



Immunochemical detection of the human epidermal growth factor receptor 2 (HER2) via gold nanoparticle-based rolling circle amplification

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Abstract

The authors describe an adapted rolling circle amplification (RCA) method for the determination of human epidermal growth factor receptor 2 (HER2). This method (which is termed immunoRCA) combines an immunoreaction with DNA based signal amplification. Gold nanoparticles (AuNPs) were loaded with antibodies against HER2 and DNA, and then fulfill the functions of recognizing HER2 and achieving signal amplification. The DNA serves as a primer to trigger RCA. This results in formation of a long DNA containing hundreds of copies of circular DNA sequence on the electrode surface. Then, molybdate is added which reacts with the phosphate group of the long DNA to generate the redox-active molybdophosphate. This, in turn, results in an increased current and, thus, in strongly increased sensitivity of the immunoassay. A linear response is linear relationship between the change of current intensity and the logarithm of the concentration in the range from 1 to 200 pg·mL⁻¹ of HER2, and the detection limit is 90 fg·mL⁻¹ (at an S/N ratio of 3). The method was applied to the determination of HER2 in breast cancer patients serum samples, and the results correlated well with those obtained by an ELISA. The method was further successfully applied to the determination of HER2 in HER2-expressed mouse breast cancer 4 T1 cells. Conceivably, this strategy may be adapted to other DNA amplification methods and also may be used for the determination of other proteins and biomarkers by using the appropriate antibodies.

Keywords ImmunoRCA · Rolling circle amplification · Human epidermal growth factor receptor 2 · Molybdate · Molybdophosphate

Introduction

There are many biological markers in serum or tissues present at too low a concentration to be detected by conventional immunoassays. Thus, establishing sensitive detection methods and effective signal amplification strategies are of great significance for extremely low level protein detection. Compared to conventional enzyme linked immunosorbent assay (ELISA) [1–5], fluorescence [4, 6] and chemiluminescence [7, 8],

electrochemistry has the advantages of relatively simple instrumentation, high sensitivity and low cost [9].

DNA based signal amplifications have been widely reported and include polymerase chain reaction (PCR) [10, 11], strand displacement amplification (SDA) [12], hybridization chain reaction (HCR) and rolling circle amplification (RCA). Among these methods, RCA is an isothermal technique that does not require expensive and complex equipment for thermal cycling or special conditions to avoid contamination [13–15]. Using a single primer, RCA generates hundreds of tandemly linked copies of the circular template DNA within a few minutes.

Human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor associated with cancer cell aggressiveness, which is related closely to cancer metastasis [16]. It is often used to determine the prognosis of breast cancer and help doctors to develop accurate treatment plans. HER2 detection is one of the essential testing items for breast cancer patients, because the HER2 positive patients are highly invasive and have higher recurrence rates. HER2 positive

Congcong Shen and Shuping Liu contributed equally to this work

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patients can employ anti-HER2 therapy by taking target HER2 drug Herceptin to control the tumor growth and improve survival rates effectively [17]. At present, the most commonly used diagnostic methods for HER2 involve fluorescent in situ hybridization (FISH) and immunohistochemistry (IHC) [18, 19]. However, FISH and IHC require invasive biopsy tissue sample, specific instruments and professional technician to complete. In addition, detection of biomarkers in serum and tumor cells has been suggested as prognostic and predictive way for cancer diagnosis and treatment [20]. With this in mind establishing sensitive and accurate method for detection of HER2 in easy access serum and cell is of great significance.

