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Determination of soluble CD44 in serum by using a label-free aptamer based electrochemical impedance biosensor

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CD44 is a promising biomarker in the diagnosis and prognosis of malignancies. The serum CD44 level is closely related to disease progression and metastasis of malignancies. It is of great clinical significance for the detection of serum soluble CD44. In this study, a facile, label-free aptamer based electrochemical impedance sensor for serum CD44 has been proposed. The aptamer showing high affinity to CD44 was immobilized on the gold electrodes through Au–S interaction. The interaction between target CD44 and the immobilized aptamer will cause a complex structure change of the aptamer, which makes the diffusion of $[Fe(CN)_6]^{3-/4-}$ toward the electrode surface easy, thus resulting in the decrease of the impedance of the system. The decreased degree of the impedance had a good linear relationship with the logarithm of the CD44 concentration in the range of 0.1–1000 ng mL^{-1} with a detection limit of 0.087 ng mL^{-1} (S/N = 3). The developed biosensor has been applied to detect CD44 in serum samples with satisfactory results.

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Introduction

Cluster of differentiation-44 (CD44) is a transmembrane glycoprotein encoded by a single gene, which belongs to the cell adhesion molecular family. CD44 plays a key role in tumour progression and metastasis, and is over-expressed in multiple carcinomas, such as breast, colon and gastric cancer. Accumulating evidence reveals that CD44 can serve as the biomarker of cancer stem cells (CSCs) which are highly malignant and associated with tumour proliferation, invasion, metastasis and drug resistance.[1] Proteolytic cleavage of the extracellular domain of CD44 can release soluble CD44, which can be found in serum.[2] Recent studies also suggest that the elevated soluble serum CD44 concentration is correlated with the disease progression and metastasis in various malignancies.[3–5] Therefore, serum CD44 can be applied as a potential biomarker in diagnosis and prognosis of malignancies. Thus, the detection of biomarker CD44 in complex serum with high sensitivity and selectivity is of important clinical significance.

The detection of the serum CD44 depends mainly on the enzyme-linked immunosorbent assay (ELISA),[6] which relies on the antibody–antigen interaction and has the character of high specificity. But the screening as well as the production process of antibodies is expensive and time-consuming. And the storage conditions for antibodies are extremely strict. These shortcomings limit their wide applications. Aptamers are a DNA or RNA oligonucleotide sequence selected by the systematic evolution of ligands by exponential enrichment (SELEX), which can specifically bind to various targets such as small molecules, protein and cells with high affinity.[7] Aptamers have outstanding advantages including quick chemical synthesis, low price, high chemical and thermal stability, easy storage and flexible modifications, which can serve as an alternative to antibodies. Many selective aptamer based biosensors had been developed for protein biomarker detection.[8–10] The aptamer specifically binding to the extracellular domain of CD44 with high affinity had been selected,[11] and this aptamer has been successfully applied to devise some biosensors for CD44 detection. For example, based on the fluorescence labelling of CD44 aptamers, Jeong and co-workers[12] developed a fluorescence biosensor to detect the CD44 extracellular domain-hyaluronic acid binding domain. The biosensors have the merits of high sensitivity and low limits of detection.

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Electrochemical impedance biosensors can directly relate the impedance change to the concentration of target objects. The biosensors have many advantages, such as convenient operation, a quick response, and high sensitivity, and are widely used in virus, cell and protein detection.[13–15] For example, Khadka R. and co-workers[16] developed an electrochemical impedance based sensor for insect odorant receptors. The odorant ligand concentration was measured through decreasing impedance corresponding to the specific binding of odorant receptors and their ligand. Lin and co-workers developed a label-free aptamer-based electrochemical impedance biosensor for microcystin-LR detection with simple procedures.[15] To the best of our knowledge, little attention had been paid to developing electrochemical impedance based biosensors for CD44 detection.

