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ORIGINAL RESEARCH

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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Yun Xing[1,2]
Xiao-Zhen Feng[2]
Lipeng Zhang[1]
Jiating Hou[2]
Guo-Cheng Han[2]
Zhencheng Chen[2]

[1]Henan Province of Key Laboratory of New Optoelectronic Functional Materials, College of Chemistry and Chemical Engineering, Anyang Normal University, Anyang, [2]School of Life and Environmental Sciences, Guilin University of Electronic Technology, Guilin, Guangxi, People's Republic of China

Abstract: Soluble beta-amyloid (Aβ) 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 Aβ oligomer with low cost and high sensitivity. In this paper, we report an electrochemical method for the detection of Aβ 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 Aβ 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 Aβ 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 (Aβ) peptide in the brain.[2,3] Aβ 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, Aβ 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 Aβ monomer with high sensitivity, such as electrochemical immunosensors, colorimetric assays, resonance light scattering and surface plasmon resonance.[10–18] However, assay of Aβ monomer only might be unable to discriminate between AD patients and healthy controls or other types of dementia because the levels of Aβ monomer may differ by gender and age.[19] Soluble Aβ oligomer comprising 50–100 Aβ monomers is believed to be neurotoxic and responsible for neuronal death in preclinical AD.[20,21] In addition, elevated levels of Aβ oligomer have been detected in the cerebrospinal fluid (CSF) of AD patients.[22,23] Therefore, the direct detection of

Correspondence: Guo-Cheng Han
School of Life and Environmental Sciences, Guilin University of Electronic Technology, Jinji Road, No. 1, Guilin, Guangxi 541004, People's Republic of China
Email yunxinghxx@163.com

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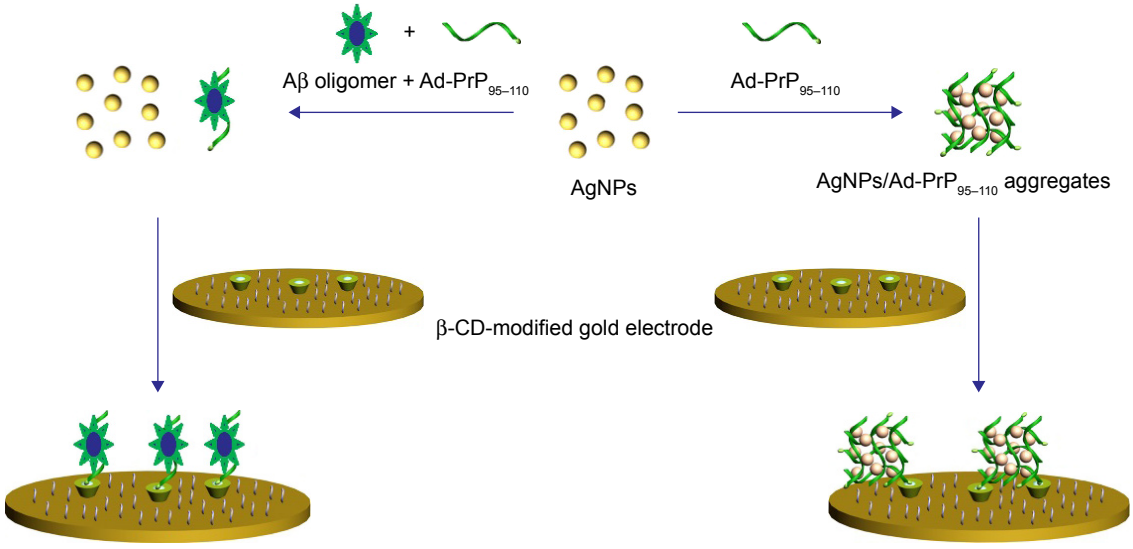
Aβ oligomer level would be more reliable for AD diagnosis than assay of its monomer.[24,25]

Recently, a few novel biosensors have been developed for the detection of Aβ 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 Aβ oligomer. Moreover, the reported antibody of Aβ oligomer also recognizes Aβ monomer and other Aβ 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 Aβ aggregates in laboratory investigation.[37,38] However, most of the dyes cannot be used to discriminate Aβ oligomer from other β-sheets of Aβ aggregates,[37] thus limiting their applications for the routine test of Aβ 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 Aβ oligomer.[39–44] The core region of PrP^C to bind with Aβ 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 Aβ oligomer/$PrP_{95–110}$ interaction is in the subnanomolar range, and the interaction is highly specific for Aβ 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 Aβ 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 Aβ oligomer/$PrP_{95–110}$ interaction and the well-defined and signal-amplified electrochemical signal of AgNP aggregates, Xia et al[59] have developed an electrochemical biosensor for the determination of Aβ 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 Aβ 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 Aβ oligomer with $PrP_{95–110}$ as the receptor and AgNP aggregates as the redox reporters.