In previous work, we have studied DNA generated electrochemical current based on the reaction of DNA, specifically, the reaction of phosphate groups on DNA with molybdate to form redox molybdophosphate [21, 22]. In this paper, a novel immunoelectrochemical assay is designed combining antibody based immunoreaction with RCA signal amplification, termed “immunoRCA” for detection of HER2. Gold nanoparticles (AuNPs) loaded with antibody and RCA primer are utilized as immunoelectrochemical probe. The assay was established based on a sandwich structure with HER2 specific peptide, HER2 molecules and anti-HER2 antibody loaded AuNPs sequentially assembled onto electrode. In presence of circular DNA, DNA polymerase and nucleotides (DNTPs), RCA was carried out that resulted in a long DNA molecule containing hundreds of copies of the complementary circular DNA sequence. Then, the phosphate groups on the DNA sequence react with molybdate can form redox molybdophosphate precipitate and generate electrochemical current. RCA enhanced the amount of DNA on electrode, and then increased the sensitivity of the assay. After the detection of standard HER2 sample, we applied this immunoelectrochemical assay to detect HER2 in serum samples, which indicated the assay results are well correlated to ELISA kit testing results. In addition, HER2 in 4 T1 cell was also analyzed, and the electrochemical current intensity was increased in accordance with the number of cell tested.

Experimental section

Materials and apparatus

The peptide (CKLRLEWNR) used for capture HER2 was obtained from GL Biochem Ltd. (Shanghai, China, <http://www.glbiochem.com/>). HER2 protein and HER2-antibody (Ab) were acquired from Abcam Co., Ltd. (Cambridge, MA, <https://www.abcam.com/>). The primer and padlock probe (the sequences were listed in Supporting Information, Table S1) were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China, <http://www.sangon.com/>). *E. coli* DNA

Ligase and *E. coli* DNA Polymerase were purchased from Takara (Dalian, China, <http://www.takara.com.cn/>). Deoxynucleotides (dNTPs) was purchased from Genview Scientific Inc. (El Monte, CA, <http://www.gen-view.com/>). Sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and 6-mercapto-1-hexanol (MCH) were bought from Sigma-Aldrich (<https://www.sigmaaldrich.com/>). The human HER2 ELISA kit was obtained from Jining Biotech Co., Ltd. (Shanghai, China, <http://shjnsjh.cn.coovee.net/>). The human serum samples (six samples from breast cancer patients) were acquired from the Second Xiangya Hospital. All stock solutions were prepared with deionized water.

All electrochemical measurements were performed on CHI-650D electrochemical workstation (Shanghai CH Instruments Co., China). A standard three-electrode system with a working electrode (gold electrode, 2 mm in diameter), a saturated Ag/AgCl reference electrode, and a platinum auxiliary electrode was utilized for electrochemical measurement. Shimadzu UV-2450 spectrophotometer was used to record UV-spectra. FEI Titan G2 60–300 transmission electron microscope was used to examine the morphologies of AuNP and other compounds. Zeta potential was tested on a dynamic light scattering spectrometer (DLS, ZEN3600). For detecting HER2 in cell lysates, the 4 T1 cells were broken with Fisher Model FB120 ultrasonic processor.

Preparation of the gold nanoparticle (AuNP) probe

Preparation of the AuNP probe by modify AuNPs with anti-HER2 antibody and RCA primer. AuNPs were synthesized based on previous literature report [23, 24]. Briefly, 3 mL sodium citrate (*w/v*, 2%) was added to 100 mL boiling and stirring HAuCl_4 (0.01%) solution and kept heated for another 10 min. With continuous stirring until cooled to room temperature, the AuNPs were synthesized. To modify antibody and RCA primer onto AuNP, firstly, antibody was added to AuNP solution to reach a concentration of $500 \text{ pg}\cdot\text{mL}^{-1}$ and shaken for 2 h. Then the RCA primer was added to the mixture at a concentration of 1 mM and shaken for 16 h. After that 1 M NaCl was added to the mixture every 10 h successfully for 3 times until the NaCl reached 0.2 M. The solution was aged at room temperature for 24 h and centrifuged (12,000 rpm) for 30 min. Finally, the product was re-dispersed in deionized water. The probe was stored at 4 °C refrigerator before use.

