Microchimica Acta (2020) 187:302
https://doi.org/10.1007/s00604-020-04273-z

ORIGINAL PAPER

Signal multi-amplified electrochemical biosensor for voltammetric determination of tau-441 protein in biological samples using carbon nanomaterials and gold nanoparticles to hint dementia

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Received: 3 November 2019 / Accepted: 11 April 2020

Abstract

A signal multi-amplified electrochemical biosensor was fabricated for tau-441 protein, a dementia biomarker. It utilizes a carbon nanocomposite film modified gold electrode. The carbon nanocomposite film was composed of multi-walled carbon nanotubes (MWCNTs), reduced graphene oxide (rGO), and chitosan (CS). For the nanocomposite film, rGO improved the dispersibility of MWCNTs, and the effective surface area of MWCNTs was increased. On the other hand, MWCNTs also increased the interlayer spacing of rGO, resulting in a thinner rGO layer. MWCNTs-rGO had a better conductivity than that of MWCNTs and rGO due to the synergy effect. Biocompatible CS was employed for immobilization of the specific antibody. Tau-441 protein was modified with gold nanoparticles (AuNPs) for signal amplification again. The response of the electrochemical biosensor is linear in the range 0.5–80 fM (0.5, 1.5, 5, 10, 40, 80 fM) with a limit of detection (LOD) of 0.46 fM, using differential pulse voltammetry (DPV) in a potential range of − 100–500 mV. The biosensor was successfully applied to the analysis of serum samples of 14 normal people, 14 mild cognitive impairment (MCI) patients, and 14 dementia patients.

Keywords Cognitive impairment · Multi-walled carbon nanotubes-reduced graphene oxide-chitosan · Synergy effect · Serum sample analysis

Introduction

Tau protein is a major component of the cytoskeleton of the nerve cell [1]. There are six tau protein isoforms. They are distinguished by different numbers of the amino-terminal region and the carboxy-terminal region. Tau protein containing amino-terminal region induces cognitive disorder [2]. Tau-441 protein is the longest tau protein isoform, including two amino-terminal regions. Compared with other tau protein isoforms, the connection between tau-441 protein and cognitive disorder has been widely investigated [3]. The cognitive disorder is a typical clinical manifestation of various types of dementia. Patients have different degrees of cognitive dysfunction before they reach the diagnostic standard for dementia. The assessment of the level of tau-441 protein is expected to reflect the degree of cognitive disorder and contribute to the early diagnosis of dementia. The cerebrospinal fluid and blood can be used as the biological samples of tau-441 protein [4]. Compared with cerebrospinal fluid, blood samples possess unique superiorities, such as convenient sampling and low invasiveness. Therefore,

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00604-020-04273-z) contains supplementary material, which is available to authorized users.

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Published online: 30 April 2020

the work focuses on assaying tau-441 protein in the blood, providing a new biological sample analysis-based opportunity for early molecular diagnosis of dementia.

Due to the impact of the blood-brain barrier, only trace of tau-441 protein exists in the blood. Sensitive technology for determination of tau-441 protein is needed. The electrochemical biosensor has unique advantages in improving sensitivity and selectivity [5–9]. It is an excellent choice for determination of tau protein. Table S1 shows electrochemical biosensors for the determination of tau-441 protein [10–13]; however, the analysis of tau-441 protein in biological samples has not been realized.

As one of the carbon nanomaterials, multi-walled carbon nanotubes (MWCNTs) are ideal electrode modification materials in biosensing technology [14–16]. MWCNTs can provide a large number of reaction sites and enough sufficient space for electrocatalytic active substances because of topological defects on the surface and large specific surface area. However, in practical applications, a single MWCNTs material has the defects of poor dispersion and easy reunion [17]. Graphene oxide (GO), another kind of carbon nanomaterial, has been reported to disperse carbon nanotubes in water under ultrasound efficiently [18]. This is because of the synergy effect between them. Also, MWCNTs can widen the layer spacing of graphene, preventing agglomeration of graphene sheets [19]. The performance of the MWCNTs-rGO nanocomposite is much better than that of a single material. However, GO has poor conductivity. Reduced graphene oxide (rGO) is obtained by reducing some oxygen-containing groups based on GO; rGO has better conductivity than GO [20]. Compared with MWCNTs-GO nanocomposite, MWCNTs-rGO nanocomposite is a better electrode modification material. And chitosan (CS) has the potential to make modified materials stably fixed on the electrode surface and immobilize the specific antibody because of its good adsorption, excellent film-forming ability, and good biocompatibility [21, 22]. Besides, AuNPs are often used to modify biomolecules (antigen or antibody) for signal amplification because of the stable physicochemical properties, facile synthesis, large specific surface area, and good electrical conductivity and biocompatibility [23–25].

