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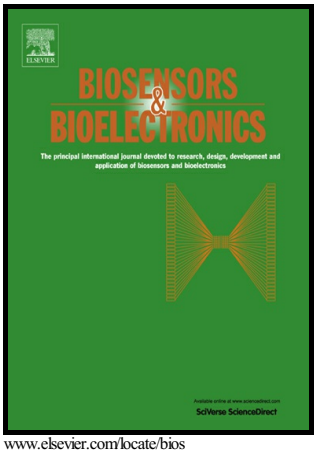

www.elsevier.com/locate/bios

PII: S0956-5663(16)30965-4
DOI: http://dx.doi.org/10.1016/j.bios.2016.09.080
Reference: BIOS9192

To appear in: *Biosensors and Bioelectronic*

Received date: 25 July 2016
Revised date: 9 September 2016
Accepted date: 23 September 2016

Cite this article as: Gangbing Zhu and Hye Jin Lee, Electrochemical sandwich type biosensors for α−1 antitrypsin with carbon nanotubes and alkalin phosphatase labeled antibody-silver nanoparticles, *Biosensors and Bioelectronic* http://dx.doi.org/10.1016/j.bios.2016.09.080

Electrochemical sandwich-type biosensors for α-1 antitrypsin with carbon nanotubes and alkaline phosphatase labeled antibody-silver nanoparticles

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Abstract

A novel sandwich-type biosensor was developed for the electrochemical detection of α-1 antitrypsin (AAT, a recognized biomarker for Alzheimer's disease). The biosensor was composed of 3, 4, 9, 10-perylene tetracarboxylic acid/carbon nanotubes (PTCA-CNTs) as a sensing platform and alkaline phosphatase-labeled AAT antibody functionalized silver nanoparticles (ALP-AAT Ab-Ag NPs) as a signal enhancer. CNTs offer high surface area and good electrical conductivity. Importantly, Ag NPs could increase the amount of ALP on the sensing surface and the ALP could dephosphorylate 4-amino phenyl phosphate (APP) enzymatically to produce electroactive species 4-aminophenol (AP). For detecting AAT based on the sandwich-type biosensor, the results show that the peak current value of AP using ALP-AAT Ab-Ag NPs as signal enhancer is much higher than that by using ALP-AAT Ab bioconjugate (without Ag NPs), the biosensor exhibited desirable performance for AAT determination with a wide linearity in the range from 0.05 to 20.0 pM and a low detection limit of 0.01 pM. Finally, the developed sensor was successfully applied to the analysis of AAT concentration in serum samples.

Keywords: Alzheimer's disease; α-1 antitrypsin; Sandwich assays; antibody functionalized silver nanoparticles; electrochemical biosensors

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1. Introduction

With the aging of the world population, Alzheimer's disease (AD) has become an increasing and serious public health problem in the industrialized world. AD is accompanied by a gradual loss of cognitive function and synaptic integrity, selective neuronal death, and the abnormal formation of neurotic and core plaques in the cerebral cortex (Liu et al., 2013; Luo et al., 2015; Yu et al., 2014). Thus achieving an early diagnosis or risk assessment of AD via the analysis of easily accessible body fluids is an essential aspect of improving upon the current standards for patient care and treatment. β-amyloid and tau proteins are the most established neurochemical indicators reported in serum to identify changing levels between healthy and AD patients (Kaushik et al., 2016; Yu et al., 2015; Zhou et al., 2015). Measurements restricted to only one or two of the protein targets are generally insufficient to provide a valid snapshot of the disease state (Kim and Lee, 2015). As one of the protease inhibitors, α-1 antitrypsin (AAT) (normal 150-350 mg/dl in serum(Ganrot and Bjerre, 1967; Mornex et al., 1986)) is the major protease inhibitor of human plasma protein, and it has been increasingly proposed as an important biomarker of AD and liver cancer in the very early stages.(Song et al., 2009) Also, AAT has been shown to be related to emphysema, liver sclerosis and other diseases (Lista et al., 2013; Song *et al.*, 2009; Zhang et al., 2011). Therefore, developing effective method to detect AAT in serum is of great significance for the early diagnosis of AD and other disease.

