a linear Stern–Volmer plot (F_0/F versus [ACh–dabcyl]) is obtained with a K_{sv} value of 1.84×10^7 M^{-1} (see the Supporting Information). Thus, the dabcyl moiety shows superquenching behavior with respect to PFP-SO$_3^-$. At a remarkably low concentration of 0.4 µM, ACh–dabcyl quenches the fluorescence of PFP-SO$_3^-$ (2.0 µM in RUs) with an efficiency of about 92% upon excitation at 376 nm. One equivalent of ACh–dabcyl could quench the fluorescence of PFP-SO$_3^-$ with more than 99% efficiency. The AChE assay shows very high sensitivity because of the extremely low background noise.

The results of the fluorescence-monitored hydrolysis of ACh–dabcyl by AChE are presented in Figure 1a. Upon the addition of AChE to the PFP-SO$_3^-$/ACh–dabcyl complex ([PFP-SO$_3^-$] = 2.0 µM, [ACh–dabcyl] = 2.0 µM) in a phosphate buffer solution (25 mM, pH 8.0) and equilibration at 37 °C, the fluorescence of PFP-SO$_3^-$ was observed to rise gradually during the first 10 min until a plateau was reached after 12 min (final [AChE]: 2.0 units mL^{-1}). This result indicates that AChE catalyzes the hydrolysis of ACh–dabcyl, which leads to a "turn-on" response of PFP-SO$_3^-$ fluorescence. The hydrolysis of ACh–dabcyl by AChE enhanced the fluorescence of PFP-SO$_3^-$ 130-fold: Thus, the PFP-SO$_3^-$/ACh–dabcyl complex has an extremely high fluorescence turn-on response for AChE. The fluorescence recovery of PFP-SO$_3^-$ depends on the concentration of AChE (Figure 1b). A decrease in the concentration of AChE leads to a slow initial cleavage reaction rate and a low level of fluorescence recovery. The limit of detection (LOD) of this assay is 0.05 units mL^{-1}, which is comparable to that of most sensitive chemiluminescence techniques.[6]

To measure the enzyme reaction kinetics, the cleavage reaction was monitored as a function of substrate concentration. A plot of the change in intensity of PFP-SO$_3^-$ emission at 424 nm versus the AChE incubation time was used to calculate the initial velocity (in M s^{-1}; see the Supporting Information). The concentration of ACh–dabcyl was calculated from the fluorescence intensity of PFP-SO$_3^-$
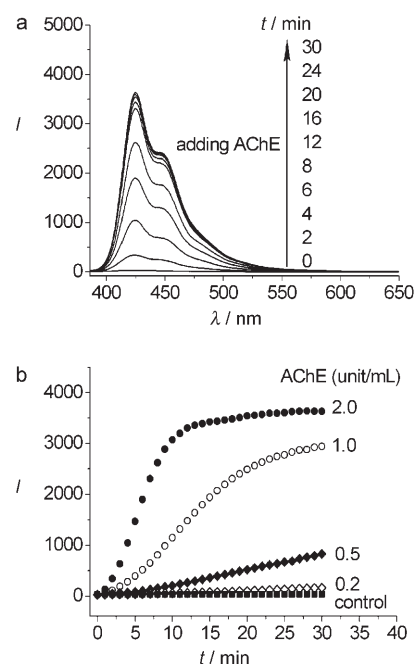

Figure 1. a) Emission spectra of PFP-SO$_3^-$/ACh–dabcyl as a function of reaction time for AChE-catalyzed hydrolysis; [PFP-SO$_3^-$] = 2 µM, [ACh–dabcyl] = 2 µM, [AChE] = 2.0 units mL^{-1}. The experiment was carried out with a fixed concentration of AChE b) Emission intensity of PFP-SO$_3^-$ at 424 nm versus reaction time in the hydrolysis of ACh–dabcyl with varying concentrations of AChE; [PFP-SO$_3^-$] = 2 µM, [ACh–dabcyl] = 2 µM, [AChE] = 0.2–2.0 units mL^{-1}. Fluorescence measurements were made in phosphate buffer solutions (25 mM, pH 8.0) with excitation at 376 nm.

from the Stern–Volmer curve at pH 8.0 for each data point. The initial reaction rate (V_0) was calculated as the slope of the plot of the concentration change of ACh–dabcyl versus reaction time for the first five data points. The plot of $1/V_0$ versus $1/[S]$ obeys the Lineweaver–Burk equation and yields the Michaelis constant[25] $K_m = 4.0$ µM (Figure 2a), which is of the same order of magnitude as those reported.[5,6] This result shows that the modification of the substrate with the dabcyl group does not affect AChE activity. It is possible to detect AChE activity with a substrate concentration of 0.2–0.6 µM with a common fluorometer within 2 min, which emphasizes the high sensitivity and rapid response of the PFP-SO$_3^-$/ACh–dabcyl complex.