Herein, the method relied on MWCNTs, rGO, CS and AuNPs as the means of signal amplification. MWCNTs-rGO-CS nanocomposite was used as the modification material of the gold electrode to improve the electrode conductivity. And tau-441 protein was modified with AuNPs to achieve the signal amplification further. The project was based on a three-electrode system, $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ as the redox probe. The interaction between tau-441 protein and the specific antibody was investigated by differential pulse voltammetry (DPV) technology. The interaction was indicated by the sensing signal. It was controlled by the formation of tau-441 protein-specific antibody complex. The signal changed when the complex formed, and the electron transfer was blocked. The signal multi-amplified electrochemical biosensor had good sensing properties for tau-441 protein from 0.5 to 80 fM. It was applied in the actual biological samples—the serum of normal people, MCI patients, and dementia patients, thus providing a new possibility for early prediction of dementia.

Experiment

Materials and instruments

The chemical reagents of analytical reagent (AR) and ultrapure water (18.25 MΩ cm) were used throughout the work. A detailed description of the chemical reagents and instruments were showed in Electronic Supporting Material.

Preparation of multi-walled carbon nanotubes-reduced graphene oxide-chitosan (MWCNTs-rGO-CS) nanocomposite

MWCNTs-rGO-CS nanocomposite was acquired by blending. The following are weighed: 4.8 mg of MWCNTs, 4.8 mg of rGO, and 19.2 mg of chitosan. And 2% acetic acid solution was configured. Then, 4.8 mg of MWCNTs, 4.8 mg of rGO, and 19.2 mg of CS were added to 4 mL of acetic acid solution (2%) followed by an ice bath ultrasonic for 1 h. The synthesized MWCNTs-rGO-CS nanocomposite was stored at 4 °C for future use.

Construction of the nanocomposite film and immobilization of the specific antibody on the gold electrode

The gold electrode was pretreated before constructing the nanocomposite film on electrode surface and glutaric dialdehyde (GLA) was selected as a linker to fix the specific antibody on the electrode surface. The specific steps were described in Electronic Supporting Material.

Preparation of the tau-441-AuNPs conjugate

AuNPs (4 nm) were synthesized by sodium borohydride method. Firstly, 18 mL of ultrapure water and 0.5 mL of chloroauric acid solution (0.01 M) were poured into a clean beaker, and mixed them with a magnetic stirrer (500 rpm), and then quickly add 0.5 ml of sodium citrate solution (0.01 M) and increased the stirring speed (1000 rpm). The mixed solution was colorless, and then add 0.5 mL of sodium borohydride solution (0.01 M, sodium borohydride solution was configured with ice water) dropwise, and stirred until the color of the solution changed from colorless to orange, and stirring was stopped. The reaction vessel was placed out of the light for 2 h. The synthesized AuNPs were mixed with 0.5 mL of

cysteamine (0.03 M), and ultrasonic heating the mixed solution (AuNPs-cysteamine) at 50 °C for 2 h. Finally, the AuNPs-cysteamine was mixed with the tau-441 protein solution (0.5–80 fM) at a volume ratio of 1:2 and incubated at 4 °C for 4 h.

Construction of the electrochemical multiple signal amplification biosensor

Six microliters of 1% BSA was added on the nanocomposite-antibody modified electrode surface for 1 h to avoid nonspecific adsorption. Subsequently, it was washed using PBS and dried using nitrogen and then incubated the modified electrode with tau-441-AuNPs conjugate for 30 min. Ten millimeters $[Fe(CN)_6]^{3-/4-}$ (1:1) were prepared as the electrochemical probe.

Electrochemical analysis

All electrical analyses in this experiment were based on a 3-electrode system using $[Fe(CN)_6]^{3-/4-}$ (1:1) as the probe. CV and EIS were used to confirm the signal amplification of MWCNTs-rGO-CS nanocomposite and the immobilization of antibody. The confirmation of the signal amplification of AuNPs and the determination of tau-441 protein were based on DPV. More details were shown in Electronic Supporting Material.

Treatment of serum samples

The serum samples were obtained from the sporadic community population (Hubei Province, China). This study was approved by the Hubei community and Wuhan University of Science and Technology, and it complies with the provisions of the Declaration of Helsinki and obtained written informed consent from all patients. More details were presented in Electronic Supporting Material.

