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In situ induced metal-enhanced fluorescence: A new strategy for biosensing the total acetylcholinesterase activity in sub-microliter human whole blood

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ABSTRACT

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities (i.e., total AChE) in human blood are biomarkers for theranostic monitoring of organophosphate neurotoxin-poisoned patients. We developed an ultra-sensitive method to detect the total AChE activity in sub-microliter human whole blood based on in situ induced metal-enhanced fluorescence (MEF). Both AChE and BChE can catalyze the hydrolysis of the acetylthiocholine (ATCh) substrate and produce positively-charged thiocholine (TCh). TCh can reverse the negatively-charged surface of core–shell Ag@SiO_2 nanoparticles (NPs). The negatively-charged fluorescent dye (8-hydroxypyrene-1,3,6-trisulfonic acid, HPTS) is then confined to the surface of Ag@SiO_2 NPs and generates an enhanced fluorescence signal in situ. Changes in the surface charge of Ag@SiO_2 NPs are monitored by Zeta potential, and the MEF effect is confirmed by the measurements of fluorescence time decay. AChE activity has a dynamic range of 0 U/mL to 0.005 U/mL and a detection limit of 0.05 mU/mL. The total AChE activity in the sub-microliter human whole blood could be determined; the results were further validated. Therefore, combining the AChE catalytic reaction with MEF provides a simple, ultra-sensitive, and cost-effective "in situ MEF" approach to determine the total AChE activity in human whole blood sample down to sub-microliters without matrix interferences. The strategy also allows potential usage in other tissues and other fields.

1. Introduction

Cholinesterase (ChE) is traditionally classified as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) based on its specificity for substrates and inhibitors (Sussman et al., 1991; Silman and Sussman, 2008). AChE splits neurotransmitter acetylcholine (ACh) and terminates its action (Rosenberry, 1975). The unbalanced transmission of cholinergic by abnormal AChE causes various disorders, most importantly, Alzheimer's disease or myasthenia gravis (Kochi et al., 2013; Gilboa-Geffen et al., 2007). BChE is less clearly defined but can partially compensate for the absence of AChE and split ACh (Chatonnet and Lockridge, 1989). AChE is mainly found at neuronal synapses in the central nervous system, at neuromuscular junctions, and the whole blood (erythrocyte membrane). While muscular and neuronal AChE is inaccessible for direct measurement, AChE in the blood is a reliable surrogate (Worek et al., 1999). Moreover, AChE and BChE activities (i.e., total AChE) in whole blood are biomarkers of organophosphate nerve agents or pesticide exposure because these compounds inhibit both ChEs (Lotti, 1995; Elsinghorst et al., 2013; Ge et al., 2013). Therefore, sensitive accurate determination of total AChE activities in whole blood is an important procedure in clinics, especially in point-of-care testing.

Several quantification techniques on AChE (Holas et al., 2012; Miao et al., 2010; Sabelle et al., 2002; Feng et al., 2007; Wang et al., 2009; Peng et al., 2009; Golub et al., 2013; Liao et al., 2013a, 2013b; Zhang et al., 2013; Garai-Ibabe et al., 2014) have been developed, each of which is suitable for different purposes and can be improved on user demand. The existing methods can solve problems in AChE activity determination. However, these methods cannot accurately measure AChE activity in low amounts of biological media without labor-intensive sample preparation procedures (Holas et al., 2012). The colorimetric Ellman method (Ellman et al., 1961) is the common standard for determining the total AChE activity in human blood. However, this method is disturbed by massive Soret absorbance of hemoglobin (Hb)

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at 412 nm and slow reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) with matrix sulfhydryl groups, and is limited by its low sensitivity (Worek et al., 1999).

When the fluorophores are near metal nanoparticles (NPs), their interactions cause the emitter to enhance fluorescence significantly (metal-enhanced fluorescence, MEF) (Lakowicz, 2001; Saboktakin et al., 2012; Cao et al., 2014). MEF is an emerging technology and has attracted significant attention as an optical platform in highly-sensitive bioassays for small bioactive molecules, proteins, and nucleic acids (Bharill et al., 2011; Li et al., 2012; Lee et al., 2011; Wang et al., 2015; Kinkhabwala et al., 2009). The fluorescence of a single molecule fluorophore positioned at a "hot spot" of the Au bowtie nanoantenna can be enhanced by 1340-fold (Kinkhabwala et al., 2009). This enhancement can significantly improve assay performance (i.e., detection sensitivity) (Zhou et al., 2012; Zhang et al., 2007; Bardhan et al., 2009; Attridge et al., 1991; Sokolov et al., 1998). However, its real application and commercial use remain challenging because of the difficulties in determining the enhancing factor and fabricating reproducible MEF substrates (Aslan et al., 2007; Ray and Lakowicz, 2013).

In this study, a unique MEF effect, induced in situ with the hydrolysis of acetylthiocholine (ATCh) on AChE catalysis and mediated by the charge reversal in the surface of Ag@SiO_2 core–shell NPs, has been proposed and verified. It is easy to determine the enhancing factor because the enhanced fluorescence signal itself is in situ output. An ultra-sensitive assay for total AChE activity in the sub-microliter blood sample has been demonstrated and validated based on the in situ output of the MEF signal of a negatively-charged fluorescent dye, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS). This method can easily evaluate the level of total AChE activity in human blood without laborious sample handling and matrix interferences.