Abbreviations: Aβ, 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 Aβ oligomer in biological samples. In the present study, we reported an innovative electrochemical method for the detection of Aβ oligomer based on the in situ formation of AgNP aggregate tags. As shown in Scheme 2, $PrP_{95–110}$ 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 Aβ oligomer, $PrP_{95–110}$ 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 Aβ oligomer sample first and follow-up incubation with AgNPs/$PrP_{95–110}$. 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 ($PrP_{95–110}$) were synthesized and purified by Synpeptide Co., Ltd (Shanghai, China). The Aβ peptide with 42 amino acid residues ($Aβ_{1–42}$), 6-mercapto-1-hexanol (MCH), tris(2-carboxyethyl)phosphine (TCEP), bovine serum albumin (BSA), immunoglobin 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).

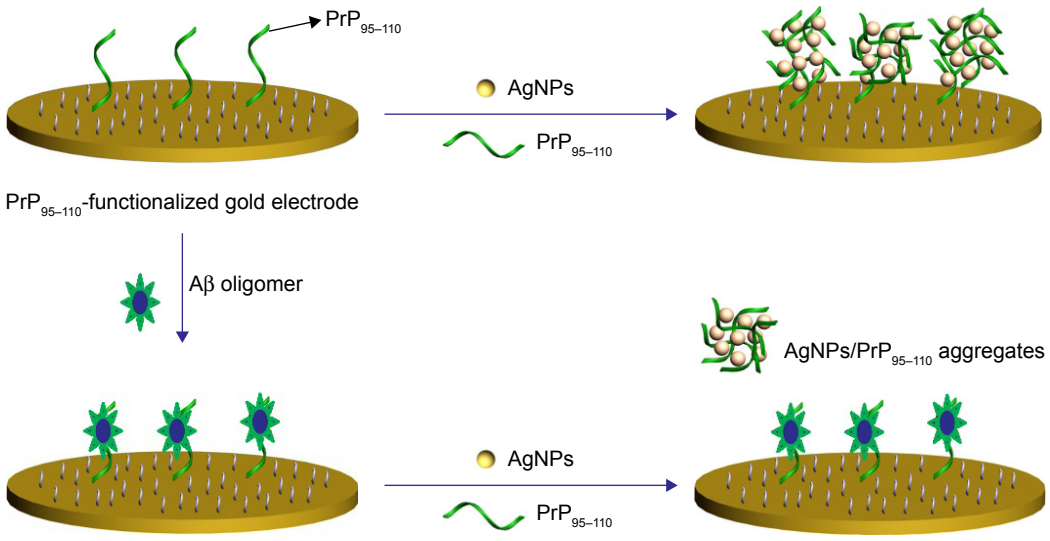
Citrate-stabilized AgNPs and soluble Aβ oligomer were prepared as in our previous report.[59] Artificial cerebrospinal fluid (aCSF) used in the determination of the samples was prepared by 150 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1 mM phosphate and 0.8 mM $MgCl_2$.[29,60]

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 Aβ oligomer on the $PrP_{95–110}$-triggered assembly of AgNPs, $PrP_{95–110}$ was mixed with Aβ oligomer for 10 min. Then, AgNPs suspension was added to the $PrP_{95–110}$ solution. After incubation for 5 min, color change



Scheme 2 Schematic illustration of the present electrochemical strategies for the detection of Aβ oligomer with $PrP_{95–110}$ as the receptor and AgNP aggregates as the redox reporters.

Abbreviations: Aβ, 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 Aβ oligomer

The cleaned gold disk electrode with a diameter of 2 mm was placed in a 100 μL phosphate-buffered saline (PBS) solution (10 mM, pH 7.2) containing 10 μM thiolated $PrP_{95–110}$ (CTHSQWNKPSKPKTNMK) and 50 μM 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 Aβ oligomer, the $PrP_{95–110}$-functionalized electrode was first immersed in a 20 μL PBS solution containing a given concentration of Aβ oligomer for 10 min, and the electrode was then rinsed thoroughly with water and exposed to 20 μL of AgNPs suspension in an opened plastic tube. This step was followed by the addition of 20 μL of $PrP_{95–110}$ 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