Results and discussion

The principle of the electrochemical biosensor

Successful determination of trace tau-441 protein in the actual samples is the hinge of its further clinical application. Signal amplification is considered to achieve the goal. In work, nanomaterials were applied for the signal amplification. As Fig. 1 shows, intermolecular force exists between MWCNTs and rGO. In this experiment, the carboxyl group of MWCNTs (purchased from the company, showed in 2.1) can be coupled with the amino group in chitosan by the electrostatic force. The MWCNTs-rGO-CS film can form through the interaction between them. Then, the gold electrode was modified with the MWCNTs-rGO-CS film by the electrostatic adsorption to increase the electrical property of the electrode. To realize the further amplification of the signal, AuNPs was used to modify tau-441 protein. In the process, the sulfhydryl group at one end of the cysteamine can form an Au–S bond with the AuNPs, and the amino group at one end of the cysteamine can be coupled with tau-441 protein. The AuNPs-modified tau-441 protein can be recognized and captured specifically by the specific antibody. The antibody was immobilized on the MWCNTs-rGO-CS modified electrode by GLA, because

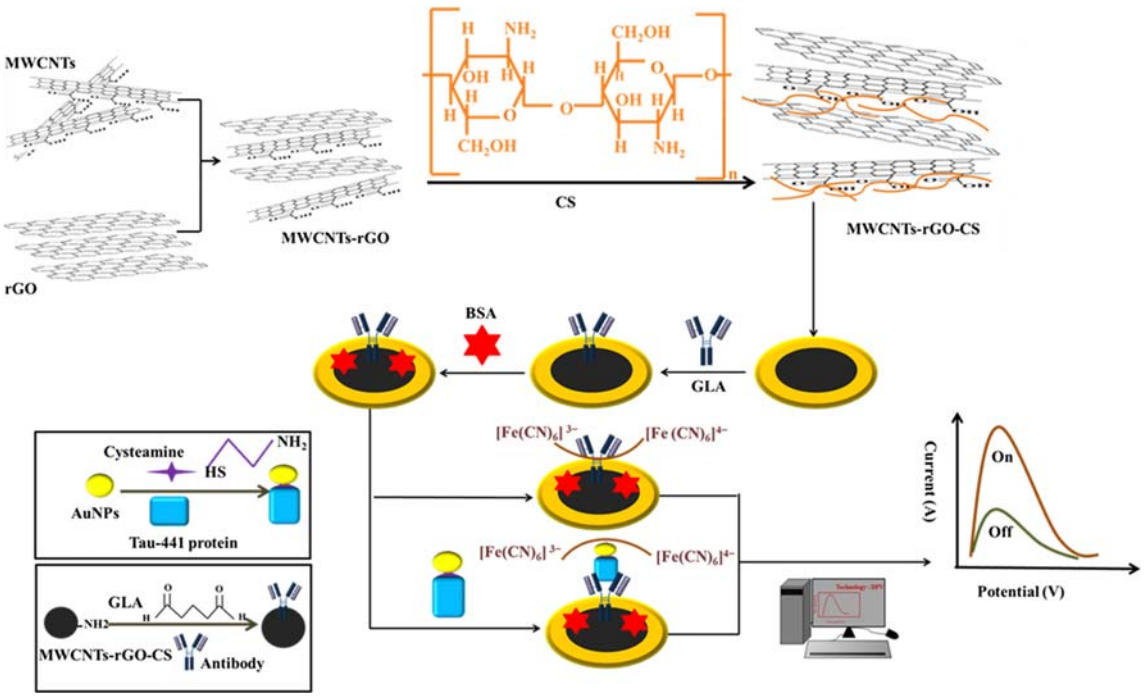

Fig. 1 The schematic diagram of the signal multi-amplified assay

the aldehyde group at both ends of GLA covalently can bond to the amino group of the CS and antibody, respectively. In this experiment, the antibody-tau-441 complex can be seen as a switch that controlled the electrical signals. There is not the antibody-tau-441 complex in the absence of tau-441 protein and the surface of the electrode undergoes the electron transfer, which is equivalent to open the channel for the electron transfer. Conversely, when tau-441 protein presents, the antibody-tau-441 complex forms. It blocks the process of the electron transfer, which is equivalent to shutting down the electron transfer channel.