In this study, a label-free aptamer based electrochemical impedance biosensor has been developed to detect serum soluble CD44. The aptamers specific to CD44 were assembled onto the gold electrode surface with Au–S bonds. In the presence of target CD44, the CD44 binding to aptamers brought about a complex structure change, resulting in the decrease of the impedance. Combining the advantages of high selectivity and affinity of the aptamer toward the target and the label-free as well as sensitive detection of electrochemical impedance spectroscopy, the proposed biosensor had been successfully applied to the determination of serum CD44 with satisfactory results.

Experimental

Materials and regents

The single-strand DNA aptamer for CD44 used in this study was chosen and designed according to the prior reported literature.[11] The aptamer was synthesized by Sangon (Shanghai, China) and purified by high-performance liquid chromatography. The sequence of the aptamer was shown below:

5′-SH-GAG ATT CAT CAC GCG CAT AGT CCC AAG GCC TGC AAG GGA ACC AAG GAC ACA GCG ACT ATG CGA TGA TGT CTT C-3′.

The aptamer was modified at its 5′-end with a thiol. NE Buffer2 (containing 1 mM DTT) was purchased from New England BioLabs Inc. (Beverly, MA). Potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate ($K_4[Fe(CN)_6]$) and potassium chloride (KCl) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Standard CD44 protein was purchased from Sino biological Inc. (Beijing, China) and dissolved in sterilized water to obtain various concentrations of standard CD44 solution. Bull Serum Albumin (BSA), hyaluronidase (HAase) and ascorbic acid (Vc) were purchased from Sigma Aldrich (St Louis, MO, USA). Carcinoembryonic antigen (CEA) and glucose (Glu) were purchased from Sangon (Shanghai, China). The human cluster of differentiation 44(CD44) ELISA Kit was purchased from Nanjing pars Biochem CO., Ltd (Nanjing, China). All chemicals used were of analytical grade and used as received. DNA solution was dissolved in NE Buffer2. The deionized water (18.2 MΩ) used throughout the experiment was purified by using a Millipore Milli-Q water purification system.

Preparation of the aptamer-based sensor

The 3 mm diameter bare gold disk electrodes were first polished on chamois leather with 1 μm, 0.3 μm and 0.05 μm Al_2O_3 powders respectively. Then, the electrodes were sonicated in deionized water for 5 min to remove residual powder. Afterwards, the electrodes were cleaned by using ethanol followed by using double-distilled water in an ultrasonic bath for 5 min respectively. Subsequently, the surface of the gold electrodes was electrochemically activated in 0.5 M H_2SO_4 solution *via* cyclic voltammetry (CV) scanning in the range of −0.2 to 1.6 V at a scan rate of 0.1 V s^{-1} until a steady reproducible cyclic voltammogram was reached.

Following the activation of the gold electrodes, 10 μL of 1 μM aptamer solution was dropped on the electrodes and incubated in an airtight container for 3 h at 37 °C to obtain the aptamer-modified Au electrodes.

Electrochemical detection

Electrochemical measurements were performed on a CHI660A electrochemical analyzer (Chenhua Instruments, Shanghai, China). Electrochemical impedance spectroscopy (EIS) studies were performed in 5 mmol L^{-1} $[Fe(CN)_6]^{3-}$/$Fe[(CN)_6]^{4-}$ solution containing 0.1 mol L^{-1} KCl with a three-electrode system: a 3 mm diameter bare gold disk electrode was used as the working electrode after pre-immobilization with aptamers, and an Ag/AgCl electrode and a Pt wire were used as the reference electrode and counter electrode, respectively. Impedance measurements were performed between 0.1 MHz and 1 Hz at a sinusoidal voltage perturbation of 5 mV amplitude.

Detection procedures

The aptamer modified electrodes were treated with various concentrations of CD44 protein at 37 °C for 2.5 h. Subsequently, the electrodes were cleaned with distilled water to remove the CD44 that was not immobilized onto the electrode surface. Finally, the EIS of the system had been tested.

