Article

Chinese Science Bulletin

Analytical Chemistry

June 2011 Vol.56 No.18: 1877–1883
doi: 10.1007/s11434-011-4505-0

Colorimetric detection of glucose and an assay for acetylcholinesterase with amine-terminated polydiacetylene vesicles

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Received January 26, 2011; accepted February 18, 2011; published online May 12, 2011

The colorimetric response of amine-terminated polydiacetylene (PDA) vesicles was initially demonstrated by varying the pH of the solution. Convenient colorimetric methods to detect glucose and acetylcholinesterase (AChE) activity were successfully established using amine-terminated PDA vesicles by taking advantage of the following features: (1) the amine-terminated PDA vesicles undergo a colorimetric transition as the pH of the solution changes; (2) glucose can be oxidized to gluconic acid in the presence of glucose oxidase; and (3) AChE catalyzes the hydrolysis of acetylcholine to acetic acid. The visual detection of glucose levels and AChE activity showed good selectivity and acceptable sensitivity. The detection limit of glucose was ~2.5 μmol/L and the level of AChE activity was assayed as low as 10.0 mU/mL. Moreover, the amine-terminated PDA vesicles can be used for screening the activity of inhibitors against AChE.

polydiacetylene vesicles, glucose, glucose oxidase, acetylcholinesterase

Citation: Xue W X, Zhang D Q, Zhang G X, et al. Colorimetric detection of glucose and an assay for acetylcholinesterase with amine-terminated polydiacetylene vesicles. Chinese Sci Bull, 2011, 56: 1877–1883, doi: 10.1007/s11434-011-4505-0

Polydiacetylenes (PDAs) are usually prepared by light irradiation of self-assembled diacetylene monomers and purification is not required because no initiators or catalysts are used in the polymerization process [1–3]. A unique feature of PDAs is the existence of two phases that exhibit different absorptions; these are referred to as the "blue phase" and "red phase" [4,5]. For example, the blue PDA-vesicles that are synthesized from amphiphilic diacetylenes after UV light irradiation in aqueous solutions can be converted into the corresponding red phase in response to appropriate stimuli [6–15]. Using this feature of PDA-vesicles, a number of chemo-/biosensors have been reported in recent years [16–26]. For example, PDA-based detection of glucose was achieved by the ligand-induced conformational changes in hexokinase immobilized on a PDA monolayer [24]. Colorimetric DNA sensors have been constructed with PDA vesicles, in which probe DNA molecules were immobilized [24]. Besides colorimetric sensing, elegant PDA-based fluorescent sensors have also been reported [25].

In this paper, we describe the colorimetric detection of glucose and acetylcholinesterase (AChE) activity using amine-terminated PDA vesicles from diacetylene **1** (Scheme 1) with a long alkyl chain and an amino group as the head-group [27–30]. The design rationale is schematically shown in Scheme 1 and explained as follows. (1) It is anticipated that the "blue-red" phase transition can also occur for the amine-terminated PDA vesicles by lowering the pH of the solution. For example, Kew et al. [9] have studied the pH response of carboxyl-terminated PDA vesicles. Here, coulombic repulsion between the adjacent ionized head groups after base-induced deprotonation is responsible for the conformational perturbation of the structure, leading to the blue-red colorimetric transition. (2) Glucose can be oxidized to gluconic acid in the presence of glucose oxidase (GOx) and as a result the pH value of the solution will decrease [31]. (3) Similarly, the hydrolysis of acetylcholine iodide catalyzed

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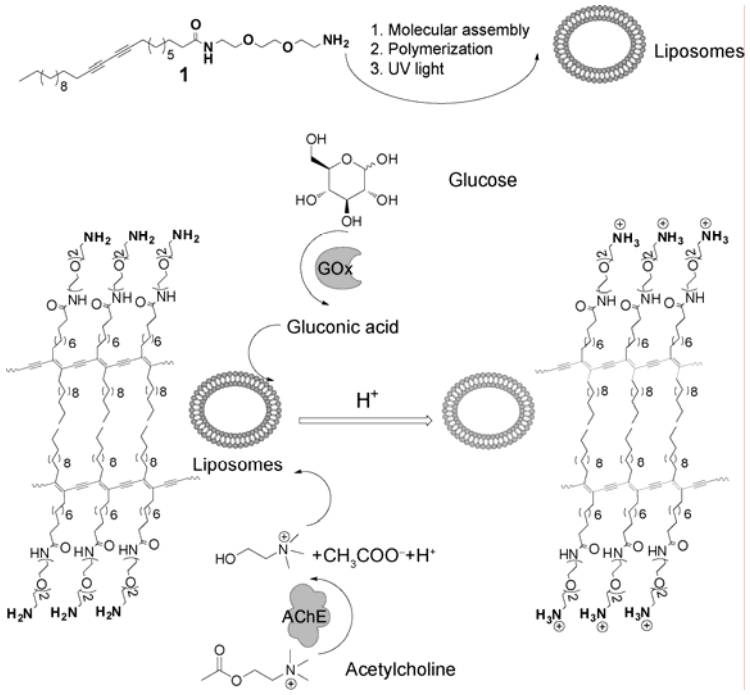