Characterization of nanomaterials

As Fig. 2 a shows, the color of the synthesized AuNPs solution is orange. There is no precipitation in the solution, and the AuNPs solution is evenly dispersed. The transmission electron microscope (TEM) image of AuNPs is shown in Fig. 2 b_1. An enlarged view of a part of the Fig. 2 b_1 is shown in Fig. 2 b_2. Fig. 2 b_1 shows that AuNPs are evenly dispersed. It can be seen that the spherical AuNPs are approximately 4 nm as shown in Fig. 2 b_2. The large specific surface area of the spherical structure is beneficial to provide more binding site and generate the electrical signal.

TEM was used to realize the morphology features of MWCNTs, rGO, and MWCNTs-rGO nanocomposite. The TEM image of MWCNTs is shown in Fig. 2 c. The image shows an apparent tubular structure, but some parts of the MWCNTs tangles up. And Fig. 2 d shows the TEM image of rGO, there is the multilayer lamellar structure, but sheets agglomerate**.** Figure 2 e shows the TEM image of MWCNTs-rGO nanocomposite. It can be observed that the dispersibility of MWCNTs of the nanocomposite **(**Fig. 2e is improved compared with single MWCNTs **(**Fig. 2c. And the rGO sheet of nanocomposite in Fig. 2 e was thinner than it in Fig. 2 d.

Electrochemical evaluation of the signal amplification and the immobilization of antibody

CV and EIS can provide information about the electrochemical property of the electrode. The electrochemical evaluation of every modification step on the gold electrode was carried out by the CV and EIS in PBS containing 10 mM $[Fe(CN)_6]^{3-/4-}$. The CV measurements of a bare gold electrode, rGO modified gold electrode, MWCNTs modified gold electrode, MWNCNTs-rGO modified gold electrode, MWCNTs-rGO-CS modified gold electrode, and MWCNTs-rGO-CS-antibody modified gold electrode are shown as Fig. 3 a. According to the CV measurement, the peak current of the electrode covered with MWCNTs or rGO both are higher than a bare gold electrode. It indicates that both MWNNTs and rGO can improve the electron transfer. But the peak current of rGO is lower than that of MWCNTs. This is probably because rGO aggregates are more severe than MWCNTs. Then, the MWCNTs-rGO was modified on the electrode, causing a followed increase in the peak current. This result

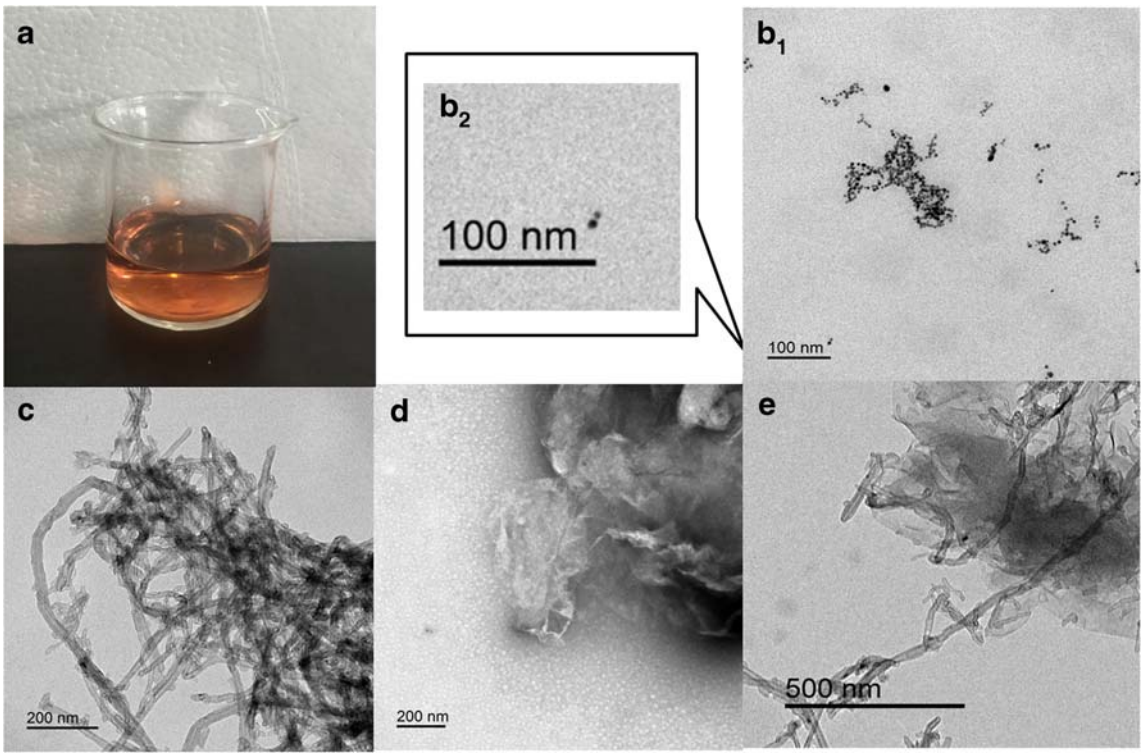

Fig. 2 **a** The photography of synthesized AuNPs. The TEM image of **b_1, b_2** AuNPs. **c** MWCNTs. **d** rGO. **e** MWCNTs-rGO

