



Gold nanoparticle enhanced hybridization chain reaction as a method for signal amplification. Application to electrochemical immunodetection of the ovarian cancer biomarker carbohydrate antigen 125

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Abstract

The authors describe a method for enhancing the hybridization chain reaction (HCR) by using gold nanoparticles (AuNPs). This can considerably improve the sensitivity of electrochemical immunoassays as demonstrated for the carbohydrate antigen 125 (CA125), a biomarker for ovarian cancer. Compared to previous HCR based assays, the DNA acting as fuel strands were immobilized onto AuNPs, so that dendrimeric like chains were formed on the electrode after HCR. The improved signal is due to the reaction of DNA on the electrode. Specifically, the reaction of the phosphate groups of DNA with molybdate forms redox-active molybdophosphate, and this generates a strong electrochemical current. The immunosensor was prepared by sequential capturing, on the electrode, (a) antibody against CA125, (b) analyte (CA125), and (c) an aptamer against CA125 to form a sandwich structure. The primer on the aptamer sequence initiates HCR by annealing to one strand of DNA on the AuNPs and to another DNA in solution. The increased loading of DNA molecules onto the electrode increases the amount of phosphate groups and subsequently increases the electrical signal. The sensitivity of the assay is found to be significantly improved compared to assays without HCR and when using conventional HCR. The immunosensor was successfully applied to the determination of CA125 in human serum samples. The detection limit (based on an S/N ratio of 3) is $50 \mu\text{U}\cdot\text{mL}^{-1}$. This indicates that this signal amplification strategy has a large potential in terms of clinical applications. It may be modified such that it also can be applied to the determination of other analytes for which proper aptamers are available.

Keywords Gold nanoparticle · Hybridization chain reaction · Molybdophosphate · Immunoassay · Protein biomarker · Carbohydrate antigen 125

Introduction

Immunoassays are traditionally used for the detection of different protein biomarkers, however, their sensitivity is often limited for the detection of low level of analyte targets due to the difficulties for signal amplification [1–6]. Signal tags usually need to be conjugated onto antibodies, which is rather complex and can affect the bioactivity of antibodies [7]. On the other hand, DNA based signal amplification are versatile and can be simply amplified. For example, widely reported

DNA signal amplification strategies include polymerase chain reaction (PCR) [8, 9], rolling circle amplification (RCA) [10, 11], hybridization chain reaction (HCR) [12, 13] and et al.

Among the above mentioned DNA signal amplification strategies, HCR has been extensively reported for the detection of different analytes in connection with various detection techniques [14, 15]. HCR is a rapid linear amplification method that is enzyme-free and can be performed at room temperature. Typically, two stable species of DNA hairpins (H1 and H2) coexist in the solution until a primer strand is introduced [16, 17]. The two hairpin DNA molecules were triggered by the primer to start autonomously a cascade of hybridization events in which both hairpins assemble into long nicked double helices analogous to alternating copolymers [18].

Previously, we reported electrochemical assays for the detection of different protein biomarkers based on DNA molecules generated electrochemical current [19, 20]. The generation of

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electrochemical current was due to the reaction of DNA phosphate backbone with molybdate to form redox molybdophosphate [21, 22]. The electrochemical current intensity was proportional to the amount of DNA molecules on the electrode surface. So DNA signal amplification strategies, such as RCA and HCR can also be combined with the DNA generated current technique to further enhance the assay sensitivity.

Here, we describe a gold nanoparticle (AuNP) enhanced HCR as a means for signal amplification in electrochemical immunoassays. In contrary to traditional HCR that H1 and H2 DNA strands are coexist in the solution, we immobilized H1 onto AuNP surface. After construction of the immunoassay based on sandwich protocol, for signal amplification, HCR was carried out by annealing of H1 immobilized on AuNP to the sticky end primer sequence of the aptamer. Then each H1 strand on AuNP can anneal H2 strand to its complementary sequence. Through this way, each aptamer molecule can initiate dendrimeric like chain reaction, not just the formation of linear double helices strand. Thus, the loading of DNA molecules were significantly increased and then the sensitivity of the immunoassay was enhanced after reaction with molybdate. Protein biomarker carbohydrate antigen 125 (CA125), which is the clinical gold standard biomarker for ovarian cancer, was chosen as a model analyte to test this signal amplification strategy [23, 24]. The resulted electrochemical immunoassay display high sensitivity, low limit of detection, and was successfully applied for detection of CA125 in human serum samples.

