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Dual Signal Amplification of Glucose Oxidase-Functionalized Nanocomposites as a Trace Label for Ultrasensitive Simultaneous Multiplexed Electrochemical Detection of Tumor Markers

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A novel tracer, glucose oxidase-functionalized nanocomposite, was designed to label the signal antibodies for ultrasensitive multiplexed measurement of tumor markers using a disposable immunosensor array. The immunosensor array was constructed by coating layer-by-layer colloidal Prussian blue (PB), gold nanoparticles, and capture antibodies on screen-printed carbon electrodes. The preparation of glucose oxidase-functionalized nanocomposites and the labeling of antibody were performed by one-pot assembly of glucose oxidase and antibody on gold nanoparticles attached carbon nanotubes. The PB immobilized on immunosensor surface acted as a mediator to catalyze the reduction of H_2O_2 produced in the enzymatic cycle. Both the high-content glucose oxidase and carbon nanotubes in the tracer amplified the detectable signal for the sandwich-type immunoassay. Using carcinoembryonic antigen and α-fetoprotein as model analytes, the simultaneous multiplexed immunoassay method using the immunosensor array and the designed tracer showed linear ranges of 3 orders of magnitude with the detection limits down to 1.4 and 2.2 pg/mL, respectively. The assay results of serum samples with the proposed method were in an acceptable agreement with the reference values. The dual signal amplification of glucose oxidase-functionalized nanocomposites provided a promising ultrasensitive simultaneous multiplexed immunoassay approach for clinical applications.

The determination of tumor markers plays an important role in screening and diagnosis of cancer.[1,2] Immunoassay based on the antibody−antigen interaction is one of the most important analytical techniques in the quantitative detection of tumor markers due to the highly specific molecular recognition of immunoreaction. In comparison with other immunological methods based on fluorescence, chemiluminescence, surface-plasmon resonance, or quartz crystal microbalance, electrochemical immunoassay has attracted considerable interest for its intrinsic advantages such as good portability, low cost, and high sensitivity.[3,4] Therefore, different electrochemical immunosensors, particularly amperometric immunosensors, have been developed and extensively applied to the determination of tumor markers.[5−8]

In order to achieve a highly sensitive electrochemical immunoassay, various signal amplification strategies have been developed. For example, a large number of oligonucleotides have been assembled on one nanoparticle probe for a biobarcode assay of protein down to an attomolar level;[9] polyelectrolyte-coated ferrocene microcrystals have been used to label antibody for highly sensitive amperometric detection by release of a large amount of ferrocene after sandwich immuno-binding.[10] All these indicate nanoparticles are excellent carriers in the amplification of the transduction of recognition events.[11−13] As one of the most popular tracer labels, enzymes, including alkaline phosphatase (AP)[14,15] and horseradish peroxidase (HRP),[8,16−20] have been immobilized

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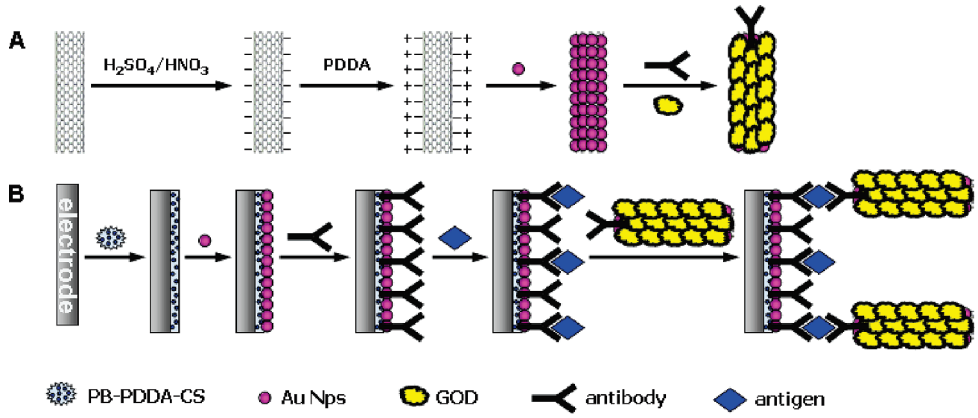
Scheme 1. Schematic Representation of (A) Preparation Procedure of GOD-Au Nps/CNTs-Ab_2 Tracer and (B) Preparation of Immunosensors and Sandwich-Type Electrochemical Immunoassay



on carbon nanotubes (CNTs),[8,14,15] bionanospheres,[16–19] and magnetic beads[20] for enhancing the enzymatically catalytical signal.