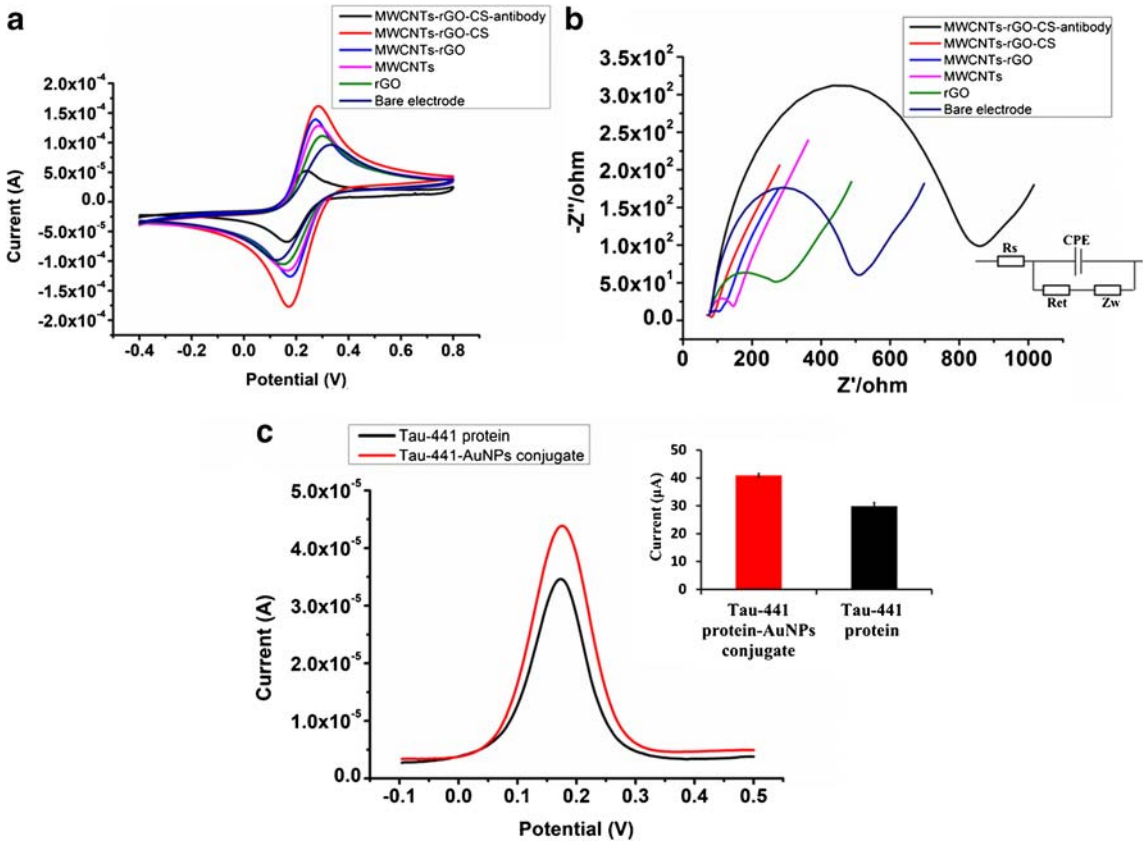

Fig. 3 **a** The CV measurement. **b** The EIS measurement of a bare gold electrode, rGO modified gold electrode, MWCNTs modified gold electrode, MWCNTs-rGO modified gold electrode, MWCNTs-rGO -CS modified gold electrode, and MWCNTs-rGO-CS-antibody modified gold electrode. **c** The DPV evaluation of the signal amplification based on AuNPs

