

A sensitive and selective electrochemical biosensor for the determination of beta-amyloid oligomer by inhibiting the peptide-triggered in situ assembly of silver nanoparticles

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Abstract: Soluble beta-amyloid (A β) oligomer is believed to be the most important toxic species in the brain of Alzheimer's disease (AD) patients. Thus, it is critical to develop a simple method for the selective detection of A β oligomer with low cost and high sensitivity. In this paper, we report an electrochemical method for the detection of A β oligomer with a peptide as the bioreceptor and silver nanoparticle (AgNP) aggregates as the redox reporters. This strategy is based on the conversion of AgNP-based colorimetric assay into electrochemical analysis. Specifically, the peptide immobilized on the electrode surface and presented in solution triggered together the in situ formation of AgNP aggregates, which produced a well-defined electrochemical signal. However, the specific binding of A β oligomer to the immobilized peptide prevented the in situ assembly of AgNPs. As a result, a poor electrochemical signal was observed. The detection limit of the method was found to be 6 pM. Furthermore, the amenability of this method for the analysis of A β oligomer in serum and artificial cerebrospinal fluid (aCSF) samples was demonstrated.

Keywords: electrochemical biosensors, Alzheimer's disease, beta-amyloid oligomer, peptide, silver nanoparticles

Introduction

Alzheimer's disease (AD), the most common neurodegenerative disorder, will affect ~66 million people globally by the year 2030.¹ A hallmark of AD is the deposition of the beta-amyloid (A β) peptide in the brain.^{2,3} A β monomer, typically comprising 39–43 amino acid residues, results from proteolytic cleavage of amyloid precursor protein (APP) by β - and γ -secretase.⁴ Furthermore, the monomers can coalesce to form small, soluble oligomeric species and then assemble into higher molecular weight fibrils. Thus, A β monomer and its aggregates have been considered not only as a therapeutic target but also as a diagnostic marker.^{5–9} There are many methods for the detection of A β monomer with high sensitivity, such as electrochemical immunosensors, colorimetric assays, resonance light scattering and surface plasmon resonance.^{10–18} However, assay of A β monomer only might be unable to discriminate between AD patients and healthy controls or other types of dementia because the levels of A β monomer may differ by gender and age.¹⁹ Soluble A β oligomer comprising 50–100 A β monomers is believed to be neurotoxic and responsible for neuronal death in preclinical AD.^{20,21} In addition, elevated levels of A β oligomer have been detected in the cerebrospinal fluid (CSF) of AD patients.^{22,23} Therefore, the direct detection of

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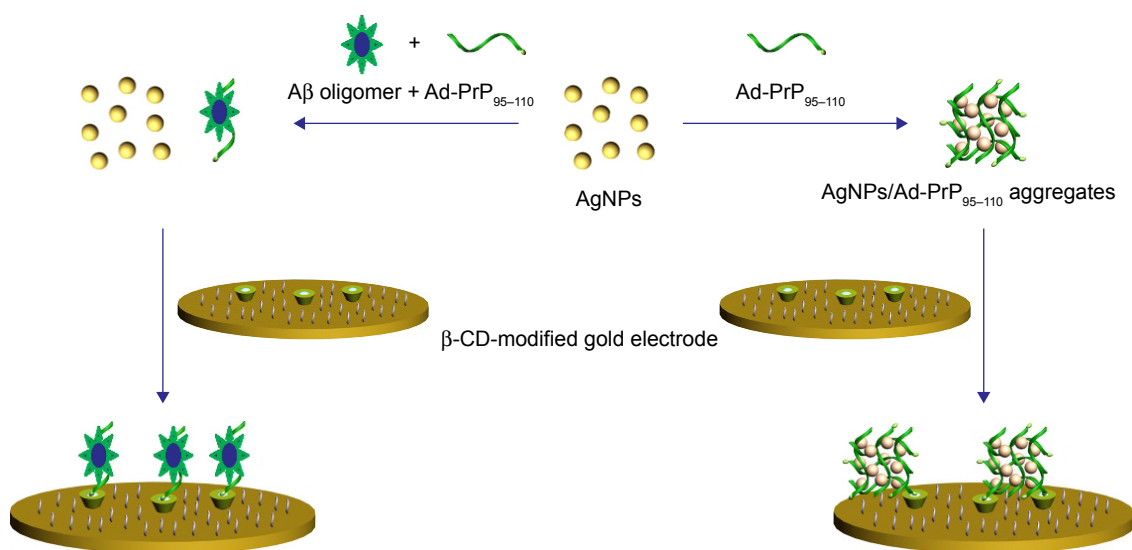
A β oligomer level would be more reliable for AD diagnosis than assay of its monomer.^{24,25}

