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TECHNICAL NOTE

Electrochemical genosensor for detection of human mammaglobin in polymerase chain reaction amplification products of breast cancer patients

Xiongwei Xu · Xiuhua Weng · Ailin Liu · Qihuang Lin · Changlian Wang · Wei Chen · Xinhua Lin

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Abstract Human mammaglobin (MG) has been found to be the most specific molecular marker for the hematogenous spread of breast cancer cells. In our study, an electrochemical impedance spectroscopic DNA biosensor was established for the detection of MG in breast cancer patients. The working conditions for the biosensor, such as immobilization time, rinse process, and hybridization process, were optimized. Under the optimal conditions, the charge transfer resistance of the proposed DNA biosensor shows excellent correlation with the amount of the complementary oligonucleotides in the range from 1.0×10^{-9} to 2.0×10^{-8}M. The detection limit is 5.0×10^{-10}M. The proposed biosensor was used to detect the polymerase chain reaction amplification products of actual clinical breast cancer samples. The results were compared with that obtained by conventional gel electrophoresis. The results indicate that the electrochemical impedance spectroscopic assay is significantly sensitive and time-saving. The simple strategy described here is expected to be used in clinical application for early diagnosis of breast cancer.

Keywords Electrochemical impedance spectroscopy · Polymerase chain reaction · Biosensor · Human mammaglobin · Breast cancer

Introduction

Breast cancer is by far the most frequently diagnosed cancer and one of the leading causes of cancer-related death among females. When the breast cancer is limited in the breast and axillary lymph nodes at the time of diagnosis, patients can always be effectively treated. The number of circulating tumor cells (CTCs) before and after the initiation of a new systemic treatment regimen is an independent predictor of progression-free survival and overall survival in patients with breast cancer. Therefore, the detection of CTCs in the peripheral blood of cancer patients may have important therapeutic and prognostic implications, which can aid clinicians in the determination of prognosis and therapeutic approach. Different molecules have been used as cancer-associated markers in the detection of circulating breast cancer cells. Among the various molecules, only carcinoembryonic antigen (CEA) and mammaglobin (MG) were not detected in the healthy lymph nodes [1]. However, MG was detected in all breast cancer cell lines, whereas CEA was found only in a subset of breast cancer cell lines [2]. In addition, Fortunato et al. found that preoperative MG values were positively correlated with disease-free survival, but CEA levels

Xiongwei Xu and Xiuhua Weng contributed equally to this work.

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X. Xu (✉) · X. Weng · C. Wang
Department of Pharmacy, First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, China
e-mail: xxw0409@sina.com

X. Weng · A. Liu · Q. Lin · W. Chen (✉) · X. Lin (✉)
Department of Pharmaceutical Analysis, Fujian Medical University, Fuzhou 350004, China
e-mail: chenandhu@163.com
e-mail: xinhua63@163.com

disclosed negative correlation with disease-free survival [3]. Therefore, MG has been found to be the most specific molecular marker for the hematogenous spread of breast cancer cells [4–8]. The current assays for the detection of MG were morphology, flow cytometry, cytogenetics, and polymerase chain reaction (PCR). However, most of these methods are costly and time-consuming. Therefore, it is important to develop effective methods for the detection of MG.

Electrochemical DNA biosensors have been attracting increased attention because of their advantages such as low cost, simple design, small dimensions, and low power requirements [9–11]. In recent years, electrochemical DNA sensors have been applied extensively in many fields, such as life science, medical science, drug screening, and so on [12–14]. Researches in this field would be beneficial to recognition of the biotic origin, inheritance, growth, and evolution, as well as creating new methods for diagnosis, treatment, and prevention of human diseases [15–17]. The typical electrochemical DNA biosensors could be prepared by immobilizing single-stranded DNA probes on different electrodes and using electroactive indicators or other methods to measure the hybridization events between the DNA probes and their complementary DNA fragments [18–25]. Of these techniques, electrochemical impedance spectroscopy (EIS) is very effective for the characterization of bio-functionalized electrodes and the detection of DNA hybridization [26–29]. Hybridization of target DNA to the immobilized probe DNA results in an increased negative charge at the sensor surface. This cause a change in charge transfer resistance (R_{ct}) that can be detected using EIS. As an indicator-free system where the recognition element and the transduction component are integrated at a single interface on the electrode surface, EIS biosensor offers a number of advantages including speed of assay and deskilled analysis. Owing to its feature, it would be beneficial to the online detection of gene recombination.

