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Self-Assembled DNA Generated Electric Current Biosensor for HER2 Analysis

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ABSTRACT:

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We have developed a new DNA self-assembly amplification technology that generates electric current to power electrochemical biosensing. The new technology was used for detection of human epidermal growth factor receptor 2 (HER2). In our technology, an aptamer was utilized both as a ligand for recognition and as a signal generating reporter. The aptasensor is based on a sandwich format: a DNA primer on a HER2 aptamer initiates auxiliary DNA self-assembly on the electrode to form a long one-dimensional DNA. The resulting DNA is then reacted with molybdate to generate electrochemical current. The sensitivity of the aptasensor with DNA self-assembly was greater than that of the aptasensor without DNA self-assembly due to the extended length of DNA strand. Aptasensor analysis of HER2 in serum of breast cancer patients and healthy individuals is highly correlated ($R^2 = 0.9924$) with ELISA measurements, with a p value of 1.37E-07. The analysis of HER2 in serum (confirmed by ELISA) suggests that HER2 levels in breast cancer patients are much higher than healthy individuals. For HER2 positive patients, the levels are higher than those of HER2 negative patients. After surgery, there is a drop of HER2 levels in serum suggesting potential clinical applications of the new self-assembled DNA electric current generating biosensor. Unlike proteins, DNA is easily amplifiable. The DNA signal amplification method presented here the aptamer is integrated into an electrochemical electrode, which enables effective current generation enabling simple protein detection for a wide range of biomedical applications.

INTRODUCTION

Breast cancer is a major health concern, affecting about 12% of women in the U.S during their lifetime. An intensively studied proto-oncogene is human epidermal growth factor receptor (HER2) gene, also known as CerbB-2, which is amplified and over-expressed in 25-30% of breast cancer, making HER2 become the most common malignant tumors marker.[1] Research data show that the concentration of HER2 in breast cancer patient's blood elevate to 15-75 ng/mL compared to 2-15 ng/mL in normal individuals.[2] In addition, some evidences indicate that HER2 is an important predictive factor for therapy of breast cancer.[3,4] The HER2 over-expression patients can improve survival rates significantly by taking the target HER2 drug Herceptin. But patients without HER2 over-expression, the effect of Herceptin will be greatly reduced.[5] Thus, it is necessary to exploit a highly accurate and specific method for diagnosis of HER2 over-expression. Current clinical diagnostic tests for HER2 involve fluorescent in situ hybridization (FISH) that verify the HER2 gene expression and immunohistochemistry (IHC) to determine the HER2 protein on cell surface. However, FISH and IHC require invasive biopsy tissue sample, professional and specific instruments, which limit the extensive application of these methods. Thus, establishing a simple, specific, sensitive and accurate method for detection of HER2 is of great significance.

While immunoassays are traditionally used for HER2 and for other protein detection, their sensitivity is often limited for the detection of low level proteins. To increase the sensitivity and ability to detect low level of proteins, signal amplification strategies were

developed including immuno-PCR [6] that combines the specificity of an immunoassay with the signal amplification of polymerase chain reaction (PCR). Although immuno-PCR is highly sensitive, it is not widely used because of its complexity. Antibodies must be conjugated to oligonucleotides, and very precise cycling temperatures are required along with special equipment to detect the amplicon. The assay can also be complicated by nonspecific PCR amplification. A different signal amplification procedure, immunoassays with rolling circle DNA amplification (immuno-RCA)[7] that utilizes a rolling circle amplification (RCA) reporter system for the detection of protein was reported. The assay produces a long DNA molecule containing hundreds of copies of the circular DNA sequence that remain attached to the antibody and that can be detected in a variety of ways. This isothermal amplification requires a complex probe to initiate the rolling circle and the conjugation of antibody and DNA, but the assay enables direct quantification and high sensitivity without specialized instrumentation.

