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Simple and sensitive fluorescence assay for acetylcholinesterase activity detection and inhibitor screening based on glutathione-capped gold nanoclusters

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ABSTRACT

A direct fluorescence turn-on method for simple and sensitive acetylcholinesterase (AChE) activity assay and AChE inhibitor screening has been developed by first using low-fluorescence glutathione-capped gold nanoclusters (GSH-AuNCs). The thiocholine produced by AChE-catalyzed hydrolysis of S-acetylthiocholine iodide could effectively enhance the fluorescence of GSH-AuNCs via Au—S bond formation. In the presence of inhibitors, AChE activity was suppressed and thus fluorescence enhancement decreased. Therefore, AChE activity assay and inhibitor screening could be performed by measuring the fluorescence intensity of the system. The linear range of the AChE activity assay was 0–30 mU mL^{-1} with a limit of detection of 0.03 mU mL^{-1} (S/N = 3). The IC_{50} values of two inhibitors (tacrine and neostigmine bromide) were 42.92 nM and 37.04 nM, respectively. The developed protocol provides a simple and sensitive platform for assaying AChE activity and screening its inhibitors.

1. Introduction

Acetylcholinesterase (AChE) exists throughout the central and peripheral nervous systems of animals, and helps maintain the levels of the neurotransmitter acetylcholine by catalyzing the hydrolysis of acetylcholine to choline [1]. A low level of acetylcholine is strongly associated with Alzheimer's disease (AD), since the hydrolysis of acetylcholine (catalyzed by AChE) can promote the aggregation of the amyloid-β peptide, which plays a crucial role in the development of AD [2]. Because of its importance in neurotransmission, AChE has been recognized as a drug target for the treatment of a variety of diseases including myasthenia gravis, postoperative ileus, bladder distention, glaucoma, and neurodegenerative diseases [3]. AChE inhibitors that penetrate the blood-brain barrier have proved useful in the symptomatic treatment of AD [2]. Therefore, the determination of AChE activity is important, and the inhibition of AChE with the aim of enhancing ACh levels is an effective avenue of research for AD therapies to pursue [4].

In recent years, many efforts have been made to detect the activity of AChE and screen its potential inhibitors. Currently, the most extensively used assays for the detection of AChE activity and inhibition are the traditional and improved Ellman methods; however, these are limited by their low sensitivity [5–8]. There are a number of fluorescent [9–21], colorimetric [22,23], and electrochemical AChE assays [24,25] being investigated. Of these, fluorescent methods are attracting increasing amounts of attention from researchers. In addition to methods based on fluorescent organic materials [9–12], other novel fluorophores such as semiconductor quantum dots [14], carbon dots [15–17], and noble metal nanoclusters [18–21] have been used to improve detection of AChE activity and screen its inhibitors.

Fluorescent noble metal nanoclusters, in particular gold nanoclusters (AuNCs), are an emerging and promising class of fluorophore that has recently been developed and used for biosensing and bioimaging [26–28]. Compared with organic fluorophores and semiconductor quantum dots, AuNCs are easy to synthesize, show excellent stability, and have good biocompatibility, all of which are beneficial for the development of AuNC-based enzyme activity assays [20,21,29–32]. Li et al. reported an AuNC-related AChE activity assay that functioned by direct fluorescence quenching of the denatured protein-protected gold nanoclusters through the binding of AChE hydrolysate to the gold core [20]. While Sun et al. used 11-mercaptoundecanoic acid (11-MUA)-protected AuNCs, which have their fluorescence suppressed by Cu^{2+} and fluorescence recovery by the thiocholine (TCh) derived from AChE

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hydrolysate capturing Cu^{2+}, to perform AChE assay [21]. Recently, Su et al. reported that thiols could be used to enhance the photoluminescence of glutathione-capped gold nanoparticles [33]. However, no direct fluorescence turn-on assays for enzyme activity using AuNCs have been reported, despite their improved sensitivity, selectivity, and ease of operation.

Therefore, the water-soluble, low-fluorescence glutathione-protected gold clusters (GSH-AuNCs) were synthesized and used as label-free fluorescent sensors for sensitive and selective detection of AChE activity in this paper. S-Acetylthiocholine iodide (ATCI) was used as the substrate, with AChE hydrolyzing ATCI to yield thiocholine and the resulted sulfhydryl group combining with the GSH-AuNCs to enhance fluorescence. The method is convenient to operate and allows rapid detection, with other advantages as its label-free nature, selectivity, and sensitivity.

2. Material and methods

2.1. Reagents and instruments

AChE (EC3.1.1.7) from *Electrophorus electricus* (200–1000 U mg^{-1}) was obtained from Sigma-Aldrich (Shanghai, China). ATCI and glutathione (GSH) were purchased from J&K Chemical Technology (Beijing, China). All other reagents were of analytical grade and used as received.