In this paper, an electrochemical impedance spectroscopic DNA biosensor was established for detection of human mammaglobin (Scheme 1). In the presence of *N*-hydrosulfosuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), amine group at the 5' end of the 25-base synthetic oligonucleotides probe was covalently immobilized onto the glassy carbon electrode. EIS was performed to monitor the hybridization process. The working conditions for the biosensor, such as immobilization time, rinse process, and hybridization process, were optimized. The performance of the proposed biosensor for synthetic oligonucleotide and the PCR amplification products of actual clinical breast cancer samples were examined.

Experimental

Apparatus

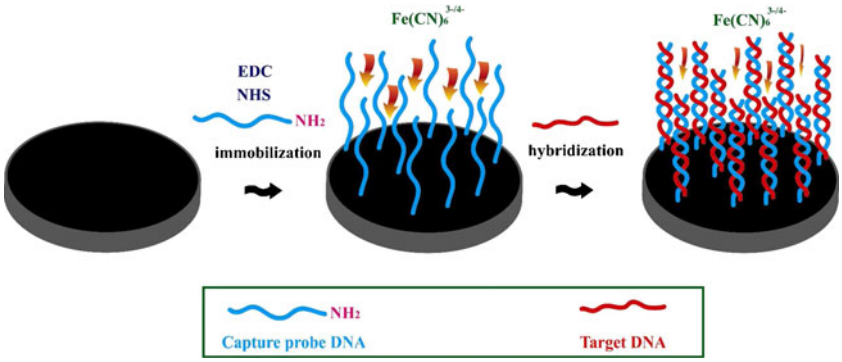
Electrochemical impedance spectroscopy was performed by using CHI 660C Electrochemical Workstation (CH Instrument, USA). The electrochemical system consisted of glassy carbon electrode (GCE, 3 mm diameter) as the working electrode, a platinum wire as the auxiliary electrode, and the reference electrode (Ag/AgCl). All potentials mentioned in this paper referred to this reference electrode.

Chemicals

The 25-base synthetic oligonucleotides were purchased from Shanghai Boshang Bioengineering Ltd. Company (Shanghai, China); their base sequences were: immobilized probe (S_1) 5'-NH_2-AGT TGC TGA TGG TCC TCA TGC TGG C-3'; target (S_2) 5'-GCC AGC ATG AGG ACC ATC AGC AAC T-3'; non-complementary (S_3) 5'-CTG GTA CAC ATA CAG ACG CAT CCA G-3'. The sequences of ssDNA probe and target nucleic acid fragment were chosen by exploring the National Center for Biotechnology Information data base. The oligonucleotides had been positively verified in respect of high selectivity and specificity to MG gene via Basic Local Alignment Search Tool (BLAST) program. All stock solutions of oligonucleotides (100 μM) were prepared with TE solution (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0) and kept frozen. Dilute solutions were prepared with 20 mM acetate buffer (pH 4.8). EDC and NHS from Sigma were used without further purification. Other all chemicals were of analytical reagent grade. Saline phosphate buffer solutions (PBS, 0.1 M, pH 7.0) were prepared by mixing the stock solutions of NaCl and NaH_2PO_4-Na_2HPO_4 and then adjusting the pH with H_3PO_4 or NaOH. All the buffer solutions contained 50 mM NaCl. Sterilized and deionized water was used in all experiments.

Fabrication of the biosensor

The GCE was polished in sequential order with 1.0, 0.3, and 0.05 μm alumina (Alfa Aesar, USA). The electrode was oxidized at 0.5 V for 1 min in 50 mM phosphate buffer solution (pH 7.40) to form the terminal carboxylic groups on GCE. After the oxidation step, the electrode was thoroughly washed with water, sonicated in ethanol, and finally dried thoroughly under N_2 flow. A versatile method for covalently attaching ssDNA to GCE was by using EDC and NHS linking reaction. The terminal carboxylic acid groups of GCE were activated by immersion in the 50 mM phosphate buffer solution (pH 7.4) containing 2 mM EDC and 5 mM NHS for 1 h. The linker/GCE was rinsed with 50 mM



Scheme 1 Scheme of the EIS DNA biosensor for MG detection

phosphate buffer (pH 7.4) to wash off the excess EDC and NHS. Then, ssDNA immobilization onto the electrode surface was performed by the following procedure—10 μl of 1.0×10^{-12}M probe DNA was pipetted onto the chemically modified GCE. After immobilization, the electrode was washed with a 0.1 % sodium dodecyl sulfate (SDS) phosphate buffer (pH 7.3) for 5 min to remove the unbound DNA probes. This electrode S_1/GCE was designated as probe electrode.