Recently, a few novel biosensors have been developed for the detection of A β oligomer, including electrochemistry,^{26–29} surface plasma resonance (SPR),³⁰ localized surface plasmon resonance (LSPR),^{24,31} fluorescence,^{32,33} nuclear magnetic resonance,³⁴ and surface-enhanced Raman spectroscopy.³⁵ These methods are feasible, but they require the use of special instruments and/or relatively expensive and variable antibodies for the capture and recognition of A β oligomer. Moreover, the reported antibody of A β oligomer also recognizes A β monomer and other A β aggregates and metabolites to some extent.³⁶ Alternatively, the organic dye-based fluorescence assays (eg, thioflavin T [ThT]) have been commonly used for monitoring the formation of A β aggregates in laboratory investigation.^{37,38} However, most of the dyes cannot be used to discriminate A β oligomer from other β -sheets of A β aggregates,³⁷ thus limiting their applications for the routine test of A β oligomer for early diagnosis of AD.

Cellular prion protein (PrP^C) is a membrane-bound glycoprotein present in the central nervous system. There is increasing evidence demonstrating that PrP^C may be a high-affinity receptor for A β oligomer.^{39–44} The core region of PrP^C to bind with A β oligomer is PrP_{95–110}, which is located within the unstructured N-terminal region of PrP^C with an amino acid sequence of THSQWNKPSKPKTNMK (PrP_{95–110}).^{39,42–45} The dissociation constant (K_d) for the A β oligomer/PrP_{95–110} interaction is in the subnanomolar range, and the interaction is highly specific for A β oligomer, but not for its monomer

and fibril.^{42,43,46} These results provide researchers a hint that PrP_{95–110} would be a good receptor for the design of novel biosensors for A β oligomer detection.

In recent years, metal nanoparticles (MNPs) have been widely used for creating effective recognition and transduction processes in chem/biosensing due to their unique physicochemical attributes.^{47–59} In particular, silver nanoparticles (AgNPs) offer clear advantages for the design of electrochemical (bio) sensors, such as a simple preparation procedure, a size-dependent optical property, facile surface modification, a high surface area and a low oxidation potential.^{55–59} Based on the specific A β oligomer/PrP_{95–110} interaction and the well-defined and signal-amplified electrochemical signal of AgNP aggregates, Xia et al⁵⁹ have developed an electrochemical biosensor for the determination of A β oligomer by using adamantine (Ad)-labeled PrP_{95–110} (Ad-PrP_{95–110}) as the receptor and AgNP aggregates as the redox reporters. In this work, the network architecture of Ad-PrP_{95–110}/AgNP nanocomposites produced in solution was introduced onto the β -cyclodextrin (β -CD)-modified electrode surface through the host–guest interaction (Scheme 1). The specific A β oligomer/PrP_{95–110} interaction made the Ad-PrP_{95–110} in solution to lose its capability to trigger the formation of AgNPs-based network architecture. This work presented a concept for converting the AgNPs-based colorimetric assay into a sensitive electrochemical analysis by simply incorporating the colorimetric principle into the electrochemical platform. The method is simple and does not require the modification of analyte-binding molecules onto the surface of nanoparticles. However, it requires the modification



Scheme 1 Schematic illustration of the previous electrochemical strategies for the detection of A β oligomer with PrP_{95–110} as the receptor and AgNP aggregates as the redox reporters.

Abbreviations: A β , beta-amyloid; PrP, prion protein; AgNP, silver nanoparticle; Ad, adamantine; β -CD, β -cyclodextrin.

of both electrode and peptide probe. More importantly, the unmodified method showed poor anti-interference ability to high concentration of salts and other components in body fluids, thus failing to determine A β oligomer in biological samples. In the present study, we reported an innovative electrochemical method for the detection of A β oligomer based on the in situ formation of AgNP aggregate tags. As shown in Scheme 2, PrP₉₅₋₁₁₀ immobilized on the electrode surface and presented in solution triggered together the in situ formation of AgNP aggregates, which produced a well-defined electrochemical signal. Once the electrode was covered with A β oligomer, PrP₉₅₋₁₁₀ on the electrode surface would lose its ability to trigger the in situ formation of AgNPs-based network architecture. To avoid the absorption of other components onto the surface of unmodified AgNPs in the real sample analysis, the competitive assay was performed by a two-step procedure: incubation of the sensing electrode with A β oligomer sample first and follow-up incubation with AgNPs/PrP₉₅₋₁₁₀. The proposed strategy not only features simple manipulation principle similar to that of colorimetric assay but also shows high sensitivity and specificity of electrochemical biosensor.

