A Highly Sensitive Gold-Nanoparticle-Based Assay for Acetylcholinesterase in Cerebrospinal Fluid of Transgenic Mice with Alzheimer’s Disease

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This study provides a highly sensitive and selective Rhodamine B-modified gold nanoparticle (RB-AuNP)-based assay with dual readouts (colorimetric and fluorometric) for monitoring the levels of acetylcholinesterase (AChE) in the cerebrospinal fluid (CSF) of transgenic mice suffering from Alzheimer’s disease (AD). The use of AuNPs as colorimetric assays has recently become an attractive system because molecular events can be easily transformed into color changes, which corresponds well with the change of absorption spectrum or surface plasmon resonance.[1] The past few years have witnessed a variety of AuNP-based colorimetric sensors for various analytes such as metal ions,[2] anions,[3] small organic compounds,[4] proteins,[5] and DNA.[6] However, most reported colorimetric detection schemes still lack sufficient sensitivity; a problem that limits the broad application of this kind of assay in complex samples for practical applications. In addition, many components such as biothiols in real samples may interfere with the detection, generating false positive results. Therefore, it is necessary to create a scheme to enhance the sensitivity and selectivity of this detection so that complex samples could be diluted many times and tiny amounts of analyte could still be effectively detected while interferences can be avoided.[7]

AChE is an enzyme that can catalytically break down acetylcholine at cholinergic synapses, resulting in the termination of synaptic transmission. Much research has provided evidence that the AChE level in the CSF of individuals suffering from AD is significantly reduced, and a reduced level of AChE correlates well with a certain degree of the cognitive impairment.[8] The low level of AChE in the CSF may indicate a brain at risk or that the person is in the preclinical stage of AD. This information could be useful for early prevention and treatment of the disease due to the fact that the current diagnosis of AD requires not only the presence of severe cognitive deficits but also post-mortem confirmation of the presence of the typical AD histopathologic changes in the brain.[9] There is still no accurate method for identifying AD in an early or asymptomatic stage of the disease.[10] More and more evidence has shown that the use of CSF biomarkers (levels of β-amyloid protein, total tau protein, and phosphorylated tau protein, etc.) on their own or in combination with neuroimaging or/and biomarkers may provide useful complementary information and thus improve the accuracy of the clinical diagnosis of AD.[11] The AChE levels in the CSF, which correlate well with the degrees of the cognitive progression of AD, could be applied as an important parameter to monitor the progression of AD and the effects of treatment, especially in conjunction with other CSF biomarkers as well as neuroimaging techniques. It is therefore important to develop reliable tools for measuring the AChE level in the CSF.[12,13]

Traditional methods to measure the level of AChE include the colorimetric method by using Ellman’s reagent[13] and the detection of hydrogen peroxide produced by oxidation of the AChE-induced choline.[14] Both methods lack sufficient sensitivity and require time-consuming procedures. In order to enhance the sensitivity, advanced chemical methods such as electrochemical probes[15] and chemiluminescent or fluorescent assays for AChE, based on organic compounds[16] and quantum dots,[17] have played important roles. These approaches, however, still lack sufficient sensitivity and require a tedious chemical synthesis.

Two kinds of AuNP-based colorimetric assays for AChE have been reported: one is based on the aggregation of AuNPs, which causes the red-shift of the absorption band along with a color change from red to blue[18] while the other relies on an AChE-catalyzed enlargement of the size of the monodispersed AuNPs, which leads to a change of the maximum absorbance.[19] However, researchers still face the challenge of improving sensitivity and accuracy of the assays and, more importantly, no report has assessed their utility in detection of analytes in real samples of complex mixtures such as the CSF or serum. Therefore, the development of sensitive and selective assays for AChE in real samples is of high demand.