As the exciton can diffuse during its lifetime along the backbone of the conjugated polymer, one would not expect to see fluorescence recovery of PFP-SO$_3^-$ until after the hydrolysis of all of the ACh–dabcyl if an excessive amount of ACh–dabcyl is bound to the polymer. Thus, the use of heavily quenched polymers to determine enzyme kinetics may underestimate initial reaction rates and affect the enzyme activity. We therefore performed measurements of enzymatic activity with the polymer PFP-COOH, which is only quenched slightly by ACh–dabcyl. A linear relationship between the fluorescence response of PFP-COOH and the concentration of ACh–dabcyl was found, with a K_{sv} value of 8.21×10^5 M^{-1} (see the Supporting Information). On the basis of these results we can be confident that there is no overlap of
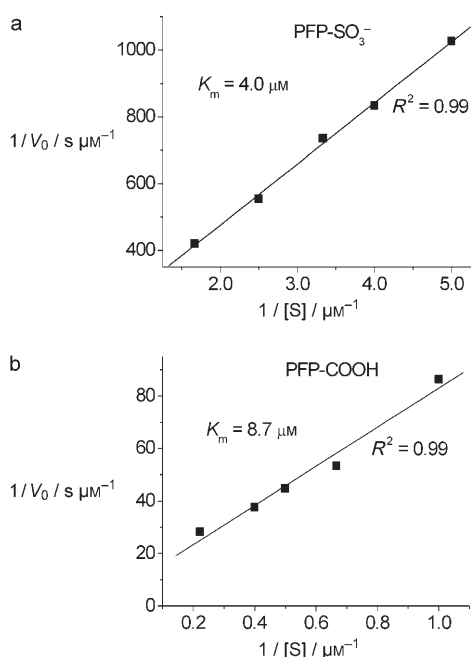
a

PFP-SO$_3^-$

$K_m = 4.0$ μM

$R^2 = 0.99$

$1/V_0$ / s μM^{-1}

b

PFP-COOH

$K_m = 8.7$ μM

$R^2 = 0.99$

$1/V_0$ / s μM^{-1}

Figure 2. a) Lineweaver–Burk plot for AChE-catalyzed hydrolysis in the presence of PFP-SO$_3^-$; [PFP-SO$_3^-$] = 2 μM, [AChE] = 1.0 unit mL^{-1}, [ACh–dabcyl] = 0.2, 0.25, 0.3, 0.4, 0.6 μM. Fluorescence measurements were made in phosphate buffer solutions (25 mM, pH 8.0) with excitation at 376 nm. b) Lineweaver–Burk plot for AChE-catalyzed hydrolysis in the presence of PFP-COOH; [PFP-COOH] = 2 μM, [AChE] = 0.25 units mL^{-1}, [ACh–dabcyl] = 1, 1.5, 2.0, 2.5, 4.5 μM. Fluorescence measurements were made in phosphate buffer solutions (5 mM, pH 8.0) with excitation at 376 nm.

PFP-COOH
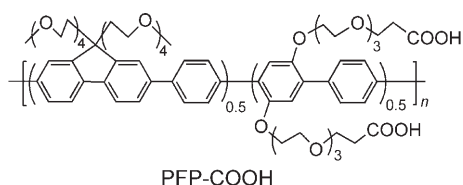
the quenching radius of multiple quenchers. The K_m value of 8.7 μM found (Figure 2b) is in good agreement with that obtained with PFP-SO$_3^-$. The results of this control experiment combined with the instant fluorescence response of the PFP-SO$_3^-$/ACh–dabcyl complex to AChE show that the PFP-SO$_3^-$/ACh–dabcyl complex can be used to measure the enzymatic kinetics of AChE as long as ACh–dabcyl is not used in excess (that is, the charge ratio of the quencher to the polymer should not be greater than 1.0).