For the construction of the calibration curve, six different aptamer-modified electrodes were incubated with different concentrations of CD44 standard solution for 2.5 h. The selectivity of the biosensor was assessed in the presence of BSA, CEA, HAase, Vc, and Glu at 37 °C. For stability analysis, the biosensors were fabricated independently and stored at 4 °C in a refrigerator for 14 days. Then the biosensors had been incubated with 10 ng mL^{-1} CD44 and the EIS has been tested. The biosensors prepared under the same conditions were incubated with the same concentration of CD44 (10 ng mL^{-1}) to test the biosensor reproducibility. All experiments were repeated three times.

Analysis of clinical serum samples

The human serum samples were obtained from the department of the clinical laboratory of the Affiliated Wuxi Maternity

and Child Health Care Hospital of Nanjing Medical University. The serum samples were stored at 4 °C before use. The electrochemical impedance measurements were performed according to the protocol as mentioned previously. All experiments were performed in accordance with the Guidelines followed at the Nanjing Medical University and approved by the ethics committee at the Affiliated Wuxi Maternity and Child Health Care Hospital of Nanjing Medical University. Informed consent was obtained from human participants of this study.

Results and discussion

Principle of the aptamer-based impedance biosensor

The principle of the aptamer-based electrochemical impedance biosensor is illustrated in Fig. 1. Firstly, the thiol-terminated aptamers were immobilized on the surface of the electrodes by Au–S covalent bonds. The aptamers on the electrode surface can prevent the external redox mediator, $[Fe(CN)_6]^{3-/4-}$, from penetrating to the gold electrode surface, thus inhibiting the electron transfer of $[Fe(CN)_6]^{3-/4-}$ toward the electrode surface and a relatively big impedance of the system can be detected. When the electrodes were incubated with the target protein CD44, the aptamers selectively binding to the CD44 could result in a complex structure change, thus leading to an easier accessibility of the electrons and a faster flow rate of redox $[Fe(CN)_6]^{3-/4-}$ toward the electrode surface. So, the impedance of the system became decreased. The decrease degree had some relationship with the CD44 concentration; based on this, a simple electrochemical impedance biosensor for CD44 can be developed.

Simple experiments have been performed to verify our assumption. As displayed in the upper right corner of Fig. 1, the bare Au electrode (Au) shows a very small semicircular domain and the impedance was very low. Immobilization of thiol-modified aptamers on the electrode surface resulted in an obvious increase of the semicircular domain (Apt/Au), suggesting the high electron-transfer resistance. The impedance decreased greatly after the aptamer-immobilized electrode was incubated with the 10 ng mL^{-1} CD44. These results indicated the feasibility of the proposed biosensor.

Optimization of experimental conditions

The amount of aptamer immobilized on the surface of the gold electrode affected greatly the performance of the sensor. The increase in the immobilization time of the aptamers will result in the increased amount of aptamers immobilized. As shown in Fig. 2A, the impedance increased considerably with the increase of immobilization time, which reached equilibrium at 3 h, indicating that the electrode surface was saturated with aptamers. Therefore, we chose 3 h as the optimum time for aptamer immobilization time.

The incubation times between the aptamer and the target also affect the performance of the biosensor. The degree of impedance decrease θ (%) was chosen as an indicator, which denoted the variation degree of the biosensor's impedance before and after incubation with CD44. It is calculated by the following equation:

$$\theta(\%) = \left(1 - \frac{R_{ct}(\text{after})}{R_{ct}(\text{before})}\right) \times 100\%$$

where R_{ct} (before) denotes the impedance of the aptamer modified gold electrode before incubation with the CD44 standard solution and R_{ct} (after) represents the impedance of the aptamer modified gold electrode after incubation with CD44.[15,17] As shown in Fig. 2B, the decreased degree of the impedance increased with the increase of the incubation time and a plateau was reached when the incubation time was 2.5 h. Thus, 2.5 h was selected as the optimal incubation time.