The hybridization was performed by immersing the probe-modified GCE electrode into 0.1 M PBS (pH 7.0) containing target DNA (sequence S_2) with different concentrations. This hybridization process was left for 20 min at 37 °C. Thus, a hybrid-modified GCE was obtained. The electrode surface was then washed with 0.1 % SDS phosphate buffer (pH 7.3) for 5 min to remove the unbound oligonucleotides. The same protocol as above was applied at probe-modified GCEs for hybridization reactions of probe with non-complementary sequences (sequence S_3) and also with bare sample.

The electrochemical characteristics of the modified electrode were measured by EIS. EIS measurements were performed at open circuit potential in 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture containing 0.1 mM KCl with the frequency ranging from 10^5 to 1.0 Hz.

Determination of PCR amplification products

The actual PCR amplification products of breast cancer patients were detected as following. Total RNA was extracted from liquid-nitrogen-frozen tissue samples by homogenization in Trizol reagent (Invitrogen). The amount of each RNA sample was determined by ultraviolet spectrophotometry at 260 nm. Each sample RNA was converted to cDNA by using oligo(dT18) primer with Superscript™ II reverse transcriptase (Invitrogen) for 60 min at 42 °C. One tenth of each RT reaction was subject to PCR analysis using the mammaglobin-specific primers R1 (5'-GACAGCGGCTTCCTTGATCC-3') and F1 (5'-GTGGCATTGTCGTCTATGAACTC-3'). Reactions were performed in a 25-μl volume containing 1× premixTaq (Takara) provided by the manufacturer. Amplification occurred for 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. Aliquots of 5 ul of the PCR products were electrophoresed on 1.5 % agarose gels containing ethidium bromide for 1 ~ 1.5 h and visualized on an ultraviolet transilluminator and photographed. The 100-bp DNA ladder (Fermentas) was used as marker. At the same time, the PCR amplification products were detected by EIS.

Results and discussion

Optimization studies of the biosensor

The working conditions for the biosensor were examined in terms of the immobilization time, rinse process, hybridization time, hybridization temperature, and bare hybridization.

The effect of the immobilization time of the probe ssDNA on the biosensor response was investigated from 5 to 120 min. The electrodes had been thoroughly rinsed to remove nonspecifically adsorbed DNA molecules before the measurements were made. The experimental results show that the charge transfer resistance increased with the immobilization time within 30 min (see Electronic Supplementary Material, Fig. S1). The change of the Nyquist diagram during the modification process is a strong proof of ss-DNA immobilization on the electrode surface. The negative-charged phosphate skeletons of DNA prevented $[Fe(CN)_6]^{3-/4-}$ from reaching the electrode surface for electron transfer during the process of redox reaction, which increased the R_{ct} of the electrode. As shown in Fig. S1, R_{ct} of the electrode reached the maximum value at 30 min. Therefore, the immobilization time of 30 min was used for the following experiments.

The effect of nonspecific adsorption of DNA on the biosensor response was investigated using the rinse times from 0 to 3 min. As shown in Fig. S2A (see Electronic Supplementary Material), the R_{ct} value decreased rapidly when the ssDNA-modified GCE was rinsed with phosphate buffer containing 0.1 % SDS (pH 7.3) to remove the non-

specific adsorbed probes. After 1 min, the R_{ct} value did not change. Consequently, the rinse time for the immobilization process was chosen as 1 min. As for the hybridization process, nonspecific adsorption of the target sequence on the electrode surface can also occur. The R_{ct} value was stable after the electrode was rinsed for 3 min (see Electronic Supplementary Material, Fig. S2B). So the optimal rinse time after hybridization is chosen as 3 min.

Other important parameters wanted to be optimized were hybridization time and temperature applied in the biosensing system. Effect of the hybridization time under different temperature on EIS signal of the biosensor was investigated. As shown in Fig. S3 (see Electronic Supplementary Material), the optimal hybridization time decreased with the increasing hybridization temperature due to the fact that higher temperature speeds the movement of DNA molecules. On the other hand, higher hybridization temperature accelerated the denaturation of dsDNA, resulting in the decreasing of the hybridization efficiency. Taking into consideration of the above two contrary factors, 37 °C and 20 min were selected as hybridization conditions for the following experiments.