Simpler assays using aptamer-based PCR and aptamer-based RCA assays have been developed. Oligonucleotide aptamers, as short pieces of nucleic acid have attracted significant attention as alternative to conventional antibodies for uses in biosensing and therapeutics.[8] The aptamers can be isolated through systematic evolution of ligands by exponential enrichment (SELEX), and then synthesized and modified automatically via a solid-phase synthesis.[9,10] Aptamer can recognize and specifically bind to given targets including peptides, proteins, small molecules and even cells, often refer to as recognition probe in biosensing.[11-16] To improve the sensitivity of aptasensor, numerous DNA

amplification methods have been developed, such as PCR,[17-19] RCA,[20-22] strand displacement amplification (SDA).[23,24] However, the aptamer-PCR process still requires precise cycling temperatures, and aptamer-RCA still requires the design of a complex probe to initiate RCA.

Other DNA amplification method includes DNA self-assembly[25-30], which does not need nuclease, and can be assembled into specific structure precisely.[31-33] The assembly of single-stranded DNA is driven by the free energy of base pair formation, with an initiator stand to trigger a chain reaction of hybridization events similar to living chain polymerization.[34] What's more, one dimensional structure is the smallest and can facilitate the electron transfer.[28]. Here we developed a novel assay for protein signal amplification based on self-assembled DNA amplification combined with DNA-generated current detection.

In previous work we utilized aptamer to generate redox electric current.[35] The DNA current generation is based on the reaction of DNA phosphate groups bound to electrochemical electrode with molybdate that can form redox molybdophosphate precipitate and generate electrochemical current.[36,37] Because for the current generation, the DNA must be bound or in very close proximity to the electrode, with the exception of RCA,HCR, none of the other protein DNA amplification can work effectively because some or most of the DNA generated released to the media and will not generate current. Since the electric current depends on the amount of the DNA phosphate groups, in this work, by amplifying the amount of DNA, we fabricated an ultrasensitive electrochemical

aptasensor for detection of HER2 based on the amplification of aptamer generated electric current by DNA self-assemble reaction.

EXPERIMENTAL SECTION

Materials and apparatus: The peptide (CKLRLEWNR) specifically binding to the tumor biomarker human epidermal growth factor receptor 2 (HER2) was synthesized by GL Biochem Ltd. (Shanghai, China). The oligonucleotides used in our study were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). HER2 was obtained from Abcam Co., Ltd. (Cambridge, MA, USA). Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) and 6-mercapto-1-hexanol (MCH) were acquired from Sigma-Aldrich. The human HER2 ELISA kit was obtained from Jining Biotech Co., Ltd. (Shanghai, China). The serum samples were got from the second Xiangya hospital that attached to Central South University. Other reagents were of analytical grade and used without further purification. All stock solutions were prepared with ultrapure water (ZOOMWO-M, 18.25 MΩ·cm@25℃).

All the electrochemical measurements were performed on a CHI-650D electrochemical workstation (Shanghai CH Instruments Co., China). A conventional three-electrode system was used with working electrode (gold electrode 2 mm in diameter), Ag/AgCl reference electrode and a platinum column auxiliary electrode. The human HER2 ELISA was performed on an ELX800 microplate reader (BioTek, USA) under 450 nm.

Electrode pretreatment: Au electrode was cleaned by immersing into fresh prepared piranha solution (the mixture of 30% H_2O_2 and concentrated H_2SO_4 at 1:3 v/v) for 5 min, and rinsed thoroughly with deionized water. Then, the electrode was polished and sonicated in ethanol and ultrapure water, followed by blowing dry with nitrogen.

Construction of aptasensor with DNA self-assembly: To construct aptasensor, a cleaned gold electrode was immersed into peptide solution (50 μg/mL) overnight at 4 ℃ to modify the HER2 specificity peptide onto gold electrode. After rinsed with ultrapure water and dried, the modified electrode was incubated with MCH (1 mM) for 1.5 h to block nonspecific sites. Then 5 μL of different concentration of HER2 solution was dropped onto electrode surface at 4 ℃ for 2 h. After thoroughly washing, 50 μM DNA (HER2 aptamer connected with DNA self-assembly primer) was added onto electrode at 4 ℃ for another 1 h, and then the electrode was washed to remove excess DNA. Subsequently, 5 μL DNA hybridization buffer (10 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl, 1 mM $MgCl_2$, pH 7.4) containing 50 μM of S1 and S2 (the sequences of DNA used in this article were listed in Table 1) was dropped on electrode and reacted at 37 ℃ for 4 h to accomplish the DNA self-assembly, and then the electrode was washed again. Finally, the Na_2MoO_4 solution (5 mM) was added onto electrode surface and reacted for 15 min before electrochemical characterization.