Construction of the immunoelectrochemical assay

The cleaned gold electrode was immersed into $10 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ peptide solution at 4 °C overnight to link HER2 specificity peptide onto the gold electrode. After thorough washing with deionized water and drying with nitrogen, the electrode was blocked by dipped into 1 mM MCH for 50 min to avoid non-specific adsorption. Then a series of different concentrations of

HER2 were dropped onto the electrode for 2 h, followed by adding AuNP probes onto the electrode for another 1 h. Then, to perform RCA, after the electrode was washed, 0.2 μM padlock probes were added onto the electrode for 2 h to hybridize with the primer on the AuNP surface. Next the solution of 2 U *E.coli* DNA Ligase, 10 \times *E.coli* DNA Ligase buffer (provided along with ligase), 10 \times BSA (0.05%) was added onto the electrode at room temperature for 1 h to make the padlock probe form a circular template. Finally, the solution of 1 U *E.coli* DNA polymerase, 10 \times *E.coli* DNA polymerase buffer (provided along with polymerase) and 1 mM dNTPs were dropped onto electrode for 2 h. After washing again, 5 mM Na_2MoO_4 solution was dropped onto the electrode and incubated for 25 min before electrochemical detection.

Detection of HER2 in human serum and 4 T1 cell

The serum of breast cancer patients were diluted with water and detected by ELISA kit and our assay.

4 T1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ penicillin and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin at 37 $^\circ\text{C}$ in a humidified atmosphere containing 5% CO_2 . Then the cultured 4 T1 cells were broken with an ultrasonic processor (the ultrasonic frequency was 20 KHz) for 40 min at an interval of 3 s in ice bath and centrifuged for 10 min. The suspended lysate was collected and diluted to a series of concentrations with saline for the detection.

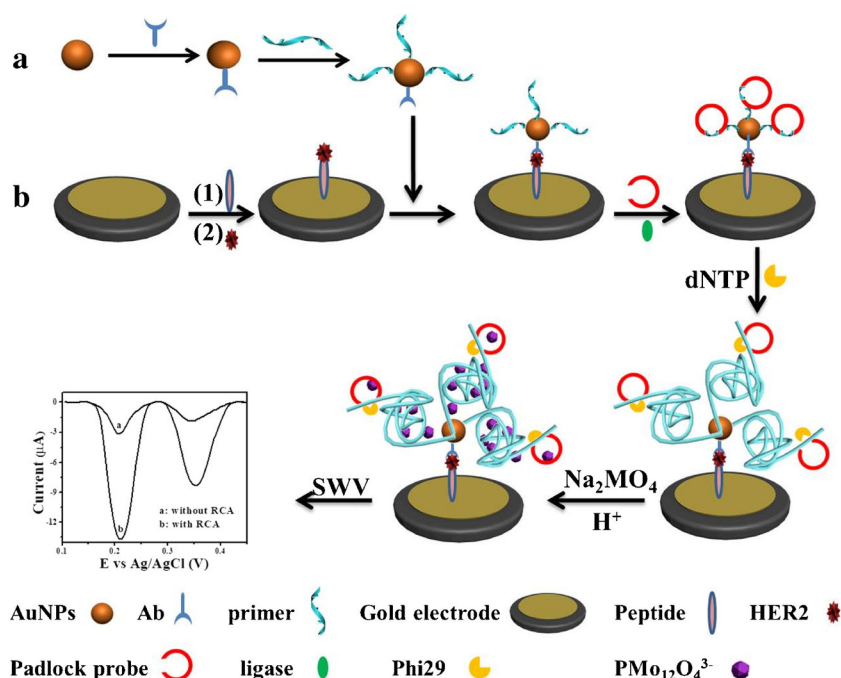
Results and discussion

Principle of the detection

In this paper, we combined rolling circle amplification (RCA) and traditional immunoreaction, termed "immunoRCA" to build an immunoelectrochemical assay for ultrasensitive detection of HER2. Scheme 1 shows the schematic representation for the synthesis of AuNP based electrochemical probe, for the preparation of the electrochemical assay and voltammetric detection process. Thiolated ss-DNA has a strong affinity for AuNPs and is quickly adsorbed. The antibodies are large molecules and leave enough space on the surface of the AuNPs to allow the subsequent adsorption of RCA primer. Stable AuNP probe was obtained by modifying both HER2 antibody and RCA primer onto AuNPs. The anti-HER2 antibody on AuNP probe was to bind with HER2 that was captured by HER2 specific peptide immobilized on gold electrode surface. Simultaneously, both the 3'- and 5'-ends of the padlock DNA can hybridize to the 3'-end of the primer (The sequences of DNA used in this article were listed in Table S1.), and resulted in a circular template for RCA. The long repeated DNA sequence, as a result of RCA, can react with molybdate, generating electrochemical current and measured by voltammetry.

