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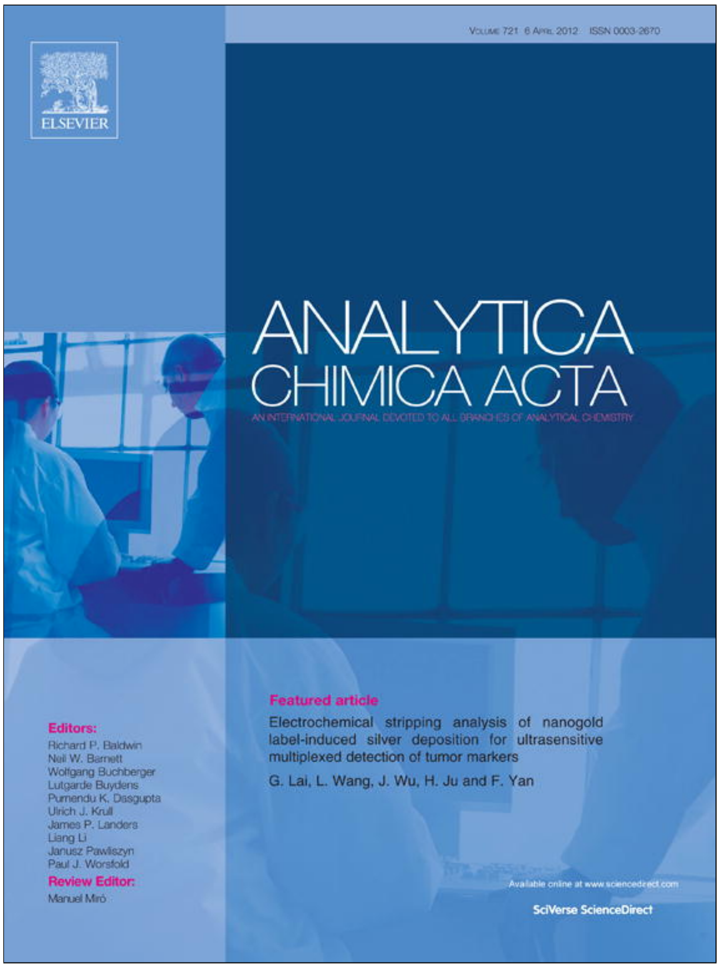

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Electrochemical stripping analysis of nanogold label-induced silver deposition for ultrasensitive multiplexed detection of tumor markers

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ABSTRACT

A multiplexed electrochemical immunoassay method was developed for simultaneous ultrasensitive measurement of tumor markers based on electrochemical stripping analysis of silver nanoparticles (Ag NPs). The Ag NPs were deposited on a disposable immunosensor array with a reduction reaction catalyzed by nanogold labels. The immunosensor array was prepared by covalently immobilizing capture antibodies on chitosan modified screen-printed carbon electrodes. Through a sandwich-type immunoreaction, antibody-functionalized Au NPs were captured onto immunosensor surface to induce the silver deposition from a silver enhancer solution. The deposited Ag NPs could be directly measured by anodic stripping analysis in KCl solution. The catalytic deposition enhanced the analytical sensitivity for detection of protein markers. The interference of dissolved oxygen could be avoided as the detection was performed with positive stripping potential range. Using carcinoembryonic antigen and α-fetoprotein as model analytes, the proposed multiplexed immunoassay method showed wide linear ranges of three orders of magnitude with the detection limits down to 3.5 and 3.9 pg mL^{-1}, respectively. The localized silver deposition, as well as the stripping detection process, eliminated completely the electrochemical cross talk between adjacent immunosensors. The immunosensor array exhibited acceptable reproducibility, stability and accuracy, showing a promising potential in multianalyte determination for clinical application.

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1. Introduction

Accurate detection of protein biomarkers, such as carcinoembryonic antigen (CEA) and α-fetoprotein (AFP), in serum and tissue through immunoassay methods shows promising applications in early cancer screening and diagnosis [1–3]. Unfortunately, most markers are not specific to a particular tumor and most cancers have more than one biomarkers associated with their incidence [4,5]. Thus, simultaneous measurement of panels of protein biomarkers, as opposed to single biomarker, may be necessary for reliable cancer detection and monitoring.