Electrochemical impedance spectroscopy (EIS) characterization: Due to EIS could provide detailed information of electrode modification, in this study, each

modification step of the aptasensor was characterized by EIS in 5 mM $Fe(CN)_6^{3-/4-}$ solution containing 100 mM NaCl.

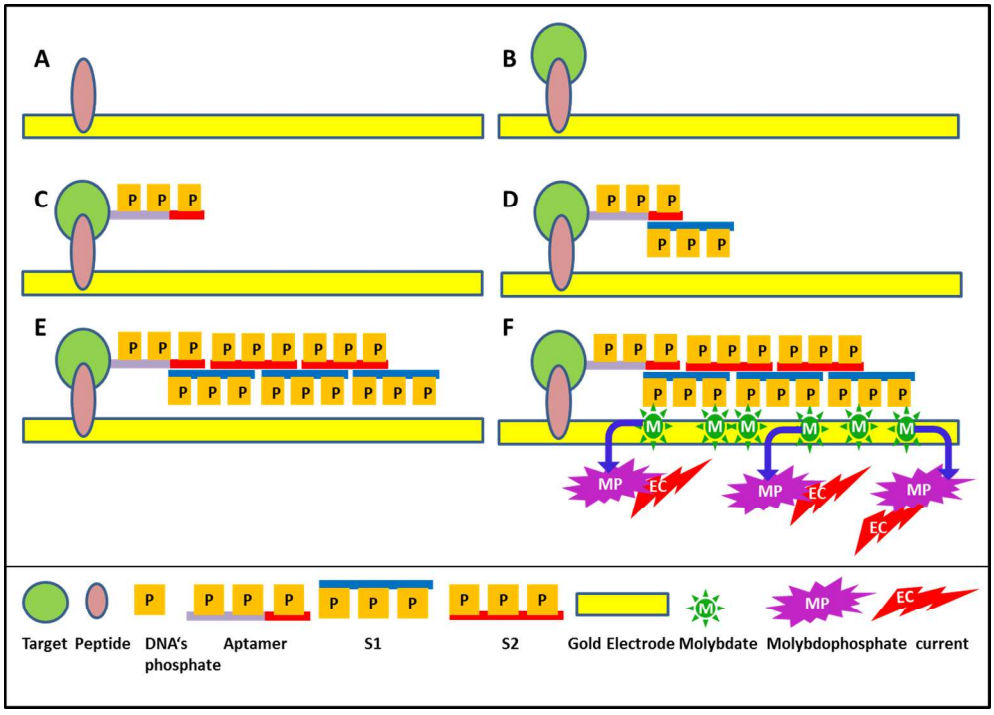
Detection serum of patients with the aptasensor: The serum samples from breast cancer patient and the normal individuals were diluted with ultrapure water. Then the serum samples were analyzed by the ELISA kit and our aptasensor.

Statistical analysis: The data was analyzed with Excel (Microsoft, Redmond, WA)

RESULTS AND DISCUSSION

The principle of the amplified DNA sensing strategy: The principle of the detection system is based on DNA self-assemble amplification in close proximity to the electrode and the reaction of molybdate with the backbone phosphate of DNA to generate electrochemical current. As illustrated in Scheme 1, the functional assay include a gold electrode on which a peptide specific to target is immobilized, for this application of the technology the binding peptide is specific for HER2 (A), the peptide capture the HER2 target (B) which is followed by binding of target-aptamer specific for HER2 to create a peptide-target-aptamer sandwich structure (C). For signal amplification, DNA self-assembly elongate the aptamer by annealing of S1 to its sticky end complementary sequence on the aptamer (D) and the annealing of S2 to its complementary sequence on S1(E). In this manner, each copy of the target can initiate a chain-like assembly of S1 and

S2 to generating a long DNA sequence, increasing the amount of DNA backbone phosphate, which can react with the molybdate to form redox molybdophosphate precipitate and generate electrochemical current (F). In the absence of targets, the DNA self-assemble process cannot be triggered. While in the presence of targets, the targets serve as initiator for DNA self-assemble amplification enabling generation of electrochemical current.