The PFP-SO$_3^-$/ACh–dabcyl complex described herein can also be used to screen AChE inhibitors. We selected for our study the two most effective inhibitors currently employed clinically for AD, galanthamine and donepezil.[26] As these inhibitors are amino salts, all experiments were performed in phosphate buffer (25 mM) at pH 7.4 to avoid their deprotonation and precipitation from solution. In the inhibition assays, solutions of AChE and the inhibitors of varying concentration were incubated at 25 °C for 15 min, then ACh–dabcyl and PFP-SO$_3^-$ were added, and the mixtures were left

for 10 min to allow hydrolysis to occur. The inhibition ability of an inhibitor is described by an IC$_{50}$ value, which refers to the inhibitor concentration required for 50% inhibition of enzyme activity. The fluorescence intensity of PFP-SO$_3^-$ in the presence and absence of the inhibitor was converted into the concentration of ACh–dabcyl for all data points by using the Stern–Volmer curve at pH 7.4. The IC$_{50}$ value was obtained from the plot of inhibition efficiency versus inhibitor concentration; the inhibition efficiency (IE) was determined by monitoring concentration changes of ACh–dabcyl by using Equation (2) in which C_0 is the initial concentration of ACh–dabcyl, and $C_{t(inhibitor)}$ and $C_{t(no\ inhibitor)}$ are the concentrations of ACh–dabcyl during the hydrolysis reaction with AChE in the presence and absence of the inhibitor, respectively.

$$IE = \frac{C_{t(inhibitor)} - C_{t(no\ inhibitor)}}{C_0 - C_{t(no\ inhibitor)}} \times 100\% \qquad (2)$$

Galanthamine and donepezil were found to inhibit AChE with IC$_{50}$ values of 12 and 23 nM, respectively (Figure 3 a,b). The PFP-SO$_3^-$/ACh–dabcyl complex is more favorable for screening inhibitors with IC$_{50}$ values at or below the nM scale, and such inhibitors are urgently required for AD treatment. Thus, the methodology described herein provides a sensitive, rapid, and convenient protocol for screening AD drugs.

In summary, we have taken advantage of the charge-reversal mechanism of a novel quencher-modified substrate combined with the light-harvesting properties of conjugated polymers to develop an assay for AChE activity and inhibitor screening. The new method has several significant features.
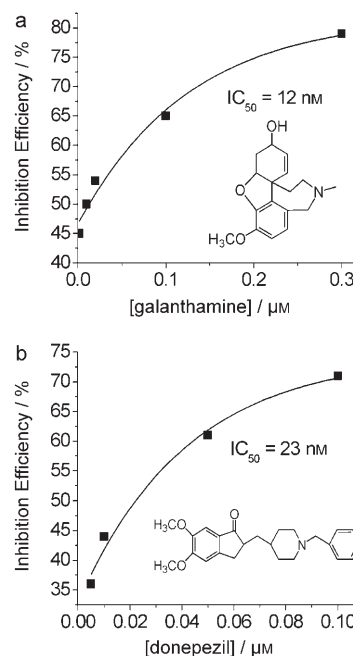
a

IC$_{50}$ = 12 nM

Inhibition Efficiency / %

[galanthamine] / μM

b

IC$_{50}$ = 23 nM

Inhibition Efficiency / %

[donepezil] / μM

Figure 3. Plots of the inhibition efficiency of a) galanthamine and b) donepezil for AChE versus the concentration of the inhibitor; [PFP-SO$_3^-$] = 2 μM, [AChE] = 1.0 unit mL^{-1}, [ACh–dabcyl] = 0.3 μM, [galanthamine] = 0.002, 0.01, 0.02, 0.1, 0.3 μM, [donepezil] = 0.005, 0.01, 0.05, 0.1 μM. Fluorescence measurements were made in phosphate buffer solutions (25 mM, pH 7.4) with excitation at 376 nm.

First, it offers a convenient "mix-and-detect" approach for the rapid and sensitive detection of AChE activity and inhibition. Second, the fluorescence intensity of PFP-SO$_3^-$ is enhanced 130-fold through the hydrolysis of ACh–dabcyl, and therefore the assay offers the benefits of extremely low background noise and high detection sensitivity. Third, the method is more favorable than existing methods for screening inhibitors with IC$_{50}$ values at or below the nM scale. Finally, this continuous method is well suited for studies of the kinetics of enzyme reactions. In principle, this sensor mechanism may prove to be highly generalizable and provide a means to monitor the activity of other enzymes (such as butyrylcholinesterase and phosphatase). Furthermore, such fluorescence-assay systems could be expanded to fluorescence-based high-throughput screening assays.