Herein, we present a highly sensitive assay for AChE by using RB-AuNPs and demonstrate its utility in sensing AChE levels in the CSF of transgenic mice suffering from AD (Scheme 1). RB is an ideal ligand for this assay, because it is water-soluble, photostable, strongly fluorescent, and readily...
adsorbs onto surfaces of AuNPs to result in quenched fluorescence. The positively charged amino groups on RB molecules are capable of recognizing the negatively charged citrate-AuNPs via electrostatic interactions, thus attaching onto surfaces of AuNPs. Simultaneously the fluorescence of RB molecules is quenched by AuNPs. Upon the addition of both acetylthiocholine (ATC, an analogue of acetylcholine) and AChE into a RB-AuNPs solution, AChE could hydrolyze ATC to generate thiocholine. Thiocholine strongly binds onto surfaces of AuNPs via the formation of Au–S bond to replace RB molecules, resulting in the desorption of RB molecules from Au surfaces to recover the fluorescence of RB (Figure 1b). At the same time, thiocholine and the residual RB molecules attached to different AuNP surfaces may be able to interact via electrostatic interaction between the quaternary ammonium group on thiocholine and the acidic group on RB and cause the aggregation of AuNPs. This process resulted in a rapid change of the absorption band as well as the color change of the AuNPs solution from red to blue (Figure 1a).

In addition, if AChE inhibitors were present, AChE fails to catalyze ATC and thus to generate the thiocholine that is required to either cause aggregation of RB-AuNPs or recover the fluorescence of RB molecules. We thus anticipate that both the color change of the AuNP solution and the fluorescence recovery of RB molecules can be applied as an effective tool to detect AChE in complex samples and screen its inhibitors. Furthermore, the two simultaneous outputs can also be used to avoid a -positive signal.

As a proof of concept, we first synthesized AuNPs using citrate as the reducing agent as well as the stabilizer. The as-prepared citrate-AuNPs were red in color and showed a typical absorption band at 520 nm, which was attributed to the surface plasmon resonance of AuNPs, which are about 13 nm in size. We prepared RB-AuNPs by allowing various final concentrations of RB (0–2.0 μM) to mix with a fixed concentration of AuNPs (5 nM) under mild stirring, the mixtures were prepared in 2.5 mM NaHCO₃-NaOH buffer (pH 10.0). The fluorescence spectra of the RB-AuNPs solutions were recorded after 2 h of equilibration. We found the optimal concentration of RB to be 1.2 μM, when very weak fluorescence was observed, indicating that no excess RB was free in the solution (Figure S1 in the Supporting Information). We reasoned that RB molecules are positively charged, thus can readily attach onto the negatively charged citrate-AuNPs via electrostatic interactions, which results in the formation of RB-AuNP assemblies and the quenched fluorescence of RB by AuNPs. The absorption band of RB-AuNPs was similar to that of the citrate-capped AuNPs, and the color of AuNPs solution remained red after modifying with RB molecules (Figure 1a).

![Scheme 1](image)

**Scheme 1.** The detection (colorimetric and fluorometric) of AChE based on RB-AuNPs. The well-dispersed RB-AuNPs (red) are induced to aggregate (purple) via electrostatic interaction in the presence of thiocholine derived from the hydrolysis of ATC catalyzed by AChE in the CSF of transgenic mice, accompanied with the fluorescence recovery of RB (the color of the stars changed from gray to green).

To confirm the proposed mechanism, we conducted zeta-potential measurements to investigate the change of the surface charge of RB-AuNPs before and after incubation with both AChE and ATC. The charge on RB-AuNPs is negative because of the acidic groups on RB. The zeta potential of well-dispersed RB-AuNPs was about −38 mV, while that of the AChE-induced aggregates of RB-AuNPs increased to be −0.27 mV (Figure S2), most likely due to the presence of positively charged quaternary ammonium groups in the thiocholines derived from the AChE-catalyzed hydrolysis of ATC. The aggregation process was also supported...
by dynamic light scattering (DLS) data (Figure S3). The average hydrodynamic diameter of well-dispersed RB-AuNPs was 21 nm, while that of the AChE-induced aggregates of RB-AuNPs increased to 400 nm, congruent with transmission electron microscopy (TEM) analysis (Figure S4a, b). In addition, we tried to analyze the ligands on surfaces of well-dispersed RB-AuNPs and their aggregates, respectively, by using high-resolution TEM. However, the organic layers on surfaces of RB-AuNPs were too thin to be observed, similar to previous reports (Figure S4c,d). The aggregation process was further confirmed by UV–vis absorption. UV–vis spectroscopy showed that with the formation of aggregates, the absorption band of RB-AuNPs at 520 nm decreased, accompanied by the emergence of a new absorption peak between 600 and 800 nm (blue curve, Figure 1a). At the same time, the detached RB molecules from Au surfaces were detected by fluorescence spectroscopy (Figure 1b). The very weak fluorescence of the attached RB molecules on AuNPs increased significantly after adding AChE and ATC. All the above-mentioned characterizations supported our proposed mechanism for this detection system.