Currently, only a few examples, including surface plasmon resonance and fluorescent sensing, have been developed for the detection of AAT (Kim and Lee, 2015; Zhang *et al.*, 2011). Herein, we presented for the first time an electrochemical method for the determination of AAT (Scheme 1). Electrochemical technologies have many advantages such as cheap and portable instruments, low

cost and rapid analysis, high sensitivity and selectivity (Ge et al., 2016; Karimi-Maleh et al., 2013; Karimi-Maleh et al., 2016; Karimi-Maleh et al., 2014a; Karimi-Maleh et al., 2014b; Zhu et al., 2015). For achieving ultrasensitive sensing AAT, we produced alkaline phosphatase-labeled AAT antibody functionalized silver nanoparticles (ALP-AAT Ab-Ag NPs) as signal enhancers: Ag NPs were introduced as the carriers to enrich ALP-AAT Ab, ALP dephosphorylates 4-amino phenyl phosphate (APP) enzymatically to produce electroactive species 4-aminophenol (AP), which could be oxidized electrochemically to 4-quinone imine, thus the peak current value of AP was monitored for the analysis of AAT concentration (Liu et al., 2014b; Liu et al., 2014a); and 3, 4, 9, 10-perylene tetracarboxylic acid/carbon nanotubes (PTCA-CNTs) as sensing platform: CNTs have high electrical conductivity (1000-2000 S/cm) and large theoretical surface areas (50-1315 m^2/g) (Chen and Chatterjee, 2013; Peigney et al., 2001; Zhu et al., 2016), the pyrenyl group of PTCA could tightly attach to the sidewall of CNTs by means of π-π stacking interactions and carboxyl could link to NH_2 modified AAT aptamer (aptamers are in vitro selected artificial nucleic acid receptors that possess high affinity and specificity toward their ligands (Zhang et al., 2008)), thus constructing an aptamer-antigen-antibody sandwich-type electrochemical biosensor for AAT. Finally, the proposed biosensor was applied to real serum samples.

2. Experimental section

2.1. Reagents and apparatus

All of the chemicals listed here were used as received unless otherwise specified: CNTs (diameter, ~20 nm; length, ~5 μm), perylene-3, 4, 9, 10-tetracarboxylic dianhydride (PTCD, Sigma-Aldrich), silver nitrate ($AgNO_3$, Sigma-Aldrich), AAT (46 kDa, R&D Systems), AAT antibody (150kDa,

R&D Systems), human serum from human male AB plasma (Sigma-Aldrich), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Thermo), N-hydroxysulfo-succinimide (NHS, Thermo), APP (LKT Laboratories, Inc.). The DNA aptamer specific to AAT (5'-H_2N GGG GCA CGT ACG GGC ATC ATA ACA ACA GGC GTG CCC C-3') was purchased from Integrated DNA Technologies (IDT). 10 mM phosphate buffered saline (PBS, pH7.4) was used as a storage buffer. AAT antibody was conjugated with ALP using an ALP labeling kit-NH_2 (Dojindo Molecular Technologies, Inc.) following the protocol provided by the company website (www.dojindo.com/store/p/47-Alkaline-Phosphatase-Labeling-Kit-NH_2. html). Blocking buffer was 10% (w/v) bovine serum albumin (BSA, J&K Scientific. Ltd) containing 0.05% Tween-20. The electrocatalytic reaction between ALP and APP was performed in 50 mM Tris buffer solution containing 10 mM KCl and 1g/L of $MgCl_2$ (pH 8.5) at room temperature (Diba et al., 2015; Nam et al., 2012). Millipore-filtered water was used to make all aqueous solutions.

All electrochemical investigations were performed using a computer controlled potentiostat (AutolabPGSTAT128N, Ecochemie) operated by General Purpose Electrochemical System (GPES) version 4.9 software package. Screen printed carbon electrode (SPCE) consisting of a carbon working electrode with a geometric area of a 28 mm^2, a Ag/AgCl Reference electrode and a carbon counter electrode were purchased from the BIO Co., Ltd. SPCE was connected with the potentiostat using a sensor connector. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were used throughout the experiments with the condition as follows: for CV, the scan rate of 50 mV/s, while for DPV, the step potential of 20 mV/s, pulse interval of 50 ms and amplitude of 50 mV.

2.2. Synthesis of PTCA-CNTs and ALP-AAT Ab-Ag NPs

PTCA-CNTs: PTCA was prepared by the hydrolysis of PTCD (Wu et al., 2011; Zhu et al., 2012).