Experimental

Reagents and instrumentations

The DNA strands used in our study were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China, <http://www.sangon.com/>). The detailed sequences of DNA used in this work are listed below. Anti-CA125 antibody and CA125 protein were obtained from Abcam (Cambridge, UK, <http://www.abcam.cn/>). Sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and chitosan were purchased from Sigma-Aldrich (<http://www.sigmaaldrich.com>). The serum samples were obtained from the Second Xiangya hospital that attached to Central South University (<http://www.xyey.com/>). All other reagents were of analytical grade and deionized water with a conductivity of 18.2 M Ω .cm was used throughout the experiment.

CA125 aptamer-primer sequence: TAATACGA CTCACTATAGGGAGACAAGAATAAACGCTCAATATC **GTTAATTCGGTTCG** (the bold part is the primer sequence).

H1: 5'-SH- TACGTGGCTTGGACCGACCGAATTAA CGATA-3'.

H2: 5'- GTCCAAGCCACGTATATCGTTAATTC GGTCG-3'.

Electrochemical measurements were performed on a CHI-650D electrochemical workstation (Shanghai CH Instruments Co., China, <http://www.chinstr.com/lxwm>). A conventional three-electrode system was used with glassy carbon electrode (GCE, 3 mm in diameter) as the working electrode, an Ag/AgCl electrode as the reference electrode and a platinum wire as the auxiliary electrode.

Preparation of the immunoassay

The immunoassay was prepared based on the sandwich protocol. To conjugate CA125 antibodies onto GCE surface, graphene oxide (GO) was dispersed into chitosan solution (0.2%) to reach a concentration of 3 mg.mL⁻¹ and 5 μL of the composite solution was dropped onto electrode surface. After dried at room temperature, the electrode was immersed into glutaraldehyde (0.25%) mixture for 30 min and then into anti-CA125 antibody solution (1 $\mu\text{g} \cdot \text{mL}^{-1}$) for 1 h. After gentle wash, the electrode was incubated with BSA (1%) solution for 30 min to block nonspecific binding sites. Then, the modified electrode was incubated with different concentrations of CA125 standard solution for 1 h. With another round of wash, subsequently, the electrode was incubated with 50 μM CA125 aptamer for 1 h. After wash again, the immunosensor was finished.

HCR reaction

To carry out HCR reaction, H1 DNA strand was immobilized onto 13 nm AuNP surface via the thiol moiety on the end of H1 DNA. Subsequently, 5 μL DNA hybridization buffer (10 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl, 1 mM MgCl₂, pH 7.4) containing H1 modified AuNPs and 50 μM of H2 was dropped on electrode and reacted at 37 °C for 2 h to accomplish the HCR. After extensive wash, the electrode was ready for measurement.

Electrochemical measurements

For electrochemical testing, 5 μL of Na₂MoO₄ solution (6 mM) was dropped onto electrode and reacted for 20 min. The electrode was then measured in 0.5 M H₂SO₄. Cyclic voltammetry (CV) measurements were performed at scan rate of 0.1 V/s. For square wave voltammetry (SWV) testing, the amplitude applied was 0.025 V with frequency of 15 Hz. The SWV current at a peak potential of 0.18 V was recorded to draw the calibration plot.