We next investigated the detection limit of this assay for AChE in aqueous solutions. To a mixture of ATC (20 μM) and RB-AuNPs solutions (5 μM) we added various amounts of AChE to final concentrations of 0, 1.0, 2.0, 3.0, 4.0, and 5.0 μM. We allowed the solutions to incubate at room temperature for 21 min. The absorption spectra were recorded every 3 min during the hydrolysis of ATC catalyzed by AChE. We note that the aggregation of RB-AuNPs was a linearly dynamic process. The degree of aggregation of AuNPs depended on the concentration of AChE, where higher concentrations of AChE induced more complete aggregation. Figure 2a shows the concentration-dependent color change, which was further demonstrated by the change of UV–vis absorption after 20 min of incubation time. With the increase of the concentration of AChE, the absorbance at 520 nm decreased gradually, along with an increase in the absorption band between 600 nm and 800 nm (Figure 2c). It is worth noting that with the addition of such low concentrations of AChE (0–5.0 μM), the absorption peaks spanning from 600 to 800 nm were unable to be observed clearly. We reasoned that the aggregation of AuNPs was incomplete, and thus the color of the solutions changed from red to purple gradually. As a result, the new peak between 600 nm and 800 nm was not so distinct as that shown in Figure 1a, where the concentration of AChE was 1 U·mL⁻¹. The changes in UV–vis absorption spectra were quantified by calculating the changes of the ratio A/D (aggregated/dispersed area) for the area under the absorption peak, while their corresponding changes in fluorescence spectra were monitored by calculating the changes of fluorescence intensity (F/F₀). The integrals for region D, (D, dispersion, absorption area spanning from 450 to 570 nm) and region A, (A, aggregation, absorption area spanning from 580 to 750 nm) under the curve were computed for all samples (Figure S5). Measuring the plots of the Δ ratio A/D versus the concentrations of AChE shows that the detection limit can reach 1.0 μM·L⁻¹ (Figure 2e). Moreover, the aggregation is a time-dependent process. Figure S6 shows the plots of the Δ ratio A/D versus the incubation time (0–21 min) for various concentrations of AChE. Larger Δ ratios A/D indicate higher degrees of aggregation. Almost all Δ ratios A/D increased with the incubation time, except that of the blank sample (0 μM·L⁻¹), which showed a negligible change in the Δ ratio A/D. We conclude that the degree of aggregation of RB-AuNPs is in proportion to the concentrations of AChE and the incubation time. The detection limit can be further improved by monitoring the fluorescence of the detached RB to as low as 0.1 μM·L⁻¹ (Figure 2b,d,f), which, to our knowledge, is much lower than most AuNP-based probes for AChE.

We evaluated the interference of this assay from other species such as biothiols and proteins, particularly those that exist in human body. Biothiols (0.1 mM for each biothiol) such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) contain thiol groups. They are similar to thiocholine that can readily adsorb onto surfaces of AuNPs via Au–S bonds, thus
potentially removing RB molecules from Au surfaces. However, biothiols, unlike ATC, were unable to cause aggregation of AuNPs, because they lacked the positively charged quaternary ammonium groups that were capable of interacting with the acidic groups on RB. Certain proteins such as human serum albumin (HSA) containing thiol groups exhibit functions similar to biothiols, i.e., they can abstract RB molecules from Au surfaces. They however also failed to induce aggregation of AuNPs due to the lack of the quaternary ammonium groups. We also investigated interferences from other proteins (1 U mL⁻¹ for each protein) that are commonly used in bioanalysis, such as phosphatase alkaline (P. Alka), phosphatase acid (P. Acid), acylase I (Acyl. I), β-galactosidase (Galac), and glucose oxidase (Glu. O). All these proteins had negligible capability in changing the color of RB-AuNPs solutions, although some of them, such as P. Alka, P. Acid and Glu. O could turn on the fluorescence. Each biothiol and protein was incubated with ATC (20 μM) and RB-AuNPs (5 nM), and we set the sample with only ATC as the blank and the presence of both AChE (0.1 U mL⁻¹) and ATC (20 μM) in RB-AuNPs solution as positive comparison. None of the biothiols and proteins were able to cause aggregation of RB-AuNPs (Figure S7), but those containing thiol groups could trigger the recovery of fluorescence of RB molecules (Figure S8), which fully agreed with our design.