Scheme 1. The principle of the amplified DNA sensing strategy utilizing DNA self-assemble amplification and the reaction of molybdate with the backbone phosphate of DNA to generate electrochemical current. A, the immobilization of peptide that specific to HER2 onto gold electrode; B, the capture of target HER2 molecules; C, the capture of

aptamer; D and E, the self-assemble of S1 and S2 DNA sequence; F, the reaction of the modified electrode with molybdate to form redox molybdophosphate precipitate and generate electrochemical current.

Table 1. The DNA used in this study

Probe	Sequence (5’ to 3)
HER2 aptamer[a]	GCAGCGGTGTGGGG**TATCGTTAATTCGGTCG**
S1[b]	TACGTGGCTTGGACCGAC**CGAATTAACGATA**
S2[c]	**GTCCAAGCCACGT**ATATCGTTAATTCGGTCG

[a] The DNA self-assemble primer is indicated with block letters,
[b] The S1 complimentary sequences for annealing are indicated with red
[c] The S2 complimentary sequences for annealing are indicated with blue

Electrochemical impedance spectroscopy (EIS) characterization of the electrode modification process: EIS can provide detailed information of electrode modification, so we used EIS to characterize each step of the aptasensor modification process. As shown in Figure 1. The increasing of electron-transfer resistance (Ret) indicated the modification of gold electrode successfully step by step. Especially, the Ret values after DNA self-assemble and after the reaction with acid molybdate were increased significantly, indicating the completion of DNA self-assemble and the formation of molybdophosphate precipitation.

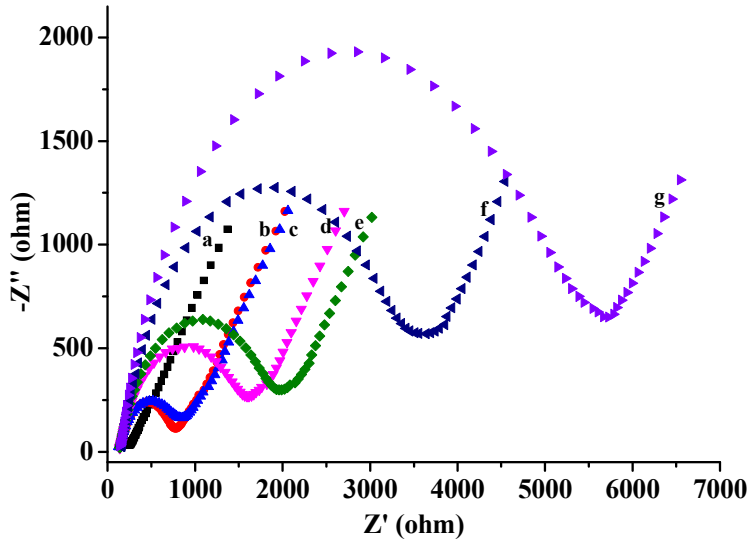


Figure 1. Nyquist plots for EIS. Each curve represented the EIS response for corresponding electrodes, bare Au (a), Au/peptide (b), Au/peptide/MCH (c), Au/peptide/MCH/HER2 (d), Au/peptide/MCH/HER2/aptamer (e), Au/peptide/MCH/HER2/aptamer/DNA self-assemble (f), and Au/peptide/MCH/HER2/aptamer/DNA self-assemble/MoO_4^{2-} (g) immersed in 5 mM $Fe(CN)_6^{3-/4-}$ containing 100 mM NaCl solution