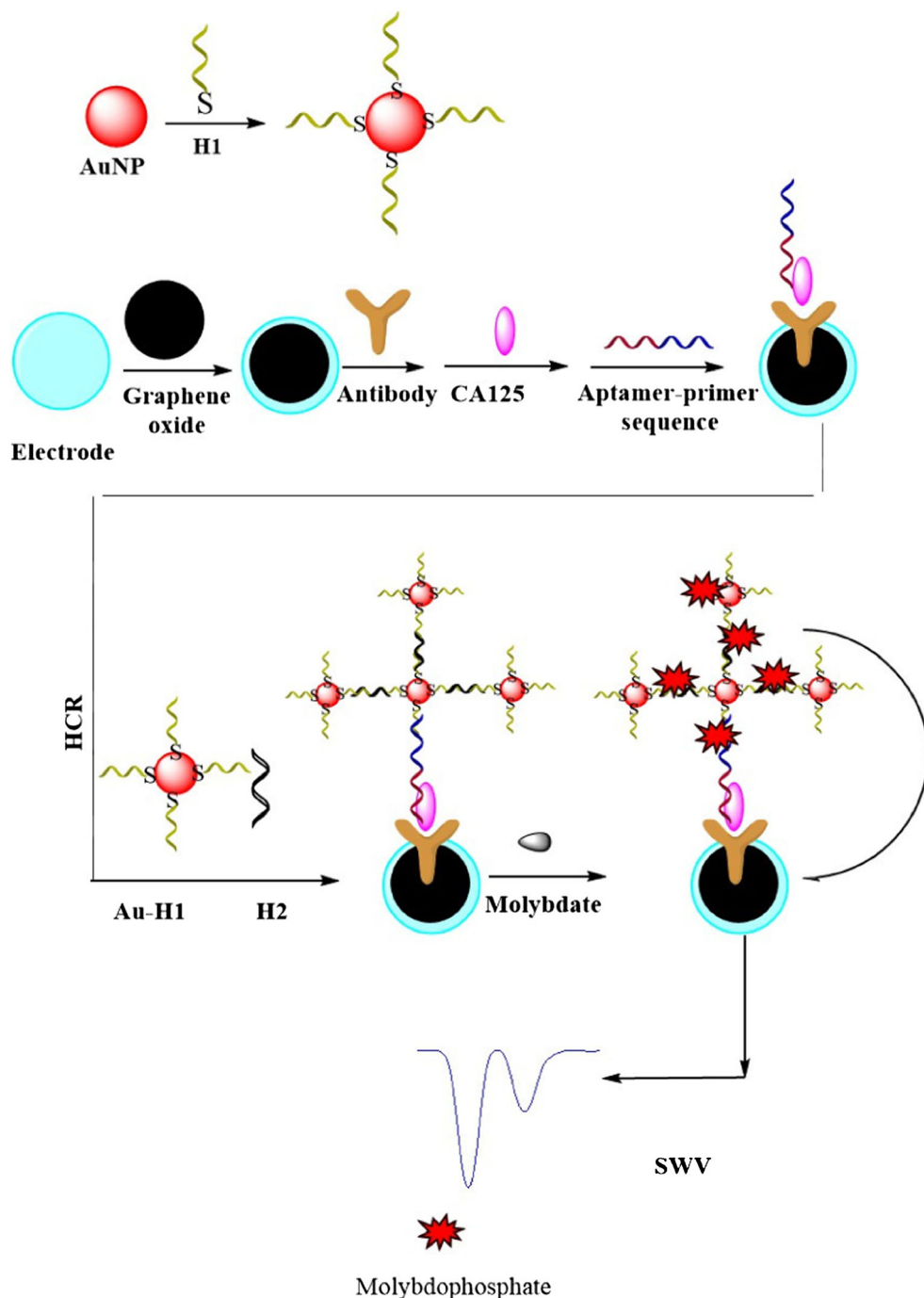
Results and discussion

Figure 1 shows the schematic representation of the immunoassay preparation and detection processes. The idea of the work is utilizing AuNP to enhance the HCR and improve the sensitivity of the immunosassay. After formation of the sandwich structure, HCR is carried out to induce dendrimeric like chain reaction due to the immobilization of H1 DNA strands on AuNPs. This is in contrary to traditional HCR that linear chain

reaction is performed. The signal of the immunoassay is due to the reaction of DNA molecules on electrode with molybdate to form redox molybdophosphate and generate electrochemical current. Since the HCR increased the amount of DNA molecules on the electrode surface, the sensitivity of the assay is significantly increased.