Recently, electrode array have shown their considerable importance in simultaneous multianalyte measurement due to their higher analytical efficiency and throughput over single-analyte assays [6–10], as well as the unique advantages of electrochemical biosensors including low cost, high sensitivity and good portability [11–14]. A single-enzyme label such as alkaline phosphatase [15–17] or horseradish peroxidase (HRP) [18,19] coupling with an enzymatic cycle is commonly used in these methods for signal tracing. Although these analytical systems were well developed, they still met the following challenges: (1) cross-talk interference, which is caused by the diffusion of electroactive product between the neighboring working electrodes and brings limitation in further miniaturization of the immunosensor array [15–17]; (2) the requirement of deoxygenation process and nitrogen atmosphere over the detection system for HRP-based electrochemical immunoassays, in which the electrochemical detection potential window for the electroactive products from HRP-based enzymatic cycle is normally more negative than −0.2 V, and dissolved oxygen can be reduced to produce interference signal [20–22]; (3) the low detection sensitivity along with the traditional single-label immunoassay, which makes it difficult to carry out the accurate and reliable detection of low abundant protein biomarkers in real serum samples [23–26].

To overcome these challenges, we previously designed several systems, such as glucose oxidase-functionalized nanocomposites coupled with a Prussia blue-mediated enzymatic cycle [27], and alkaline phosphatase-labeled antibodies-functionalized gold nanoparticle (Au NP) bioconjugates along with enzyme-catalyzed silver deposition [28], to develop ultrasensitive multiplexed electrochemical immunoassays. As tracing tags, the noble-metal NPs have superior abilities of low cost, good stability, and convenient operability and functionalization over enzyme labels [29–32]. Different from Au NPs, a normally used trace label that generally

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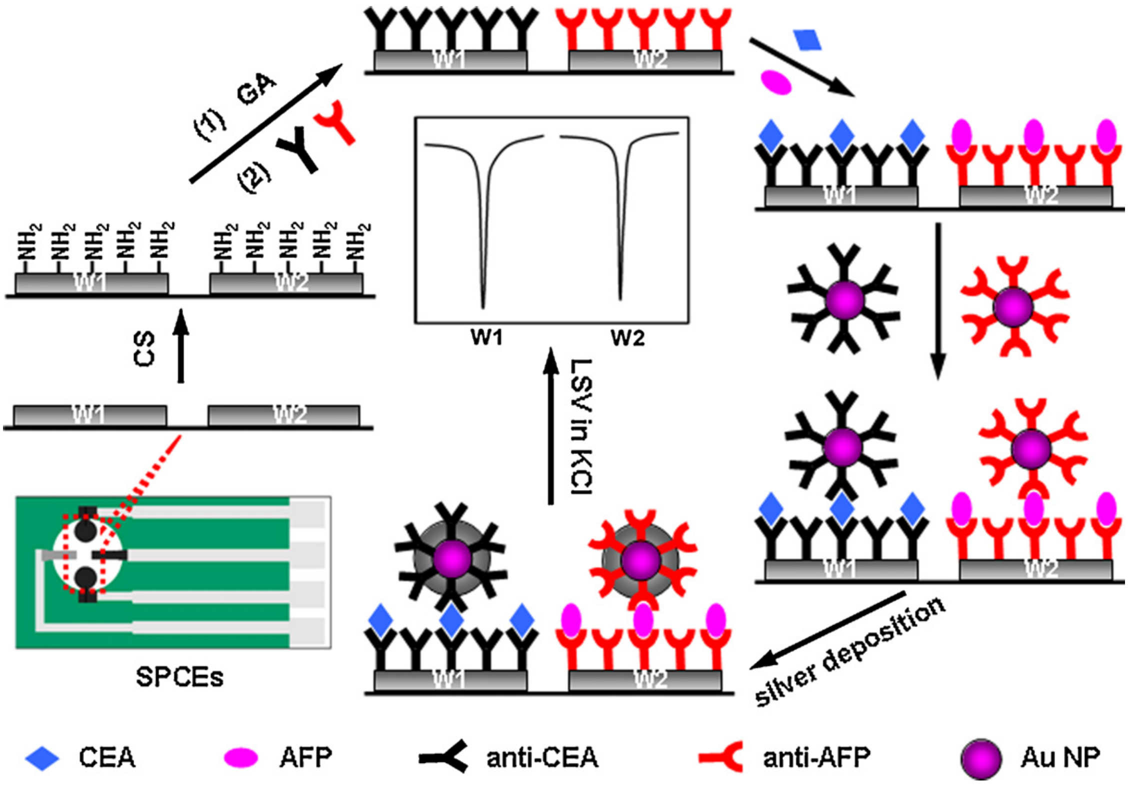