The use of dual readouts in this assay is a remarkable advantage over most reported assays that only have one output signal. As mentioned above, biothiols have the similar function as that of thiocholine in the fluorescence assay, i.e., removing RB from surfaces of AuNPs, thus leading to recovery of fluorescence, but they are unable to cause aggregation of AuNPs, which was monitored by the naked eye. Both the condition of recovery of fluorescence and the condition of color change of solutions have to be met to indicate the presence of AChE-catalyzed thiocholine. This requirement effectively avoids interference from biothiols, thus guaranteeing the accuracy of the detection results.

Encouraged by the above-mentioned investigations, we evaluated if the probe we described here could be utilized to monitor AChE in complex samples such as CSF. CSF, an ideal source that reflects the metabolic and pathological states of the central nervous system more directly than any other bodily fluids, is more accessible and less costly than neuroimaging that is sometimes used for clinical diagnosis of AD. In order to demonstrate the practical potential of this assay for AChE in CSF, we obtained transgenic mice prone to age-related learning and memory deficits. SAM prone/8 (SAM-P8) is a common model of age-related dementia of the Alzheimer phenotype, in whose CSF the level of AChE is much lower than that in the SAM resistant/1 (SAM-R1), which is another species of SAM that shows normal aging characteristics. We treated SAM-P8 mice with neostigmine (a known drug for the treatment of AD in the clinic) by high (5.0 mg kg⁻¹ day⁻¹) and low (0.05 mg kg⁻¹ day⁻¹) doses respectively for 10 days, and chose those treated with the equal volumes of sodium phosphate buffer (pH 7.4) as controls.

Because this assay is highly sensitive and selective, a very small amount of sample is required for the detection. We just need to add 0.5 μL of each CSF sample into a 1.0 mL mixture of ATC (20 μM) and RB-AuNPs (5 nM), and incubate them for 20 min. The addition of such a little amount of CSF sample can not only avoid the possible interference from biothiols in CSF, but also make the color changes of AuNP solutions and their corresponding fluorescence recovery amongst the four samples more apparent than those with a large amount of added CSF samples. Upon the addition of a large amount of CSF the changes in color and fluorescence recovery amongst the four samples were similar, i.e., the color of the four samples changed to purple, and, correspondingly, their fluorescence recovered in a similar level. Therefore, after adding 0.5 μL of each CSF sample into the mixture of ATC and RB-AuNPs, the degrees of color change amongst the CSF samples were different (top in Figure 3a). For the SAM-R1 sample (sample 1), the color of the solution changed from red to purple within 20 min, while that of the SAM-P8 (sample 2) with the same treatments remained red. For the SAM-P8 samples treated with neostigmine, the degrees of aggregation of AuNPs for the high dose (sample 3) was higher than that of the low dose (sample 4; sample 3 was more purple in color than sample 4), both of which are, however, more purple than that of the SAM-P8 treated with sodium phosphate buffer (pH7.4), and less purple than that of the SAM-R1. We ascribed the color change to the concentrations of AChE in

Figure 3. Monitoring the level of AChE in CSF of transgenic mice. a) Top: color change after adding four samples (1, 2, 3, 4), to each of which (0.5 μL) was added into 1.0 mL mixture of RB-AuNPs (5 nM) and ATC (20 μM). Bottom: the four CSF samples were pretreated with 100 nM of galantamine, and then RB-AuNPs (5 nM) and ATC (20 μM) was added. b) Fluorescent images of (a). c) Absorption responses of (a). d) The corresponding changes of fluorescence intensity of (b). 1: SAM-R1 sample treated with the equal volume of sodium phosphate buffer (pH 7.4); 2: SAM-P8 sample treated with the equal volume of sodium phosphate buffer (pH 7.4); 3: SAM-P8 sample treated with high dose (5.0 mg kg⁻¹ day⁻¹) of neostigmine; 4: SAM-P8 sample treated with low dose (0.05 mg kg⁻¹ day⁻¹) of neostigmine.
different samples. As published results showed that the level of AChE in CSF of AD brain is significantly decreased, we reasoned that the level of AChE in SAM-P8 sample without treatment of neostigmine is much lower than that of the SAM-R1 sample, thus the former remained red while the latter caused the complete aggregation of AuNPs. After 10 days of persistent treatment with neostigmine, SAM-P8 mice treated with a high dose of neostigmine become more active in their behaviors and their CSF also contain a higher level of AChE than those treated with low dose of neostigmine. This result is exciting because the levels of color change of solutions correlate well with the doses of treated drug.