is created by the synergy effects between MWCNTs and rGO. On the one hand, rGO improved the dispersibility of MWCNTs and the effective area of MWCNTs increased to make MWCNTs possess higher electrocatalytic activity. On the other hand, due to the doping of MWCNTs, the layer spacing of rGO was enlarged to avoid rGO agglomeration. The peak current of MWCNTs-rGO-CS modified gold electrode is higher than the peak current of MWCNTs-rGO modified gold electrode. The superb dispersing ability of CS possibly contributes to the result. Thus, the signal amplification was achieved step by step. A decrease of the peak current value of MWCNTs-rGO-CS-antibody modified gold electrode is observed, indicating the antibody immobilized on the electrode surface formed a steric hindrance and affected the electron transfer. As Fig. 3 b shows, the resistance of a bare gold electrode, rGO modified gold electrode, MWCNTs modified gold electrode, MWNCNTs-rGO modified gold electrode, and MWCNTs-rGO-CS modified gold electrode decreases gradually. And the resistance of MWCNTs-rGO-CS-antibody modified gold electrode increases. The same conclusion can be reached by the EIS measurement. It is that MWCNTs-rGO-CS enhanced the electrical conductivity, and the antibody was successfully immobilized to the electrode surface.

Tau-441 protein was modified with AuNPs for the further signal amplification. In the section, the signal amplification of AuNPs was researched by DPV using the MWCNTs-rGO-CS-antibody modified gold electrode in PBS containing 10 mM $[Fe(CN)_6]^{3-/4-}$. The peak current of tau-441-AuNPs conjugate is higher than it of tau-441 protein, as shown in Fig. 3(c). The electrical signal increases obviously on account of the AuNPs. A large number of electrical signal binding sites and vast specific surface area of AuNPs contribute to the result.

Optimization of the experiment conditions

In this work, the volume of MWCNTs-rGO-CS, the incubation time of antibody with tau-441 protein, the volume ratio between tau-441/AuNPs, and the sample pH were optimized using DPV in PBS containing 10 mM $[Fe(CN)6]^{3-/4-}$ to obtain a satisfying signal. As Fig. S1 shown, the optimal volume of MWCNTs-rGO-CS, the incubation time of antibody with tau-441 protein, the volume ratio between tau-441/AuNPs, and the sample pH were 6 μL, 30 min, 2:1, and 7.4. More descriptions were introduced in Electronic Supporting Material.

Evaluation of the property of the electrochemical biosensor

The analysis performance-line range, a limit of detection (LOD) and sensitivity in PBS

DPV was used to determinate tau-441 protein using $[Fe(CN)_6]^{3-/4-}$ (10 mM) as the probe. As Fig. 4 shows, the excellent response of DPV is observed at the concentrations from 0.5 to 80 fM of tau-441 protein. The peak current of the redox probe decreases with the increase of tau-441 protein concentration. Tau-441 protein was recognized and captured by the specific antibody, and antibody-tau-441 complex formed on the electrode surface. The complex can be seen as an electronic transfer channel switch. When the complex was formed, the electron transfer channel was turned off and it blocked the electron transfer, giving rise to a decreased electrical signal. As more complexes formed, more electron transfer channels were turned off and the electrical signal decreased even more.

As Fig. 4 shows, the changed electrical signal is linear with the logarithm (log) of the concentration of tau-441 protein, and the linear range is from 0.5 to 80 fM. Take the log of the tau-441 protein concentration as the x-axis, and ΔI as the y-axis. ΔI was obtained by multiple measurements ($n \geq 3$). The regression equation (y (μA) = 1.7692 × (fM) + 11.622 with $R^2 = 0.9942$) can be acquired. In accordance with the International Union of Pure and Applied Chemistry (IUPAC), the limit of detection (LOD) was calculated to 0.46 fM. The LOD is lower than those of the previous reports, as shown in Table S1. And the sensitivity is 25.27 μA fM^{-1} cm^{-2} (the surface area was obtained by CV using 5 mM $K_3[Fe(CN)_6]$ as a probe at different scan rates. The following Randles–Sevcik equation was used, $Ip = 2.69 \times 10^5 n^{3/2} A C_0 D^{1/2} \nu^{1/2}$ (Ip is anodic peak current, n which refers to the electron transfer is 1, C_0 is the concentration of $K_3[Fe(CN)_6]$, ν refers to the scan rate, and D is 0.76×10^{-5} cm^2 s^{-1} which represents the diffusion coefficient. According to the slope of the Ip and $\nu^{1/2}$ relation, the surface area was calculated as 0.07 cm^2). The electrochemical multiple signal amplification assay based on DPV had the ability to analyze trace tau-441 protein. This is because the electrode modification material (MWCNTs-rGO-CS) and AuNPs. MWCNTs and rGO both have good electrical conductivity, and can enhance the electron transport speed. When MWCNTs and rGO were mixed with CS, the dispersion performance was even better. The MWCNTs-rGO-CS nanocomposite can improve the conductivity of the gold electrode. Also, AuNPs contributed to the signal amplification further.