Scheme 1 Colorimetric detection of glucose and the assay for measuring acetylcholinesterase activity by combining the use of the amine-terminated polydiacetylene vesicles and the enzymatic reactions.

by AChE will lead to the generation of acetic acid and accordingly the solution will become acidic [32]. Therefore, it is possible to establish the colorimetric detection of glucose and the activity of AChE with amine-terminated PDA vesicles in the presence of GOx and acetylcholine iodide.

1 Experimental section

1.1 Materials

Acetylcholine iodide, glucose, fructose, mannose, galactose and maltose were obtained from Alfa-Aesar, whereas acetylcholinesterase and GOx from *Electrophorus electricus* were purchased from Sigma-Aldrich. The 10, 12-tricosadiynoic acid (98%), *N*-hydroxysuccinimide (NHS) and 2,2′-(ethylenedioxy)diethylamine (EDEA) were purchased from Sigma-Aldrich. Ultrapure water was obtained using a Millipore filtration system. The amine-terminated diacetylene monomer **1** (10, 12-pentacosa-diynoic acid-2-2′(ethylenedioxy) bis (ethylamine)), was obtained by reacting PCDA-NHS (*N*-hydroxysuccinimide ester of 10,12-pentac-osadiynoic acid) with an excess amount of EDEA (2,2′-(ethylene-dioxy) diethylamine) in methylene chloride, as previously described [27] . All experiments were carried out in ultra-high pure (UHP) water, except the pH responsiveness of the PDA absorption spectrum which was carried out in MES (2-(*N*- morpholino) ethanesulfonic acid, 10.0 mmol/ L) buffer (10.0 mmol/L, MES = 2-(*N*-morpholino) ethanesulfonic acid).

1.2 Apparatus

Absorption spectra were measured on a U-3010 (Hitachi) instrument. All measurements were carried out at room temperature using cells with 1.0 cm optical path lengths.

1.3 Preparation of vesicles

Diacetylene monomer **1** was dissolved in chloroform in a test tube. The solvent was evaporated by a stream of N_2 gas and deionized water was added to the test tube to give the desired concentration of **1** (2.0 mmol L^{-1}). The resulting suspension was sonicated for 5.0 min at ~80°C. After sonication, the solution was filtered to remove the aggregates with a 0.8-μm filter and cooled at 4°C overnight. PDA vesicles were prepared by UV light (254 nm, 1.0 mW cm^{-2}) irradiation of the solution for 90 s.

To quantify the extent of the blue-red color transition for the PDA vesicles, the CR (the colorimetric response) was calculated using the following equation [33]:

$$CR(\%) = [(PB_0 - PB_1)/PB_0] \times 100$$

where $PB=A_{blue}/(A_{blue}+A_{red})$, A_{blue} and A_{red} are the absorbance values at 630 nm for the blue phase and 545 nm for the red phase, respectively. PB_0 and PB_1 refer to the blue/red ratios in the absence and presence of stimuli, respectively.