Before characterization of the performance of the immunoassay, initially, the immunoassay preparation and AuNP based HCR process were characterized by electrochemical impedance spectroscopy (EIS) measurements in 5 mM $[\text{Fe}(\text{CN})_6]^{3-}$

Fig. 1 Schematic representation of the immunoassay preparation and detection processes



$-/4$ solution. As shown in Fig. 2, compared to the bare electrode (curve a), the electron-transfer resistance of the electrode is increased after the antibody immobilization (curve b), the capture of CA125 as well as aptamer (curve c), the HCR (curve d) and the reaction with molybdate (curve e). Especially, there is a significant increase of the resistance values after HCR and after the reaction with molybdate, indicating the successful carry out of HCR and the formation of molybdophosphate precipitation on the electrode.

Then, the effect of the AuNP enhanced HCR on the improvement of the sensitivity of the immunoassay was studied in detail. Three immunoelectrodes were prepared for the detection of $1 \text{ U}\cdot\text{mL}^{-1}$ of CA125. The first immunoelectrode was based on aptamer along for signal amplification, the second was based on traditional HCR (with H1 and H2 DNA strands in solution) for signal amplification and the third was based on AuNP enhanced HCR for signal amplification. The square wave voltammetry (SWV) responses of the three immunoelectrodes are shown in Fig. 3. As can be seen, of the first immunoelectrode, two small current peaks are observed at around 0.20 and 0.32 V (curve a). The generated electrochemical current is ascribed to the reaction of aptamer phosphate backbone with molybdate that generated redox molybdophosphate [25–29]. The peak current is relatively small is because the amount of aptamer captured onto electrode surface and then the amount of phosphate groups is limited. After the traditional HCR was carried out, for the second immunoelectrode, the current intensity is increased about 3 times (curve b). This result proved the successful assemble of the DNA molecules onto electrode surface via HCR. The increased loading of DNA onto electrode resulted in enhanced electrochemical current intensity [30, 31]. Of the third immunoelectrode based on AuNP enhanced HCR, it can be seen that the

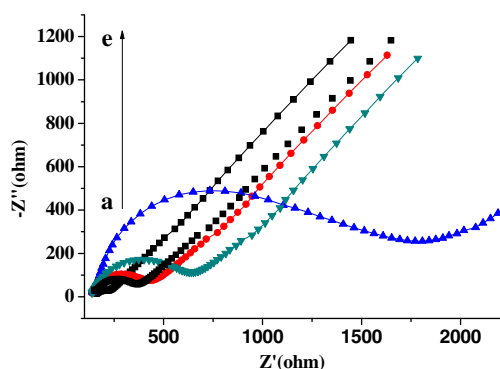


Fig. 2 Electrochemical impedance characterization of the immunoassay preparation process. Curve a to e represent the electron-transfer resistance of the bare electrode (curve a), the electrode after the antibody immobilization (curve b), after the capture of CA125 as well as aptamer (curve c), after HCR (curve d) and after the reaction with molybdate (curve e)

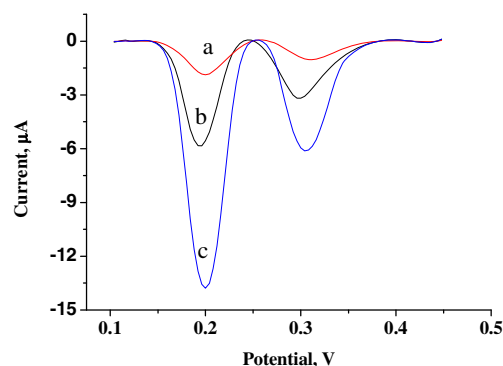


Fig. 3 SWV responses of immunoelectrodes for $1 \text{ U}\cdot\text{mL}^{-1}$ of CA125 based on aptamer along (a), traditional HCR (b) and AuNP enhanced HCR (c) for signal amplification

electrochemical current intensity is about 3 times to that based on traditional HCR. This data demonstrated that compared to traditional HCR, the AuNP based HCR further increased the loading of DNA molecules onto electrode, probably due to the formation of dendrimeric like DNA network.

