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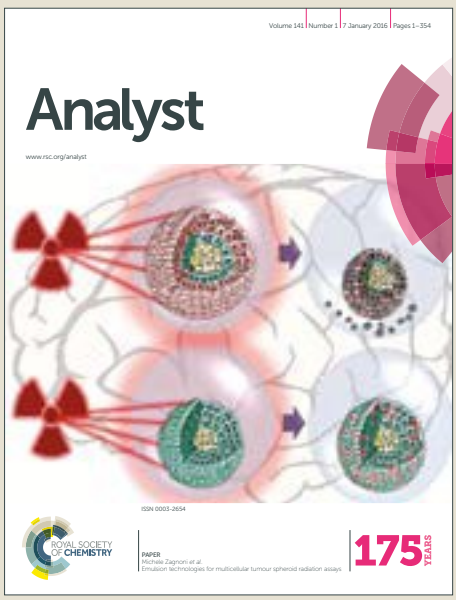




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# Novel electrochemical aptamer-antibody sandwich assay for the detection of tau-381 in human serum

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**Abstract:** Tau protein plays a crucial role in the pathogenesis of Alzheimer's disease (AD). However, the assay to detect low concentrations of tau protein is a great challenge for early diagnosis of AD. We will outline a novel aptamer-antibody sandwich assay based on an electrochemical biosensor for the detection of tau-381 in human serum. To improve the detection sensitivity, the aptamer-antibody sandwich assay for the detection of tau-381 was developed by using tau antibody (anti-tau) and aptamer specific to tau-381 as the recognition element and cysteamine-stabilized gold nanoparticles (AuNPs) as the signal amplification. Differential pulse voltammetry (DPV) was employed to record the signal response of tau-381 with different concentrations. The tau-381 concentration ranged from 0.5 pM to 100 pM. The responses of DPV measurements showed excellent results in this dynamic range. This simple, rapid, highly sensitive and specific assay gave a low limit of detection (LOD) of 0.42 pM for tau-381. The feasibility and reliability of the assay were verified by testing tau-381 in human serum from patients with AD. Thus, this method could prove valuable in diagnosing AD within the early stages of the disease.

**Keywords:** Alzheimer's disease; aptamer-antibody sandwich assay; electrochemical biosensor; tau-381; human serum

## 1. Introduction

Nowadays, Alzheimer's disease (AD) affects 47 million people worldwide,[1] with numbers expected to increase to more than 131 million by 2050 due to an aging population,[2] making this a severe threat to human health.[3] Thus, early diagnosis of AD is key for prevention and treatment.

Tau protein is an established pathological hallmark of AD,[4] and tau-381 is one of six tau isoforms ranging from 352 to 441 amino acids in length,[5] with crucial implications in the early diagnosis of AD as a critical biomarker. Tau protein levels in cerebrospinal fluid (CSF) increase with age. Meta-analyses of studies confirmed that a boundary of 4.3 pM tau in CSF can differentiate AD cases with 92% sensitivity and 89% specificity [6], and B. Olsson et al. demonstrated that blood levels of tau protein were significantly associated with AD.[7] Only few electrochemical methods have been reported for detection of tau protein, which are simple，rapid and have high specificity.[8-11] Table 1 lists the comparisons of different biosensors for tau detection. However, the main difficulty with a real sample analysis is the detection of ultra-trace tau-381 using the biosensor.

**Table 1.** The comparisons of different biosensors for tau detection

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| Target | Biosensor specification | Electrochemical techniques | Linear rang of detection | Detection limit | Reference |
|---|---|---|---|---|---|
| | Tau–Au surface | CV and EIS | 0.2-1.0 μM | 0.2 μM | 8 |
| Tau-441 | Oriented antibodies(39E10) | EIS and CV | 0.01 pM-10 nM | 0.03 pM | 9 |
| | Anti-Tau nanoimmunosensor | DPV and EIS | 0.5-15.1 nM | 0.15 nM | 10 |
| T-Tau | Anti-T-Tau (T-Tau antibody ) | DPV | 1000 pg/mL-100,000 pg/mL | – | 11 |
| Tau-381 | aptamer-antibody sandwich | EIS, CV and DPV | 0.5 pM -100 pM | 0.42 pM | This work |

CV: Cyclic Voltammetry; EIS: Electrochemical Impedance Spectroscopy; DPV: Differential Pulse Voltammetry.

Aptamers are used in biosensors for AD-related research because of high affinity and specificity to targets.[12] Taking the urgency of tau-381 quantitative detection and the advantages of aptamers into consideration, we introduce a novel electrochemical assay based on aptamer-antibody sandwich and the development of an electrochemical biosensor for sensitive determination of tau-381. This approach combines the advantages of high affinity of aptamer with the signal amplification of the gold nanoparticles (AuNPs), resulting in ultra-sensitive detection means for tau-381 in human serum.

## 2. Experiment

### 2.1 Reagents

In this study, all reagents were analytical reagent (AR) grade and obtained from commercial sources. 3-mercaptopropionic acid (MPA), $HAuCl_4 \cdot 3H_2O$, sodium citrate, cysteamine (CS), bovine serum albumin (BSA), N1-((ethylimino)methylene)-N3 (EDC) and N-Hydroxysuccinimide (NHS) were from Sigma Aldrich. Aptamer 5'-$H_2$N-GCGGAGCGTGGCAGG-3' was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Antibody tau (anti-tau) and tau-381 protein were provided by Abcam. $NaBH_4$, KOH, $H_2SO_4$, $HNO_3$, $K_4Fe(CN)_6$, $K_3Fe(CN)_6$, ethanol and other reagents were acquired from Sinopharm Chemical Reagent Co., Ltd. (China).