Experimental Section

PFP-SO$_3^-$ was synthesized according to a procedure described in the literature.[22] 6-(p-Methyl red)aminohexanoic acid was prepared from p-methyl red according to a procedure described in the literature.[27] The synthesis and characterization of PFP-COOH are described in the Supporting Information. Choline bromide was prepared from 2-bromoethanol by treatment with trimethylamine at room temperature. Acetylcholinesterase was purchased from Sigma, and the solution of the enzyme was cooled in ice before use. Stock solutions of the enzyme in Tris-HCl buffer (20 mM, pH 7.8; Tris = tris(hydroxymethyl)aminomethane) were prepared immediately before use and maintained below 4°C. Enzyme assays were performed in phosphate buffer solution (25 mM, pH 8.0 for kinetics assays, pH 7.4 for inhibition assays). Fluorescence spectra were obtained at 37°C by using a Hitachi F-4500 fluorometer equipped with a Xeron-lamp excitation source. The sample (1 mL) was placed in a 3-mL polystyrene cuvette. The excitation wavelength was 376 nm. The water was purified by using a Millipore filtration system.

Synthesis of ACh–dabcyl: A solution of 6-(p-methyl red)aminohexanoic acid (38 mg, 0.1 mmol) and carbonyldiimidazole (24 mg, 0.15 mmol) in DMSO (0.5 mL) was stirred under N$_2$ for 30 min. The reaction mixture was then transferred with a syringe to a flask containing a solution of choline bromide (10 mg, 0.08 mmol) and DBU (21 μL, 0.14 mmol) in DMSO (0.25 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 column chromatography on silica gel (eluent: CH$_2$Cl$_2$/MeOH/H$_2$O 65:25:4) to give ACh–dabcyl (15 mg, 34%) as a red solid. ^{1}H NMR (400 MHz, D$_2$O): δ = 7.70 (d, J = 7.6 Hz, 2H), 7.55–7.51 (m, 4H), 6.57 (d, J = 8.3 Hz, 2H), 4.48 (t, J = 2.8, 2.2 Hz, 2H), 3.65 (t, J = 3.7, 1.8 Hz, 2H), 3.31 (t, J = 3.8, 2.3 Hz, 2H), 3.14 (s, 9H), 2.88 (s, 6H), 2.41 (t, J = 7.4, 7.0 Hz, 2H), 1.60–1.57 (m, 4H), 1.35–1.25 ppm (m, 2H). ^{13}C NMR (150 MHz, D$_2$O): δ = 174.5, 167.5, 154.2, 152.1, 142.3, 133.3, 127.9, 125.3, 121.8, 111.0, 67.8, 58.1, 53.8, 39.9, 39.3, 33.4, 28.7, 26.0, 24.9 ppm. MS (ESI): m/z 468.4 ([M−Br]$^+$).

Fluorescence quenching: ACh–dabcyl was added in a number of batches to the solution of PFP-SO$_3^-$ ([PFP-SO$_3^-$] = 2.0 μM) in phosphate buffer (25 mM, pH 8.0 or pH 7.4) at room temperature, and the fluorescence spectra of the resulting mixtures were measured. The K_{sv} value was calculated from the first five acquisition points by using the Stern–Volmer equation [Eq. (1)].

Fluorescence recovery: AChE (2.0 units mL^{-1}) was added to a solution of ACh–dabcyl (2.0 μM) and PFP-SO$_3^-$ (2.0 μM) in phosphate buffer (25 mM, pH 8.0). The resulting solution was incubated at 37°C for a certain period of time (from 0 to 30 min), and its fluorescence spectrum was then measured. The fluorescence intensity of PFP-SO$_3^-$ at 424 nm was then plotted as a function of the incubation time of AChE. The limit of detection (LOD) of this method is obtained from

Equation (3)[28] in which S_0 is the standard deviation of the background and S is the sensitivity.

$$LOD = 3\frac{S_0}{S} \tag{3}$$

Kinetic assay: ACh–dabcyl (0.2, 0.25, 0.3, 0.4, and 0.6 μM) and then AChE (1.0 unit mL^{-1}) were added to PFP-SO$_3^-$ (2.0 μM) in phosphate buffer (1 mL, 25 mM, pH 8.0) in five 3-mL polystyrene cuvettes. The cuvettes were incubated at 37°C, and fluorescence spectra were recorded at 20-s intervals over 400 s with excitation at 376 nm. The fluorescence intensity at 424 nm was plotted against the incubation time of AChE. The substrate concentration was calculated from the fluorescence intensities by using the Stern–Volmer curve. The initial reaction rate was calculated by using the first five acquisition points. A plot of $1/V_0$ versus $1/[S]$ was used to calculate the Michaelis constant (K_m).

AChE inhibition: Inhibitors were preincubated at varying concentrations (0–0.3 μM for galanthamine, 0–0.1 μM for donepezil) with AChE (1 unit mL^{-1}) in phosphate buffer solution (1 mL, 25 mM, pH 7.4) at 25°C for 15 min, then PFP-SO$_3^-$ (2.0 μM) and ACh–dabcyl (0.3 μM) were added, and the resulting solutions were incubated at 37°C for 10 min. Fluorescence spectra were then measured, and the fluorescence intensities of PFP-SO$_3^-$ in the presence and absence of the inhibitors were converted into the concentration of ACh–dabcyl by using the Stern–Volmer curve at pH 7.4.