Analytical characteristics of the biosensor

As shown in Fig. 1, curves a and b were the Nyquist diagrams of 10 mM $[Fe(CN)_6]^{3-/4-}$ at bare GCE and probe–DNA-modified GCE, respectively. Compared with bare GCE, the R_{ct} of probe–DNA-modified GCE was enlarged obviously. The change of the R_{ct} value during the modification processes was a strong proof to confirm that probe-DNA has been immobilized on the electrode surface. The negatively charged phosphate backbone of the probe DNA prevented $[Fe(CN)_6]^{3-/4-}$ from reaching the electrode surface during the processes of the redox reaction and led to a much larger R_{ct} value. After hybridization process, the R_{ct} of the biosensor (curve c in Fig. 1) was increased significantly. Consequently, the measurements could well distinguish between single- and double-stranded series of artificial samples of mammaglobin in breast cancer metastasis.

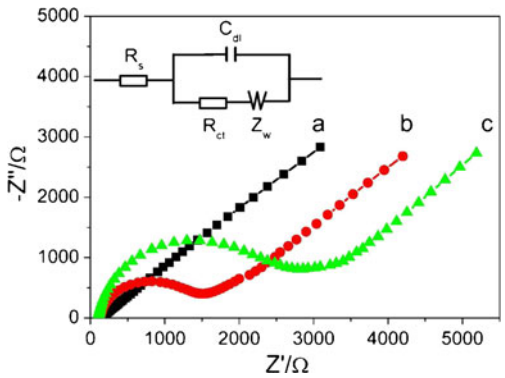


Fig. 1 Nyquist plots recorded at *a* GCE, ss-DNA-modified electrode *b* before and *c* after hybridization. *Inset* is the equivalent circuit model used to fit the impedance data

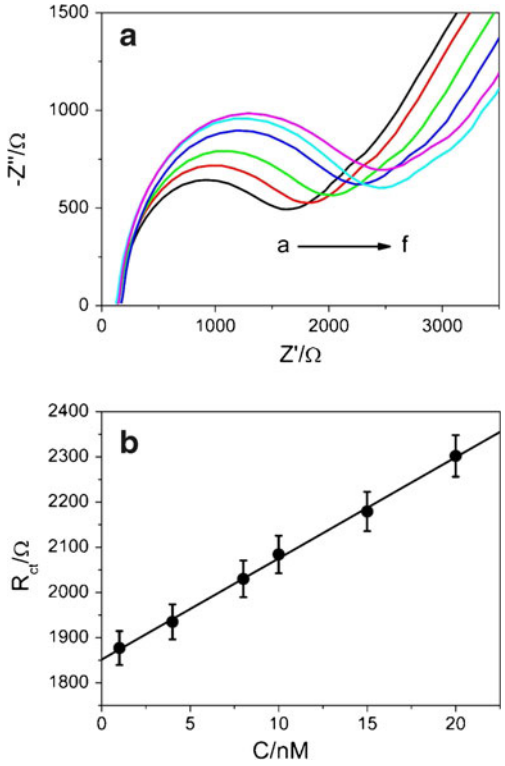


Fig. 2 **a** Nyquist plots of ssDNA/GCE after hybridization with its complementary MG gene-fragment-specific sequence of different concentrations—1.0×10^{-9}M (*a*), 4.0×10^{-9}M (*b*), 8.0×10^{-9}M (*c*), 1.0×10^{-8}M (*d*), 1.5×10^{-8}M (*e*), and 2.0×10^{-8}M (*f*). **b** The plot of R_{ct} versus complementary DNA concentration

Under the optimal conditions, the sensitivity of this assay was investigated by hybridizing with target DNA sequence with different concentration (Fig. 2a). As shown in Fig. 2b, the R_{ct} value shows excellent correlation with the amount of the complementary oligonucleotides in the range from 1.0×10^{-9} to 2.0×10^{-8}M. The regression equation was $R_{ct}(\Omega)=1851.61+22.37C(nM)$ and the correlation coefficient

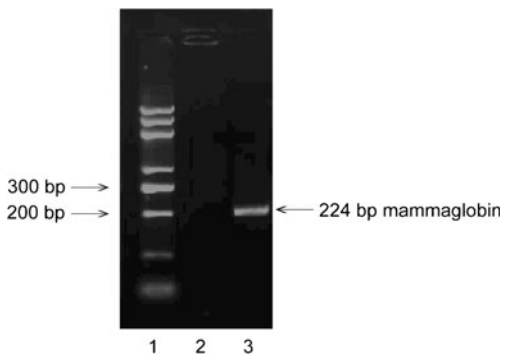


Fig. 3 The gel electrophoresis of mammaglobin mRNA RT-PCR amplification products in human breast tissue. (*1*) Marker (50, 100, 200, 300, 400, 700, 800, and 900 bp), (*2*) negative sample, (*3*) positive sample