2 Results and discussion

2.1 Preparation of the amine-terminated PDA vesicles and the CR to pH change

The amine-terminated PDA vesicles were prepared from diacetylene **1** containing one amino group (Scheme 1) according to the reported procedure [27]. Briefly, diacetylene

1 was dissolved in UHP water with the aid of ultrasonic treatment, and the aqueous solution was exposed to UV light (254 nm) for 90 s. The as-prepared PDA vesicles were blue in color and exhibited strong absorptions around 630 nm. The absorption spectra of the PDA vesicles were measured in MES buffer solutions of different pH values. As depicted in Figure 1(A), the absorption band around 630 nm decreased gradually, and concomitantly a new absorption band around 545 nm emerged. The intensity of the band at 545 nm increased gradually by decreasing the pH of the aqueous solutions. The color of the PDA vesicles gradually changed from blue to red as the pH was lowered. Moreover, the CR decreased almost linearly with the pH change in the aqueous solutions of the PDA vesicles (Figure 1(B)). Previous studies suggest that this spectral variation indicates the transformation of the blue-phase of PDA vesicles into the corresponding red-phase [27–30]. This structural transformation is probably induced by the protonation state of the amino groups of the vesicles in acidic solutions. Here, the electronic repulsion among the positively-charged head groups may alter the interactions of alkyl chains, and accordingly the conformation of the backbone of polydiacetylene would change.

2.2 Colorimetric detection of glucose

Glucose can be easily oxidized to generate gluconic acid and H_2O_2 in the presence of GOx. Consequently, an absorption spectral change (the "blue-red" phase transition) should occur for the amine-terminated PDA vesicles following the addition of glucose in the presence of GOx. In Figure 2(A), the absorption signal around 630 nm gradually weakened in intensity and an absorption signal at ~545 nm emerged, and the intensity of this absorption increased as the concentration of glucose increased (in the presence of GOx). Such absorption spectral variation was found to be dependent on the concentration of GOx in the solution. Figure S1 shows the plots of CR, which reflects the absorption spectral variation for PDA vesicles *vs.* the reaction time (0–15 min) for the PDA vesicle solutions containing glucose in the presence of different concentrations of GOx (0.0, 0.01, 0.05, 0.08 and 0.10 mg/mL). Clearly, the changes in the *CR* values were more remarkable for the vesicle solutions containing higher concentrations of GOx. However, as shown in Figure S2, the absorption change of the PDA vesicles was

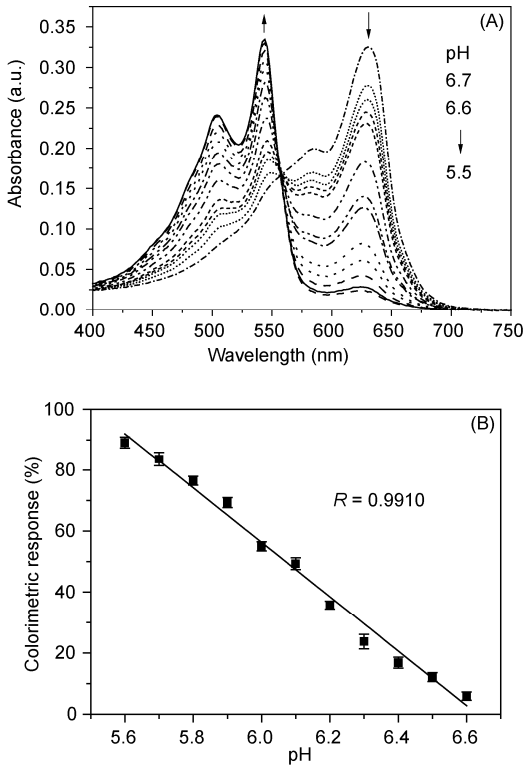

Figure 1 (A) The absorption spectra of the PDA-vesicles (50.0 μmol/L) in solutions of different pH values (6.7 to 5.5); (B) variation of the colorimetric response of the PDA-vesicles *vs.* the pH. All experiments were carried out in MES buffer (10 mmol/L) at 25°C.