### 2.2 Pretreatment of the gold electrode

The gold electrode was polished with 0.3 μm alpha-alumina powder and then cleaned ultrasonically with ethanol and deionized water for 3 minutes，subsequently immersed in turn to 2 M KOH solution, 0.92 M $H_2SO_4$ solution and 0.8 M $HNO_3$ solution for 15 minutes, and rinsed with copious amount of deionized water between each step. After drying with nitrogen, the gold electrode was placed in 0.5 M $H_2SO_4$ via cycling between the potential +0.4 to +1.7 V until a reproducible CV was obtained. Then, it was rinsed with deionized water, and dried in a steam of nitrogen. This pretreatment led to an increase in electrode catalytic activity and a decrease in electrode charge transfer resistance.

The MPA modified electrode via an Au-S covalent bond was prepared by placing the gold electrode in 250 mM solution of MPA in 100% ethanol for 24 h at room temperature, rinsed with deionized water, and blown dry with nitrogen.

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## 2.3 Preparation of aptamer-Au-CS bioconjugate

The stabilized AuNPs with a size of 4 nm were synthesized as follows[13]: 0.5 mL of 0.01 M $NaBH_4$ solution was freshly prepared, and added to a mixture solution composed of 0.5 mL of 0.01 M $HAuCl_4$ trihydrate solution, 0.5 mL of 0.01 M sodium citrate solution and 18 mL of deionized water. The mixture solution was mixed and stirred until the solution color changed from colourless to orange. Stirring was stopped and the reaction vessel was left undisturbed for 2h. Fig. 1A exhibites the transmission electron microscope (TEM) image of AuNPs and illustrates that synthesized AuNPs are of a size of 4nm. Then, 0.5 mL of 0.03 M CS was added into AuNPs solution, the mixture (Au-CS solution) was heated at 50℃ for 2h. 17 μL of aptamer aqueous solution (100 μM) was added into the 2.0 mL of Au-CS solution and incubated for 3h (140 rpm) at room temperature and the bioconjugate of aptamer-Au-CS was obtained. In the chemical synthesis procedure, CS was immobilized onto the surfaces of the AuNPs through Au-S covalent bonds, and thus the —$NH_3^+$ terminus of CS would impart positive charges to the AuNPs by the protonation of CS. The signal amplification of AuNPs is demonstrated in Fig. 1B, which confirms that AuNPs can increase signal and improve the electron transfer efficiency.

## 2.4 Fabrication of the aptamer-antibody sandwich assay

The MPA-modified electrode was incubated in 0.1 M phosphate buffer solution (PBS) at pH 7.4 containing EDC (0.25 M) and NHS (0.05 M) for 5 h to create the carboxyl coupling reaction. Then, the electrode was rinsed with 0.1 M PBS and dried by nitrogen. 5 μL of 0.05 mg/mL anti-tau was dropped onto the electrode surface and dried overnight at 4℃ to minimize its potential to become part of the rate-limiting component in the bio-recognition mechanism. After washing with 0.1 M PBS (pH 7.4), 5 μL of 1% BSA solution was added to avoid the non-specifc binding, followed by washing with PBS and dried again under nitrogen flow. The detection of tau-381 based on the aptamer-antibody sandwich assay was subjected to successive incubations in the mixture of tau-381 (0.5~100.0pM) and aptamer-Au-CS bioconjugate for 45 min at room temperature. The responses of the sandwich strategy were recorded by DPV to detect tau-381. Fig. 2 shows the aptamer-antibody sandwich assay developed for tau-381 detection.

## 2.5 Electrochemical measurements

A CHI 660E electrochemical workstation (CH Instruments, Inc.) was used for EIS, CV and DPV. Electrochemical measurements were performed in the solution of 10mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ (1:1 ratio) in PBS pH 7.4 with a three electrode system (the gold electrode as the working electrode, the platinum wire and Ag/AgCl (3M KCl) as the counter and reference electrode respectively). EIS and CV were used to confirm the layer-by-layer construction of the electrode, the EIS parameters were at the initial potential of +0.2 V, 1 Hz low frequency, 0.005 V amplitude and 30 s quiet time, and the scans of CV were conducted between −0.4 V and +0.8 V. DPV was employed to evaluate the electrochemical behavior of tau-381, the DPV parameters were at the initial potential of -0.1V, +0.5 V final potential, 0.05 V pulse amplitude and 0.05 s pulse width.

# 3. Results and discussion

## 3.1 Construction of the gold electrode confirmed by EIS and CV

The gold electrode was assembled as described in Section 2.2. Nyquist plots of the measured EIS data was showed in Fig. 3A. The values increased with a layer by layer by the increasing height and diameter of the semi-circular Nyquist traces, corresponding to increased capacitance and resistance of the biosensor surface. The

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current of CV changed after each layer was assembled (Fig. 3B), namely bare gold, self-assembled monolayers (SAM) of MPA, anti-tau, tau-381, aptamer and aptamer-Au-CS bioconjugate, which shows the successful construction of the working electrode. In the testing process, the transfer of electrons between the redox ions $K_4Fe(CN)_6/K_3Fe(CN)_6$ and modified electrodes caused the redox reaction: $Fe[(CN)_6]^{3-} + e^- \rightarrow Fe[(CN)_6]^{4-}$. Therefore, a continuous decrease in peak value was observed with the Aptamer-Au-CS bioconjugate binding to the MPA-modified electrode surface, because of the electron exchange hindrance between $K_4Fe(CN)_6$/ $K_3Fe(CN)_6$ and the gold electrode. This was caused by the formation of the negatively charged interface and the stereo-hindrance effect.