Signal amplification by DNA self-assembly: To study the principle of electrochemical signal generation and the efficiency of signal amplification by DNA self-assembly, the aptasensor was characterized by cyclic voltammetry (CV) and square wave voltammetry (SWV). As shown in Figure 2A, the bare gold electrode reacted with only sodium molybdate has no redox peaks (curve a). However, of the aptasensor for 100 pg/mL HER2, there arose two pairs of redox peaks when reacted with sodium molybdate. In addition, the

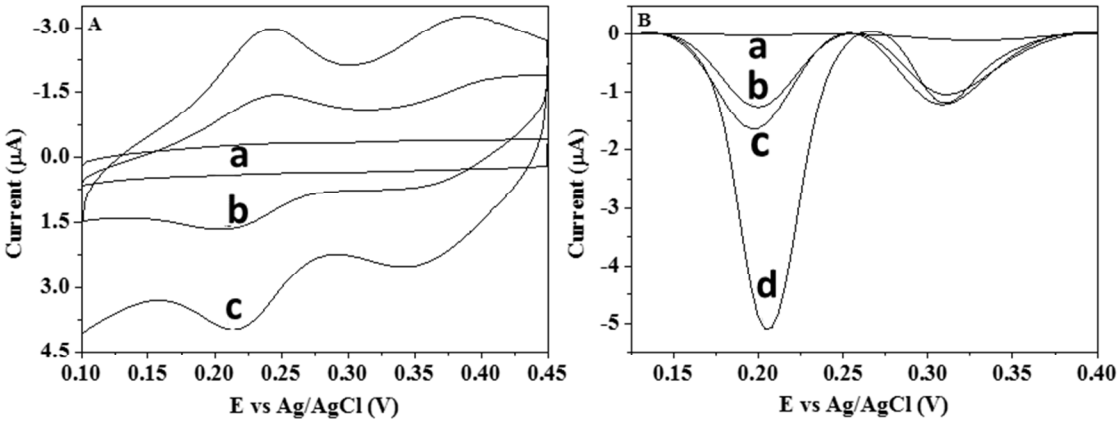
peaks after DNA self-assemble (c) were increased obviously than that without DNA self-assemble (b). The two pairs of redox peaks (0.22 V and 0.37 V) can be attributed to the electrons transfer within molybdophosphates (equation II and III) after reaction of phosphate with acid molybdate (equation I). [38-40]

$$12MoO_4^{2-} + 24H^+ + PO_4^{3-} = PMo_{12}O_{40}^{3-} + 12H_2O \quad (1)$$

$$PMo_{12}O_{40}^{3-} + 2e + 2H^+ = H_2PMo_2^{V}Mo_{10}^{VI}O_{40}^{3-} \quad (2)$$

$$H_2PMo_2^{V}Mo_{10}^{VI}O_{40}^{3-} + 2e + 2H^+ = H_4PMo_4^{V}Mo_8^{VI}O_{40}^{3-} \quad (3)$$

The SWV response of bare electrode (a), aptasensor for 10 pg/mL HER2 (b) and 100 pg/mL HER2 (c), as shown in Figure 2B, suggest the current intensity increased with the increasing of the concentration of HER2. Especially, for detection of 100 pg/mL HER2, the peak current was about 3.5 times higher after DNA assemble (d) compared to that



before DNA

Figure 2. (A) CV response of aptasensor after reaction with molybdate in 0.5 M H_2SO_4. (a) bare electrode. (b) 100 pg/mL HER2 without DNA self-assemble. (c) 100 pg/mL HER2 with DNA self-assemble. (B) SWV response of aptasensor after reaction with

molybdate in 0.5 M H_2SO_4. (a) bare electrode. (b) 10 pg/mL HER2 without DNA self-assemble. (c) 100 pg/mL HER2 without DNA self-assemble. (d) 100 pg/mL HER2 with DNA self-assemble.

Optimization of the reaction time for DNA self-assemble: Considering the sensitivity of aptasensor and the experimental time, it is important to optimize the time for the DNA self-assemble reaction. As shown in Figure 3A, with the extension of reaction time, the current intensity at 0.2 V was increased. However, the current increased slowly from 4-6 h. So to achieve high sensitivity while considering the experiment time, 4 h was selected for our experiment. Through such signal amplification strategy, the sensitivity of the aptasensor was increased significantly.