Characterization of the AuNP probe synthetic process and the RCA

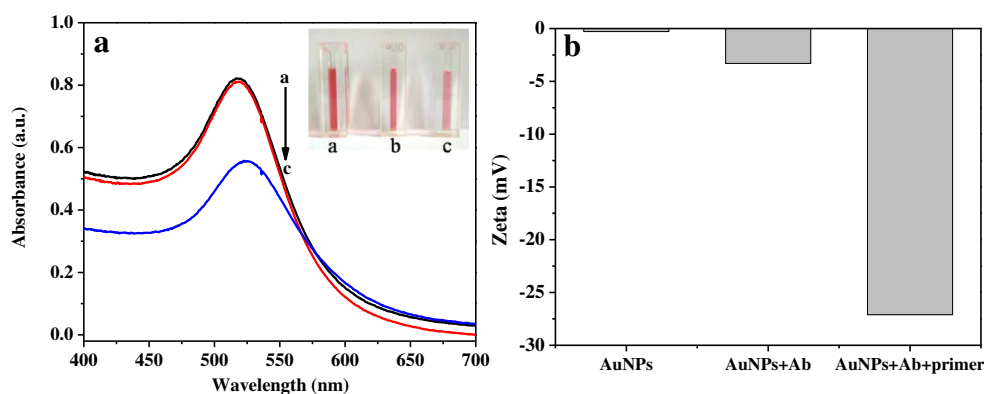
AuNPs were synthesized by reducing HAuCl_4 with trisodium citrate, and then modified with antibody and RCA primer in



Scheme 1 Immunoelectrochemical detection principle for HER2 with AuNP-based rolling circle amplification reaction. **a** Modification of AuNPs with HER2 antibody and RCA primer. **b** Illustration of the

preparation process of immunoelectrochemical assay, and detection of HER2 using prolonged DNA, which react with molybdate to generate electrochemical current

Fig. 1 Characterization of AuNP probe (a): UV-vis absorption of AuNPs (curve a), AuNPs modified with HER2 antibody (curve b), and AuNPs modified with both antibody and RCA primer (curve c). The insert picture shows the color change of the corresponding solutions under visible light. **b**: The change of zeta potential during the AuNP modification process



succession. As shown in Fig. 1a, for UV-Vis characterization, compared to AuNPs (curve a), the modification of HER2 antibody onto AuNPs (curve b) does not bring significant changes to either the intensity or position of the plasma bands. After RCA primer conjugation, the absorption intensity reduced and the absorption position shows a little red shift, from around 518 nm to 524 nm (curve c), indicating AuNPs formed aggregation slightly. This may be attributed to low concentration of antibody and negative charged DNA. The color of solution a is almost the same with solution b, only the color of solution c become a little shallow, which is consistent with the result of UV-Vis spectrum. In addition, we used zeta potential to characterize the synthesis of AuNP probe. It can be seen the AuNPs is negative charged. The modification of low concentration of antibody made the potential of AuNPs become more negative charged. With the conjugation

of RCA primer, the negative charge increases obviously (Fig. 1b and Supporting Information, Fig. S1.). The above data suggest the AuNPs was successfully modified with antibody and RCA primer successfully.