In clinical analysis, simultaneous determination of panels of tumor markers possesses important application to the screening and diagnosis of cancer.[1,21] This can be performed by the use of electrode arrays and single-enzyme label.[4,22] A series of iridium oxide electrode arrays has been successfully used for simultaneous immunoassay of multiple proteins by covalent binding or physical adsorption of different capture reagents on corresponding working electrodes to recognize the tracer.[21,23,24] These methods add AP substrate in the solution to obtain detection signal and, thus, require proper spatial separation of the individual transducers to eliminate the cross-talk problem. Several disposable immunosensor arrays have also been constructed for simultaneous detection of tumor markers with screen-printed carbon electrode arrays (SPCEs) by the use of an HRP as a tracer.[22,25–27] Because the tracer[22,27] or the tracer and redox mediator[25,26] are coimmobilized on the individual working electrode of the arrays, these immunosensor arrays avoid electrochemical cross talk among immunosensors. However, the detection sensitivity of these arrays is limited due to the nonenzymatic detection[22,27] and competitive immunoassay format.[25,26] Furthermore, dissolved oxygen greatly interferes with the detection associated with HRP, and a deoxygenation process and nitrogen atmosphere over the detection system are required. These drawbacks limit their clinical application, particularly in early screening of cancer disease and point-of-care diagnosis.

This work designed a novel tracer, glucose oxidase (GOD)-functionalized CNTs, to label the signal antibody (Ab_2) and developed an ultrasensitive immunoassay method by combining the dual signal amplification of the tracer with a Prussian blue (PB)-mediated electron transfer process. A novel immunosensor array was constructed by coating layer-by-layer colloidal PB, gold nanoparticles (Au Nps), and capture antibodies on SPCEs (Scheme 1). The immobilized PB could not only eliminate the electrochemical cross talk but also avoid the interference of dissolved oxygen with detectable current signal by a PB-mediated electron transfer process to catalyze the reduction of H_2O_2 produced in a GOD cycle at an individual working electrode. Using carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) as model analytes, an ultrasensitive multiplexed immunoassay method was, thus, proposed for simultaneous determination of panels of tumor markers. The tracer nanoparticle probe labeled antibody prepared by one-pot assembly of GOD and the antibody on Au Nps attached CNTs could provided dual signal amplification for the multiplexed immunoassay. This assay approach showed a great potential in clinical applications and detection of low-abundant proteins.

EXPERIMENTAL SECTION

Materials and Reagents. Mouse monoclonal capture and signal anti-CEA antibodies (clone no. 27D6 and 28E4) were purchased from Shuangliu Zhenglong Biochem. Lab (Chengdu, China). Mouse monoclonal capture and signal anti-AFP antibodies (clone no. 274-1 and 179-2) were purchased from Boson Biotechnology Co. Ltd. (Xiamen, China). CEA and AFP standard solutions of concentrations from 0 to 75 and 0 to 500 ng/mL were from ELISA kits of CEA and AFP, respectively, which were supplied by Fujirebio Diagnostics AB (Göteborg, Sweden). Glucose oxidase (GOD, from *Aspergillus niger*), poly(diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW: 200 000−350 000), *o*-dianisidine, bovine serum albumin (BSA), and chitosan (CS, ≥85% deacetylation) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Multiwalled carbon nanotubes (CNTs, CVD method, purity ≥98%, diameter 60−100 nm, and length 1−2 μm) were purchased from Nanoport Co. Ltd. (Shenzhen, China). 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, Milli-Q, Millipore) was used in all assays. The clinical serum samples were from Jiangsu Institute of Cancer Research. All other reagents were of analytical grade and used as received.