[1] Z. S. Khachaturian, *Arch. Neurol.* **1985**, *42*, 1097.

[2] C. G. Ballard, *Eur. Neurol.* **2002**, *47*, 64.

[3] N. C. Inestrosa, A. Alvarez, C. A. Perez, R. D. Moreno, M. Vicente, C. Linker, *Neuron* **1996**, *16*, 881.

[4] J. M. Ellis, *JAOA* **2005**, *105*, 145.

[5] G. L. Ellmann, K. D. Courtney, V. Andres, R. M. Featherstone, *Biochem. Pharmacol.* **1961**, *7*, 88.

[6] A. G. Hadd, S. C. Jacobson, J. M. Ramsey, *Anal. Chem.* **1999**, *71*, 5206.

[7] M. Guardigli, P. Pasini, M. Mirasoli, A. Leoni, A. Andreani, A. Roda, *Anal. Chim. Acta* **2005**, *535*, 139.

[8] V. Pardo-Yissar, E. Katz, J. Wasserman, I. Willner, *J. Am. Chem. Soc.* **2003**, *125*, 622.

[9] V. Pavlov, Y. Xiao, I. Willner, *Nano Lett.* **2005**, *5*, 649.

[10] D. T. McQuade, A. E. Pullen, T. M. Swager, *Chem. Rev.* **2000**, *100*, 2537.

[11] B. Liu, G. C. Bazan, *Chem. Mater.* **2004**, *16*, 4467.

[12] H. A. Ho, A. M. Béra, M. Leclerc, *Chem. Eur. J.* **2005**, *11*, 1718.

[13] F. He, Y. Tang, M. Yu, F. Feng, L. An, H. Sun, S. Wang, Y. Li, D. Zhu, G. C. Bazan, *J. Am. Chem. Soc.* **2006**, *128*, 6764.

[14] Y. Tang, F. Feng, F. He, S. Wang, Y. Li, D. Zhu, *J. Am. Chem. Soc.* **2006**, *128*, 14972.

[15] U. H. F. Bunz, *Chem. Rev.* **2000**, *100*, 1605.

[16] K. P. R. Nilsson, O. Inganäs, *Nat. Mater.* **2003**, *2*, 419.

[17] L. H. Chen, D. W. McBranch, H. L. Wang, R. Helgeouson, F. Wudl, D. G. Whitten, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12287.

[18] B. S. Gaylord, A. J. Heeger, G. C. Bazan, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 10954.

[19] F. Feng, Y. Tang, F. He, M. Yu, X. Duan, S. Wang, Y. Li, D. Zhu, *Adv. Mater.* **2007**, DOI: 10.1002/adma.200700165

[20] L. An, Y. Tang, F. He, F. Feng, S. Wang, *J. Mater. Chem.* **2007**, DOI: 10.1039/b706268k.

[21] a) M. R. Pinto, K. S. Schanze, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7505; b) K. E. Achyuthan, T. S. Bergstedt, L. Chen, R. M. Jones, S. Kumaraswamy, S. A. Kushon, K. D. Ley, L. Lu, D. McBranch, H. Mukundan, F. Rininsland, X. Shi, W. Xia, D. G. Whitten, *J. Mater. Chem.* **2005**, *15*, 2648.

[22] H. D. Burrows, V. M. M. Lobo, J. Pina, M. L. Ramos, J. S. de Melo, A. J. M. Valente, M. J. Tapia, S. Pradhan, U. Scherf, *Macromolecules* **2004**, *37*, 7425.

[23] E. D. Matayoshi, G. T. Wang, G. A. Krafft, J. Erickson, *Science* **1990**, *247*, 954.

[24] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum, New York, **1999**.

[25] D. L. Nelson, M. M. Cox, *Lehninger Principles of Biochemistry*, 3rd ed., Worth Publisher, New York, **2000**.

[26] W. Luo, Q. Yu, S. S. Kulkarni, D. A. Parrish, H. W. Holloway, D. Tweedie, A. Shafferman, D. K. Lahiri, A. Brossi, N. H. Greig, *J. Med. Chem.* **2006**, *49*, 2174.

[27] T. M. Rose, G. D. Prestwich, *ACS Chem. Biol.* **2006**, *1*, 83.

[28] B. R. Eggins, *Chemical Sensors and Biosensors*, Wiley, Chichester, England, **2002**.