To prove the accomplishment of RCA, we used the formed AuNP probe to initiate RCA in centrifuge tube, and then the morphology and size of the AuNPs were characterized by TEM. As shown in Fig. 2a, AuNPs display uniform sphere structure with size around about 13 nm. After modified by antibody and RCA primer, the size of the AuNPs is increased to around 15 nm (Fig. 2b). As the RCA reaction performed, the diameter of AuNPs is further increased to around 17 nm (Fig. 2c). Electrochemical impedance spectroscopy (EIS) can also provide detailed information about the electrode modification process [25], so we also used EIS to characterize the RCA

Fig. 2 TEM images of (a): AuNPs, (b): AuNP after modification with antibody and primer, c: after RCA. **d**: Nyquist plots for EIS, a: bare electrode; b: detection of 200 $\text{pg}\cdot\text{mL}^{-1}$ HER2 without RCA; c: detection of 200 $\text{pg}\cdot\text{mL}^{-1}$ HER2 with RCA

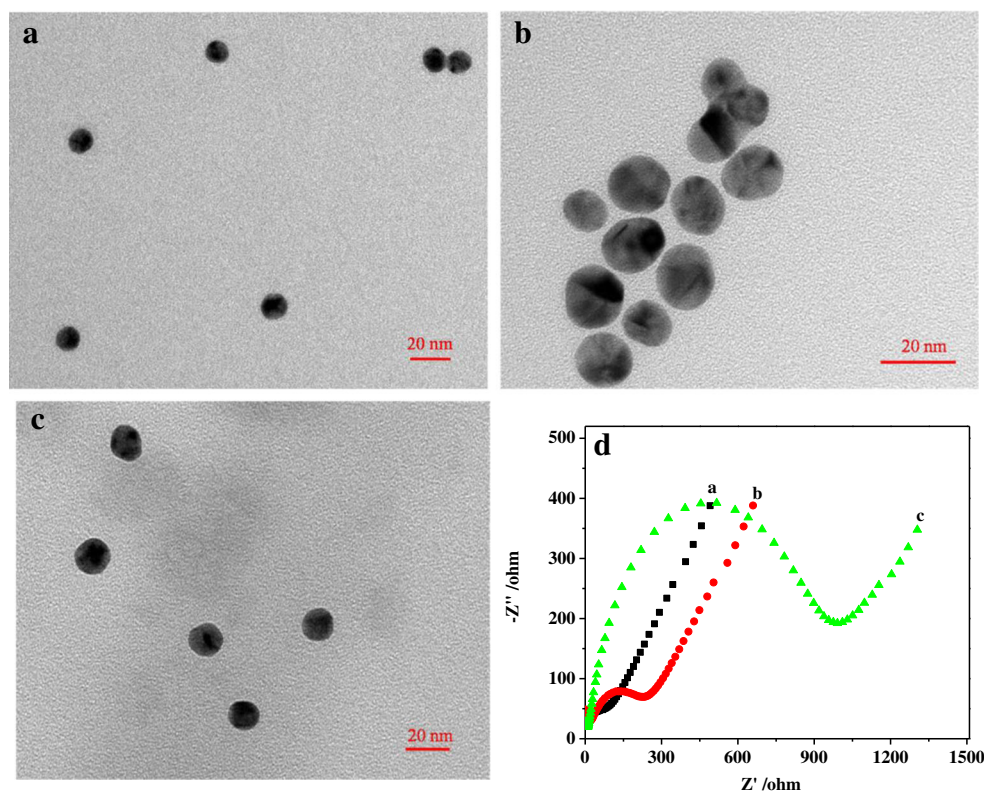
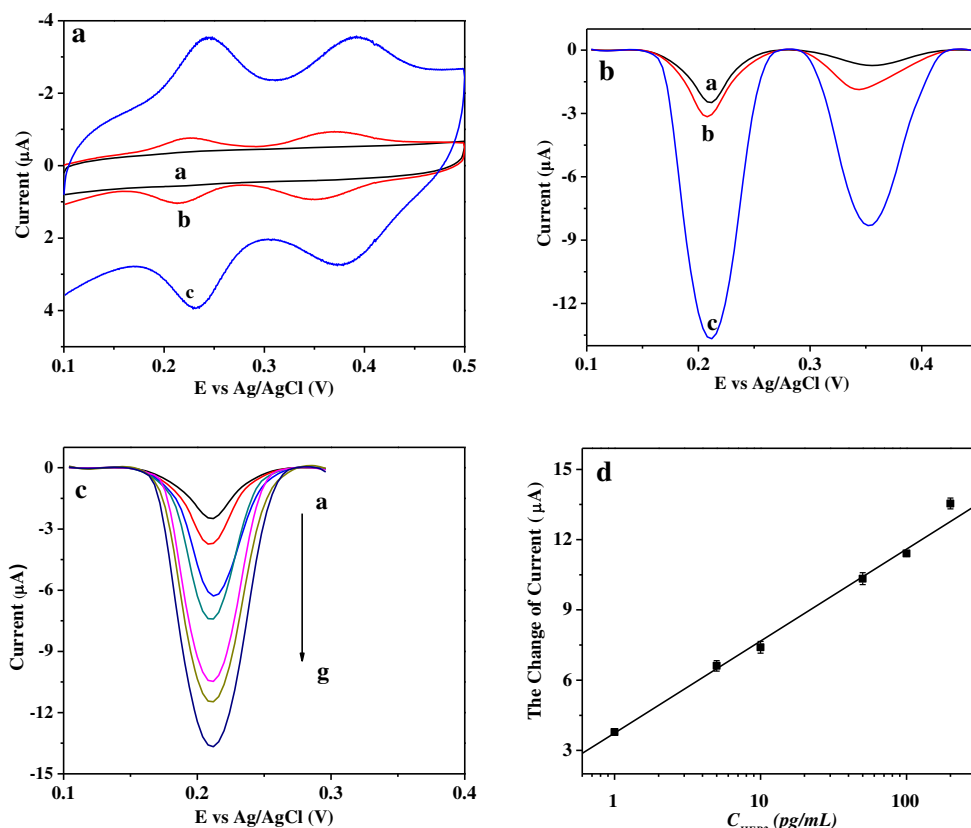