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Phosphate buffered saline (PBS, 0.05 M) of various pHs were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and 0.1 M KCl was added as the supporting electrolyte. The washing buffer was PBS (0.05 M, pH 7.0) containing 0.05% (w/v) Tween 20 (PBST). Blocking solution was 2% (w/v) BSA containing 0.05% Tween 20.

Apparatus. All electrochemical measurements were performed with a Uniscan PG580RM multichannel potentiostat (Uniscan Instruments, U.K.). The reference levels of the tumor markers in the human serum samples were detected with an automation electrochemiluminescent analyzer (Elecsys 2010, Roche). Scanning electron micrographs (SEM) were obtained with a Hitachi S-3000N scanning electron microscope (Japan) at an acceleration voltage of 10 kV. The determination of enzymatic kinetic parameters was performed with a UV-3600 UV−vis spectrophotometer (Shimadzu, Japan).

Preparation of GOD-Au Nps/CNTs-Ab_2 Bioconjugate. First, the colloidal Au Nps of 13 nm diameter were prepared according to the previous protocol.[28] Briefly, 100 mL of 0.01% $HAuCl_4$ solution was boiled with vigorous stirring, and 2.5 mL 1% trisodium citrate solution was quickly added to the boiling solution. When the solution turned deep red, indicating the formation of Au Nps, the solution was left stirring and cooling down.

The preparation procedure of the GOD-Au Nps/CNTs-Ab_2 bioconjugate is showed in Scheme 1A. The template of CNTs was first treated with 3:1 H_2SO_4/HNO_3 in sonication for 4 h. The resulting dispersion was filtered and washed repeatedly with water until pH was about 7.0. This procedure shortened CNTs, removed metallic and carbonaceous impurities, and generated carboxylate groups on the CNTs surface. Next, 0.5 mg/mL of the carboxylated CNTs was dispersed into a 0.20% PDDA aqueous solution containing 0.5 M NaCl by 30 min sonication to give a homogeneous black suspension. Residual PDDA polymer was removed by high-speed centrifugation, and the complex was thrice washed with water to obtain PDDA-functionalized CNTs. Then, the PDDA functionalized CNTs (0.75 mg) were dispersed in 9.0 mL of as-prepared colloidal Au Nps and stirred for 20 min. After centrifugation, light purple Au Nps/CNTs composites were obtained, which were further washed with water and redispersed in 2.5 mL of 50 mM pH 9.0 Tris−HCl solution. At room temperature, 375 μL of 2 mg/mL GOD and 15 μL of 0.5 mg/mL Ab_2 were added to 0.5 mL of the Au Nps/CNTs composite. The mixture was gently mixed for 3 h and centrifuged at 3500 rpm for 15 min at 4 °C. After centrifugation, the obtained bioconjugate was washed with washing buffer and resuspended in 100 μL of PBST containing 0.2% BSA as the assay solution. Prior to use, this solution was immediately 5-fold diluted with PBST.

Preparation of Immunosensor Array. The SPCEs containing two graphite working electrodes (W1 and W2, diameter: 2 mm), a Ag/AgCl reference, and a graphite auxiliary electrode were prepared with screen-printing technology according to our previous report.[25] The insulating layer printed around the working area constituted an electrochemical microcell. Nanosized PB colloid protected by PDDA and CS (PB-PDDA-CS) was prepared with the method similar to Yuan's group.[29] In these experiments, 4 mL of 0.025 M $K_3[Fe(CN)_6]$ solution was slowly mixed with 16 mL of a solution containing 6.25 mM $FeCl_2 \cdot 4H_2O$, 0.4% PDDA, and 0.15 wt % CS (1% HAc solution) under vigorous stirring at room temperature. Upon mixing, the corresponding solution gradually turned dark blue, indicating the formation of colloidal PB-PDDA-CS.