The PTCA-CNTs were prepared as follows: 50 mg pristine CNTs were ultrasonicated in 50 mL of ethanol containing 20 mg PTCA for 2 h and stirred continuously for 6 h at room temperature. After that, the mixture was filtered through a nylon membrane and washed several times with ultra-pure water and ethanol. Finally, the obtained product was dried in a vacuum oven overnight.

ALP-AAT Ab-Ag NPs: The colloidal Ag NPs were prepared firstly according to previous reports (Lai et al., 2011; Lin et al., 2015). Then, the resulting Ag NPs were further functionalized by adding of 2 μL MPA (pH=7.0) into 5 mL Ag NPs. After chemisorbed MPA to Ag NPs through Ag-S covalent bond, EDC and NHS were then used to activate carboxyl group in the MPA-Ag NPs. A 30 μL (2 μM) of ALP-AAT Ab was next mixed with activated MPA-Ag NPs and the resulting solution was rotated for 2 hour under room temperature to form ALP-AAT Ab functionalized Ag NPs, which was denoted as ALP-AAT Ab-Ag NPs and stored in 4°C when not in use.

2.3. Construction of sandwich-type electrochemical biosensor for AAT

Firstly, 10 μL of PTCA-CNTs suspension (1.0 mg mL^{-1}) was dropped on SPCE surface. After air-dried for 20 min, a 50 μL mixed solution of EDC/NHS was casted on PTCA-CNTs/SPCE surface for 1 h and followed by rinsing with water and drying. Next, a 2 μL aliquot of 10 nM aptamer was spread over the chip surface by covering with a piranha treated glass cover slip for 3h. Following AAT aptamer immobilization, a drop of 2 μL BSA blocking solution was applied to the assay to eliminate the non-specific binding effects and blocking the remaining active sites, and then the chip was washed with PBS and ready to use.

For detecting AAT protein, a 2.0 μL aliquot of different concentrations of AAT protein was drop casted and spread on the AAT aptamer-CNTs/SPCE and incubated for 1 h (Depending on their sequence, aptamers fold into specific shapes which strongly interact (*e.g.* electrostatically, Van der

Waal interactions) at a specific epitope on the protein surface). After washing the chip with PBS, 2.0 μL of ALP-AAT Ab-Ag NPs was drop casted and spread on the AAT protein/aptamer/SPCE surface and incubated for a minimum 1h followed by rinsing with Tris buffer. This results in the formation of the surface sandwich complex of ALP-AAT Ab-Ag NPs/AAT protein/AAT aptamer-CNTs/SPCE which was assembled in an electrochemical cell containing a total volume of 5.0 mL of Tris buffer containing APP. The electrocatalytic reaction of surface bound ALP sandwich complex with APP was monitored using CV and DPV.

3. Results and discussion

3.1. Sandwich-type assay methodology for AAT

The preparation of the sandwich-type assay and the combination of ALP-AAT Ab-Ag NPs and AAT aptamer used for the determination of AAT target molecules is highlighted in Scheme 1. PTCA-CNTs were prepared first and dropped on SPCE and then subsequently activated with EDC/NHS to enable the covalent immobilization of AAT specific DNA aptamer. The sequential adsorption of AAT protein and ALP-AAT Ab-Ag NPs conjugate on to the DNA aptamer/CNTs resulted in the formation of a surface ALP-AAT Ab-Ag NPs/AAT protein/AAT aptamer-CNTs complex. The electrochemical response of AP produced enzymatically from APP was then used for the quantitative analysis of AAT target. In the sandwich-type assay, the Ag NPs as the carriers could enrich ALP-AAT Ab amount, and the CNTs providing high electrical conductivity and large theoretical surface areas, thus further improve the sensitivity for detecting AAT.

3.2. Characterization of PTCA-CNTs and ALP-AAT Ab-Ag NPs

FT-IR spectroscopy was used to characterize the PTCA-CNTs. As shown in Figure S1, the pure CNTs only exhibit typical C=C conjugation (1585 cm^{-1}) and C–C band (1122 cm^{-1}), *etc.* After

modifying CNTs with PTCA, the PTCA-CNTs show the broad stretching bands for O-H at 3162 cm^{-1} and C=O at 1763 cm^{-1} assigned to the carboxyl groups of PTCA, suggesting that the successful functionalization of the CNTs by PTCA via π-π interactions between the pyrenyl group of PTCA and the surface of CNTs (Wu *et al.*, 2011; Yi et al., 2016; Zhu *et al.*, 2012). The dispersibility of the PTCA-CNTs in water was also evaluated (Figure S2). It can be observed that the dispersion ability of the pristine CNTs in aqueous solution is rather poor due to the strong attractive interaction between the hydrophobic sidewalls of the CNTs. However, for the PTCA-CNTs, a dark and homogeneous solution was observed and no obvious precipitates appeared. This implies that the PTCA-CNTs have an excellent dispersibility in water, which is important for efficiently inhibiting their aggregation and thus enhancing the utilization of nanohybrids, as well as being beneficial towards improving the preparation reproducibility of the sensor.