After proved the AuNP based HCR can enhance the sensitivity of the immunoassay, we studied the effect of the HCR time on the sensitivity of the immunoassay. For the detection of $1 \text{ U}\cdot\text{mL}^{-1}$ of CA125, with the increase of the HCR time, the sensitivity of the immunoassay is increased continually. However, after 2 h, further increase of the reaction time resulted in no obvious enhancement of the sensitivity (Fig. 4). So the HCR time of 2 h was selected for the following experiments.

Under the optimized experimental conditions, different concentrations of CA125 standard solutions were then analyzed by the immunoassay to test the linear range of the immunoassay. From Fig. 5, we can see that the SWV peak current of the immunoassay at 0.2 V is increased with the increase of CA125 concentrations. A linear range between the current response of the immunoassay and the logarithm of concentrations of CA125 in the range from $100 \mu\text{U}\cdot\text{mL}^{-1}$ to $10 \text{ U}\cdot\text{mL}^{-1}$ is obtained (inset of Fig. 5) with sensitivity of $198 \mu\text{A}\cdot\text{U}\cdot\text{mL}^{-1}$

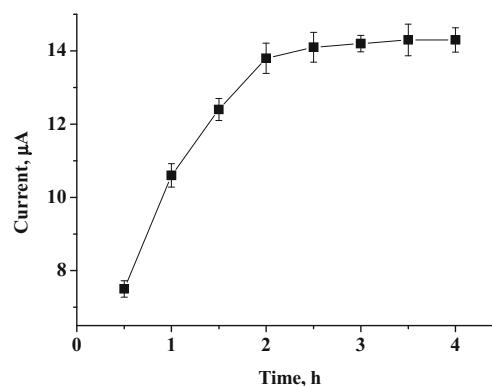


Fig. 4 Effect of HCR time on the sensitivity of the immunoassay towards detection of $1 \text{ U}\cdot\text{mL}^{-1}$ of CA125

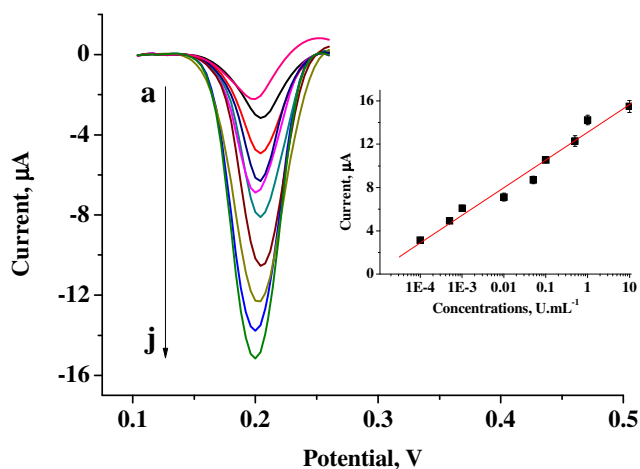


Fig. 5 SWV responses of the immunoassay to different concentrations of CA125, from a to j, 0 to 10 U.mL⁻¹. The inset is the calibration plot of the immunosensor to different concentrations of CA125. The current intensity at 0.2 V is acquired to establish the calibration plot

·cm⁻². The detection limit of the immunoassay that calculated based on a S/N ratio of 3 is 50 µU.mL⁻¹.

When comparing the performance of the immunoassay with literature reports for the detection of CA125, we can see the performance of our immunoassay is better than literature reports (Table 1). The good performance of our immunoassay, including high sensitivity and wide linear range is mainly ascribed to the AuNP enhanced HCR and the DNA molecules generated electrochemical current.