$PrP_{95–110}$-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 $PrP_{95–110}$, 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 $PrP_{95–110}$ 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 $PrP_{95–110}$.[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 $PrP_{95–110}$-triggered AgNPs assembly. When $PrP_{95–110}$ was first mixed with Aβ 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 Aβ oligomer–$PrP_{95–110}$ complex. Furthermore, these results were confirmed by the TEM observations: aggregated AgNPs in the presence of $PrP_{95–110}$ only (Figure 1B) and dispersed AgNPs in the presence of Aβ oligomer/$PrP_{95–110}$ (Figure 1C). We also found that Aβ monomer and fibril did not inhibit the $PrP_{95–110}$-triggered red shift of AgNPs absorbance, which agrees with the previous report.[59] These results confirmed that only Aβ oligomer inhibited the $PrP_{95–110}$-induced assembly of AgNPs, which is contributed to the strict dependence of the recognition of $PrP_{95–110}$ on the secondary structure of Aβ.

Electrochemical analysis

Herein, we suggested that $PrP_{95–110}$ both on electrode and in solution could trigger the in situ formation of AgNP

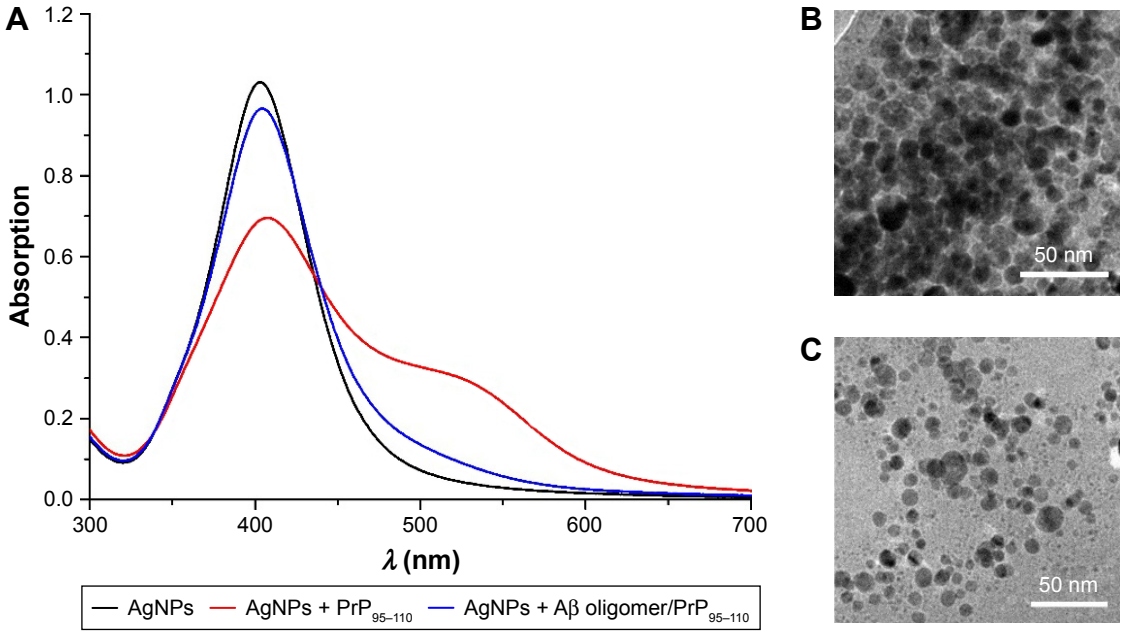


Figure 1 Characterization of AgNPs in the presence $PrP_{95–110}$ or Aβ oligomer/$PrP_{95–110}$.
Notes: (**A**) UV–Vis absorption spectra of AgNPs in the absence and presence of $PrP_{95–110}$ or Aβ oligomer/$PrP_{95–110}$. TEM images of AgNPs in the presence of $PrP_{95–110}$ (**B**) or Aβ oligomer/$PrP_{95–110}$ (**C**). The concentrations of AgNPs, $PrP_{95–110}$ and Aβ sample (equivalent monomer) were 2.4 nM, 0.1 μM and 2 μM, respectively.
Abbreviations: UV, ultraviolet; Vis, visible; AgNP, silver nanoparticle; PrP, prion protein; Aβ, beta-amyloid; TEM, transmission electron microscope.