Fig. 1. Schematic representation of preparation of immunosensor array and detection strategy by linear-sweep stripping voltammetric analysis of Ag NPs catalytically deposited on the immunosensor surface by gold nanolabels.

requires a chemical [29,30] or electrochemical [31,32] oxidation pretreatment prior to detection of electrochemical signal, silver nanoparticles (Ag NPs) can be directly measured through electrochemical stripping analysis [33–35].

Here, combining the excellent stability of nanogold label with the convenient stripping analysis of Ag NPs and a disposable immunosensor array, a multiplexed electrochemical sandwich-type immunoassay method was developed for simultaneous ultrasensitive detection of CEA and AFP by the catalytic deposition of Ag NPs by the captured Au NPs (Fig. 1). Both the silver deposition enhancement and the well-defined silver stripping peak in KCl solution improved the analytical sensitivity. The positive stripping potential range excluded the interference of dissolved oxygen. Moreover, the localized deposition and stripping processes avoided cross talk completely. Thus, the proposed method provides great promise for clinical diagnosis application.

2. Materials and methods

2.1. Reagents and apparatus

Mouse monoclonal anti-CEA antibody (clone no. 27D6 and 28E4) and mouse monoclonal anti-AFP antibody (clone no. A14C11 and A46C9) were purchased from Shuangliu Zhenglong Biochem. Lab (Chengdu, China). CEA and AFP standard solutions were from ELISA kits of CEA and AFP, respectively, which were supplied by Fujirebio Diagnostics AB (Göteborg, Sweden). Chitosan (CS, ≥85% deacetylation), bovine serum albumin (BSA) and silver enhancer solution were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Glutaraldehyde (GA, 25% aqueous solution) was purchased from Alfa Aesar China Ltd. Chloroauric acid ($HAuCl_4{\cdot}4H_2O$) and trisodium citrate were obtained from Shanghai Reagent Company (Shanghai, China). Ultrapure water obtained from a Millipore water purification system (≥18 MΩ cm, Milli-Q, Millipore) was used in all assays. The clinical serum samples were from Jiangsu Institute of Cancer Prevention and Cure. All other reagents were of analytical grade and used as received. A Tris–HNO_3 buffer (50 mM, pH 7.2) was prepared and used as working solution. The washing buffer was 50 mM Tris–HNO_3 containing 0.05% (w/v) Tween-20. 50 mM Tris–HNO_3 containing 2% (w/v) BSA was used as blocking solution. A mixture solution of 20-fold diluted silver enhancer solution A and B was freshly prepared for silver deposition.

The morphology of the Au NPs was examined using a JEM 2100 high-resolution transmission electron microscope (TEM, Japan). All electrochemical immunoassays were performed on a CHI 660B electrochemical workstation (Chenhua, Shanghai, China). The reference levels of the tumor markers in the human serum samples were detected with an automation electrochemiluminescent analyzer (Elecsys 2010, Roche).

2.2. Preparation of Au NP-labeled antibodies

The colloidal Au NPs of 13-nm diameter (Fig. 2) were firstly prepared according to the previous protocol [27]. 10 μg of anti-CEA or anti-AFP signal antibody was added to 1.0 mL of colloidal Au NPs adjusted to pH 9.0 with 0.1 M K_2CO_3 and gently mixed for 60 min at room temperature. After centrifugation at 4800 rpm for 30 min, the supernatant containing the excessive antibody was discarded and the soft sediment was washed with 50 mM pH 7.2 Tris–HNO_3. After another centrifugation and discarding the supernatant, the resulting Au NP-labeled antibodies were resuspended in 1.0 mL of 50 mM pH 7.2 Tris–HNO_3 containing 1.0% BSA and stored at 4 °C. The concentration of gold nanolabel was calculated to be 3.6 nM, which was coincident with the previous report [36].