Experimental section

Chemicals and materials

Peptides with the sequences of CTHSQWNKPSKPKTNMK and THSQWNKPSKPKTNMK (PrP₉₅₋₁₁₀) were synthesized and purified by Synpeptide Co., Ltd (Shanghai, China). The A β peptide with 42 amino acid residues (A β ₁₋₄₂), 6-mercapto-1-hexanol (MCH), tris(2-carboxyethyl)phosphine (TCEP), bovine

serum albumin (BSA), immunoglobulin G (IgG), lysozyme, thrombin, serum and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All other chemicals were of analytical grade and provided by Beijing Chemical Reagent Co. Ltd (Beijing, China).

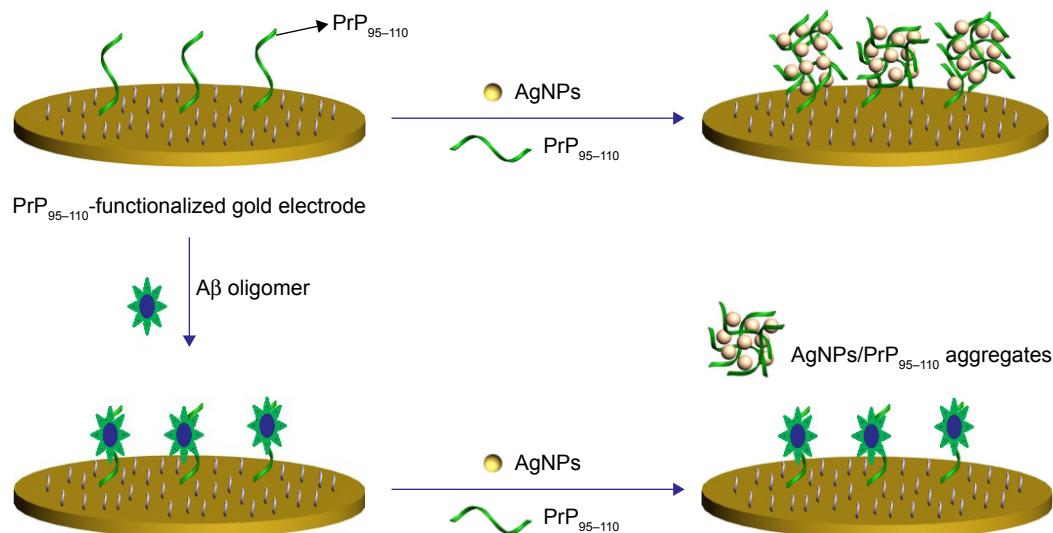
Citrate-stabilized AgNPs and soluble A β oligomer were prepared as in our previous report.⁵⁹ Artificial cerebrospinal fluid (aCSF) used in the determination of the samples was prepared by 150 mM NaCl, 3 mM KCl, 1.4 mM CaCl₂, 1 mM phosphate and 0.8 mM MgCl₂.^{29,60}

Instruments

The ultraviolet (UV)/visible (Vis) spectra were collected on a Cary 60 spectrophotometer using a 1-cm quartz spectrophotometer cell. The atomic force microscopy (AFM) images were taken using a Dimension Edge microscope (Bruker Nano Inc., Santa Barbara, CA, USA) equipped with a tapping mode. The transmission electron microscope (TEM) images were taken using an FEI Tecnai G2 T20 TEM (Hillsboro, OR, USA). The electrochemical experiments were carried out using a CHI-660E (CH Instruments, Shanghai, China) electrochemical workstation. Platinum wire was used as the auxiliary electrode. The reference electrode was Ag/AgCl.

Stability of AgNPs

To examine the inhibition of A β oligomer on the PrP₉₅₋₁₁₀-triggered assembly of AgNPs, PrP₉₅₋₁₁₀ was mixed with A β oligomer for 10 min. Then, AgNPs suspension was added to the PrP₉₅₋₁₁₀ solution. After incubation for 5 min, color change



Scheme 2 Schematic illustration of the present electrochemical strategies for the detection of A β oligomer with PrP₉₅₋₁₁₀ as the receptor and AgNP aggregates as the redox reporters.

Abbreviations: A β , beta-amyloid; PrP, prion protein; AgNP, silver nanoparticle.

was observed with the naked eye and the photograph was taken by a digital camera. UV/Vis absorption spectra were collected using the spectrophotometer.

Electrochemical detection of A β oligomer

The cleaned gold disk electrode with a diameter of 2 mm was placed in a 100 μ L phosphate-buffered saline (PBS) solution (10 mM, pH 7.2) containing 10 μ M thiolated PrP₉₅₋₁₁₀ (CTHSQWNKPSKPKTNMK) and 50 μ M TCEP overnight. After the formation of peptide self-assembled monolayers (SAMs), the electrode was washed with water and then soaked in a 1 mM MCH solution for 30 min. For the detection of A β oligomer, the PrP₉₅₋₁₁₀-functionalized electrode was first immersed in a 20 μ L PBS solution containing a given concentration of A β oligomer for 10 min, and the electrode was then rinsed thoroughly with water and exposed to 20 μ L of AgNPs suspension in an opened plastic tube. This step was followed by the addition of 20 μ L of PrP₉₅₋₁₁₀ to incubation for 10 min. After being rinsed with water, the electrode was placed in a 1 M KCl solution for linear sweep voltammetry (LSV) measurement.