aggregates on the electrode surface. When $PrP_{95–110}$ immobilized on the electrode surface interacted with Aβ 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 $PrP_{95–110}$-functionalized electrode with AgNPs/$PrP_{95–110}$ 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 $PrP_{95–110}$ 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/$PrP_{95–110}$ network architecture. When the electrode was incubated with Aβ oligomer, followed by incubation with $PrP_{95–110}$/AgNPs (green curve), the current dropped almost to the background level. This indicated that the binding of $PrP_{95–110}$ to Aβ oligomer inhibited the in situ formation of AgNPs/$PrP_{95–110}$ 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, $PrP_{95–110}$ and Aβ oligomer (one-step method). Thus, the two-step method performed by incubation of the sensor electrode with Aβ oligomer first and follow-up incubation with AgNPs/$PrP_{95–110}$ (green curve) is more sensitive than the one-step method. The result is understandable since large amount of $PrP_{95–110}$ in solution would preferentially bind to Aβ oligomer, thus hampering the formation of Aβ oligomer/$PrP_{95–110}$ 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.

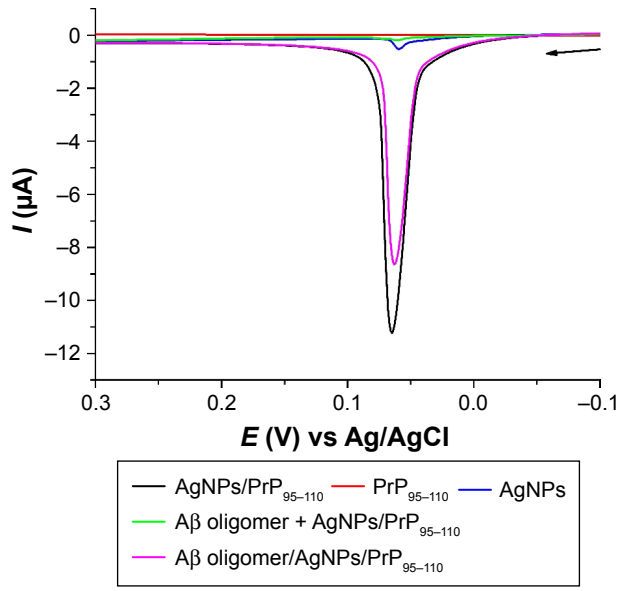


Figure 2 The LSV responses of the $PrP_{95–110}$-functionalized electrodes after incubation with AgNPs/$PrP_{95–110}$ (black curve), $PrP_{95–110}$ (red curve), AgNPs (blue curve), Aβ oligomer and AgNPs/$PrP_{95–110}$ (green curve) and the mixture of Aβ oligomer/$PrP_{95–110}$/AgNPs (magenta curve).
Notes: The arrow indicates the scan direction. The concentrations of AgNPs, $PrP_{95–110}$ and Aβ sample were 2.4 nM, 0.1 μM and 2 μM, respectively.
Abbreviations: LSV, linear sweep voltammetry; PrP, prion protein; AgNP, silver nanoparticle; Aβ, beta-amyloid.

Optimization of experimental conditions

A higher concentration of $PrP_{95–110}$ can make the aggregation of AgNPs more powerful. However, a higher concentration of $PrP_{95–110}$ in solution would compete with the anchored $PrP_{95–110}$ on the electrode surface to bind with AgNPs, thus hampering the in situ formation of the AgNPs/$PrP_{95–110}$ network architecture. Thus, we first investigated the effect of the concentration ratio of $PrP_{95–110}$ to AgNPs ([$PrP_{95–110}$]/[AgNPs]) on the oxidation current (I_{pa}). It was found that I_{pa} initially increased with the increasing [$PrP_{95–110}$]/[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 Aβ oligomer, the concentrations of AgNPs and $PrP_{95–110}$ were kept at 1.2 and 100 nM, respectively.

With the increase in incubation time, Aβ monomers can assembly spontaneously into oligomeric and fibrous species. We also studied the influence of Aβ incubation time on the formation of Aβ oligomer and the inhibition of $PrP_{95–110}$-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 Aβ 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 Aβ oligomer was performed. As shown in Figure 5A, I_{pa} decreased with increasing Aβ oligomer concentration ([Aβ], equivalent monomer) varying from 0 to 2 μM. The relative standard deviations (RSDs) are all <13% for assay of the same Aβ oligomer sample at three different electrodes in parallel. The acceptable reproducibility demonstrated that multiple electrodes can be