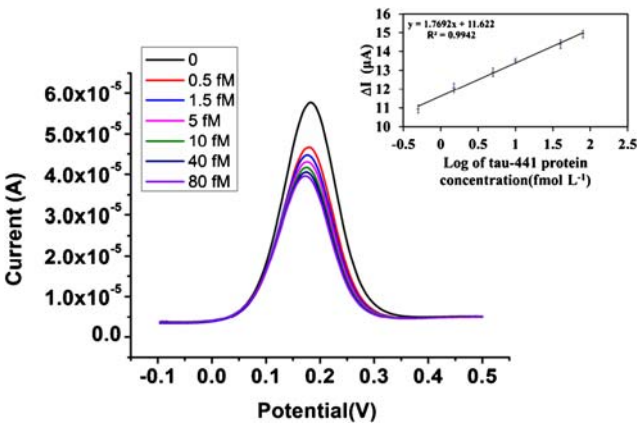

Fig. 4 The DPV measurement of tau-441 protein in PBS (0, 0.5, 1.5, 5, 10, 40, 80 fM) and the calibration plot between the log of the tau-441 protein concentration and ΔI

The specificity, the stability and the reproducibility

The interference experiment can reflect the specificity. Glucose (Glu), ascorbic acid (AA), L-cysteine (L-cys), and human serum albumin (HSA) are common substances in the blood, and α-synuclein (α-Syn) is an essential biomarker of Parkinson's disease. In this study, the above five molecules were selected as interfering substances to investigate the specificity of the electrochemical biosensor. Tau-441 protein (5 fM) was mixed with Glu (500 fM), AA (500 fM), L-cys (500 fM),α-Syn (500 fM), and HSA (500 fM), respectively. Tau-441 protein and these mixtures were tested by the signal multi-amplified assay. The peak current (41.44 ± 0.16 μA) of the tau-441 protein alone showed little difference compared with the peak current of tau-441 protein mixed with the interfering substance (39.93 ± 0.99 μA of tau-441 + Glu, 39.46 ± 0.17 μA of tau-441 + AA, 40.4 ± 1.2 μA of tau-441 + L-cys, 40.2 ± 1.5 μA of tau-441 + α-Syn, 39.7 ± 1.4 μA of tau-441 + HSA, $n = 3$), as shown in Fig. S2.

To investigate the stability of the MWCNTs-rGO-CS-antibody modified gold electrode, the modified electrode was assessed every 2 days. The modified electrode was stored at 4 °C. The peak current reserves 99.3% of its initial response after 3 days storage, 99.23% after 5 days storage, 97.11% after 7 days storage, 94.35% after 9 days storage, and 92.86% after 11 days storage, as shown in Fig. S3. The result indicates that the modified electrode had acceptable stability for more than a week.

In order to evaluate the reproducibility between different electrodes, three gold electrodes were tested. The three gold electrodes were respectively modified with 6 μL of the MWCNTs-rGO-CS nanocomposite. Then the same concentration of tau-441 protein was measured by three electrodes. The measurement result is shown in Fig. S4. The relative

Table 1 The measurement of recoveries (%) and RSD (%)

Sample	Added concentration (fM)	Found (fM)	Recoveries (%)	RSD (%)
1	1.5	1.36	90.67%	3.78
2	10	10.26	102.26%	1.96
3	40	40.93	102.33%	1.33

standard deviation of current density is 4.74% (the electrode surface area was calculated by Randle-Sevcik equation), demonstrating an acceptable reproducibility.

The accuracy and the precision

In this part, the signal multi-amplified assay was used to determinate the known low, medium, and high concentrations of tau-441 protein (1.5 fM, 10 fM, 40 fM). Each concentration was measured several times ($n = 3$). The results are shown in Table 1. The recoveries are between 90.67 and 102.33% with relative standard deviation (RSD) less than 5%, indicating that the electrochemical biosensor had a satisfying accuracy and precision.