The preparation of the immunosensor array was illustrated in Scheme 1B. First, 1.0 μL of the resulting PB-PDDA-CS stock solution was applied to each working electrode of the SPCEs previously electrochemically pretreated for 2 min at an anodic potential of 1.3 V in 0.1 M H_2SO_4. After drying in air, 10 μL of colloidal Au Nps was applied to the working area for a 6 h assembly. After washing with PBST and pH 7.0 PBS successively, 0.5 μL of 0.5 mg/mL anti-CEA and anti-AFP antibody (Ab_1) were applied to the corresponding working electrodes of W_1 and W_2 repeatedly and incubated in a 100% moisture-saturated environment overnight at 4 °C. Subsequently, the resulting electrodes were washed three times with PBST and PBS to remove the physically absorbed Ab_1, incubated with blocking solution for 60 min at room temperature to block possible remaining active sites against nonspecific adsorption, and then washed with PBST and PBS again to form the immunosensor array.

Measurement Procedure. To carry out the immunoreaction and electrochemical measurement, the immunosensor array was first incubated with a 10 μL drop of the mixture of CEA and AFP standard solutions or serum samples with different concentrations for 40 min at room temperature, followed by washing with PBST and PBS for 1.5 min each. Next, it was further incubated with 10 μL of 1:5 diluted GOD-Au Nps/CNTs-Ab_2 bioconjugate for 40 min at room temperature, followed by washing with PBST and PBS for 3 min each. Finally, 40 μL of pH 6.5 PBS containing 10 mM glucose was dropped onto the electrochemical microcell as detection solution. Differential pulse voltammetry (DPV) from 300 to −200 mV (vs Ag/AgCl) with a pulse amplitude of 50 mV and a pulse width of 50 ms was performed to record the electrochemical responses at W1 and W2 for simultaneously quantitative measurement of CEA and AFP.

RESULTS AND DISCUSSION

Characterization of GOD-Au Nps/CNTs-Ab_2 Bioconjugate. Recent research has indicated that CNTs can be used as carriers to load numerous enzymes by a layer-by-layer assembly or covalent linkage for construction of biosensors or biocatalysts, and cationic polyelectrolyte PDDA can wrap on the sidewall surface of the carboxylated CNTs for loading of negatively charged enzyme molecules.[30] The carboxylated CNTs obtained by chemical oxidation showed a homogeneous surface and good dispersion (Figure 1a). After positively charged PDDA-modified CNTs were formed, the assembly of negatively charged Au Nps appeared larger in size and showed a distinctively more densely packed morphology (Figure 1b). The Au Nps could compactly and uniformly attach on the surface of the CNTs by the PDDA bridge. The uniformly distributed Au Nps could selectively attach protein

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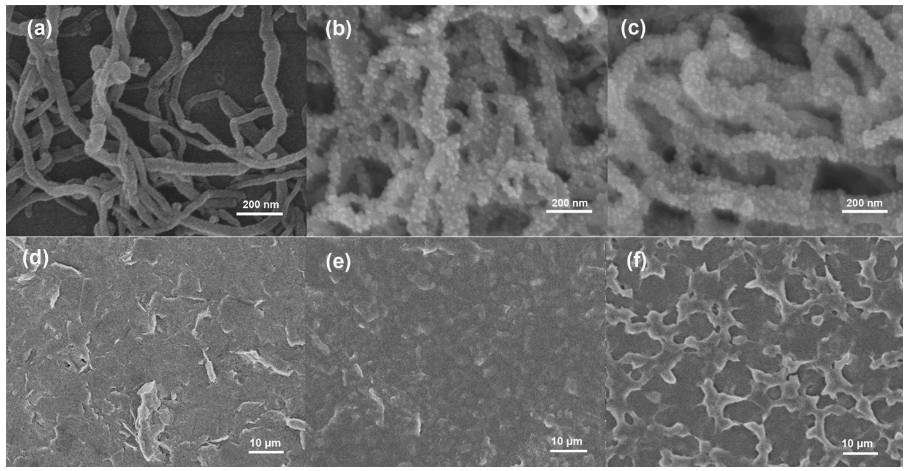
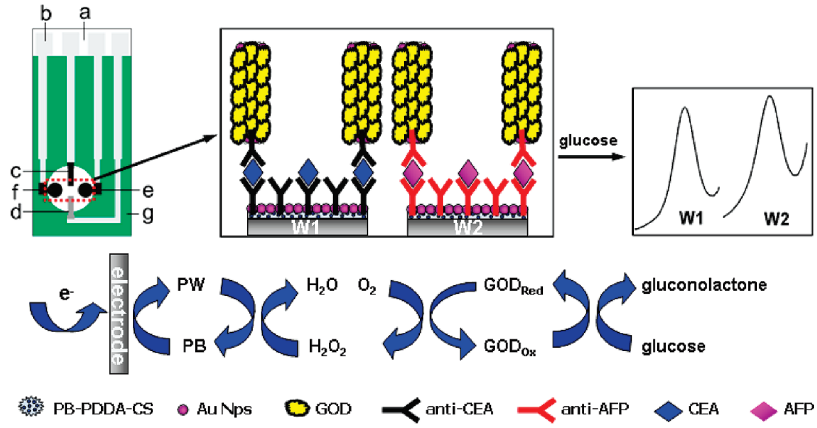