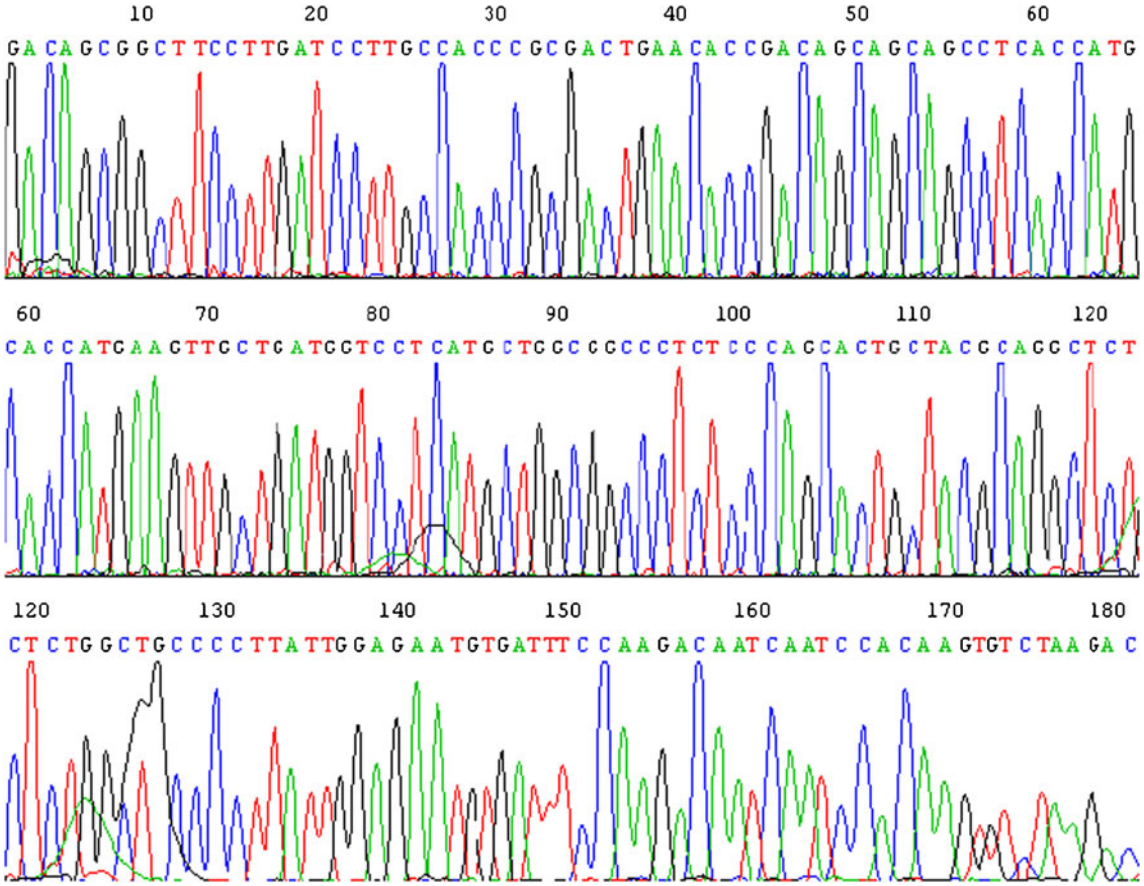


Fig. 4 Gene sequencing *graph* of the mammaglobin mRNA RT-PCR amplification product. Base sequence: GACAGCGGCT TCCTTGATCC TTGCCACCCG CGACTGAACA CCGACAGCAG CAGCCTCACC ATGAAGTTGC TGATGGTCCT CATGCTGGCG GCCCTCTCCC AGCACTGCTA CGCAGGCTCT GGCTGCCCCT TATTGGAGAA TGTGATTTCC AAGACAATCA ATCCACAAGT GTCTAAGAC

r=0.9992. A detection limit of this electrochemical DNA biosensor for determining the MG gene fragment-specific sequence was determined to be 5×10^{-10}M using 3σ, where σ was the standard deviation of the blank solution with eight parallel measurements. These experimental results indicated that the designed electrochemical DNA biosensor could be applied in the detecting of MG marker genes in breast cancer metastasis.