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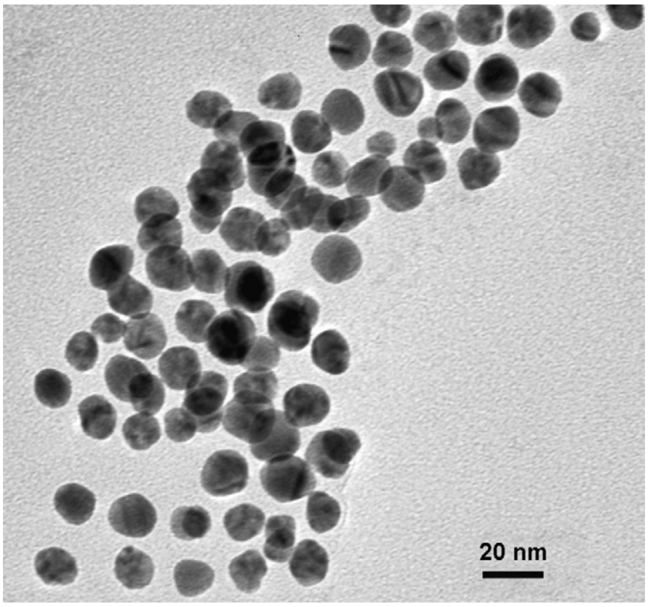

Fig. 2. TEM image of the prepared Au NPs.

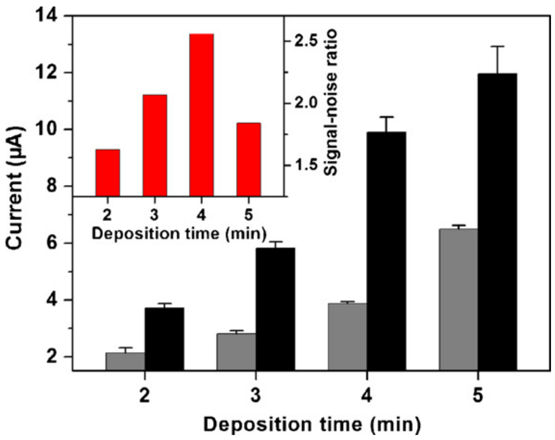

Fig. 3. Effect of silver deposition time on stripping-current response of Ag NPs for 5.0 ng mL^{-1} AFP (in black) and blank control (in gray) in 1.0 M KCl. Inset: signal–noise ratio at different deposition times.

2.3. Preparation of immunosensor array

The screen-printed carbon electrodes (SPCEs) containing two graphite working electrodes (W1 and W2, diameter: 2 mm), a graphite auxiliary electrode and a silver pseudo-reference electrode were prepared according to our previous report [18]. The insulating layer printed around the working area constituted the electrochemical microcell.

The immunosensor array was constructed by immobilizing the corresponding capture antibodies on the working electrodes of SPCEs through CS coating and GA cross-linking [28]. Firstly, 1.0 μL of 0.25 mg mL^{-1} CS was coated on the working electrodes and dried at room temperature. After activating with 2.5% GA (in 50 mM pH 7.4 phosphate buffer) for 2 h and washing with water, 1.0 μL of 0.5 mg mL^{-1} anti-CEA and anti-AFP capture antibodies were applied to the corresponding working electrodes W1 and W2, respectively, and reacted at room temperature for 60 min and then at 4 °C overnight in a 100% moisture-saturated environment. Subsequently, excess antibodies were washed by washing buffer and pH 7.2 Tris–HNO$_3$, and a drop of 15 μL of blocking solution was applied to the array and incubated for 60 min at room temperature to block the possible remaining active sites against non-specific adsorption, followed by washing with washing buffer and pH 7.2 Tris–HNO$_3$. The resulting immunosensor array was stored at 4 °C in dry prior to use.