Results and discussion

PrP₉₅₋₁₁₀-triggered AgNPs aggregation

As shown in Figure 1A, the AgNPs solution showed an absorption peak at 404 nm (black curve), which is ascribed to the surface plasmon resonance of AgNPs. With the addition of PrP₉₅₋₁₁₀, the original absorbance of AgNPs at 404 nm

decreased, while a new absorbance peak at 525 nm appeared (red curve). The red-shifted band demonstrated that PrP₉₅₋₁₁₀ triggered the aggregation of AgNPs. The aggregation is attributed to the electrostatic interaction between the negatively charged citrate-capped AgNPs and the positively charged lysine residues in PrP₉₅₋₁₁₀.⁵⁹ We also found that the absorption intensity of AgNPs at 525 nm increased and reached a plateau value within 7 min, indicating the achievement of the PrP₉₅₋₁₁₀-triggered AgNPs assembly. When PrP₉₅₋₁₁₀ was first mixed with A β oligomer, only one absorption peak at 404 nm was observed (blue curve) with the addition of the mixed solution to AgNPs suspension. It is indicative of a good dispersion of AgNPs in the presence of the A β oligomer-PrP₉₅₋₁₁₀ complex. Furthermore, these results were confirmed by the TEM observations: aggregated AgNPs in the presence of PrP₉₅₋₁₁₀ only (Figure 1B) and dispersed AgNPs in the presence of A β oligomer/PrP₉₅₋₁₁₀ (Figure 1C). We also found that A β monomer and fibril did not inhibit the PrP₉₅₋₁₁₀-triggered red shift of AgNPs absorbance, which agrees with the previous report.⁵⁹ These results confirmed that only A β oligomer inhibited the PrP₉₅₋₁₁₀-induced assembly of AgNPs, which is contributed to the strict dependence of the recognition of PrP₉₅₋₁₁₀ on the secondary structure of A β .

Electrochemical analysis

Herein, we suggested that PrP₉₅₋₁₁₀ both on electrode and in solution could trigger the in situ formation of AgNP

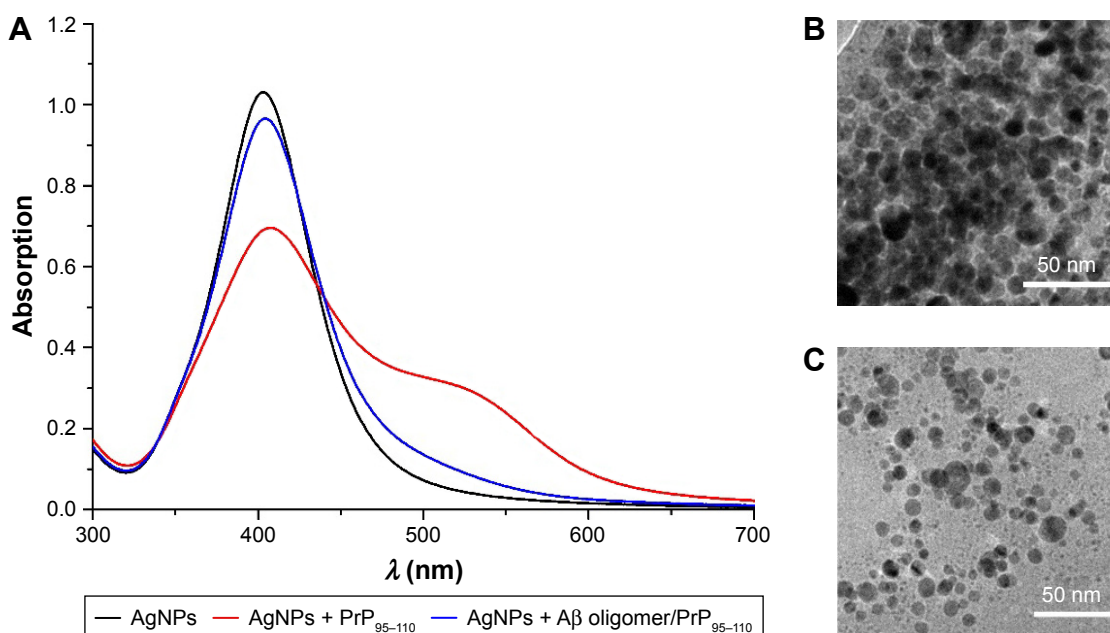


Figure 1 Characterization of AgNPs in the presence PrP₉₅₋₁₁₀ or A β oligomer/PrP₉₅₋₁₁₀.