Fig. 3 **a:** CV responses of the immunoelectrochemical assay after react with molybdate: (a) bare electrode; (b) assay for 200 pg·mL⁻¹ HER2 without RCA; (c) assay for 200 pg·mL⁻¹ HER2 with RCA. **b:** The corresponding SWV responses of the assay. **c** SWV responses of the assay to different concentrations of HER2, from (a) to (g): 0, 1, 5, 10, 50, 100, 200 pg·mL⁻¹. **d** The calibration plot. Supporting electrolyte, 0.5 M H₂SO₄



reaction. In Fig. 2d, we can see that compared to bare gold electrode, the resistance value of the immunoelectrochemical assay for the detection of HER2 is increased (curve a and b) and further enhanced significantly after RCA (curve c), which prove the RCA reaction was performed successfully in both the centrifuge tube and on electrode surface.

Feasibility study and signal amplification by AuNP-based rolling circle amplification (RCA)

According to previous work, phosphate on DNA can react with molybdate and form redox molybdophosphates, which can generate two pairs of redox peaks at around 0.22 V and 0.37 V [26–29]. To demonstrate the feasibility of the

immunoelectrochemical assay for HER2 detection and the efficiency of signal amplification by AuNP-based RCA, the immunoelectrochemical assay was characterized by cyclic voltammetry (CV) and square wave voltammetry (SWV). As shown in Fig. 3a. There is no redox peak when only sodium molybdate is covered on bare electrode (curve a). However, of the assay for 200 pg·mL⁻¹ HER2 detection, two pairs of relatively weak redox peaks are appeared (curve b), which proved the successfully capture of the AuNP probe onto the electrode through immunoreaction and the reaction of the primer on AuNP with molybdate. The current intensity is rather weak because the amount of primer on AuNP is limited. Of the same assay for 200 pg·mL⁻¹ HER2 detection, after RCA, it can be seen the peak current is increased obviously, which is

Table 1 Compared the analytical performance of our immunoelectrochemical assay with other kinds of assay for HER2 detection