For characterizing ALP-AAT Ab-Ag NPs, UV–vis spectrophotometer was applied to identify the optical absorption properties of Ag NPs and ALP-AAT Ab-Ag NPs at an optical wavelength of 300-900 nm (Figure 1). It was noted from curve ***a*** that the spectra of Ag NPs shows absorption peaks at 398 nm. When AgNPs were functionalized with ALP-AAT Ab, this band is found to extend to 412 nm (curve ***b***), which reflect the variation of the local dielectric function of functionalized AgNPs versus as-prepared Ag NPs. The transmission electron microscopy (TEM) images of Ag NPs and ALP-AAT Ab-Ag NPs were shown in Figure S3.

3.3. Electrochemical sensing of AAT

Signal amplification is critical to obtain high sensitivity and low detection limit for the sandwich-type electrochemical biosensor. To investigate the signal amplification of the proposed protocol, the sensors were utilized for the detection of AAT with two types of labeled probes,

including ALP-AAT Ab and ALP-AAT Ab-Ag NPs bioconjugate (Figure 2). During the measurement process, the same batch biosensors were incubated with 30 pM AAT protein in the absence and presence of 100 μM APP. Curve ***a*** and ***b*** show CVs before and after the addition of APP, respectively. As shown in Figure 2, the use of ALP-AAT Ab-Ag NPs bioconjugate offered much greater oxidation peak current of AP (~8 times) than that obtained with ALP-AAT Ab bioconjugate. These indicated that ALP-AAT Ab-Ag NPs exhibits efficient catalysis towards APP to produce AP than that of directly using ALP-AAT Ab, illustrating that the synergistic action of Ag NPs and ALP could extremely amplify the electrochemical signal, and the sandwich-type assay of ALP-AAT Ab-Ag NPs/AAT protein/AAT aptamer-CNTs has excellent electrochemical sensing performance for AAT.

Since the DPV shows much higher current sensitivity and better resolution compared to CV, the electrocatalytic reaction of the ALP and APP with 0.5 pM AAT was investigated using DPV to further demonstrate the electrochemical sensing performance of the proposed sandwich-type assay (Figure 3). In the presence of 100 μM APP, it is noted that there is an oxidation peak of AP observed at -0.1 V (vs Ag/AgCl) based on the ALP-AAT Ab /AAT protein/AAT aptamer-CNTs sandwich-type assay, whereas for the ALP-AAT Ab-Ag NPs/AAT protein/AAT aptamer-CNTs, the peak current of AP increased remarkably, suggesting that Ag NPs could help the enrichment of ALP-AAT Ab and further improve the sensitivity.

The concentration of the enzyme substrate is an important factor that needs to be considered when assessing the sensitivity of the biosensor. Figure S4 shows sensor performance of ALP-AAT Ab-Ag NPs/AAT protein/AAT aptamer-CNTs assay at different concentration of APP with a fixed concentration of AAT protein at 7 pM. The sensing response increased when the APP concentration increased from 25 μM to 100 μM, but no significant changes in the response was obtained above

100 μM APP. Thus, an APP concentration of 100 μM was used for all subsequent measurements.

The sensing performance of biosensors for different concentrations of AAT was studied in Tris buffer solution containing 100 μM APP by using DPV. As shown in Figure 4, the DPV peak current increased with respect to the AAT concentrations, and exhibited a linear relationship between the peak current and AAT concentration ranging from 0.05 to 20 pM. The linear regression equation was I (μA) = 0.8234+0.3977C_{AAT} (pM, R^2=0.9968) with a detection limit of 0.01 pM. These results indicated that the sensor response is reliable and can be used for the determination of AAT concentration.

