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Label-free detection of acetylcholinesterase and its inhibitor based on the *in situ* formation of fluorescent copper nanoparticles

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ABSTRACT

The poly(thymine) (poly T) can effectively template the *in situ* formation of copper nanoparticles (CuNPs) within several minutes under ambient conditions, offering great potential as fluorescence probe for biochemical analysis without complicated modifications. However, the exploration of poly T-templated CuNPs (poly T-CuNPs) for biochemical applications is still at its very early stage. Herein, a novel fluorescent assay has been developed for acetylcholinesterase (AChE) and its inhibitor detection based on poly T-CuNPs. In the absence of AChE, the high affinity between Cu^{2+} and thymine leads to the formation of fluorescent CuNPs. In the presence of AChE, the fluorescence of poly T-CuNPs is quenched based on the reaction between Cu^{2+} and thiocholine generating from the hydrolysis of ATCh by AChE. This detection assay is simple without the requirement for complex labeling of probe DNA and the multiple preparation procedure of fluorescent compounds. The detection assay is highly sensitive for sensing AChE in the concentration ranging from 0.11 to 2.78 mU mL^{-1} with a detection limit of 0.05 mU mL^{-1} and is feasible for screening AChE inhibitor. This method paves a new way for exploring the biosensing applications of the poly T-CuNPs.

1. Introduction

Several-atom noble-metal fluorescent nanoparticles have stimulated extensive interests due to their excellent optical, physical and electrical properties for the wide application in the field of bioassays as an promising kind of fluorophores [1–5]. Especially, DNA-scaffolded fluorescent nanoparticles have shown the great potential in biochemical applications, mainly because they exhibit fascinating properties, such as low toxicity, remarkable water-solubility, tunable fluorescence emission, facile synthesis, large Stokes shifts, high photostability and good biocompatibility [5,6]. For example, DNA-templated silver nanoclusters have been successfully used to detect a large variety of analytes, such as heavy metal ions [7,8], histidine [9], bioactive thiols [10,11], enzyme activities [12,13], DNA [4,14], micro RNA [15,16], cancer cells [17] and cocaine [18]. Recently, the obtained fluorescent copper nanoparticles (CuNPs) have been utilized for various targets detection since the report that double-strand DNA (dsDNA) can act as an efficient template for the formation of CuNPs [19–22].

Lately, Qing et al. reported that single-strand poly(thymine) (poly T) DNA can be employed as an ideal template for the preparation of fluorescent CuNPs [23]. Interesting, the obtained poly T-templated CuNPs (poly T-CuNPs) shows several extraordinary properties: (1) Its preparation procedure is simple by just mixing several reagents; (2) the formation of CuNPs is time-saving, which can be completed within several minutes after the reaction beginning; (3) the synthesis conditions are mild that make it convenient and reproducible; (4) the large MegaStokes shifting of the obtained fluorescent CuNPs enables the removal of strong background signal of complex biological systems, providing an opportunity for detection of targets from complex biological media. Therefore, the poly T-CuNPs holds great potential for constructing novel platforms for biochemical analysis as an *in situ* synthetic nanoprobe. The poly T-CuNPs have been utilized for the detections of protein [6], enzymes [24–26], heavy metal ion [27–29], DNA [30], melamine [31], hydrogen peroxide [32] and biothiols [33]. Despite these studies, the exploration of the applications of poly T-CuNPs remains at its very early stage.

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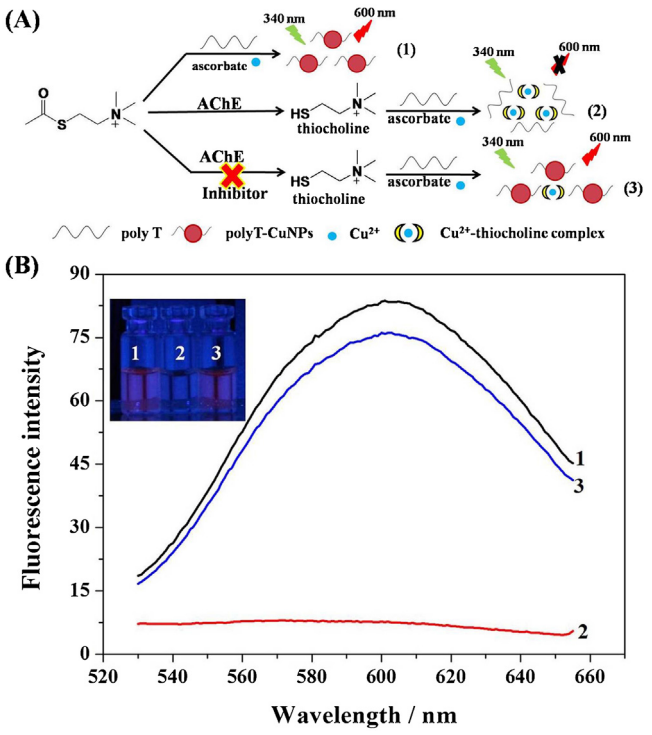

Fig. 1. (A) Schematic illustration of the poly T-CuNPs based fluorescent assay for AChE and its inhibitor detection. (B) Fluorescence spectra of poly T-CuNPs in the presence of 0.11 mM ATCh (1), 0.11 mM ATCh and 2.78 mU mL^{-1} AChE (2), 0.11 mM ATCh, 2.78 mU mL^{-1} AChE and 55.56 nM neostigmine (3). Inset shows the corresponding photographs under UV light (365 nm).