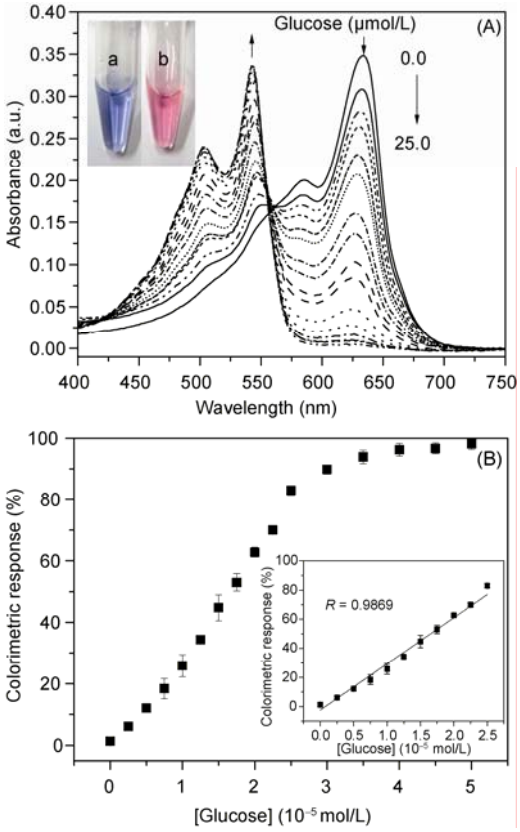

Figure 2 (A) The absorption spectra of a PDA vesicles solution (50.0 μmol/L) containing GOx (0.1 mg/mL) following the addition of different amounts of glucose (from 0 to 25.0 μmol/L); each solution was incubated for 15 min at room temperature. The inset shows the color change for the PDA vesicles (50.0 μmol/L) containing GOx (0.1 mg/mL) in the absence (a) and presence (b) of glucose (25.0 μmol/L); photos were taken after incubation at 25 °C for 15 min. (B) The plot of the colorimetric response of the PDA vesicles (50.0 μmol/L) containing GOx (0.1 mg/mL) *vs.* the concentration of glucose. Each solution was incubated at room temperature for 15 min before recording the absorption spectrum. All the experiments were carried out in UHP water.

negligible when either GOx or glucose was added.

To detect glucose with the amine-terminated PDA vesicles, the concentration of GOx must be fixed. Figure 2(A) shows the absorption spectra of the PDA vesicles (50.0 μmol/L) containing GOx (0.1 mg/mL) in the presence of different amounts of glucose after incubating at 25 °C for 15 min. After the addition of glucose, the absorption around 630 nm started to decrease and that at ~545 nm increased gradually. The plot of the corresponding CR *vs.* the concentration of glucose is presented in Figure 2(B). The CR increased linearly as the concentration of glucose increased (i.e. 0 to 25.0 μmol/L), with a detection limit of 2.5 μmol/L. The initial blue solution of the PDA vesicles (50.0 μmol/L) containing GOx (0.1 mg/mL) changed to the color red after 25.0 μmol/L of glucose was present and the solution was incubated for 15 min at 25°C, as shown in the inset of Figure 2(A). Therefore, it can be concluded that naked-eye detection of glucose can be performed with the amine-terminated PDA vesicles. Moreover, the detection limit and range can be modulated by adjusting the concentrations of the PDA vesicles and GOx.

Absorption spectra of the PDA vesicles in the presence of GOx after the addition of excess amounts of fructose, mannose, galactose and maltose were recorded to demonstrate the selectivity of the visible detection of glucose. Figure 3 shows the respective CR of the PDA vesicles in the presence of 1.0 equivalent (equiv.) of glucose and 10.0 equiv. of other saccharides. Clearly, significant colorimetric change was only detected for the sample that contained glucose. Thus, a simple, sensitive and selective colorimetric assay for glucose detection can be established by combining the use of the amine-terminated PDA vesicles and GOx.

It should be noted that various methods for glucose detection have been described. These include fluorescence [34,35] and surface plasmon resonance spectroscopic methods [36,37] as well as electrochemical approaches [38,39]. Nonetheless, the colorimetric detection of glucose based on amine-terminated PDA vesicles has the following advantages: (1) visible detection; (2) high selectivity; and (3) acceptable sensitivity.

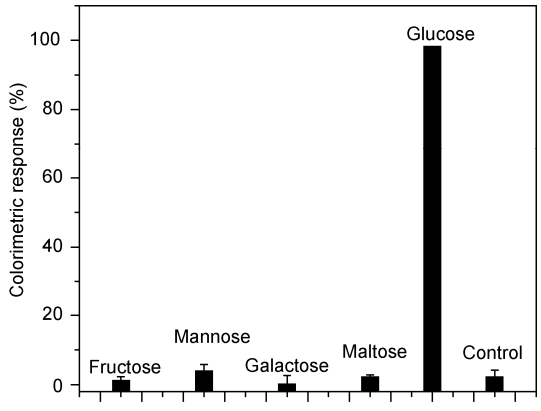

Figure 3 Variation in the colorimetric response for the PDA vesicles solution (50 μmol/L) containing GOx (0.1 mg/mL) following the addition of 1.0 equiv. of glucose and 10.0 equiv. of fructose/mannose/galactose/maltose; each solution was incubated at room temperature for 15 min. All experiments were carried out in UHP water.