Analytical performance of the aptasensor to HER2: To study the analytical performance of the aptasensor to HER2, different concentrations of HER2 were analyzed by our constructed aptasensor with SWV measurement. From Figure 3B, it is clear that the peak current at about 0.2 V is increased with the increasing of HER2 concentration. This result was because for higher concentration of HER2, more aptamers with DNA self-assemble primers were linked onto electrode and then promoted even more DNA molecules self-assembled onto electrode. Higher amount of DNA molecules on the electrode resulted in higher peak current when reacted with molybdate. From the curves, it can be seen that the current peak of blank control (a) was about 2.5 μA after DNA

self-assemble, which was larger than the aptasensor for 100 pg/mL HER2 without DNA assemble (Figure 2B, curve c). This phenomenon could be attributed to a small amount of aptamer nonspecific adsorbed onto the surface of gold electrode. As shown in Figure 3B, the peak current increased as the concentration of HER2 changed from 0 to 100 pg/mL. The insert of Figure 3B shows linear relationship between the change of peak current and the logarithm of HER2 concentration in the range from 1 to 100 pg/mL, with a linear correlation coefficient of 0.998. A detection limit of 0.047 pg/mL was obtained based on S/N=3. Compared with other biosensors for HER2 detection, such as our previous work (detection range of 0.01-5 ng/mL, LOD= 5 pg/mL)[35] and piezoelectric microcantilever sensors (detection range of 0.05-2 ng/mL)[4], this aptasensor display higher sensitivity and lower detection limit.

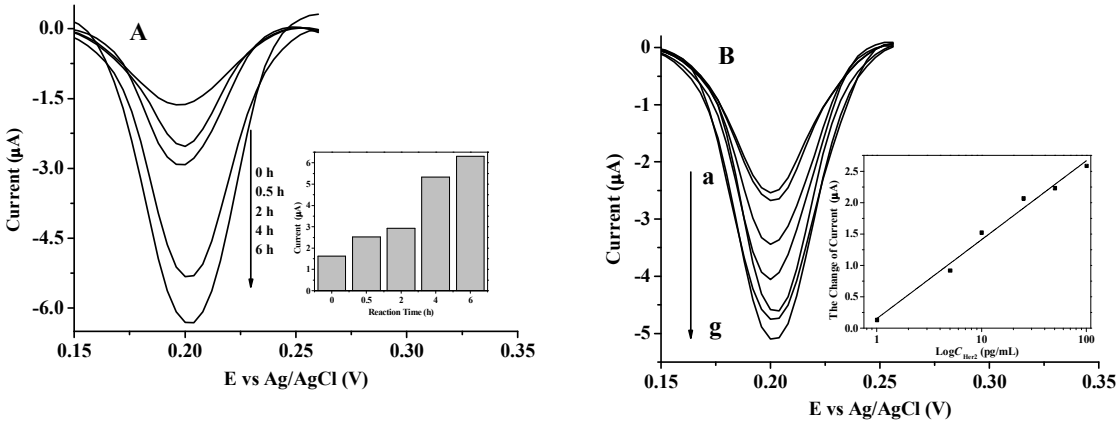


Figure 3. Analytical performance of the aptasensor to HER2. (A) Optimization of the reaction time for DNA self-assemble. (B) SWV response of developed aptasensor to different concentration of HER2 with DNA self-assemble in 0.5M H_2SO_4, from a to g, 0, 1,

5, 10, 25, 50, 100 pg/mL. The inset is the signal of the changed current at 0.204 V to the logarithm of the concentration of HER2.