Ultra-pure water (18.2 MΩ cm) was produced by a Millipore purification system (Millipore, Bedford, MA, USA) and used to prepare all aqueous solutions. UV–vis absorption spectra were measured using a TU-1901 spectrophotometer (Puxi Analytic Instrument Ltd., Beijing, China). Fluorescence measurements were performed using a LS-55 luminescence spectrometer (Perkin-Elmer Company, USA) in a 300-μL quartz cuvette. Transmission electron microscopy (TEM) images were collected using a Tecnai G2 F20 (FEI) with an accelerating voltage of 200 kV.

2.2. Preparation and separation of GSH-AuNCs

The synthesis of GSH-AuNCs was based on a previously reported method [33]. In brief, GSH-AuNCs precursors were prepared by mixing aqueous solutions of $HAuCl_4$ (25 mL) and GSH (25 mL) in a 1:1 molar ratio (final concentrations, 5.0 mM each) in a 100-mL round-bottomed flask. After the mixture turned almost colorless (~10 min), it was heated at 80 °C for 2 h without any stirring, after which it had turned yellow. The yellow solution was cooled and filtered through a 0.22-μm membrane to remove any bulk gold. After this, the same volume of absolute ethanol was added to the filtrate. The turbid solution that was obtained was precipitated by centrifugation at 8000 rpm for 15 min, and then dried in a desiccator overnight.

2.3. Determination of AChE activity with GSH-AuNCs

For AChE activity assay, 30 μL of 4 mg mL^{-1} GSH-AuNCs, 12 μL of 10 mM ATCI, and 20 μL of various concentrations of AChE were mixed and then added phosphate-buffered saline (PBS, 5.0 mM, pH 7.0) to 240 μL. The final mixtures were kept at 37 °C in a water bath for 35 min. The change in the fluorescence intensity of the solution was recorded.

2.4. Detection of AChE activity in rat brain preparations and human erythrocytes

The rat brain tissue was gained from the school of physical education, Shaanxi normal university. The brain tissue was ground, diluted with PBS solution (5.0 mM, pH 7.0), and then centrifuged at 3000 rpm for 15 min. The rat brain preparation (10 mg mL^{-1}) was obtained from the supernatant. The human blood was gained from healthy volunteer. The blood was centrifuged at 3000 rpm for 20 min and the erythrocytes were obtained from the sediment. The AChE activity in the rat brain preparation and human erythrocyte samples was determined by using proposed GSH-AuNCs fluorescence method as aforementioned procedure after dilution with PBS solution.

2.5. AChE inhibitor assay

Inhibitors were pre-incubated at varying concentrations (0–200.0 nM for tacrine and 0–500.0 nM for neostigmine bromide) with AChE (20 mU mL^{-1}) in PBS (5.0 mM, pH 7.0) at 37 °C for 10 min. After this, 30 μL of 4 mg mL^{-1} GSH-AuNCs and 12 μL of ATCI (10 mM) were added and the final volume was adjusted to 240 μL. The mixtures were kept at 37 °C in a water bath for 35 min, after which the change in the fluorescence intensity of the solution was recorded.

3. Results and discussion

3.1. Characterization of GSH-AuNCs

GSH-AuNCs were synthesized as reported in the literature [33]. The obtained GSH-AuNCs were light yellow and exhibited very low emission under UV (365 nm) light. The UV–vis spectrum of the GSH-AuNCs showed absorption below 400 nm, but no well-defined adsorption peaks were observed. The as-prepared GSH-AuNCs showed low fluorescence, with excitation and emission wavelengths of ~400 and 590 nm, respectively. Morphological characterization by TEM imaging verified that the sample consisted of nanoclusters with an average size of approximately 4 nm. The GSH-AuNCs were stable for more than 6 months when sealed and stored in a dark place at 4 °C. They did not disperse well in deionized water, but were well dispersed in 5 mM PBS, pH 7.0.

3.2. Principle of AChE activity monitoring and inhibitor screening

In the presence of sufficient thiols, strongly fluorescent AuNCs can be synthesized under certain conditions [34–37]. However, when insufficient thiols are used, low fluorescence AuNCs are produced. Low-fluorescence AuNCs are mainly comprised of "thiol-insufficient" Au species, and additional thiols can be efficiently attached to the "unsaturated" surface of the AuNCs via Au—S bond formation, accompanied by significant fluorescence enhancement [33]. Fig. 1 shows that ATCI and AChE had no noticeable influence on the fluorescence spectrum of GSH-AuNCs when added separately. However, when ATCI and AChE were simultaneously present, the fluorescence of GSH-AuNCs was clearly enhanced, as it was in the presence of GSH. This shows that the fluorescence intensity of the GSH-AuNCs was enhanced by the additional thiocholine produced by the AChE-catalyzed hydrolysis of ATCI.

When an AChE inhibitor was added to the above system, AChE activity would be inhibited, reducing its ability to catalyze the hydrolysis of ATCI into thiocholine. Based on this mechanism, a simple and sensitive method for monitoring AChE activity and screening inhibitors could be developed, as shown in Fig. 2.

3.3. Optimal conditions for AChE assay

3.3.1. Optimal dose of ATCI

GSH-AuNCs (0.5 mg mL^{-1}) and AChE (30 mU mL^{-1}) were mixed with different concentrations of ATCI. Fig. 3(a) shows the variation of fluorescence with substrate concentration. The results showed