Acetylcholinesterase (AChE), a critical enzyme in the central and peripheral nervous system, plays a significant role in the regulation of the levels of the neurotransmitter acetylcholine (ACh) by specifically catalyzing the hydrolysis of ACh to choline and acetic acid [34,35]. It is widely accepted that Alzheimer's disease (AD) is associated with the low level of ACh in the hippocampus and cortex since the hydrolysis of ACh by AChE can accelerate the aggregation of amyloid-β peptide [36]. As a result, AChE inhibitors applied to increase the ACh concentration are currently used for the treatment of AD. However, excess of ACh causes neuromuscular paralysis, which is harmful to human [37]. Therefore, the construction of AChE activity and its inhibitor assays have been intensively investigated. To date, several analytical approaches such as Ellman's method [38], colorimetric method [39,40], fluorescent method [41,42], electrochemical method [43] and chemiluminescent method [44] have been developed. Among these methods, fluorescent method has drawn more attention due to its higher sensitivity, simplicity and rapidity [45]. However, most developed fluorescent methods require complex labeling or time-consuming and sophisticated synthesis process. Therefore, the development of simple and sensitive fluorescent method for AChE activity and its inhibitor is of great importance.

In this study, we design a convenient fluorescent assay for AChE activity and its inhibitor sensing by using poly T-CuNPs as the *in situ* fluorescent nanoprobe. In the absence of AChE, the poly T DNA of 40 mer (T40) can be used as the template for the formation of fluorescent CuNPs with bright fluorescence. However, the Cu^{2+} can react with thiocholine obtained from the hydrolysis reaction of acetylthiocholine chloride (ATCh) by AChE and then fails to form the CuNPs. Consequently, the AChE activity can be identified by the fluorescence changes of poly T-CuNPs.

2. Experimental

2.1. Materials and apparatus

The oligonucleotide poly T40 (5′-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3′) was synthesized by Sangon Bioltechnology (Shanghai) Co., Ltd. and purified by HPLC. AChE (from Electrophorus electricus), glucose oxidase (GOx), horseradish peroxidase (HRP), alkaline phosphatase (ALP), S1 nuclease, lysozyme, papain, proteinase K, cysteine (Cys), glutathione (GSH), homocysteine (Hcy) and histidine (His) were purchased from Sigma-Aldrich. ATCh and neostigmine bromide were obtained from Aladdin Reagent Company. All reagents were analytical grade and used as received. Ultrapure water produced by a Milli-Q system was used throughout this work.

The fluorescence measurements were carried out on a RF-5301PC fluorescence spectrophotometer (Shimadzu, Japan). The fluorescence emission spectra were collected from 530 to 655 nm at room temperature with a 340 nm excitation wavelength. The E_x and E_m slits were set at 5 and 20 nm, respectively.

2.2. Fluorescent AChE activity and inhibitor assay procedures

For AChE activity detection, 45 μL of phosphate buffer (10 mM, pH 8.0), 5 μL of 4 mM ATCh and 5 μL of different concentrations of AChE were mixed and incubated at 37 °C for 30 min. After that, 100 μL of phosphate buffer (10 mM, 150 mM NaCl, pH 7.8), 10 μL of 0.5 mM $CuSO_4$, 10 μL of 10 μM T40 and 10 μL of 15 mM sodium ascorbate were added and incubated for 35 min in dark at room temperature (20 °C) and the fluorescent spectrum of the mixture was recorded immediately.

For AChE activity inhibitor detection, 40 μL of phosphate buffer (10 mM, pH 8.0), 5 μL of 0.1 U mL^{-1} AChE and 5 μL of different concentrations of neostigmine were firstly incubated at 37 °C for 10 min. Then, 5 μL of 4 mM ATCh was added and the obtained mixture was incubated at 37 °C for another 30 min. Finally, 100 μL of phosphate buffer (10 mM, 150 mM NaCl, pH 7.8), 10 μL of 0.5 mM $CuSO_4$, 10 μL of 10 μM T40 and 10 μL of 15 mM sodium ascorbate were added and incubated for 35 min in dark at room temperature and the fluorescent spectrum of the mixture was recorded immediately.

3. Results and discussion

3.1. The design principle of the developed assay

In this work, a novel fluorescent assay for AChE activity and its inhibitor is developed based on the poly T-CuNPs. The detection mechanism of this assay is shown in Fig. 1. In this detection system, the poly T40 single strand DNA (ssDNA) can act as an efficient template for the formation of fluorescent CuNPs through the reduction by sodium ascorbate. As shown in Fig. 1B, when excited at 340 nm, the obtained CuNPs shows strong fluorescence at 600 nm (corresponding to step (1) shown in Fig. 1A). Upon addition both AChE and ATCh to the system, the fluorescence of poly T-CuNPs is greatly quenched due to the high affinity of Cu^{2+} to thiol group of thiocholine generated from ATCh under the catalytic hydrolysis of AChE (corresponding to step (2) shown in Fig. 1A). In the presence of AChE inhibitor, the hydrolysis activity of AChE to ATCh is inhibited. As a result, the quenched fluorescence is recovered due to the absence of thiocholine (corresponding to step (3) shown in Fig. 1A). When ATCh, AChE or its inhibitor is individually introduced, no obvious change appears in the fluorescence intensity of poly T-CuNPs (Fig. S1). Based on the above facts, a simple and sensitive fluorescent assay for AChE activity and its inhibitor detection is developed.