The selectivity of HER2 aptasensor: As can be seen from above results, our aptasensor was sensitivity, moreover, its specificity was not sacrificed to obtain the remarkable detection limit. In present study, we selected six proteins as the potential interferents, including mucin 1 (MUC1), human immunoglobulin G (human IgG), p53, β-Site amyloid precursor protein cleaving enzyme 1 (BACE1), protein kinase A (PKA) and glutathione (GSH), which exist in human serum or known as other biomarkers of cancer. Figure 4 show the responses of the aptasensor to these compounds. The current displayed were compared to background control (sample without HER2). Compared to 0.1 ng/mL HER2, the responses of the aptasensor to the above interferents were negligible, despite of the larger concentrations of MUC1, human IgG, GSH, PKA and BACE1 tested. This phenomenon proves that the aptasensor has high specificity which can be ascribed to the specific identification of HER2 to its aptamer and the corresponding peptide.

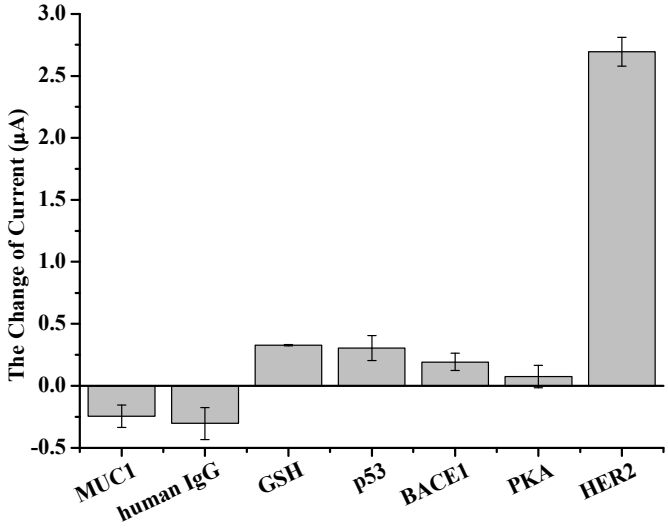


Figure 4. The selectivity of HER2 aptasensor. SWV response of the aptasensor to different proteins, from left to right, 1 ng/mL MUC1, 10 μg/mL human IgG, 0.1 mg/mL GSH, 0.1 ng/mL p53, 1 U/mL BACE1, 1 U/mL PKA and 0.1 ng/mL HER2.

Testing HER2 in breast cancer patients and healthy individual's serum by the aptasensor: Based on the above data, the aptasensor was suitable to detect HER2 in standard solutions. However, as an important prognostic marker for invasive breast cancer, the monitoring of HER2 in serum has significant clinical impact. In present study, we obtained 6 serum samples from 4 breast cancer patients and 2 healthy individuals. Of the 4 breast cancer patients, two are HER2 positive [CerbB-2(3+/2+)] (3+/2+ means the level of HER2 expression, while 3+ means the expression of HER2 is higher than 2+) and two are HER2 negative [CerbB-2(0/-)] demonstrated by immunohistochemistry. The

corresponding paraffin-embedded sections of breast cancer patients were shown in supporting information (Figure S1).

Table 2. Comparison of aptasensor detection with ELISA kit for detecting of HER2 in human serum. Analysis of 4 breast cancer patients, two are HER2 positive [CerbB-2(3+/2+)] and two are HER2 negative [CerbB-2(0/-)] demonstrated by immunohistochemistry. The controls are non-breast cancers samples.

Sample no.	Immunohistochemistry		ELISA kit (ng/mL)	Aptasensor (ng/mL)	Relative error (%)
1	CerbB-2(2+)	pre-operation	39.5	38.176	-3.35
		post-operation	31.35	27.52	-12.2
2	CerbB-2(3+)	pre-operation	55.67	51.792	-6.97
		post-operation	45.432	40.19	-11.5
3	CerbB-2(-)		24.445	20.662	-15.5
4	CerbB-2(0)		23.48	20.165	-14.1
5	non-breast cancers		7.512	7.173	-4.51
6	non-breast cancers		12.472	9.006	-27.8

The serum samples were analyzed by our electrochemical aptasensor and commercial ELISA kit. As shown in Table 2, the HER2 levels obtained by our aptasensor were in good agreement with ELISA results. Linear regression analysis (Figure 5) shows a strong positive correlation between the results of the two methods (R^2 = 0.9924) with highly significance p value (1.37E-07). The data suggest that the HER2 levels of breast cancer patient are much higher than healthy individuals, and the concentrations of HER2 for HER2 positive patient are higher than HER2 negative patient. What is more, the data also indicated for HER2 positive patient, after surgery, there is a drop of the HER2 level in serum. These results suggested that our aptasensor has enough sensitivity and accuracy to detect the HER2 in serum samples and has great potential in clinical applications.