## 3.2 Optimization of experimental conditions

To maximize the biosensor response, pH of CS solution was optimized, incubation time of aptamer-Au-CS bioconjugate and incubation time of aptamer-antibody sandwich were assessed in 10 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$. The current $\Delta I = I_p - I_0$ was applied, where the $I_p$ and $I_0$ represented the peak current in the presence and the absence of the analyte, respectively. The effect of pH of CS solution was investigated from pH 4 to 6 as shown in Fig. 4A. The ΔI value gradually increased with increasing pH from 4 to 5, and then it began to decrease. Thus, pH 5 of cysteamine solution was a better choice owing to protonation of CS under acidic conditions. The effect of incubation time of aptamer-Au-CS bioconjugate (Fig. 4B) and incubation time of aptamer-antibody sandwich (Fig. 4C) were studied, and the ΔI value reached the highest point when over 3h and 45 min incubation time, respectively. Hence, incubation time of aptamer-Au-CS bioconjugate and aptamer-antibody sandwich was 3 h and 45 min. Different concentrations of anti-tau were examined (Fig. 4D) and the optimal concentration of 0.05mg/mL of anti-tau was used in the experiments. The effect of ratio of tau-381 and the aptamer-Au-CS bioconjugate was then performed in Fig. 4E (1:5, 1:2, 1:1, 2:1 and 5:1), and the optimum ratio of tau-381 and the aptamer-Au-CS bioconjugate (1:1 v/v) was the better experimental condition.

## 3.3 Measurement of tau-381 in PBS solution

The electrode was incubated with successive concentrations of tau-381 (0~100 pM) for 45 min each using 10 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ as a redox probe by DPV (1:1 ratio of tau-381 and the aptamer-Au-CS bioconjugate), followed by flood washing with PBS. The anodic peaks corresponding to oxidation of tau-381 was observed at potential of approximately 0.21 V. The oxidation peak current increased with its concentration (Fig. 5A). Tau-381 could interact with aptamer-antibody sandwich selectively by functional groups，because of the specificity of the aptamer and antibody. The combination of the aptamer-Au-CS bioconjugate on the anti-tau modified surface allowed for an increase within the effective surface area on the electrode and enhancing electron transfer due to the accumulation of tau-381 onto the modified electrode surface, and the electrodeposition of AuNPs improved the electron transfer efficiency on the electrode surface, hence, the value of current increased with increasing concentration. Fig. 5B shows the calibration curve based on multiple experimental runs (n>3). A linear regression equation of $y(\mu A) = 7.9674x(pM) + 2.9256$ ($R^2 = 0.9992$) was obtained, where the horizontal axis for the tau-381 concentration was in logarithmic scale, and the vertical axis was ΔI. The limit of detection (LOD) calculated was 0.42 pM according to International Union of Pure and Applied Chemistry (IUPAC).

## 3.4 Biosensor accuracy precision and selectivity

To prove whether the novel biosensor could detect clinical samples sensitively and specifically, we used three different concentration serum samples. The testing results provided recoveries from 97.1% to 102.0%, relative standard deviations (RSD) less than 5.7% (Table 2), which indicated that the method had an acceptable accuracy

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and precision. The selectivity test is also important and necessary for biosensor. Ascorbic acid (AA), L-Cysteine (L-cys), glucose (Glu) and tau-441 usually co-exist with in human serum of AD. In order to evaluate the selectivity of the biosensor, the response values of the potential interferences were employed at the gold electrode by aptamer-antibody sandwich assay. The electrode was incubated in 10 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ with sequential additions of 10 pM tau-381 with 100 pM interfering species (AA, L-cys, Glu and tau-441) for 45 min. Compared to tau-381 (Fig. 6), the response current of interfering substances (AA, L-cys, Glu and tau-441) were 2.3%, 3.4%, 2.8%, 2.4% and 2.5% (less than 5.0%), respectively. The results showed that good selectivity could be applied for tau-381detection.

**Table 2.** The detection of tau-381 in human serum using the aptamer-antibody sandwich assay (n>3)

| Sample no. | Initial concentration （pM） | Added (pM) | Found (pM) | Recoveries (%) | RSDs (%) |
|---|---|---|---|---|---|
| 1 | 0.43 | 1.0 | 1.45 | 102.0 | 4.8 |
| 2 | 0.43 | 30.0 | 30.97 | 101.8 | 5.2 |
| 3 | 0.43 | 80.0 | 78.13 | 97.1 | 5.7 |

## 3.5 Application of biosensor in human serum samples

The sample used in this research was human serum collected from the Community health service center (CHSC) of Dongbao district, Jingmen City, Hubei Province, China. This study was approved by the Ethic Committees in CHSC and in Wuhan University of Science and Technology, which conforms to the provisions of the Declaration of Helsinki, and written informed consent was obtained from all patients. Human serums of AD patient groups were diluted 1/100 in 0.1M PBS, then detected by an aptamer-antibody sandwich assay. The control group included participants without AD; they had their serum levels monitored, using the same approach to detect. Fig. 7 illustrated that serum tau-381 levels were significantly higher in AD groups than those in the control group. The relative tau-381 can be estimated by measuring the ΔI value due to the equation relationship. In two AD groups, the concentrations of serum tau-381 were 76.97 pM (red line) and 316.29 pM (blue line), respectively (n>3). Tau-381 was successfully detected in human serum by the novel aptamer-antibody sandwich assay, suggesting the biosensor's capability of working in complex blood samples.