Notes: (A) UV-Vis absorption spectra of AgNPs in the absence and presence of PrP₉₅₋₁₁₀ or A β oligomer/PrP₉₅₋₁₁₀. TEM images of AgNPs in the presence of PrP₉₅₋₁₁₀ (B) or A β oligomer/PrP₉₅₋₁₁₀ (C). The concentrations of AgNPs, PrP₉₅₋₁₁₀ and A β sample (equivalent monomer) were 2.4 nM, 0.1 μ M and 2 μ M, respectively.

Abbreviations: UV, ultraviolet; Vis, visible; AgNP, silver nanoparticle; PrP, prion protein; A β , beta-amyloid; TEM, transmission electron microscope.

aggregates on the electrode surface. When PrP₉₅₋₁₁₀ immobilized on the electrode surface interacted with A β oligomer, it lost the ability to trigger the in situ formation of AgNPs-based network architecture. To demonstrate the feasibility of our design, LSV was used to measure the oxidation current of AgNPs. As shown in Figure 2, incubation of the PrP₉₅₋₁₁₀-functionalized electrode with AgNPs/PrP₉₅₋₁₁₀ resulted in the appearance of a well-defined oxidation peak at ~65 mV (black curve), which is attributed to the solid-state Ag/AgCl reaction from AgNPs. However, no oxidation peak was observed when the functionalized electrode was incubated with PrP₉₅₋₁₁₀ itself (red curve), and only a small oxidation peak was observed when the electrode was incubated with AgNPs only (blue curve). These results demonstrated that the strong oxidation peak in the black curve should be attributed to the formation of the AgNPs/PrP₉₅₋₁₁₀ network architecture. When the electrode was incubated with A β oligomer, followed by incubation with PrP₉₅₋₁₁₀/AgNPs (green curve), the current dropped almost to the background level. This indicated that the binding of PrP₉₅₋₁₁₀ to A β oligomer inhibited the in situ formation of AgNPs/PrP₉₅₋₁₁₀ network architecture on the electrode surface. Additionally, we found that a slight decrease in the current was observed (magenta curve) when the sensor electrode was incubated with the mixed solution comprising AgNPs, PrP₉₅₋₁₁₀ and A β oligomer (one-step method). Thus, the two-step method performed

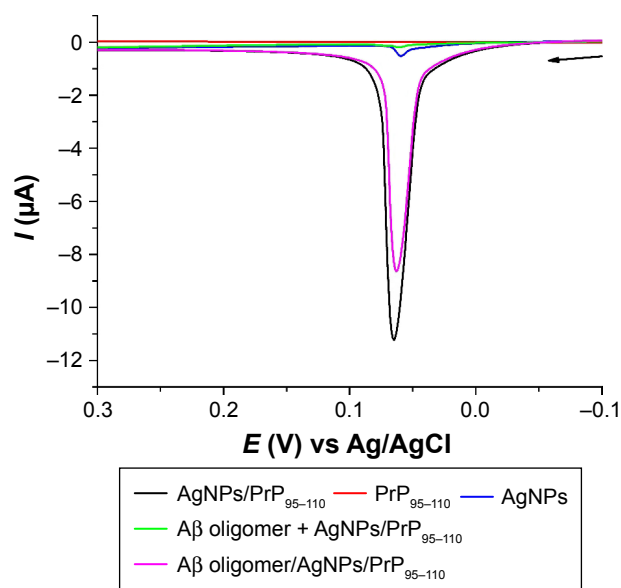


Figure 2 The LSV responses of the PrP₉₅₋₁₁₀-functionalized electrodes after incubation with AgNPs/PrP₉₅₋₁₁₀ (black curve), PrP₉₅₋₁₁₀ (red curve), AgNPs (blue curve), A β oligomer and AgNPs/PrP₉₅₋₁₁₀ (green curve) and the mixture of A β oligomer/PrP₉₅₋₁₁₀/AgNPs (magenta curve).

Notes: The arrow indicates the scan direction. The concentrations of AgNPs, PrP₉₅₋₁₁₀ and A β sample were 2.4 nM, 0.1 μ M and 2 μ M, respectively.

Abbreviations: LSV, linear sweep voltammetry; PrP, prion protein; AgNP, silver nanoparticle; A β , beta-amyloid.

by incubation of the sensor electrode with A β oligomer first and follow-up incubation with AgNPs/PrP₉₅₋₁₁₀ (green curve) is more sensitive than the one-step method. The result is understandable since large amount of PrP₉₅₋₁₁₀ in solution would preferentially bind to A β oligomer, thus hampering the formation of A β oligomer/PrP₉₅₋₁₁₀ on the electrode surface and facilitating the in situ assembly of AgNPs. Furthermore, other components in biological samples may absorb on the surface of unmodified AgNPs to reduce the selectivity of biosensor.⁵⁹ Therefore, the competitive assay was performed by the two-step procedure.