2.4. Measurement procedure

To carry out the immunoreaction and electrochemical measurement, the immunosensor array was firstly incubated in a 15-μL drop of the mixture of CEA and AFP standard solutions or serum samples for 40 min, followed by washing with washing buffer and pH 7.2 Tris–HNO$_3$. Then, it was incubated in 15 μL of the mixture of prepared Au NP-labeled anti-CEA and anti-AFP antibodies with same volume for 40 min. After washing with washing buffer and Tris–HNO$_3$, 15 μL of silver deposition solution was delivered to the electrochemical microcell for 4 min (under dark), followed by rinsing with water. Subsequently, linear sweep voltammetry (LSV) was performed from −0.15 to 0.25 V at 50 mV s^{-1} in 1.0 M KCl solution to record the stripping currents at W1 and W2 for simultaneous detection of CEA and AFP. All steps were carried out at room temperature.

3. Results and discussion

3.1. Immunoassay through stripping analysis of deposited Ag NPs

The preparation process of the immunosensor array and the detection strategy based on sandwich-type electrochemical immunoassay are shown in Fig. 1. Through a sandwich-type immunoreaction, the antibody-functionalized Au NPs could be captured on the immunosensor surface by the formation of immunocomplex. After the addition of silver deposition solution onto the electrode surface, the attached Au NPs could act as nuclei

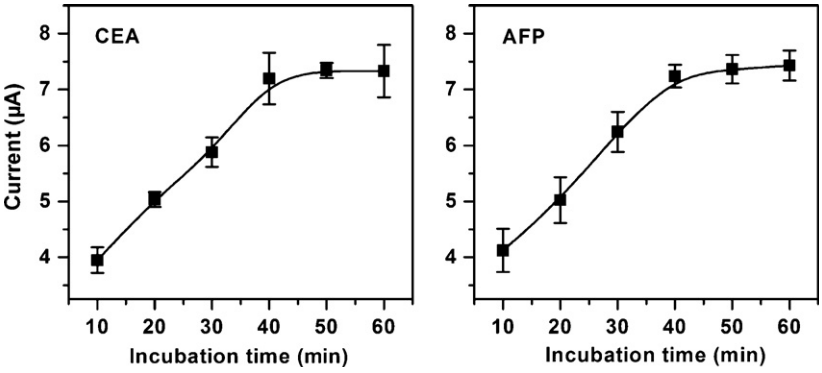

Fig. 4. Effect of incubation time on stripping-current response of Ag NPs for 0.5 ng mL^{-1} CEA and AFP.

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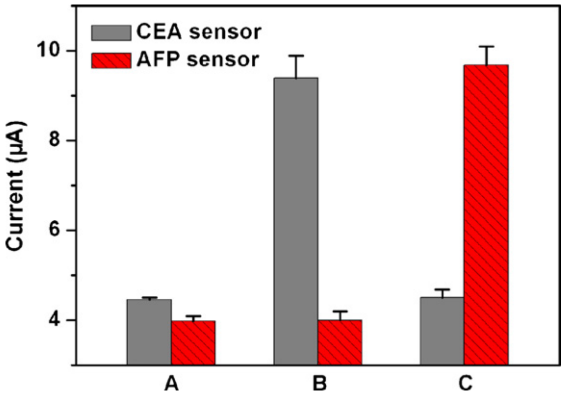

Fig. 5. Linear-sweep stripping voltammetric responses of Ag NPs on the immunosensor array incubated with blank control (A), 5.0 ng mL^{-1} CEA (B) and AFP (C) solutions, respectively.

and catalysts to induce the reduction reaction of silver ion from the silver deposition solution [37]. The higher the concentration of the analytes, the more amount of Au NPs was captured on the sensor surface to produce more Ag NPs. Finally, anodic stripping analysis of the quantitatively deposited Ag NPs on the corresponding immunosensors was used for simultaneous detection of CEA and AFP. The analytical sensitivity of the proposed method for tumor markers was greatly improved by both the Au NPs-induced silver deposition procedure and the well-defined stripping sharp peak of the deposited Ag NPs in KCl solution due to the Ag/AgCl solid-state voltammetric process [28,33]. By combining the proposed detection strategy with a disposable immunosensor array, an ultrasensitive multiplexed electrochemical immunoassay method was thus developed.