Method/probe	Linear range(ng·mL ⁻¹)	Detection limit (pg·mL ⁻¹)	references
Electrochemistry/ AuNPs-dC20	1.0 × 10 ⁻³ -1	0.5	[1]
Electrochemistry/ dC12	0.01-5	5	[21]
Electrochemistry/Ag deposition	5.0 × 10 ⁻⁴ -50.0	0.02	[30]
Impedimetric immunosensor	0.01-100	10	[31]
Enzyme-free fluorescence ELISA	2.5 × 10 ⁻² -5	9.65	[32]
Capacitance	0.1-10	100	[25]
Mass-based/glasspiezoelectric microcantilever		5000	[33]
Optical /opto-fluidic ring resonator	13-100		[34]
Fluorescence	6.25 × 10 ³ -3.125 × 10 ⁴	4.75 × 10 ⁷	[35]
Electrochemistry/AuNPs based RCA	1.0 × 10 ⁻³ -0.2	0.09	Our work

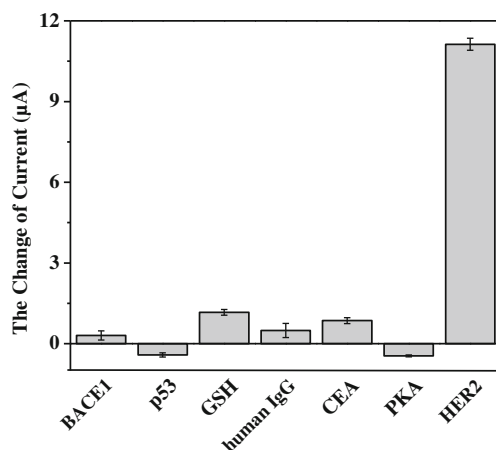


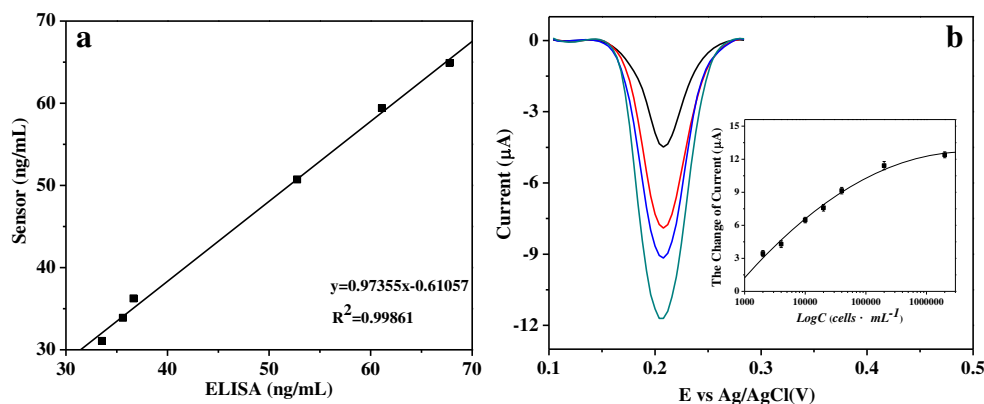
Fig. 4 The selectivity study: SWV response of the immunoelectrochemical assay to different proteins

about 4 times higher than that before RCA (curve c). SWV was also utilized to characterize the assay and the results in Fig. 3b are in agreement with the CV results. From the above data, we can conclude that the immunoelectrochemical assay is feasible for detection of HER2, and the RCA can significantly enhance the sensitivity of the assay. The reaction time of RCA affects the performance of RCA, which was optimized to be 2 h (Supporting Information, Fig. S2).