Optimization of experimental conditions

A higher concentration of PrP₉₅₋₁₁₀ can make the aggregation of AgNPs more powerful. However, a higher concentration of PrP₉₅₋₁₁₀ in solution would compete with the anchored PrP₉₅₋₁₁₀ on the electrode surface to bind with AgNPs, thus hampering the in situ formation of the AgNPs/PrP₉₅₋₁₁₀ network architecture. Thus, we first investigated the effect of the concentration ratio of PrP₉₅₋₁₁₀ to AgNPs ($[\text{PrP}_{95-110}]/[\text{AgNPs}]$) on the oxidation current (I_{pa}). It was found that I_{pa} initially increased with the increasing $[\text{PrP}_{95-110}]/[\text{AgNPs}]$ ratio until the maximal value appeared at 83:1 (Figure 3A). Furthermore, the dependence of I_{pa} on the AgNPs concentration was examined. It was found that I_{pa} increased upon increasing concentrations of AgNPs and began to level off beyond 1.2 nM (Figure 3B). Thus, in the following quantitative assays of A β oligomer, the concentrations of AgNPs and PrP₉₅₋₁₁₀ were kept at 1.2 and 100 nM, respectively.

With the increase in incubation time, A β monomers can assembly spontaneously into oligomeric and fibrous species. We also studied the influence of A β incubation time on the formation of A β oligomer and the inhibition of PrP₉₅₋₁₁₀-triggered assembly of AgNPs. As shown in Figure 4, the lowest points of the currents are in the range of 16–24 h, indicating the optimal incubation time for the formation and detection of A β oligomer. In the following quantitative assays, 20 h was set as the optimized time for oligomer preparation.

Sensitivity and selectivity

Under the optimized experimental conditions, the quantitative detection of A β oligomer was performed. As shown in Figure 5A, I_{pa} decreased with increasing A β oligomer concentration ($[\text{A}\beta]$, equivalent monomer) varying from 0 to 2 μ M. The relative standard deviations (RSDs) are all <13% for assay of the same A β oligomer sample at three different electrodes in parallel. The acceptable reproducibility demonstrated that multiple electrodes can be

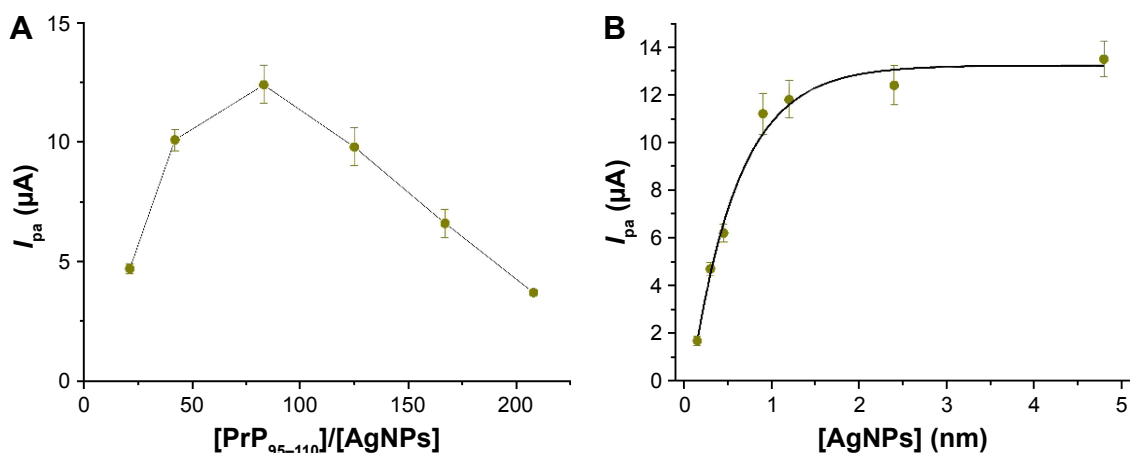


Figure 3 Dependence of the current on the concentration ratio of PrP₉₅₋₁₁₀ to AgNPs (A) and the AgNPs concentration (B).

Notes: In (A), the AgNPs concentration was kept at 2.4 nM and the concentration of PrP₉₅₋₁₁₀ was increased from 0.05 to 0.5 μM (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μM). In (B), the concentration ratio of PrP₉₅₋₁₁₀ to AgNPs was kept at 83:1 and the AgNPs concentration was increased from 0.15 to 4.8 nM (0.15, 0.3, 0.45, 0.9, 1.2, 2.4 and 4.8 nM). I_{pa} , oxidation current.