3.4. Selectivity, reproducibility and stability

The selectivity of the developed biosensor for AAT was investigated via performing an additional series of control measurements in the presence of an excess of common interfering serum proteins containing immunoglobulin G (IgG) and immunoglobulin E (IgE). As shown in Figure 5, in each case, DPV measurements in the presence of a 100-fold excess concentration of each serum protein species in addition to the mixture of 700 pM IgG or 700 pM IgE with 7 pM AAT were compared for separately obtained DPV data of 7 pM AAT only. The approach by Macca and Wang (Maccà and Wang, 1995) was employed to evaluate the selectivity coefficient (log $k_{i,g}$) for the determination of AAT (Equation (1)):

$$k_{i,g} = (I_t - I_i)c_i/I_i c_j \qquad (1)$$

where, i and j represents the target and the interfering molecule, respectively. I_t is the sum of current responses from both the analyte (I_i) and interfering molecule (I_j). c represents the concentration of i

and j species. The results show that the log $k_{i,g}$ value for IgG and IgE obtained were ~-3.55 and -3.25, respectively, which indicate the excellent selectivity of biosensors towards the AAT target.

The reproducibility and stability of the proposed immunosensor were also evaluated (Figure S5). The results reveal that the sensor has a satisfying reproducibility with a relative standard deviation of 5.2% for 8 parallel measurements with different sensors. On the other hand, after the assembled sensor was stored at 4°C in PBS for one week, only a small current decrease (about 6.3%) was observed. And 87% of the initial response was remained after storing 6 weeks. These suggest our sandwich-type biosensor has good reproducibility and stability.

3.5. Determination of AAT in serum samples

As a final demonstration, the sensor was applied to analyze AAT concentrations in serum solutions. It was anticipated that a relatively high AAT concentration is present in received serum. After diluting 10^7 times, the detected AAT concentration in serum was 3.5 pM, indicating that the original concentration of AAT in serum is 35.0 μM. Then, certain amounts of AAT were spiked in the diluted samples and measured by the proposed method (Table S1). From Table S1, it is noted that the recoveries of 95-102% are obtained, suggesting that the proposed method is suitable for the analysis of AAT protein in practice.

4. Conclusions

In summary, this article demonstrated for the first time an electrochemical method for the determination of AAT based on a sandwich-type assay featuring PTCA-CNTs as a sensing platform and ALP-AAT Ab-Ag NPs as a signal enhancer. The CNTs not only facilitated electrons transfer but also provided a large surface area for the immobilization of abundant AAT aptamer. Also PTCA could provide a bridge between CNTs and aptamer. For the ALP-AAT Ab-Ag NPs, Ag NPs could

increase the amount of ALP which could increase the catalytic reaction toward APP to produce AP, thus enhancing the response signal. The results show that the developed biosensor has excellent specificity, stability, cheap, reproducibility and high sensitivity for AAT. It is believed that the developed biosensor has a great potential application in AAT detection for future point-of-care diagnostics of AD and other disease.

Acknowledgments

This research was supported by the National Research Foundation (NRF) of Korea funded by the Ministry of Science, ICT, and Future Planning (grant number: NRF-2015R1A2A1A15052198).

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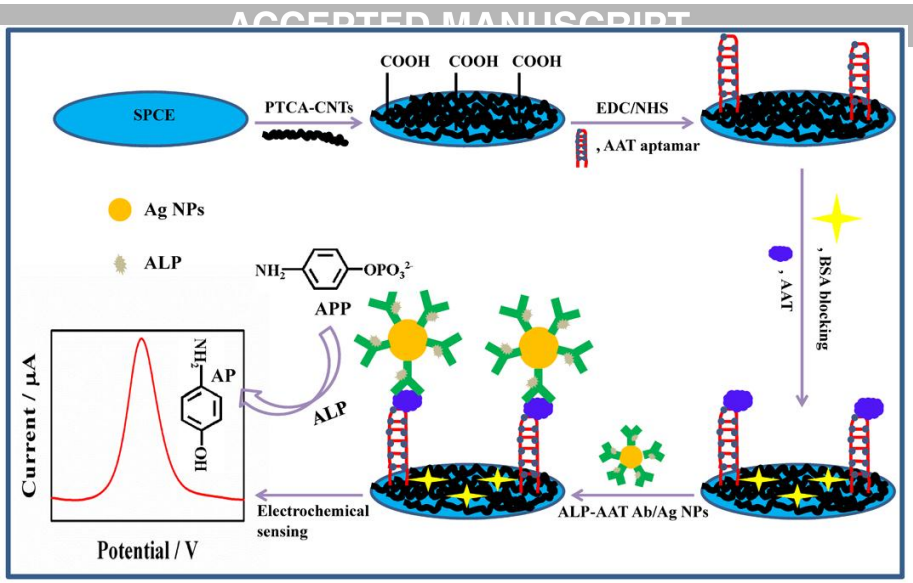

Scheme 1. Schematic illustration of the developed sandwich-type electrochemical biosensor.