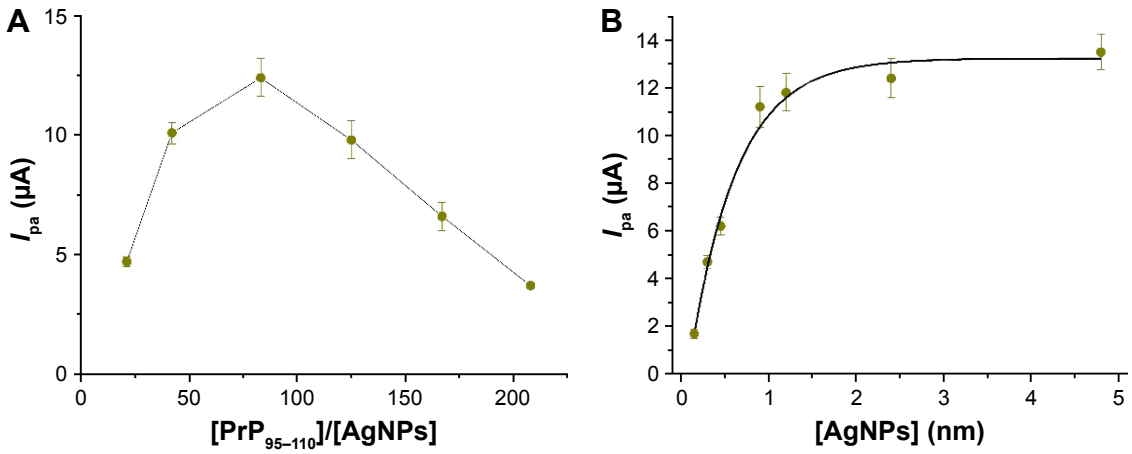


Figure 3 Dependence of the current on the concentration ratio of $PrP_{95–110}$ to AgNPs (**A**) and the AgNPs concentration (**B**).
Notes: In (**A**), the AgNPs concentration was kept at 2.4 nM and the concentration of $PrP_{95–110}$ was increased from 0.05 to 0.5 μM (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μM). In (**B**), the concentration ratio of $PrP_{95–110}$ 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; Aβ, 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 Aβ oligomer, respectively), was used to evaluate the sensor performances. As shown in the inset, ΔI_{pa} is proportional to [Aβ] in a linear range of 0.01–200 nM. The regression equation was found to be ΔI_{pa} =0.289+0.045 [Aβ] (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

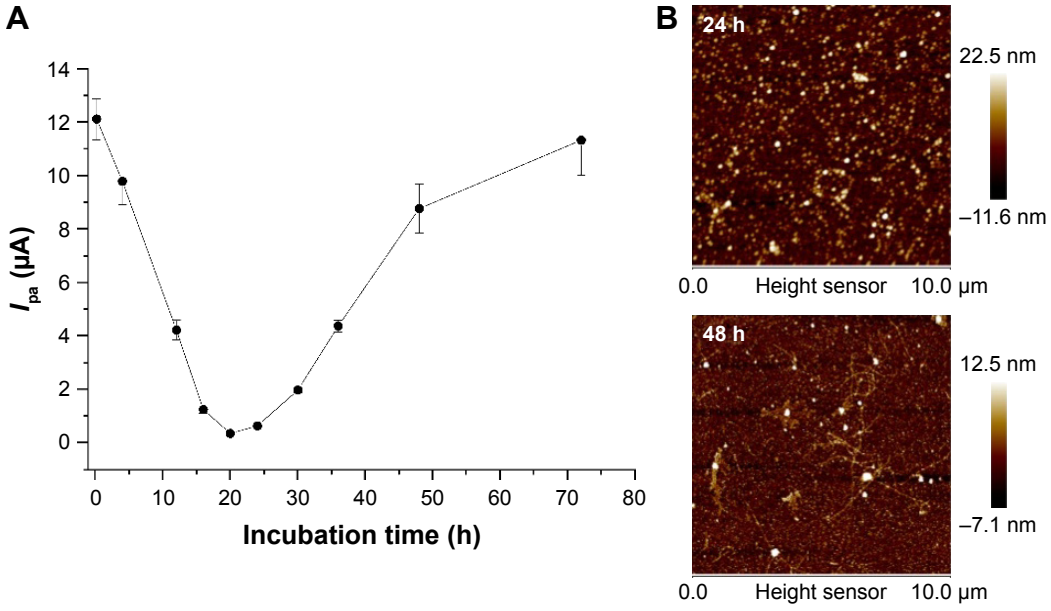


Figure 4 Influence of Aβ incubation time on the formation of Aβ oligomer and the current.
Notes: (**A**) Dependence of the current on the incubation time for the preparation of Aβ oligomer. The final concentrations of AgNPs, $PrP_{95–110}$ and Aβ sample were kept at 1.2 nM, 100 nM and 1 μM, respectively. (**B**) AFM images of the mica substrate after incubation with Aβ samples pre-incubated for 24 and 48 h. I_{pa}, oxidation current.
Abbreviations: Aβ, beta-amyloid; AgNP, silver nanoparticle; PrP, prion protein; AFM, atomic force microscopy.