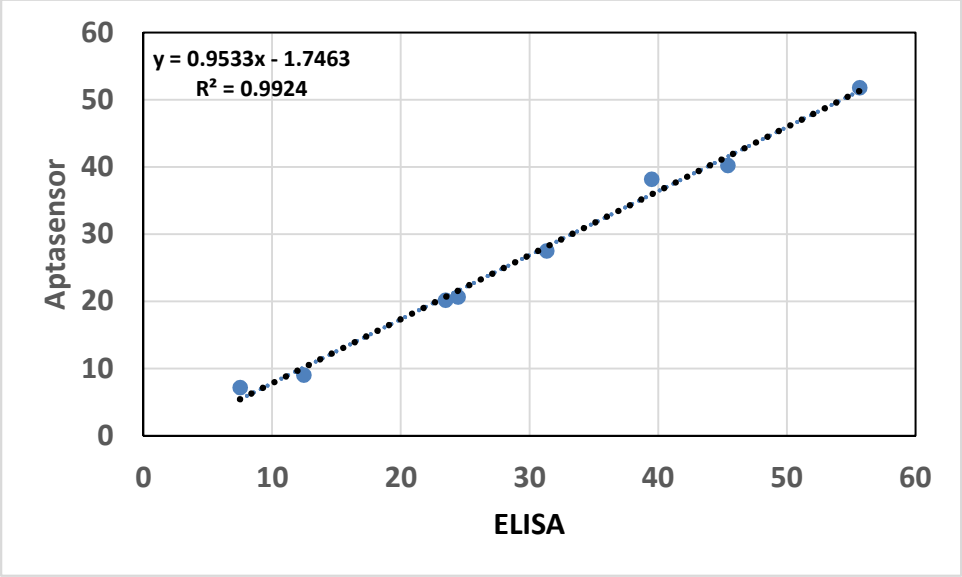


Figure 5 Regression analysis of HER2. HER2 in human serum was measured by ELISA and by aptasensor

CONCLUSIONS

HER2 as an important prognostic factor for invasive breast cancers, affect the choice of treatment for breast cancer patients, especially the molecular targeted therapy for the patients of HER2 positive patients. In this work, we developed an electrochemical aptasensor based on DNA generated electric current with DNA self-assemble for signal amplification to detect HER2 both in standard solution and serum of patients. The reaction of phosphate groups on DNA backbone with molybdate resulted in redox current. Double function of aptamer as recognize probe and signal reporter simplified the construction of the aptasensor. DNA self-assemble extends the DNA chain greatly without enzyme, which increased the sensitivity and lowered the detection limit of the aptasensor. In view of these advantages, this enzyme-free ultrasensitive electrochemical aptasensor has great potential in the breast cancer diagnostics and clinical analysis. The new method has potential advantages over ELISA. It is enzyme-free and unlike other protein based detection, DNA based detection can be easily amplified to improve detection level. Several other DNA amplification methods, such as PCR or RCA can theoretically be combined with the DNA generated current technique to increase detection sensitivity. Since for current generation, the DNA must be bound or in very proximity to the electrode, the aptamer was integrated into an electrode enabling very simple DNA based signal amplification detection and because of its simplicity, the technology can be utilized for wide applications in various proteins biosensing applications.

ACKNOWLEDGMENT

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ASSOCIATED CONTENT

Supporting Information

Additional figures showing paraffin-embedded sections of breast cancer patients analyzed by immunohistochemistry. This material is available free of charge via the Internet at http://pubs.acs.org

Notes

The authors declare no competing financial interests.

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