Calibration curve

The proposed biosensor was used to detect a variety of concentrations of the target protein CD44 standard solution under the optimized conditions. Fig. 3A shows the corresponding

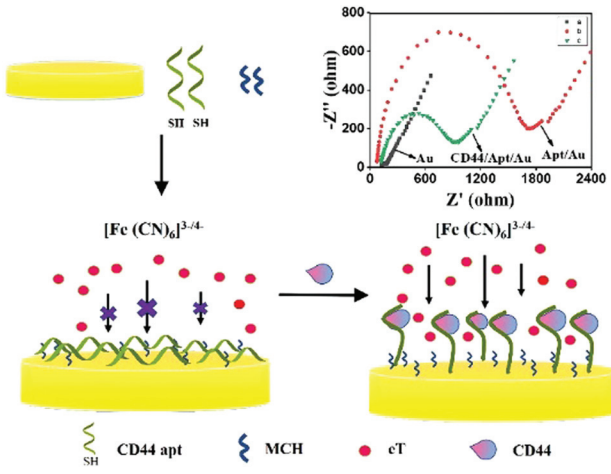

Fig. 1 Mechanism of the electrochemical impedance biosensor for CD44 detection.

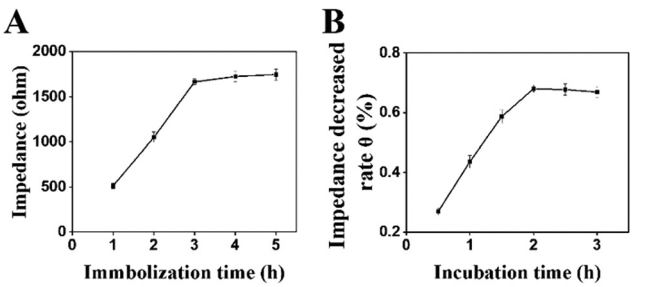

Fig. 2 (A) The change of impedance at different aptamer-immobilization times. (B) The change of impedance at different CD44 incubation times. The concentration of CD44 was 50 ng mL^{-1}. All impedances were recorded in a 5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol L^{-1} KCl, using a frequency range of 0.1 Hz–10^4 Hz at open circuit potential with an AC amplitude of 5 mV. The error bars are the standard deviation of three independent measurements (n = 3).

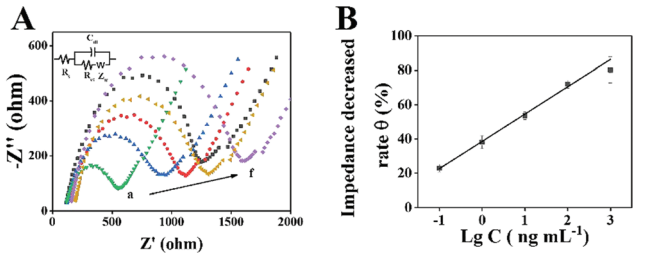

Fig. 3 (A) The Nyquist plots of biosensors at different concentrations of CD44 standard solution: (a) 1000.0 ng mL^{-1}; (b) 100.0 ng mL^{-1}; (c) 10.0 ng mL^{-1}; (d) 1.0 ng mL^{-1}; (e) 0.1 ng mL^{-1}; (f) 0.0 ng mL^{-1}. The inset exhibits an equivalent circuit model of the biosensor. (B) The linear relationship between the degree of impedance decrease θ and lg of the CD44 concentration (lg C). The error bars in B are the standard deviation of three independent measurements (n = 3).