Besides high sensitivity and low limit of detection, good selectivity and reproducibility are also of great importance for application of the immunoassay in real sample analysis. Good selectivity is to minimize interference of other substances in the serum sample on the precise detection of target analyte. The responses of the immunoassay to potential interfering proteins, such as α-fetoprotein (AFP), carcinoembryonic antigen (CEA), human IgG, human epidermal growth factor receptor 2 (HER2) and human serum albumin (HSA) were tested. The 1 U.mL⁻¹ of CA125 were mixed with 10 ng.mL⁻¹ of the above mentioned interfering proteins and then analyzed by the immunoassay. The signal variation due

Table 1 An overview of literature reports for electrochemical determination of CA125

Signal probe	Linear range	Detection limit	Reference
Au/Pd composites	10 mU.mL ⁻¹ to 200 U.mL ⁻¹	5.4 mU.mL ⁻¹	[32]
Alkaline phosphatase	5 to 1000 U.mL ⁻¹	1.3	[33]
Gold nanostructure	10–100 U.mL ⁻¹	5.5 U.mL ⁻¹	[34]
g-C ₃ N ₄	1 mU.mL ⁻¹ to 5 U.mL ⁻¹	400 µU.mL ⁻¹	[35]
AuNP based HCR	100 µU.mL ⁻¹ to 10 U.mL ⁻¹	50 µU.mL ⁻¹	This work

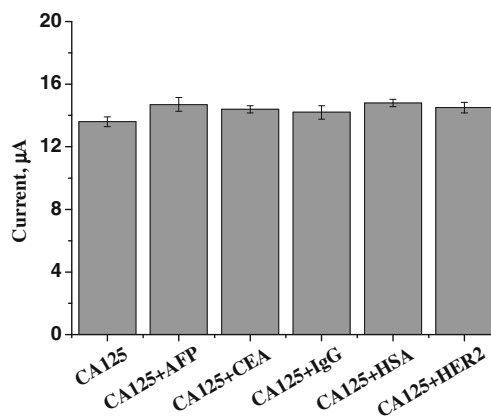


Fig. 6 Responses of the immunoassay to 1 U.mL⁻¹ of CA125 and the mixture of 1 U.mL⁻¹ of CA125 with 10 ng.mL⁻¹ of different proteins

to the presence of interfere proteins were within 10% of that without interfering proteins, demonstrating good selectivity of the immunoassay (Fig. 6). In addition, to study the reproducibility of the immunoassay, five immunoassays were prepared for the detection of 1 mU.mL⁻¹ and 1 U.mL⁻¹ of CA125. The relative standard deviation (RSD) of the testing result is 5.6 and 4.5%, respectively, indicating good reproducibility of the immunoassay.

Finally, to test potential clinical application of the immunoassay, clinical serum samples were obtained and then analyzed by the immunoassay. Eight human serum samples were collected. Before tested by the immunoassay, the serum samples were diluted with phosphate buffer. The level of CA125 in the serum samples that measured by the immunoassay were compared with those obtained by ELISA kit. From Fig. 7, we can see that the two results agreed well. A straight line was obtained by plotting the two CA125 values with a correlation coefficient of 0.991, demonstrating reliability of the immunoassay testing results.

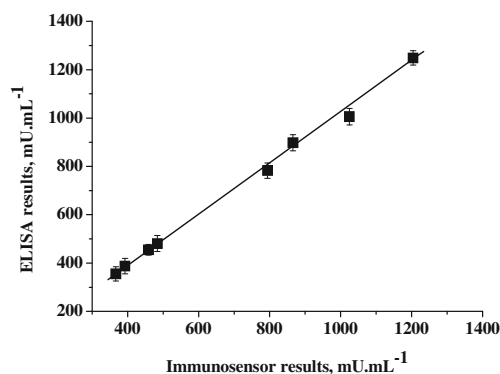


Fig. 7 Compare the CA125 level in serum samples determined by the immunoassay with those determined by ELISA

Conclusion

In summary, we studied a new HCR, that is AuNP enhanced HCR as signal amplification strategy for electrochemical immunoassays. The new signal amplification strategy was tested for detection of CA125 as a model analyte. The reaction of DNA molecules on electrode with molybdate leads to formation of molybdophosphate and generation of electrochemical current. Experimental data indicated that AuNP enhanced HCR improved sensitivity of the immunoassay compared to traditional HCR. This method expanded HCR as signal amplification strategy and will find wide applications in different bioassays. However, the detailed mechanism of the AuNP enhanced HCR was not studied in this work, which needs further research.

Compliance with ethical standards The author(s) declare that they have no competing interests.

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