## 4. Conclusion

A novel electrochemical biosensor for the sensitive and selective quantification of tau-381 using aptamer-antibody sandwich assay was introduced in this work. The interaction between aptamer-antibody sandwich and tau-381 was investigated by using EIS, CV and DPV techniques. The assay was used to detect human serum samples with excellent results. The results suggest that this biosensor could be applied successfully to early diagnosis's of AD and clinical analysis.

## Conflicts of interest

There are no conflicts to declare.

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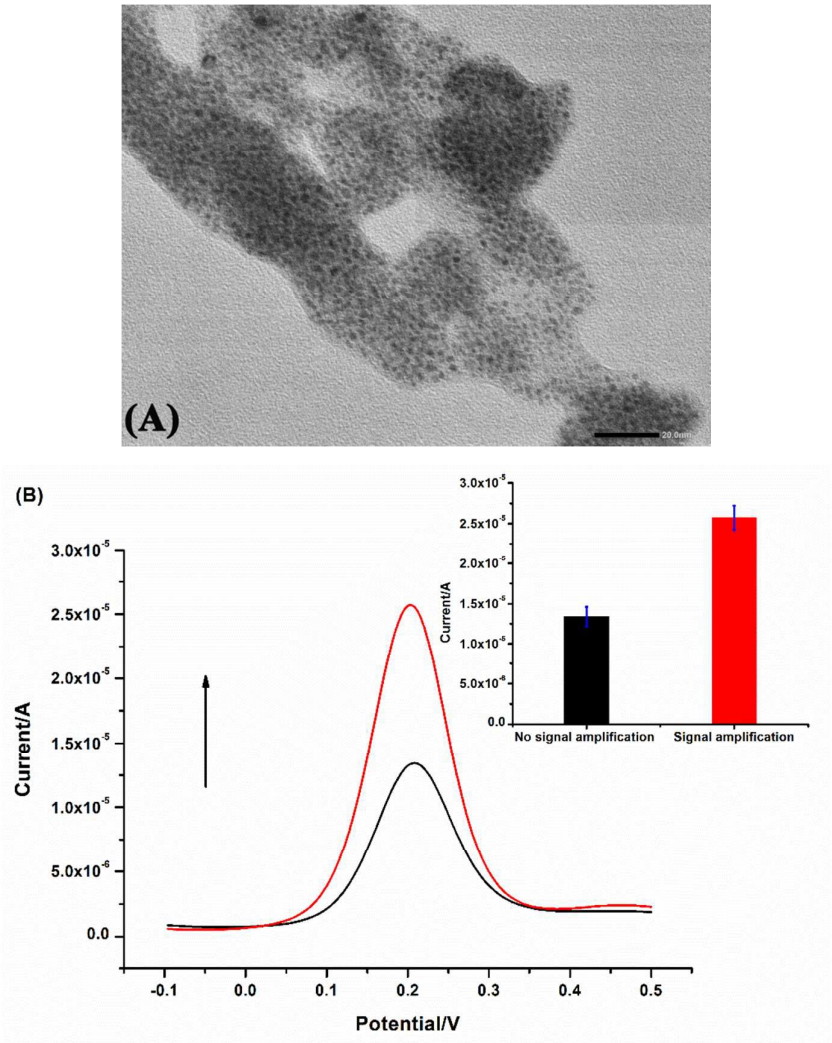


Fig. 1. (A) The characterization of the AuNPs by TEM; (B) The signal amplification of AuNPs verified by DPV.

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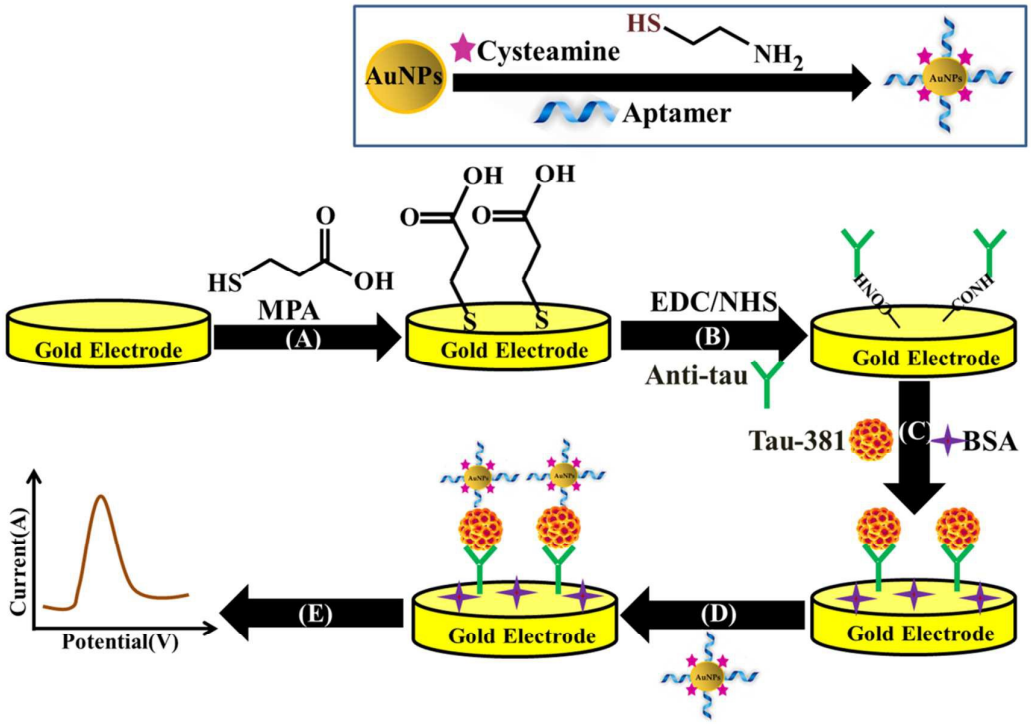