3.2. Optimization of detection conditions

To obtain excellent analytical performance, the effect of silver deposition time on stripping peak current was studied. After sandwich-type immunoreactions, 15 μL of silver deposition solution, which was prepared by mixing equal volume of 20-fold diluted silver enhancer solutions A and B, was dropped on the immunosensor array for silver deposition with different times. As shown in Fig. 3, the obtained stripping-current response increased greatly with the increasing silver deposition time. However, longer time of silver deposition also produced higher background current, leading to a decrease of the signal–noise ratio after the deposition time of 4 min. Thus, 4 min was adopted as the optimal silver deposition time in this work.

The incubation time is also an important parameter affecting the analytical performance of immunoassay. With an increasing incubation time, both the stripping voltammetric responses for CEA and AFP increased and trended to the constant values after an incubation time of 40 min (Fig. 4), indicating the saturated formation of the sandwich immunocomplex. Thus, an incubation time of 40 min was selected for the sandwich-type immunoassay.

3.3. Evaluation of cross-reactivity and cross talk

The cross-reactivity between analytes and noncognate antibodies was investigated. On the immunosensor array the capture antibodies for CEA and AFP were immobilized on W1 and W2 separately. The cross-reactivity was evaluated by comparing the silver stripping currents on the immunosensor array incubated with blank solution, 5.0 ng mL^{-1} CEA or AFP, respectively. As expected, both the CEA and AFP immunosensors only showed obvious stripping responses towards corresponding proteins (Fig. 5), indicating

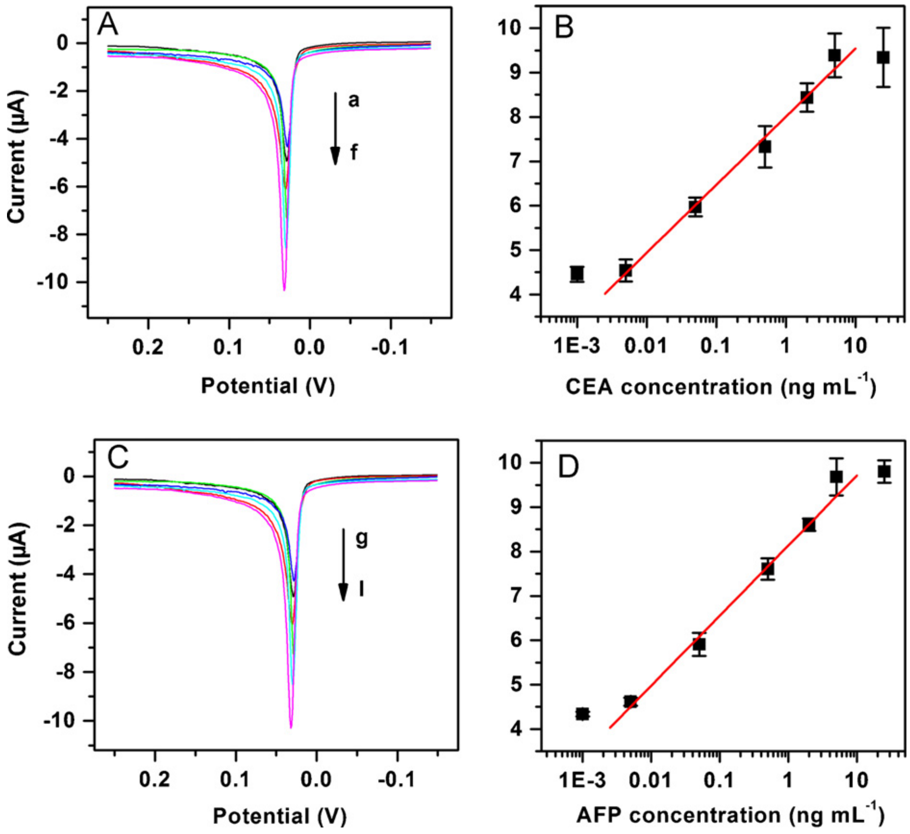

Fig. 6. Linear-sweep stripping voltammetric curves of Ag NPs (A and C) and calibration curves (B and D) for simultaneous multiplexed detection of CEA (A and B) and AFP (C and D) using the proposed strategy. Curves a–f and g–l are the LSV responses for CEA and AFP at concentrations from 0 to 5.0 ng mL^{-1} at W1 and W2, respectively.