Nyquist plot. To obtain impedance, the spectra were analysed by adopting a Randles equivalent circuit model, as shown in the inset of Fig. 3A. R_s is the electrolyte solution resistance, R_{ct} is the surface electron-transfer resistance, Z_w is the Warburg impedance and C_{dl} is the constant phase element related to double layer capacitance. Fig. 3B demonstrates that there is a linear relationship between the degree of impedance decrease (θ) of the biosensor and the lg of CD44 concentration (lg C) in the range of 0.1–1000.0 ng mL^{-1}. The linear regression equation was:

$$\theta(\%) = 0.16 \lg C + 0.385, R^2 = 0.992$$

where C represents the concentration of CD44. The limit of detection (LOD) was calculated to be 0.087 ng mL^{-1} (defined as S/N = 3), lower than the serum level of CD44 in healthy people (normal serum range: 10.0–325.0 ng mL^{-1}).[18–20]

Table 1 displays the comparison of the available methods for serum CD44 detection and our impedance biosensor. Compared with the results obtained in our work, some detection methods may exhibit much wider linear ranges and lower LOD values.[21–24] Our biosensor, however, exhibits the advantages of simple preparation, convenient detection and low cost. The developed biosensor, considering the CD44 level in normal serum, was sensitive enough to detect soluble CD44 in serum.

The selectivity, stability and reproducibility of the proposed biosensor

To investigate the selectivity of the prepared aptamer-based impedance biosensor, BSA, HAase, Vc, Glu, and CEA existing in human serum are chosen as model interferences (Fig. 4A). The concentrations of BSA, HAase, Vc, Glu, and CEA were settled as 5.0×10^6 ng mL^{-1}, 1.0×10^3 ng mL^{-1}, 1.0×10^3 ng mL^{-1}, 5.0×10^6 ng mL^{-1} and 96.0 ng mL^{-1} respectively, which were far higher than those in serum.[25–29] As shown in Fig. 4A, no remarkable responses had been detected when the aptamer-based biosensor was incubated with these interferences. However, an apparent impedance decline was observed in the presence of target CD44 (10 ng mL^{-1}). What's more, if the interferents had been mixed with 10 ng mL^{-1} CD44 firstly, a high impedance change was observed. These results indicated that the proposed biosensor has a high selectivity toward CD44 detection.

The stability of the biosensor was examined. Three electrodes were fabricated independently under the same conditions and kept at 4 °C in the refrigerator for 2 weeks. No significant change had been detected compared with the freshly prepared one, which indicated that the biosensor has good stability. Additionally, the reproducibility was assessed by recording the impedance spectra of five different aptamer-immobilized electrodes which were incubated with the same concentration of CD44 protein (10 ng mL^{-1}) (shown in Fig. 4B). The relative standard deviation was 3.96% (n = 5). These results indicated the good reproducibility of the proposed biosensor.

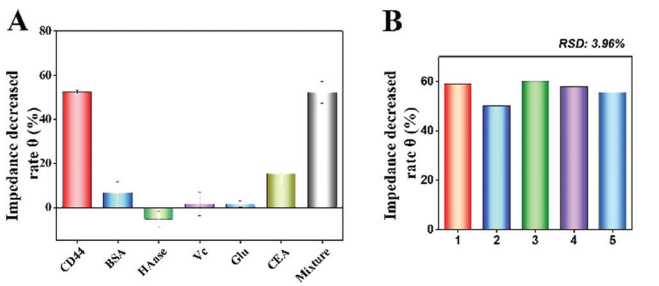

Fig. 4 The selectivity and reproducibility of the aptamer-based biosensor. (A) Degree of impedance decrease at 10.0 ng mL^{-1} CD44 and 5.0 × 10^6 ng mL^{-1} bovine serum albumin (BSA), 1.0 × 10^3 ng mL^{-1} hyaluronidase (HAase), 1.0 × 10^3 ng mL^{-1} L-ascorbic acid (Vc), 5.0 × 10^6 ng mL^{-1} glucose (Glu), 96.0 ng mL^{-1} carcinoembryonic antigen (CEA), mixture. (B) The impedance spectra of the five different aptamer-modified electrodes at the same concentration (10.0 ng mL^{-1}) of CD44. The error bars in A are the standard deviation of three independent measurements (n = 3).