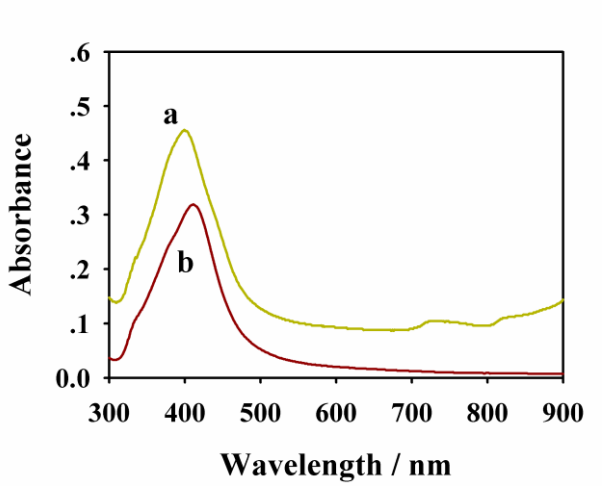

Figure 1. UV spectra of Ag NPs (a) and ALP-AAT Ab/Ag NPs (b).

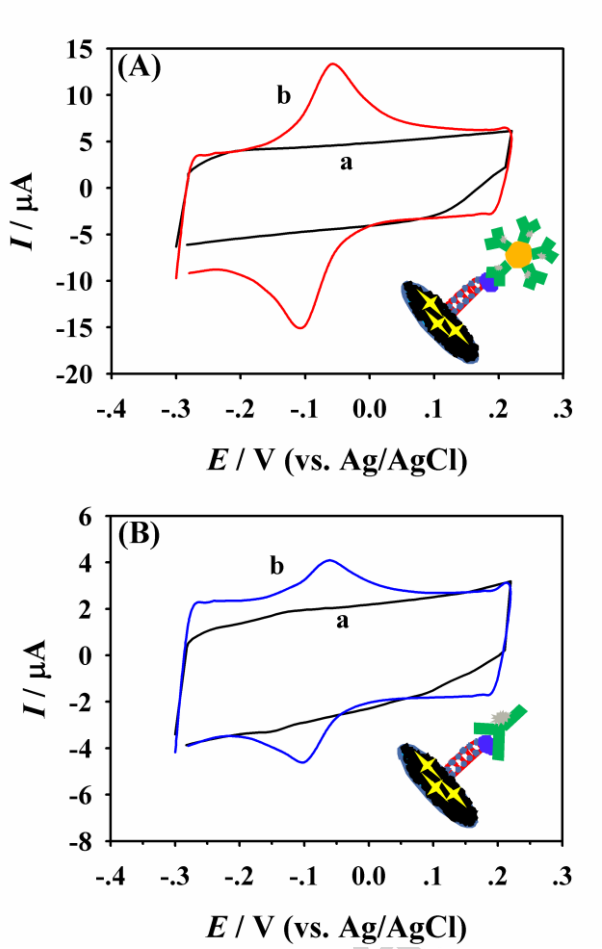

Figure 2. CV response of the different sandwich format immunosensors in the absence (curve a) and presence of 100 μM APP (curve b) in 50 mM Tris (pH 8.5) by using various signal tags: (A) ALP-AAT Ab/Ag NPs and (B) ALP-AAT Ab bioconjugate.