Analysis of the biological samples-human serum

Figure 5 a shows the signal of different concentrations of tau-441 protein (0.5, 1.5, 5, 10, 40, and 80 fM) in serum using the signal multi-amplified assay. As the concentration of tau-441 protein increases, the electrical signal decreases, and it is similar to the determination of tau-441 protein in PBS. This is because the antibody-tau-441 complex can block the electron transfer pathway, leading to the decreased peak current. And the regression equation between the log of the concentration of tau-441 protein and ΔI was derived. The regression equation is y (μA) = 3.8626 × (fM) + 13.002 with R^2 = 0.994, indicating the fitting effect is relatively good. According to Fig. 5 a, ΔI (I_0-$I_{[tau-441]}$) of tau protein in serum is higher than that in PBS at the same concentration. A similar result was also reported in the previous research [26]. As Fig. 5 a shows, the signal value (I_0) of serum without tau-441 protein is similar to that of PBS without tau-441 protein, indicating that the proteins in the serum have little interference. However, when the same content of tau-441 protein was added to PBS and serum, ΔI (I_0-$I_{[tau-441]}$) in serum> ΔI (I_0-$I_{[tau-441]}$) in PBS. This is because the serum can provide a more suitable environment to make tau-441 protein have a better spatial structure, leading to a stronger binding between tau-441 and antibody. It can more effectively block the electron transfer, thus $I_{[tau-441]}$ in serum < $I_{[tau-441]}$ in PBS, causing ΔI (I_0-$I_{[tau-441]}$) in serum > ΔI (I_0-$I_{[tau-441]}$) in PBS.

In order to prove the practicability of the method further, the method was applied in the human serums of normal persons, MCI patients (whose state is an intermediate state between normal aging and dementia), and dementia patients (Took Alzheimer disease (AD) which is a common type of dementia patient as the research object). As Fig. 5 b shows, each cylinder in the figure represents one human serum sample (normal person in purple, MCI patient in orange, and dementia patient in green). A clear increasing trend of ΔI among the three types of serum samples can be seen. The ΔI average value of 14 normal persons, 14 MCI patients, and 14 dementia patients were 11.93 ± 0.18 μA, 15.07 ± 0.38 μA, 17.98 ± 0.42 μA (the number of measurements per sample, $n = 3$) in order and the ΔI increased in turn. Thus, the concentration of tau-441 protein was increasing with the increase of the degree of cognitive impairment. And it can be observed that ΔI of normal people, MCI patients, and dementia patients all have fluctuated. This indicates that the expression of tau-411 varied in individuals from normal people to dementia patients. This electrochemical multiple signal amplification assay was successfully applied for determination of tau-441 protein in human serum, providing new potential for early prediction of dementia.

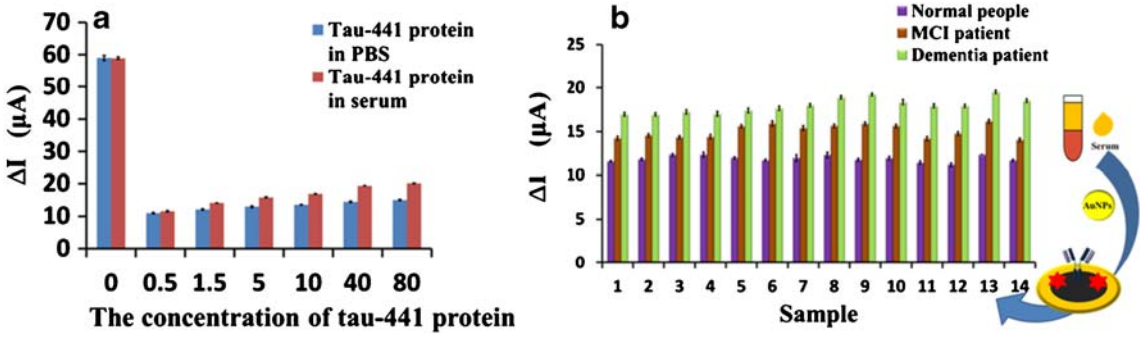

Fig. 5 **a** Electrical signal response values of tau-441 protein in different environments (PBS and serum). **b** The DPV measurement of tau-441 protein in serum of the normal person, MCI patient and dementia patient

Conclusion

In this research, a multiple signal amplification method was developed to determine tau-441 protein. An excellent conductive carbon nanocomposite film was modified on the gold electrode surface. Due to the synergy effect, the conductivity of MWCNTs-rGO nanocomposite was great than that of single MWCNTs and rGO. The electrical conductivity increased further because of the addition of CS. Tau-441 protein was modified with AuNPs to amplify the signal further. The LOD is as low as 0.46 fM. The assay of tau-441 protein exhibited a satisfactory specificity, stability, and reproducibility. The method successfully was applied to the biological sample, namely, the serum of the normal people, MCI patients, and dementia patients.

Acknowledgments Z.G. thanks the "ChuTian Scholar" Project Award support, Hubei Province, P.R. China.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict interest.

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