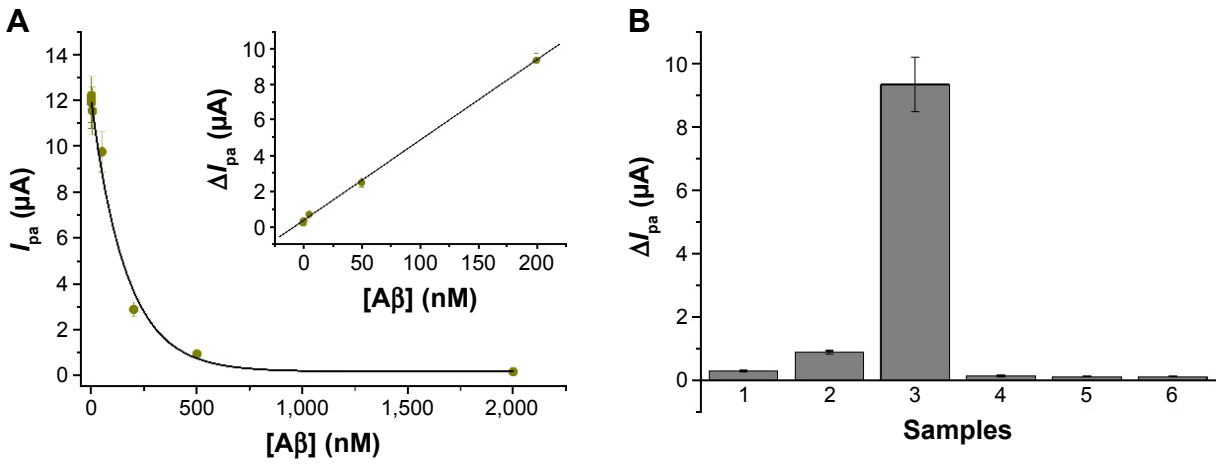


Figure 5 Sensitivity and selectivity.
Notes: (**A**) Dependence of the current on the concentration of Aβ sample (0.01, 0.2, 5, 50, 200, 500 and 2,000 nM). The inset shows the linear dependence of the current change on the concentration of the Aβ sample. (**B**) Selectivity of the sensing protocol (bar 1, Aβ monomer; bar 2, Aβ fibril; bar 3, Aβ oligomer; bar 4, BSA; bar 5, IgG and bar 6, thrombin). The concentration of the Aβ sample was 200 nM and that of BSA, IgG and thrombin was 1 μM. I_{pa}, oxidation current.
Abbreviations: Aβ, beta-amyloid; BSA, bovine serum albumin; IgG, immunoglobin 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 Aβ oligomer. The physiological content of Aβ in a normal human CSF is in the range of nanomolar, and a higher concentration of Aβ oligomer is present in AD patients.[2] Thus, the proposed method is promising to detect Aβ oligomer in body fluids.

To explore the specificity of our method, Aβ monomer, Aβ 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/$PrP_{95–110}$ on the sensor surface. Therefore, the proposed electrochemical method showed extraordinary selectivity toward the detection of Aβ oligomer. The high selectivity could be principally attributed to the strong and specific binding capacity of $PrP_{95–110}$ to Aβ oligomer.

Assay of Aβ oligomer in serum and aCSF

To demonstrate the viability of our method for real sample assay, the content of Aβ 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 Aβ oligomer varied from 86% to 109%. The acceptable values implied that the proposed method could provide a potential platform for the detection of Aβ oligomer in CSF and serum samples of AD patients.

Conclusion

This work presented an innovative electrochemical method for the detection of Aβ oligomer by inhibiting the in situ formation of AgNPs-based network architecture on the electrode surface. The Aβ 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 Aβ 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 Aβ oligomer. In view of the high

Table 1 Results of the proposed method for the detection of Aβ 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: Aβ, beta-amyloid; aCSF, artificial cerebrospinal fluid.

toxicity of soluble Aβ 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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