In order to further confirm the results of color changes, the ratios of A/D were used to measure the degree of aggregation of AuNPs (Figure 3c, black bars). To ensure that the aggregation was specifically caused by AChE in CSF, we pretreated the four CSF samples with 100 μM of galantamine (another kind of commercial AD drug) to inhibit AChE, and the degree of RB-AuNPs solutions indeed remained red (bottom images in Figure 3a) and the ratios A/D displayed negligible difference (Figure 3c, red bars). Moreover, the recovery of RB fluorescence for the four CSF samples was still different, higher levels of AChE in SAM-R1 CSF induced stronger fluorescence recovery (top images in Figure 3b), and pretreatment of the CSF samples with AD drug could prevent the turn-on of fluorescence (bottom images in Figure 3b). The fluorescence variations were confirmed by comparing the F/Fo values of the CSF samples (Figure 3d).

To establish calibration curves to quantitatively measure the levels of AChE in CSF using this present assay, we need to find the smallest volume of SAM-P8 CSF sample as the background where the AChE level is undetectable. The background samples were then spiked with various concentrations of AChE. As shown in Figure S9, this smallest volume of CSF sample was determined to be 0.125 μL (incubated with a 1.0 mL mixture of ATC (20 μM) and RB-AuNPs (5 μM)) and then used as the background to establish the calibration curves. With a standard addition method, we established two calibration curves: one was based on the ratios A/D, the other was based on the F/Fo values. As shown in Figure S10, the sensitivity of this assay based on the F/Fo values was higher than that based on the A ratios A/D and, moreover, the detection range of this assay based on the F/Fo values was wider than that based on the A ratios A/D. Thus, we chose the calibration curve based on the F/Fo values to measure the AChE levels in the CSF samples. Owing to the F/Fo-value-based calibration curve (Figure S10b) and the changes of the F/Fo values (Figure 3d) for CSF samples, the AChE levels in the four CSF samples can be calculated approximately to be 3.1 U mL−1, 0.56 U mL−1, 1.1 U mL−1, and 0.85 U mL−1. These results indicate that the treatment of AChE inhibitors could cause a dose-dependent variation of the AChE levels in CSF, which agreed with the reported results. To the best of our knowledge, this is the first report in which an AuNP-based assay was utilized to monitor AChE in CSF samples of clinical relevance.

In conclusion, we present a highly sensitive and selective assay with dual readouts (colorimetric and fluorometric) for AChE based on RB-AuNPs. The detection limit is 0.1 nM mL−1, much lower than that of reported assays for AChE. We demonstrate the practical application of this assay by measuring the levels of AChE in CSF of transgenic mice and monitoring the disease progression and drug treatment effects for AD. We believe that this RB-AuNP-based assay could be useful for monitoring AChE in human CSF for early diagnostics and prognostics of AD, especially in combination with other currently existing neuroimaging techniques and CSF biomarkers as well as other settings such as lab-on-a-chip systems.

Experimental Section

**Preparation of Transgenic Senescence-Accelerated Mouse CSF Samples:** SAM-P8 and SAM-R1 mice at 3 months of age were obtained from the Department of Laboratory Animal Science, Peking University Health Science Center (Beijing, China). All mice were maintained in an AAALAC-accredited facility and the use of animals was approved by the Animal Care and Use Committee of National Center for Nanoscience and Technology (2010-0013). SAM-P8 mice were treated with high doses (5.0 mg kg−1 day−1) and low doses (0.05 mg kg−1 day−1) of neostigmine for 10 days, and meanwhile both SAM-P8 and SAM-R1 treated with sodium phosphate buffer (pH 7.4) were used as controls. The positive and negative control groups were randomly chosen. After anaesthetising each mouse with 0.8 mL of 3.5% chloral hydrate dissolved in normal saline, the CSF samples were collected using a microinjection, whose needle was connected with a plastic pipe terminated in another needle. The CSF samples were centrifugalized at 1000 rpm for 5 min, the resulting supernatants were collected for the following detection experiments.

Other experimental details are provided in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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[22] a) M. Stobiecka, J. Deeb, M. Hepel, Biophys. Chem. 2010, 146, 98;