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Table 1
Assay results of CEA and AFP in clinical serum samples using the proposed and reference methods (in ng mL^{-1}).

Serum sample	CEA			AFP		
	Proposed method	Reference method	Relative error (%)	Proposed method	Reference method	Relative error (%)
1	3.24	2.95	9.8	1.71	1.91	−10.5
2	3.15	3.20	−1.6	2.61	2.88	−9.4
3	2.26	2.11	7.1	5.26	4.85	8.5

that the cross-reactivity between the two antibodies towards noncognate proteins was negligible. In addition, the silver deposition enhancement and stripping analysis occurred only on the corresponding immunosensor surface, which resulted in a localized deposition and stripping reaction at each electrode. Therefore, no electrochemical cross talk occurred among neighboring electrodes, and the multianalyte immunoassay could be performed in a single run.

3.4. Analytical performance

With a sandwich-type immunoassay format, the quantitatively deposited Ag NPs on the immunosensors could be easily detected by anodic stripping voltammetric analysis in KCl solution. Under the optimal conditions, the current responses from the sharp stripping peaks of the immunosensor array increased linearly with increasing concentrations of analytes, which could be used for simultaneous detection of CEA and AFP. Both calibration plots showed good linear relationship between the peak currents and the logarithm values of the concentrations of CEA and AFP in the range from 5.0 pg mL^{-1} to 5.0 ng mL^{-1} (Fig. 6). The correlation coefficients were 0.9975 and 0.9954 for CEA and AFP, with the limits of detection of 3.5 and 3.9 pg mL^{-1} at a signal-to-noise ratio of 3, respectively. By comparison with the detection limits of 1.1 and 1.6 ng mL^{-1} for human IgG and goat IgG, respectively, obtained in previous studies with Au NPs label-based stripping analysis [32], this work not only simplified the detection process but also greatly improved the sensitivity. In addition, such detection limits were also lower than that with other signal amplification strategies [38–41].

The interassay precision of the immunosensor array was examined with two panels of proteins at different concentrations. Each panel was measured for five times using five arrays. The coefficients of variation were 5.9% and 5.3% for 0.05 ng mL^{-1} CEA and AFP, and 4.7% and 7.9% for 0.5 ng mL^{-1} CEA and AFP, respectively. In addition, the immunosensor array could be stored in dry at 4 °C. In this way, over 90% of the initial responses were remained after two weeks storage for both CEA and AFP. These results indicated the immunosensor array had acceptable stability and reproducibility.

3.5. Application in analysis of serum samples

To evaluate the analytical reliability and application potential of the developed immunosensing method, the assay results of CEA and AFP in human serum samples using the proposed method were compared with the reference values obtained by commercial electrochemiluminescent single-analyte tests. The results were listed in Table 1. Acceptable results with relative errors less than 10.5% for both CEA and AFP detection were achieved, indicating good accuracy of the proposed method for clinical sample detections.

4. Conclusions

An ultrasensitive multiplexed electrochemical immunoassay method is proposed by combining gold nanolabel-induced silver deposition with convenient stripping analysis on a disposable immunosensor array. The silver deposition enhancement, along with the electrochemical stripping analysis of the deposited Ag NPs in KCl solution, not only improves the analytical sensitivity but also simplifies the detection. The positive stripping potential range excludes the interference of dissolved oxygen. The localized deposition and stripping detection avoids completely the cross talk. The proposed immunoassay method shows excellent analytical performance for simultaneous measurement of CEA and AFP with high sensitivity, wide linear range, convenient operability, and acceptable reproducibility, stability and accuracy, thus provides a great promise in clinical application.

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