2. Experimental

2.1. In situ fluorescence enhancement for detecting AChE activity

Core–shell Ag@SiO_2 NPs were prepared with 50 nm Ag core (Supporting information S2) and 8 nm SiO_2 shell (Supporting information S3) based on the method described in previous literature (Aslan et al., 2007; Qin et al., 2010; Stöber et al., 1968), with slight modifications. The extinction of the prepared Ag@SiO_2 NPs solution is kept constant (optical density of 0.55 at 433 nm) so that the concentration of Ag@SiO_2 NPs remains the same (~0.013 nM, Supporting information S3) in all experiments. In applying AChE activity assay, 750 μL of 20 nM HPTS was first added into 3.0 mL of prepared Ag@SiO_2 NPs solution and mixed well. Different amounts of AChE and 5.0 mM sodium phosphate buffer (pH=7.5) were then introduced into 595 μL of the mixture and mixed completely. Next, 3.0 μL of 0.2 mM ATCh was added and the final sample volumes were adjusted to 600 μL. Afterwards, the sample mixture was incubated at 25 °C and fluorescence spectra were recorded for a certain period. The enhanced fluorescence intensity at 510 nm was plotted as a function of incubation time. The fluorescence intensity change per minute was calculated from the slope of each straight line, which represented AChE activity. The enzyme activity was corrected for the nonspecific substrate hydrolysis with a control sample containing ATCh without AChE. The Ellman method was used to validate the activity of commercial AChE (Supporting information S4).

2.2. Determining the total AChE activity in sub-microliter human blood samples

Five whole blood samples were obtained from the antecubital veins of healthy volunteers at the Third Affiliated Hospital, Xi'an Jiaotong University using sodium citrate as an anticoagulant. The whole blood was diluted in water 100-fold with 0.03% Triton X-100. Total AChE activity was then measured at 510 nm and 25 °C. As a typical trial, 750 μL of 20 nM HPTS was added into 3.0 mL of prepared Ag@SiO_2 NPs solution (~0.013 nM, Supporting information S3) and mixed completely. Next, 10 μL of diluted blood sample was introduced into 587 μL of the mixture. Finally, 3.0 μL of the substrate (0.2 mM ATCh) was added. The emission spectrum was scanned at different time periods. The improved Ellman method (Supporting information S5) was used to determine and validate the total AChE activity in the blood samples. An automatic hemocytometer was used to detect Hb concentration.

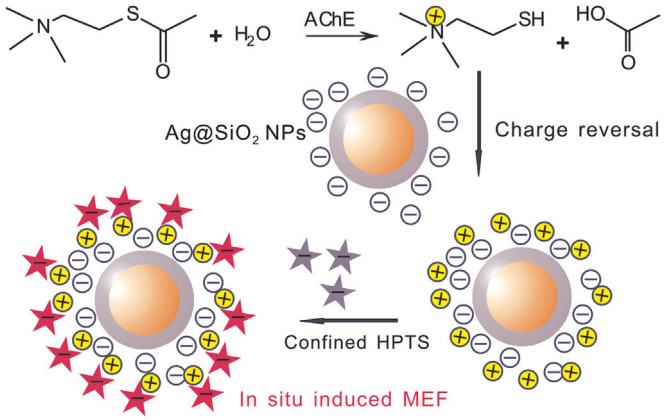
3. Results and discussion

3.1. Concept and experimental verification

For suitable MEF, the optimal diameter of Ag NPs was from 50 nm to 70 nm (Lakowicz et al., 2008) and the optimal distance for Ag NP-induced MEF was approximately between 7 nm to 10 nm (Lakowicz et al., 2004) and 5 nm to 11 nm (Bardhan et al., 2009; Li et al., 2011). Core–shell Ag@SiO_2 NPs were employed for ultrasensitive MEF with numerous fluorophores to easily control the distance with nanometer accuracy (Aslan et al., 2007). The silica shell produced a controlled dielectric environment that improved precision in MEF-based sensing. The fluorescence signal increased when the fluorophore was fixed to the outer-silica shell. The fluorescence enhancement factor was optimized by modulating the shell thickness (Arifin and Lee 2013; Akbay et al., 2012).

Scheme 1 illustrates the new concept for detecting AChE activity. The surface of the core–shell Ag@SiO_2 NPs is negatively charged. AChE can catalyze ATCh hydrolysis to thiocholine (TCh), which is positively charged due to the presence of positively charged quaternary ammonium groups in the thiocholines. Thus, the surface charge of core–shell Ag@SiO_2 NPs is reversed because of the electrostatical adsorption of positively-charged TCh. The surface charge reversal proves that negatively charged fluorescent molecule HPTS can be confined to the surface of core–shell Ag@SiO_2 NPs through electrostatic interaction. Thus, a MEF effect is generated in situ and the output signal of the enhanced fluorescence is collected to determine AChE activity. Although the use of core–shell Ag@SiO_2 NPs-based MEF to detect adenosine-5′-triphosphate and pH has been previously reported (Bai et al., 2013; Lu et al., 2014), none of the related assays combined biochemical or chemical reactions to mediate the MEF effect in situ.

HPTS consists of a pyrene core with three sulfonate groups and a single hydroxyl group (Fig. 1 shows the HPTS structure, inset)



Scheme 1. Principle of in situ induced MEF for determining the total AChE activity.