2.3 Colorimetric assay for AChE activity and inhibitor-screening

Acetylcholine (ACh) is known to be a good substrate for AChE, because it is easily hydrolyzed to choline and acetic acid by AChE. Thus, it is expected that the absorption spectrum of the amine-terminated PDA vesicles will change after mixing ACh with AChE. Figure 4(A) shows the absorption spectra of the PDA vesicles (50 μmol/L) in the presence of ACh (40 μmol/L) and AChE (40 mU/mL) after various incubation times. The absorption around 630 nm gradually weakened, whereas the absorbance at ~545 nm increased. Such spectral variation was more remarkable when the incubation time was increased. This is ascribed to the generation of acetic acid from the hydrolysis of ACh catalyzed by AChE; as a result the amine head-groups of the PDA vesicles become protonated and accordingly the "blue-phase" of PDA transforms into the corresponding "red-phase" [4]. In fact, obvious color changes occurred for the PDA vesicles containing ACh after the addition of AChE and further incubation (Figure 4(B)). The initial vesicle solution (50 μmol/L) containing ACh (40 μmol/L) was

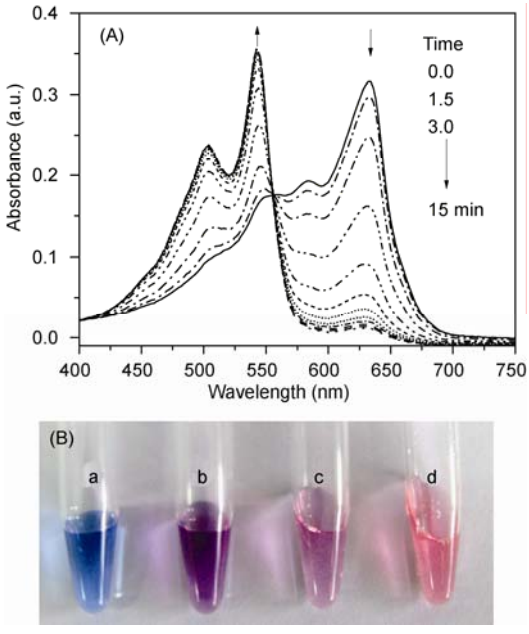

Figure 4 (A) Absorption spectra of PDA vesicle solutions (50 μmol/L) in the presence of ACh (40 μmol/L) and AChE (40 mU/mL) following incubation for different times at room temperature. (B) Photos of the PDA vesicle solutions (50 μmol/L) and those after the addition of ACh (40 μmol/L) and different amounts of AChE: a, 0; b, 20; c, 40; d, 80 mU/mL; each solution was incubated for 15 min at 25°C. All experiments were carried out in UHP water.

blue in color; however, the solution became red following the addition AChE (80 mU/mL) and incubated at 25°C for 15 min (Figure 4(B)-d). If lower amounts of AChE were added, the contrast in color change was not as apparent, but it was distinguishable as shown in Figure 4(B)-b and c, for which the concentrations of AChE in the solutions were 20 and 40 mU/mL, respectively. The absorption spectral change in the PDA vesicles was negligible when AChE or ACh was added (Figure S3).

The absorption spectra of the PDA vesicles (50 μmol/L) in the presence of ACh (40 μmol/L) were measured after introducing different amounts of AChE and further incubation for different times. As anticipated, the absorption spectra varied after the addition of AChE and further incubation. Figure 5(A) shows the variation in the CR, which measures the absorption spectral change for the PDA vesicles as mentioned above, *vs.* the reaction time (0–15 min) in the presence of different concentrations of AChE. The degree of CR increase by prolonging the reaction time was clearly more significant when the concentration of AChE in the PDA vesicles solution was higher, as depicted in Figure 5(A). For example, the CR value increased rapidly and remained essentially unchanged after incubation for ~6.0 min when the concentration of AChE reached 80.0 mU/mL. Therefore, a colorimetric assay for AChE activity was established with the amine-terminated PDA vesicles. Moreover, an AChE concentration as low as 10.0 mU/mL can be assayed under the current conditions.