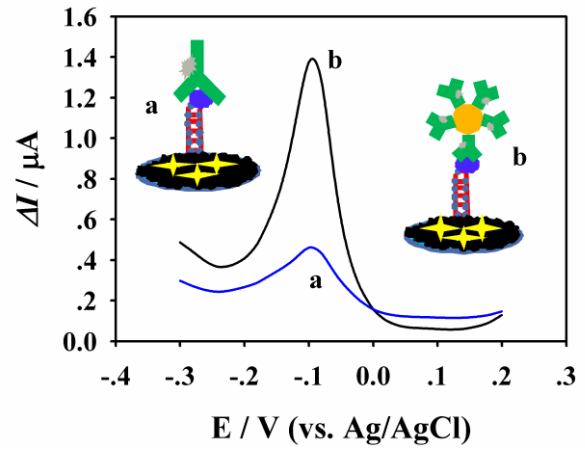

Figure 3. DPV responses of the sandwich format biosensors for 0.5 pM AAT by using (a) ALP-AAT Ab and (b) ALP-AAT Ab/Ag NPs bioconjugate signal tags in the presence of 100 mM APP in 50 mM Tris (pH 8.5).

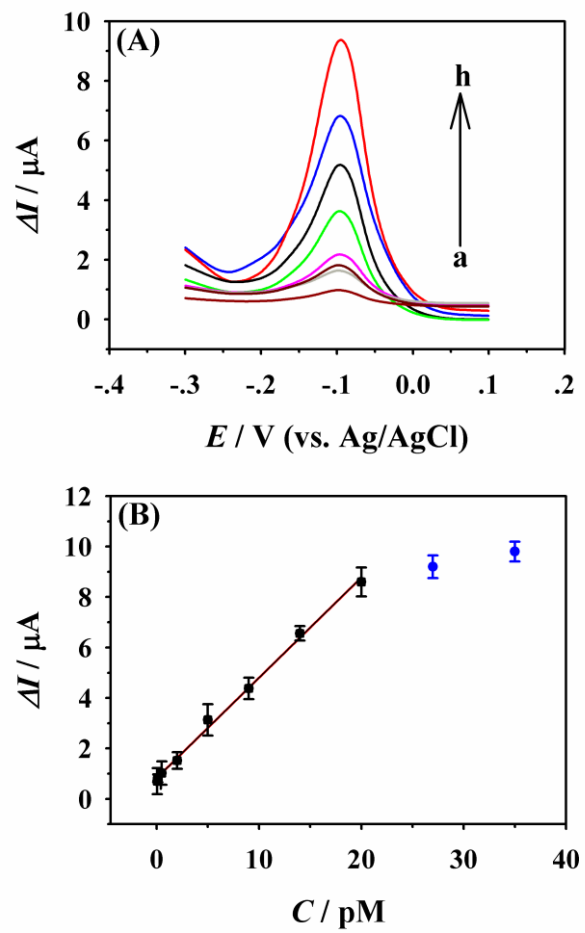

Figure 4. (A) DPV responses of the proposed biosensor after incubation with different concentrations of AAT (from a to h): 0.05, 0.1, 0.5, 2, 5 9, 14, and 20 pM. (B) Calibration curve for the determination of AAT using ALP-AAT Ab/Ag NPs bioconjugate for signal amplification.

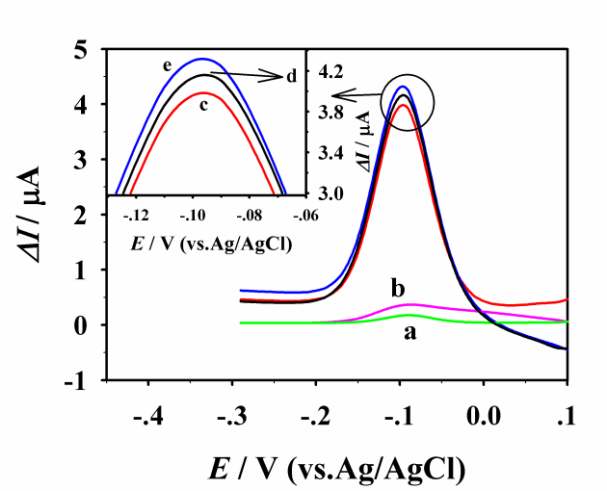

Figure 5. DPV responses of the developed sensor with different analytes: (a) 700 pM IgG, (b) 700 pM IgE, (c) 7 pM AAT, (d) a mixed solution of 700 pM IgG and 7 pM AAT, and (e) a mixed solution of 700 pM IgE and 7 pM AAT.

Highlights

>Electrochemical method was used to detect α-1 antitrypsin for the first time

>A sandwich-type immunosensor was constructed by using carbon nanotubes as platform and alkaline phosphatase-labeled antibody-silver nanoparticles as signal enhancers

>The sensor shows excellent electrochemical sensing performance for α-1 antitrypsin