Fig. 2. Schematic representation of tau-381 detection with the novel aptamer-antibody sandwich assay: (A) Self-assembly of of MPA at gold electrode surface; (B) EDC/NHS activation and anti-tau antibody immobilization; (C) Free sites blocking with BSA followed by immune reaction with tau-381; (D) Affinity interaction the aptamer-Au-CS bioconjugate; (E) DPV assessment of tau-381 levels in human serum.

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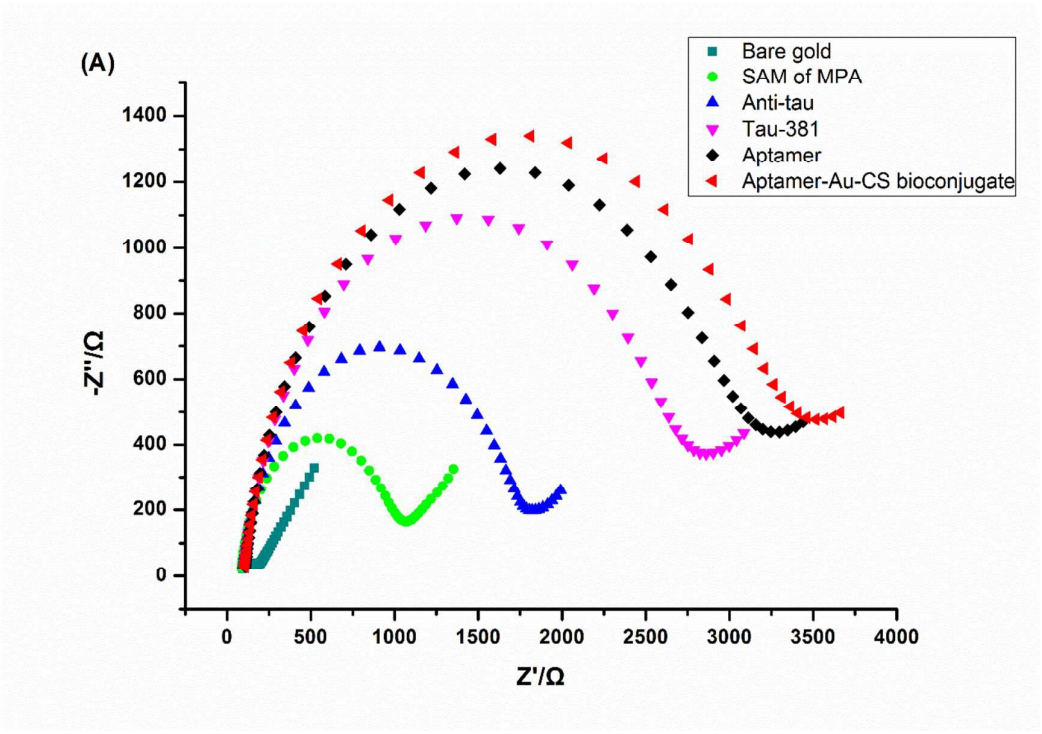