Analytical performance of immunoelectrochemical assay to HER2

To test the analytical performance of the assay for HER2 detection, a series of different concentrations of HER2 were detected by the immunoelectrochemical assay. As depicted in Fig. 3c, the SWV responses of the assay at 0.2 V is increased with the concentrations of HER2 rising from 0 $\text{pg}\cdot\text{mL}^{-1}$ to 200 $\text{pg}\cdot\text{mL}^{-1}$. This result is because higher concentration of target HER2 resulted in more AuNP probes captured onto gold electrode through immunoreaction, and then triggered more RCA reaction, leading to higher DNA loading on the electrode. There is a linear relationship between the change of current intensity and logarithm of HER2 concentration in the range from 1 to 200 $\text{pg}\cdot\text{mL}^{-1}$ (Fig. 3d

Fig. 5 The detection of HER2 in real samples. **a**: The detection of HER2 in serum of breast cancer patient by our assay and ELISA kit. **b**: HER2 analysis in different concentrations of 4 T1 cell. The insert is fitting plot



with equation of $y = 3.9268x + 3.74231$, $R^2 = 0.993$). Based on $S/N = 3$, the detection limit of this immunoelectrochemical assay is calculated as $0.09 \text{ pg}\cdot\text{mL}^{-1}$. The sensitivity is high enough for the detection of HER2 in diluted serum samples as normal individuals have a HER2 concentration between 2 and 15 $\text{ng}\cdot\text{mL}^{-1}$ in the blood, and breast cancer patients have blood HER2 levels from 15 to 75 $\text{ng}\cdot\text{mL}^{-1}$ [21]. On the basis of the above results, the immunoelectrochemical assay demonstrated lower detection limit compared to other assay for HER2 detection reported previously (Table 1).

Selectivity and reproducibility of the immunoelectrochemical assay to HER2

Selectivity and reproducibility are also of great importance for the assay. To test the selectivity of this assay, we selected six proteins including 1 $\text{U}\cdot\text{mL}^{-1}$ β -site amyloid precursor protein cleaving enzyme 1 (BACE1), 0.1 $\text{ng}\cdot\text{mL}^{-1}$ p53, 0.1 $\text{mg}\cdot\text{mL}^{-1}$ glutathione (GSH), 10 $\mu\text{g}\cdot\text{mL}^{-1}$ human immunoglobulin G (human IgG), 2.5 $\text{ng}\cdot\text{mL}^{-1}$ carcinoembryonic antigen (CEA) and 1 $\text{U}\cdot\text{mL}^{-1}$ protein kinase A (PKA), which exist in human serum. As seen in Fig. 4, compared to 200 $\text{pg}\cdot\text{mL}^{-1}$ HER2, the responses of the assay to the above interfering proteins are negligible, which is due to the specific interaction of HER2 with antibody and capture peptide. This result demonstrate that the assay has good selectivity despite the concentration of interferences are much larger than HER2.

In addition, we studied the reproducibility of the assay by testing 10 $\text{pg}\cdot\text{mL}^{-1}$ and 50 $\text{pg}\cdot\text{mL}^{-1}$ of HER2 independently 3 times. The relative standard deviation (RSD) of the testing results is 3.39% and 2.48%, respectively, indicating the results of assay was quite reproducible.

Detection of HER2 in human serum and 4 T1 cell

To verify the clinical application of our assay, six serum samples were collected from the breast cancer patients and then detected by the assay and commercial HER2 ELISA kit. As seen in Fig. 5a, the linear regression indicates the concentrations of HER2 detected by our assay were in good agreement

to the results of ELISA kit. Furthermore, HER2 in 4 T1 cell lysate were also detected by our assay. As shown in Fig. 5b, the current intensity enhanced with the increased of cell number, and the insert photograph proved the change of current is linear with logarithm of cell number within a certain range. These results suggest that the assay can detect HER2 in both serum and cancer cells, indicating the assay has great potential in clinical application.

Conclusion

In summary, we utilized AuNP as the carrier for anti-HER2 antibody and RCA primer to combine RCA with traditional immunodetection to build an immunoelectrochemical assay for ultrasensitive detection of HER2. Based on the AuNP probe, the detection of HER2 was converted to the detection of DNA amount on electrode. Compared to existing methods, this assay has advantages of high sensitivity and low detection limit. With these advantages, the immunoelectrochemical assay can detect very low concentration of HER2 in serum and cancer cell, which has great potential in cancer diagnostics and clinical analysis. However, the RCA process is still a little time consuming. This immunoRCA strategy can be easily adapted to other DNA signal amplification methods and find wide applications.

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

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