Table 1 Some representative detection strategies for CD44

Detection methods	Detection strategy	Recognition unit	Target	LOD (ng mL^{-1})	Linear range (ng mL^{-1})	Ref.
Differential pulse voltammetry	ITO-HA	HA	Serum CD44	0.587	1.0–10000.0	21
	ITO-MWCNTs-PDDA-HA	HA	Serum CD44	0.00594	0.01–100.0	23
Photocurrent responses	ITO/TiO_2/PDA-HA-PEG	HA	Serum CD44	0.00044	0.005–500.0	22
Sandwich ELISA	Ag–Ab	CD44v3-10-Fc	CD44 v3	6.20	0–125.0	24
Electrochemical impedance spectra	Au-Apt	Apt	Serum CD44	0.087	0.1–1000.0	This work

Table 2 Analysis of the soluble CD44 level in human serum samples by the proposed biosensor and CD44 ELISA kit

Sample no.	CD44 level (mean ± SD, ng mL^{-1}, n = 3)		Average CD44 level[c] (mean ± SD, ng mL^{-1})
	Biosensor	ELISA	
N1[a]	68.8 ± 10.0	64.4 ± 7.1	
N2	89.3 ± 17.6	83.8 ± 1.3	82.5 ± 11.9*
N3	89.4 ± 8.5	77.2 ± 2.7	
P1[b]	93.3 ± 11.6	80.7 ± 1.7	139.5 ± 30.4
P2	117.6 ± 6.6	109.4 ± 2.0	
P3	126.1 ± 4.9	122.8 ± 1.4	
P4	136.4 ± 11.1	124.5 ± 5.5	
P5	152.0 ± 8.9	135.5 ± 8.7	
P6	151.5 ± 2.8	138.0 ± 4.2	
P7	183.2 ± 6.6	174.5 ± 1.7	

[a] Serum samples N1–N3 were collected from healthy people. [b] Serum samples P1–7 were taken from the patients with breast cancer. [c] The average value of the healthy people group (N1–N3) and the patient group with breast cancer (P1–P7) detected by the biosensor. *$p < 0.05$ statistically significant difference to the patient group.

The application in serum samples

It is of great significance to study the possibility of real application of the proposed aptamer-based sensor. CD44s in ten human serum samples were analysed by the proposed biosensor. Besides, ELISA assays were carried out to detect serum soluble CD44 concentration. The results are shown in Table 2. The average serum soluble CD44 concentration of healthy people was 82.5 ± 11.9 ng mL^{-1} (n = 3) and the average level of patients with breast cancer was 139.5 ± 30.4 ng mL^{-1} (n = 7). T test results between the healthy people and patients with breast cancer were found to show statistically significant differences ($p < 0.05$), thereby exhibiting the potential of soluble CD44 as the biomarker of malignancies. What's more, the results obtained with the proposed biosensor were in close agreement with those determined by the ELISA. These results proved the accuracy and reliability of the proposed method. Compared with the ELISA, the aptamer-based impedance biosensor has the merits of a convenient procedure and simple equipment. Furthermore, compared to the expensive antibody, the aptamer is of low price, has high chemical stability and simple preparation.

Conclusions

A novel aptamer-based electrochemical impedance biosensor for selective and sensitive detection of serum soluble CD44 had been developed. The binding of CD44 to the aptamer on the gold electrodes, due to the improved electron transfer of $[Fe(CN)_6]^{3-/4-}$, resulted in the impedance decrease. The level of serum CD44 was detected directly based on the degree of impedance decrease. This highly specific and sensitive biosensor exhibits the advantages of antibody-free and label-free detection, simple preparation and quick detection. The constructed biosensor provided a new simple method for serum CD44 determination.

Conflicts of interest

There are no conflicts to declare.

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