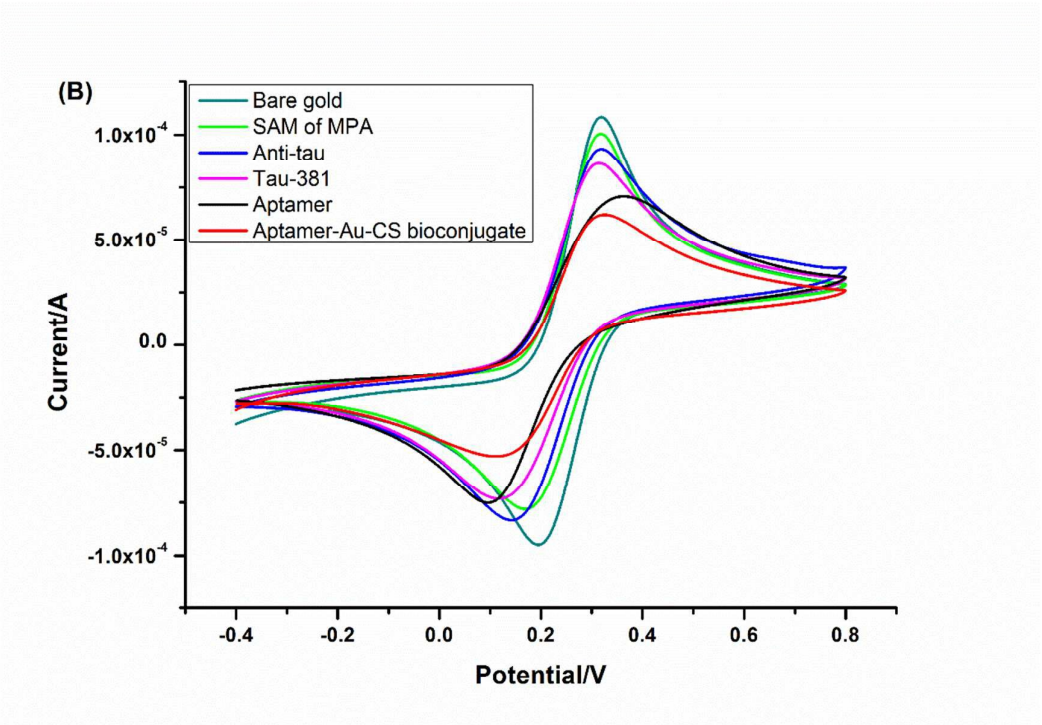


Fig. 3. (A) The characterization of each layer of the gold electrode verified by EIS; (B) The construction of the gold electrode confirmed by CV. EIS and CV of 10 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ (1:1 ratio) in PBS (pH 7.4) recorded at: Bare gold (dark cyan), SAM of MPA (green), Anti-tau (blue), Tau-381 (magenta), Aptamer (black), Aptamer-Au-CS bioconjugate (red).

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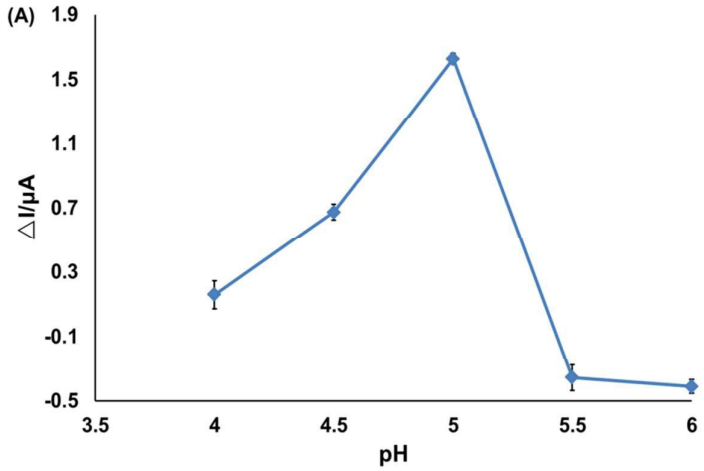