ACh is a central neurotransmitter and its hydrolysis catalyzed by AChE is an important process that regulates the neutral response system [40]. Clinical treatment of Alzheimer's disease is mainly based on AChE inhibitors [41]. Therefore, the development of a reliable assay method for measuring AChE activity and screening for AChE inhibitors is of significant importance. AChE activity and inhibition behaviors are traditionally monitored with absorption spectroscopy using the Ellman's reagent [42]. Fluorometric approaches with good sensitivity have been reported for screening AChE inhibitors [43–45]. Chemiluminescent probes are also available for AChE activity assays and inhibitor screening [46]. The current colorimetric assay for AChE and inhibitor screening has the advantages having a visible assay for AChE and inhibitor screening that is fast and easily-operated.

This convenient colorimetric assay for monitoring AChE activity with PDA vesicles was further explored as a potentially suitable assay for screening AChE inhibitors. Neostigmine, a well-known inhibitor for AChE [47], was selected as an example to demonstrate the application of the PDA vesicles in screening AChE inhibitors. The color of the PDA vesicles solution changed from blue to red after introducing AChE to the solution containing ACh as detailed above. However, the rate of color change slowed upon the addition of neostigmine to the vesicles solution. At the highest neostigmine concentration examined the color of the vesicles solution remained essentially blue under the same conditions (Figure 5(C)). These results showed that neostigmine inhibits the activity of AChE and thus the hydrolysis of ACh into acetic acid is dramatically reduced. As a result of less acetic acid being produced, less absorption change is observed because the pH has not changed significantly. Therefore, it is not only possible to conveniently perform a colorimetric assay for AChE activity with the PDA vesicles and ACh, but it is also possible to screen for

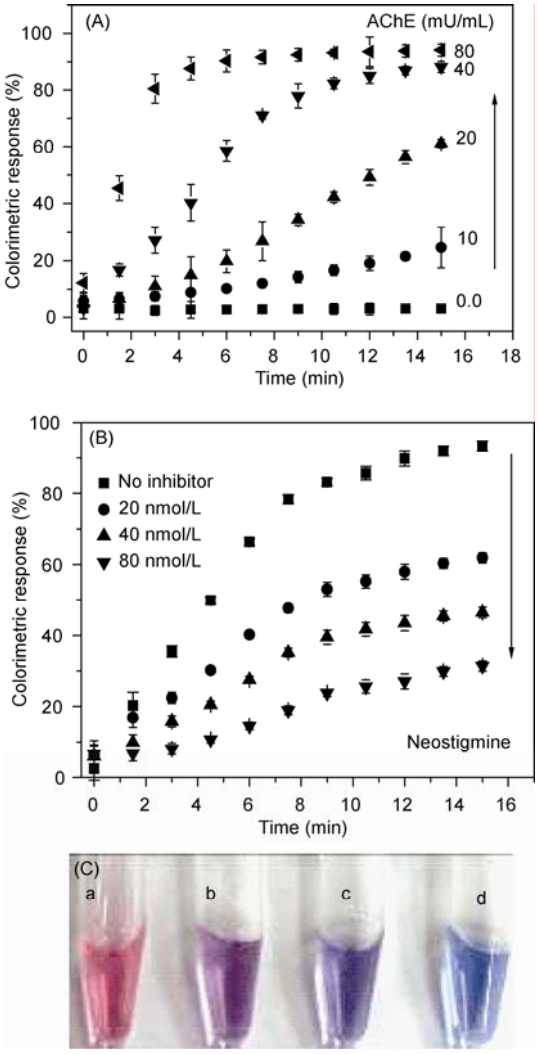

Figure 5 (A) Variation in the colorimetric response *vs.* the reaction time for the PDA vesicles solutions (50 μmol/L) containing acetylcholine (40 μmol/L) and different amounts of AChE (0, 10, 20, 40 and 80 mU/mL); each solution was incubated for 15 min at 25°C. (B) Variation in the colorimetric response *vs*. the reaction time for the PDA vesicles (50 μmol/L) containing acetylcholine (40 μmol/L), AChE (40 mU) and different concentrations of neostigmine (0, 20, 40 and 80 nmol/L); each solution was incubated for 15 min at 25°C. (C) Photos of the PDA vesicles solutions (50 μmol/L) containing ACh (40 μmol/L), AChE (40 mU/mL) and different amounts of neostigmine: a, 0; b, 20; c, 40; and d, 80 nmol/L. Each solution was incubated for 15 min at 25°C. All experiments were carried out in UHP water.

the activity of AChE inhibitors with the PDA vesicles.