Detection of the PCR samples

As shown in Fig. 3, PCR amplification products were studied by gel electrophoresis. With the positive sample a bright

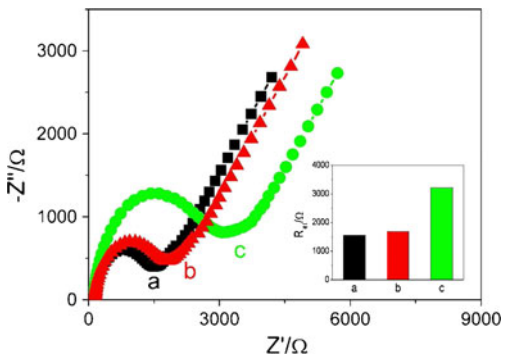


Fig. 5 Nyquist plots of ssDNA/GCE *a* before and after hybridization with PCR amplification products of *b* negative real sample and *c* positive real sample. *Inset*: the *bar graph* of R_{ct} of ssDNA/GCE *a* before and after hybridization with PCR amplification products of *b* negative real sample and *c* positive real sample

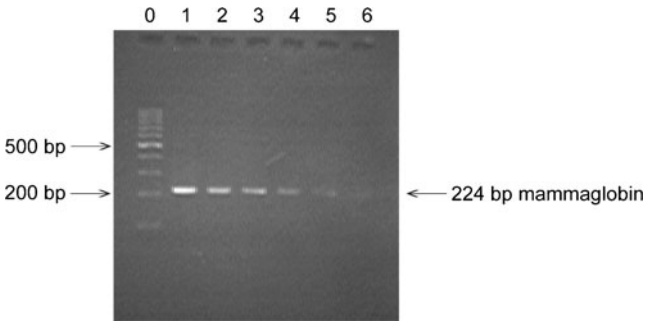


Fig. 6 The gel electrophoresis of human breast tissue mammaglobin mRNA RT-PCR amplification product with different dilution. (*0*) Marker (100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 bp), (*1–6*) PCR product with different dilutions of 1, 1/2, 1/4, 1/8, 1/16, and 1/32

strap appeared from the electrophoretogram while from the negative control sample not any strap emerged. The result indicated that the primer used in the experiment would amplify 224-bp positive gene fragment specifically. The sequence of PCR amplification product of the positive sample was then determined and shown in Fig. 4. The results indicated that the sequence matched the record in the genebank completely.

The specificity of the electrochemical biosensor for the PCR products was investigated by comparing the detection results of positive sample and negative control. As shown in Fig. 5, the R_{ct} of the biosensor was almost unchanged after being incubated with negative control, indicating that no hybridization occurred at the electrode surface while, in the case of positive sample, an obvious increase in R_{ct} was observed. In the experiment, the EIS could be well distinguished between positive and negative samples of mammaglobin in breast cancer.

The sensitivity of this assay for PCR amplification products was investigated and compared with that of gel electrophoresis. The stock solution was diluted to 1/2, 1/4, 1/8, 1/16, and 1/32, respectively. The results indicated that the fluorescence signals observed from gel electrophoresis weakened along with the increasing of the dilution factors (Fig. 6). When the solution was diluted to 1/16, no obvious fluorescence signal could be observed. However, the electrical signal change of the EIS biosensor was still significant when the solution was diluted to 512 times. Meantime, the R_{ct} value shows excellent correlation with the amount of the complementary oligonucleotides in the real samples in the range of 1.0×10^{-9}~2.0×10^{-8}M (r=0.9992). Therefore, compared with gel electrophoresis, the sensitivity of the EIS assay increased nearly 30 times. Therefore, this electrochemical method is a better choice to identify the samples when the result of gel electrophoresis is negative.

Conclusion

In this paper, an electrochemical impedance spectroscopic DNA biosensor was established for detection of human mammaglobin in breast cancer patients. The working conditions for the biosensor, such as immobilization time, rinse process, and hybridization process, were examined. Under the optimal conditions, the charge transfer resistance of the proposed biosensor shows excellent correlation with the amount of the complementary oligonucleotides in the range from 1.0×10^{-9} to 2.0×10^{-8}M, and the detection limit is 5.0×10^{-10}M. When used to detect the PCR amplification products of actual clinical breast cancer samples, the proposed biosensor is significantly sensitive and time-saving as compared with conventional gel electrophoresis. The simple strategy described here is expected to be used in clinical application for early diagnosis of breast cancer.

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