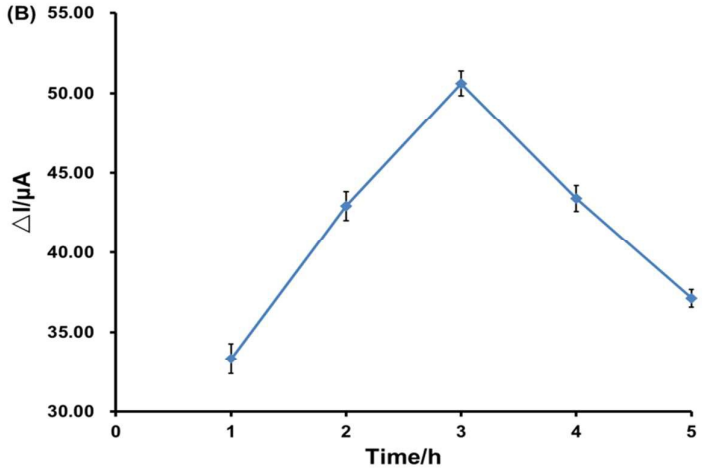


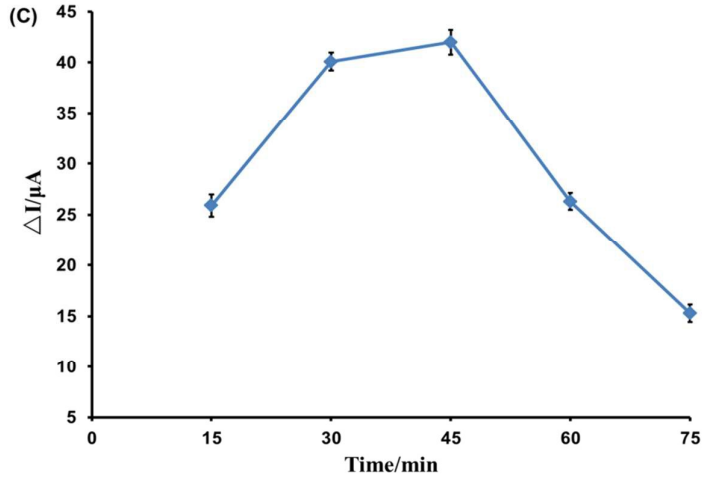

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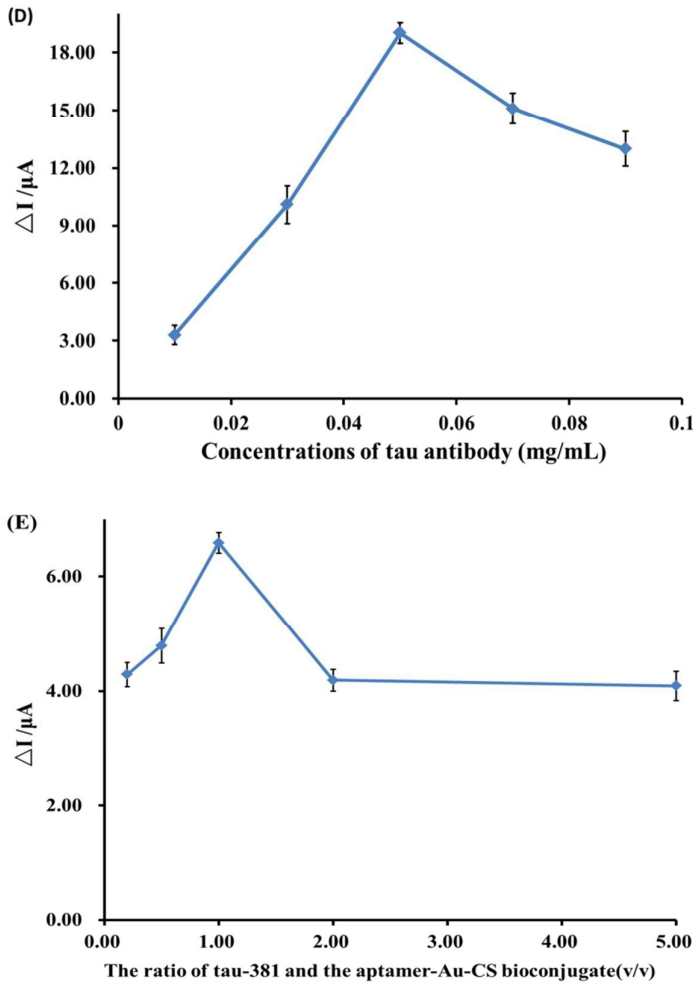


Fig. 4. Optimization data for: (A) pH of cysteamine solution; (B) Incubation time of aptamer-Au-CS bioconjugate; (C) Incubation time with 10 pM tau-381; (D) Different concentrations of anti-tau with 10 pM tau-381; (E) The ratio of tau-381 and the aptamer-Au-CS bioconjugate.

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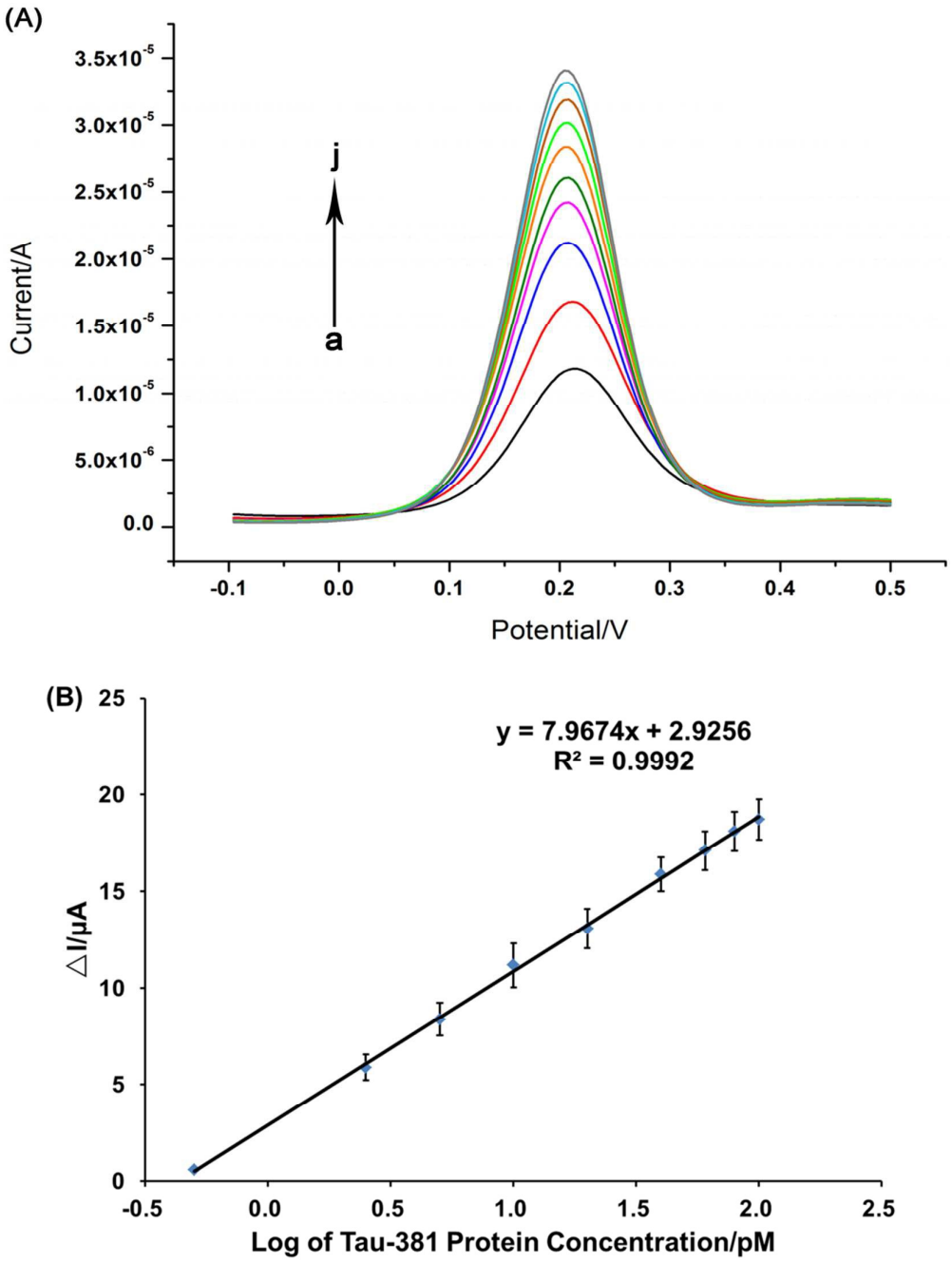


Fig. 5. (A) DPV measurement of tau-381 over different concentrations in 0.1 M PBS solution (a-j: 0, 0.5, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100.0 pM tau-381); (B) The calibration curve of ΔI value and Log of tau-381 protein concentration in 0.1 M PBS solution.