Absorption spectra of the PDA vesicles containing ACh (20 μmol/L) and AChE (40 mU/mL) in the presence of different concentrations of neostigmine (0, 20, 40 and 80 nmol/L) were measured after the solutions were incubated for different times to quantify the inhibition efficiency of neostigmine toward AChE. Clearly, variation in the CR *vs.* the reaction time became smoother after the addition of neostigmine compared with that in the absence of neostigmine (Figure 5(B)). For example, when the concentration of neostigmine in the solution reached 80.0 nmol/L, the CR value was essentially unchanged even if the PDA vesicles solution was incubated for 15 min. On the basis of the plot of the CR (measured after the incubation for 6 min) *vs.* the concentration of neostigmine (Figure 6), the corresponding IC_{50} was estimated to be 31 nmol/L. This value is different from the IC_{50} values reported before [47]. However, this difference can be accounted for because the IC_{50} values usually increase as the enzyme concentration increases.

3 Conclusion

In summary, a convenient colorimetric approach for the detection of glucose and a colorimetric assay for monitoring AChE activity were successfully established using amine-terminated PDA vesicles. The colorimetric methods take advantage of the following features: (1) the amine- terminated PDA vesicles exhibit a color transition as the pH value of the solution decreases; (2) glucose can be oxidized to gluconic acid in the presence of GOx; and (3) AChE catalyzes the hydrolysis of acetylcholine into acetic acid. The "coupling" of the PDA vesicles with reactions catalyzed by particular enzymes offers a visual detection approach to assay for glucose and AChE with good selectivity and acceptable sensitivity. The detection limit of glucose is about 2.5 μmol/L, whereas the limit for measuring AChE activity is ~10.0 mU/mL. Moreover, the results clearly demonstrate the usefulness of this convenient assay for screening the potency of AChE inhibitors. Given its simplicity and easy operation, this method may be suitable for high-throughput screening of AChE inhibitors and other relevant drug discovery platforms.

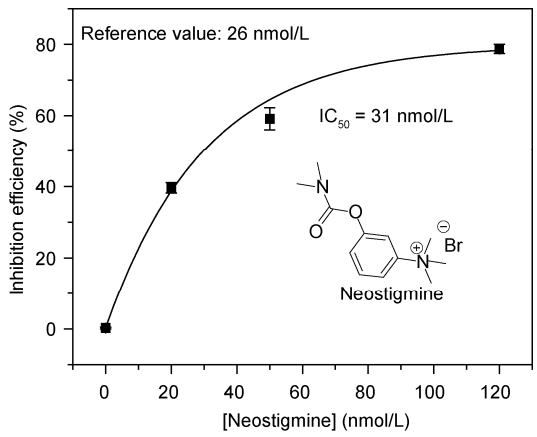

Figure 6 Inhibition efficiency of neostigmine toward AChE *vs.* the concentration of neostigmine. The data were obtained with the PDA vesicles (50 μmol/L), ACh (40 μmol/L) and AChE (40 mU/mL) in the presence of different concentrations of neostigmine (0, 20, 40, 80 nmol/L).

The work was supported by the National Natural Science Foundation of China (21075126)*, the National Basic Research Program of China* (*2010CB933502*).

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Supporting Information

Figure S1 Variation of the colorimetric re-sponse (CR) vs. the reaction time for the PDA-vesicles and glucose (40.0 μmol/L) in the presence of different concentrations of GOx (0.0, 0.01, 0.05, 0.08 and 0.10 mg/mL).

Figure S2 (A)Absorption spectra of PDA vesicles containing only glucose (a), only GOx (b) and those after glucose was oxidized in the presence of GOx for 20 min at 25°C (c); the solution under investigation was composed of PDA vesicles (50.0 μmol/L), glucose (40.0 μmol/L), and GOx (0.08 mg/mL).

Figure S3 Absorption spectra of PDA vesicles containing only acetylcholine (black), only AChE (green) and that after acetylcholine was hydrolyzed in the presence of AChE (40.0 mU/mL) for 20.0 min at 25°C (red); the solution under investigation was composed of PDA vesicles (50.0 μmol/L), acetylcholine (40.0 μmol/L), and AChE (40.0 mU/mL).

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