Abbreviations: PrP, prion protein; AgNP, silver nanoparticle; A β , beta-amyloid.

prepared concurrently for the analysis of many different samples. Herein, the current change ΔI_{pa} ($I_{pa} - I_{pa}'$, where I_{pa} and I_{pa}' represent the current in the absence and presence of A β oligomer, respectively), was used to evaluate the sensor performances. As shown in the inset, ΔI_{pa} is proportional to [A β] in a linear range of 0.01–200 nM. The regression equation was found to be $\Delta I_{pa} = 0.289 + 0.045 [A\beta]$ (nM). The detection limit was estimated to be 6 pM by measuring the sensor response to a dilution series and determining the target smallest concentration at which the sensor response is

clearly distinguishable from the response to a blank solution. This value is comparable to that achieved by the AgNPs- or AuNPs-based LSPR techniques (0.1 or 1.5 pM),^{24,31} and is significantly lower than that achieved by other methods, including molecular beacon (MB; 3.57 nM)-based,⁶ graphene oxide (1 nM)-based and CdTe quantum dots (QDs)-based fluorescent assays,⁴⁶ square wave voltammetry (48 pM),²⁷ electrochemical impedance spectroscopy (100 pM),⁴⁵ magnetic bead-droplet immunoassay (2.22 mM)⁶¹ and surface-enhanced Raman spectroscopy (0.1 μM).³⁵ Moreover, our

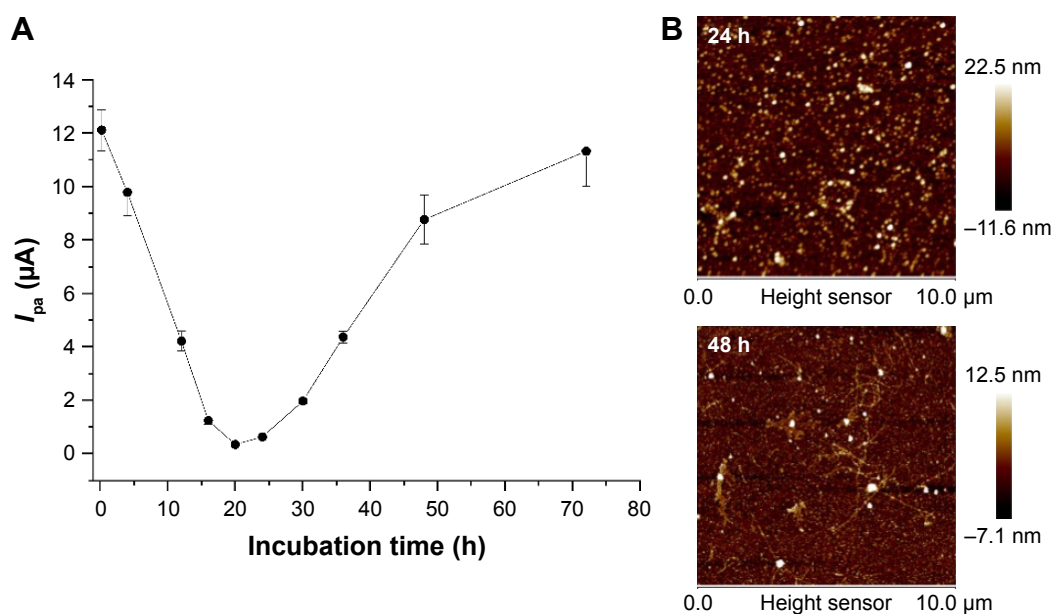


Figure 4 Influence of A β incubation time on the formation of A β oligomer and the current.

Notes: (A) Dependence of the current on the incubation time for the preparation of A β oligomer. The final concentrations of AgNPs, PrP₉₅₋₁₁₀ and A β sample were kept at 1.2 nM, 100 nM and 1 μM , respectively. (B) AFM images of the mica substrate after incubation with A β samples pre-incubated for 24 and 48 h. I_{pa} , oxidation current.

Abbreviations: A β , beta-amyloid; AgNP, silver nanoparticle; PrP, prion protein; AFM, atomic force microscopy.