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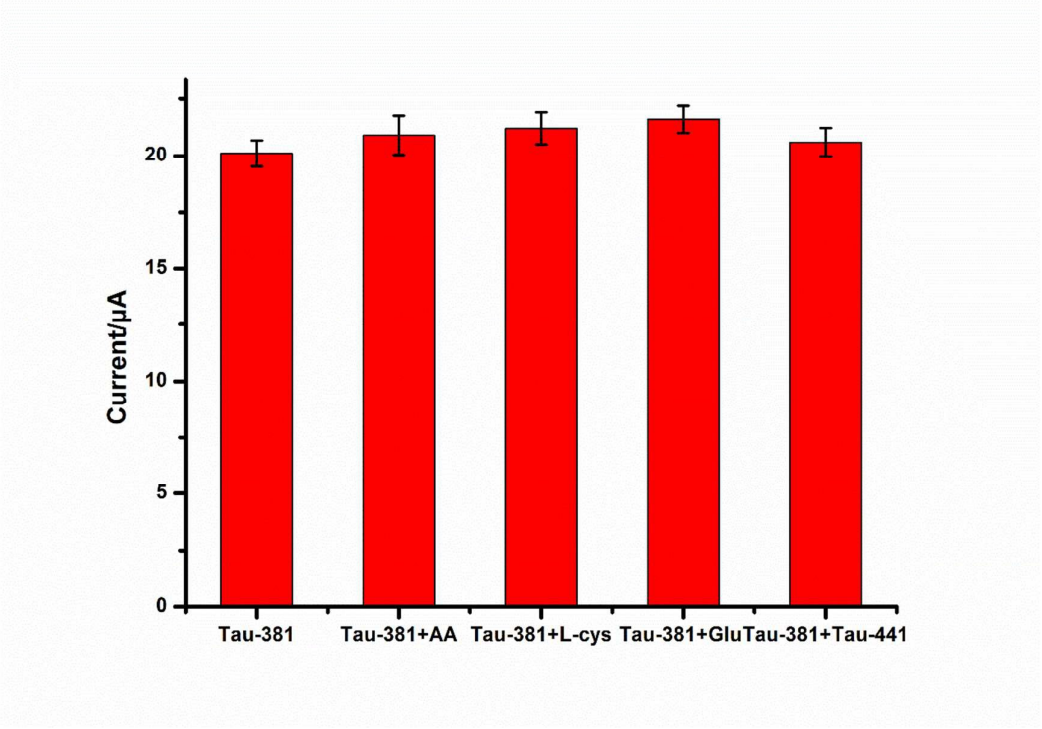


Fig. 6. The DPV response of biosensor to 10 pM tau-381, 10 pM tau-381 + 100 pM AA, 10 pM tau-381+ 100 pM L-cys, 10 pM tau-381+ 100 pM Glu and 10 pM tau-381+ 100 pM tau-441.

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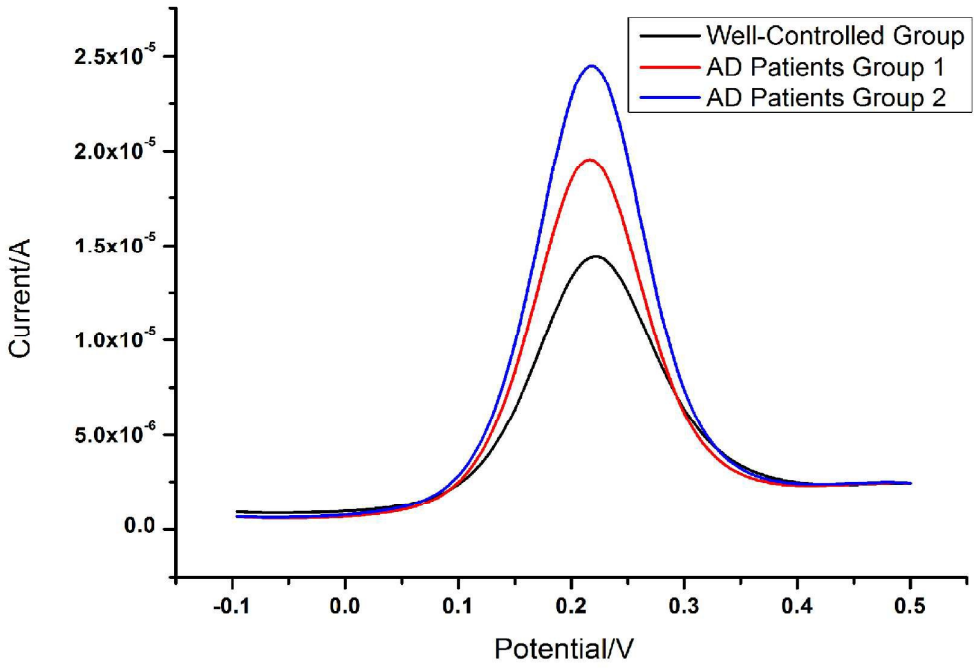


Fig. 7. The verification test of tau-381 in human serum from patients with AD.