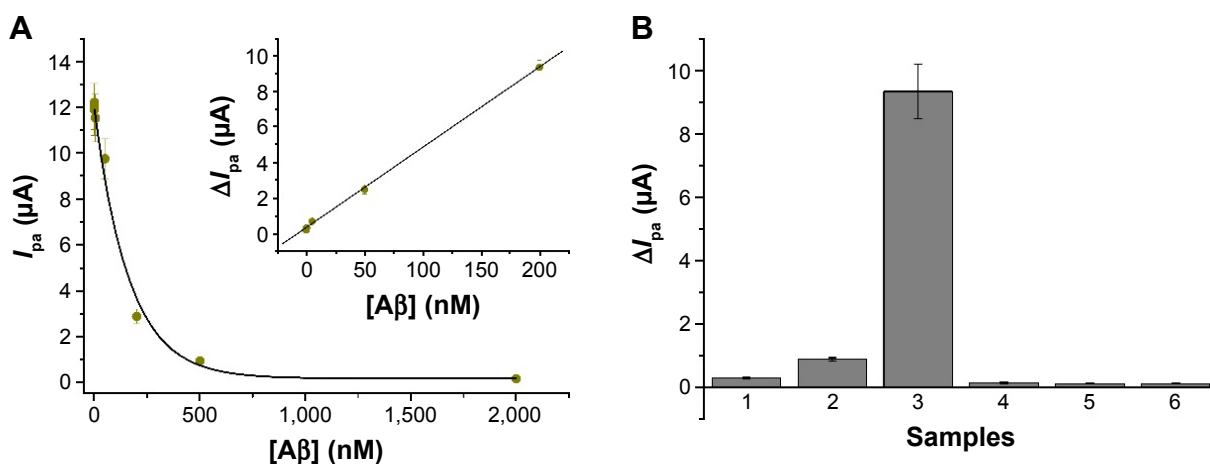


Figure 5 Sensitivity and selectivity.

Notes: (A) Dependence of the current on the concentration of A β sample (0.01, 0.2, 5, 50, 200, 500 and 2,000 nM). The inset shows the linear dependence of the current change on the concentration of the A β sample. (B) Selectivity of the sensing protocol (bar 1, A β monomer; bar 2, A β fibril; bar 3, A β oligomer; bar 4, BSA; bar 5, IgG and bar 6, thrombin). The concentration of the A β sample was 200 nM and that of BSA, IgG and thrombin was 1 μ M. I_{pa} , oxidation current.

Abbreviations: A β , beta-amyloid; BSA, bovine serum albumin; IgG, immunoglobulin G.

method required very simple sample handling procedure and obviated the modification of nanoparticles and the utilization of expensive and variable antibodies for the capture and recognition of A β oligomer. The physiological content of A β in a normal human CSF is in the range of nanomolar, and a higher concentration of A β oligomer is present in AD patients.² Thus, the proposed method is promising to detect A β oligomer in body fluids.

To explore the specificity of our method, A β monomer, A β fibril and three interfering proteins (BSA, IgG and thrombin,) were tested. As shown in Figure 5B, compared to the control, only the fibril control caused a significant change in the current. This is probably due to the existence of a small amount of unfibrillar oligomer in the solution. The other four interferences did not cause significant change in the current. The result demonstrated that the tested interferences did not prevent the assembly of AgNPs/PrP₉₅₋₁₁₀ on the sensor surface. Therefore, the proposed electrochemical method showed extraordinary selectivity toward the detection of A β oligomer. The high selectivity could be principally attributed to the strong and specific binding capacity of PrP₉₅₋₁₁₀ to A β oligomer.

Assay of A β oligomer in serum and aCSF

To demonstrate the viability of our method for real sample assay, the content of A β oligomer in aCSF and 20% serum was determined by the standard addition method. The accuracy of the assay was evaluated by determining the recovery for the spiked sample. As shown in Table 1, the recoveries for assays of three different concentrations of A β oligomer varied from 86% to 109%. The acceptable values implied

that the proposed method could provide a potential platform for the detection of A β oligomer in CSF and serum samples of AD patients.

Conclusion

This work presented an innovative electrochemical method for the detection of A β oligomer by inhibiting the in situ formation of AgNPs-based network architecture on the electrode surface. The A β oligomer-binding peptide was used as the recognition element. The proposed electrochemical method not only features simple manipulation principle and easy detection procedure similar to that of colorimetric assay but also shows high sensitivity and specificity. The detection limit of this method for A β oligomer detection is 6 pM, which is comparable to or lower than that achieved by the previously reported methods. However, our method is rapid (<30 min) and label free, obviates the modification of nanoparticles for signal amplification and does not require the utilization of expensive and variable antibodies and enzymes for the capture and recognition of A β oligomer. In view of the high

Table 1 Results of the proposed method for the detection of A β oligomer in aCSF and serum

Sample	Added (nM)	Found (nM)	Recovery (%)
1 (aCSF)	1	0.89	89
2 (aCSF)	20	21.8	109
3 (aCSF)	50	52.4	104.8
4 (serum)	1	0.86	86
5 (serum)	20	17.9	89.5
6 (serum)	50	43.7	91.4

Abbreviations: A β , beta-amyloid; aCSF, artificial cerebrospinal fluid.

toxicity of soluble A β oligomer in the brains of AD patients, the proposed biosensor could potentially serve as a viable alternative for facile clinical diagnosis of AD. The result also demonstrated that the bare AgNPs-based colorimetric assay can be converted into an electrochemical analysis with improving specificity. Moreover, this proposed detection principle should be valuable for developing label-free optical platforms with multiplexed aptameric peptide microarrays.

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Disclosure

The authors report no conflicts of interest in this work.

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