Figure 1. SEM images of CNTs (a), Au Nps/CNTs (b), GOD-Au Nps/CNTs-Ab_2 nanocomposite (c), bare SPCEs (d), PB-PDDA-CS/SPCEs (e), and Ab_1/Au Nps/PB-PDDA-CS/SPCEs (f).

Scheme 2. Schematic Representation of a Multiplexed Electrochemical Immunoassay with an Immunosensor Array and Electrochemical Response Mechanism[a]



[a] (a) Nylon sheet, (b) silver ink, (c) graphite auxiliary electrode, (d) Ag/AgCl reference electrode, (e) W1, (f) W2, and (g) insulating dielectric.

molecules for the biofunctionalization of the CNTs.[31] After GOD and Ab_2 were attached to the Au Nps/CNTs composites, the latter increased in their diameters and the surface appeared richer in texture (Figure 1c). These results indicated that the GOD and Ab_2 molecules had loaded onto the CNTs to form GOD-Au Nps/CNTs-Ab_2 bioconjugate.

To characterize the catalytic performance of the obtained bioconjugate, its Michaelis constant (K_m) and catalytic rate constant (k_{cat}) were estimated by photometry.[32] Based on the Lineweaver−Burke plot, the values of K_m, v_{max}, and k_{cat} were found to be 0.55 ± 0.07 mM, 1.24 ± 0.14 μM min^{-1} mg^{-1}, and 19.7 ± 1.87 min^{-1} (n = 5), respectively. The value of K_m was much smaller than those of 5.85 and 3.74 mM for free GOD and GOD-attached Au Nps,[32] indicating the increased affinity to glucose. The value of v_{max} was 5 times that of free GOD and comparable with that of GOD-attached Au Nps,[32] indicating good catalytic performance.

Characterization of Immunosensor Array. PB is a well-known "artificial peroxidase" which can selectively electrocatalyze the reduction of H_2O_2.[33] This property has extensively been used for preparation of glucose biosensors by coimmobilization of GOD.[34−37] According to a previous report,[29] the as-prepared PB colloid showed good dispersibility and stability due to the presence of the protective medium of PDDA and CS. The PDDA-CS-protected PB could be directly immobilized on the electrode surface to obtain a stable PB film by virtue of the excellent film-forming ability of CS and the electrostatic interaction between cationic PDDA and oxidized pretreatment electrode surface. The positively charged PDDA and the amino groups of CS could then attach Au Nps for assembly of antibody. In comparison with the bare electrode surface (Figure 1d), the PB-PDDA-CS coated surface showed a smoother and more uniform three-dimensional porous structure (Figure 1e). After Au Nps were attached on the surface, the SEM image showed smaller pore distribution (not

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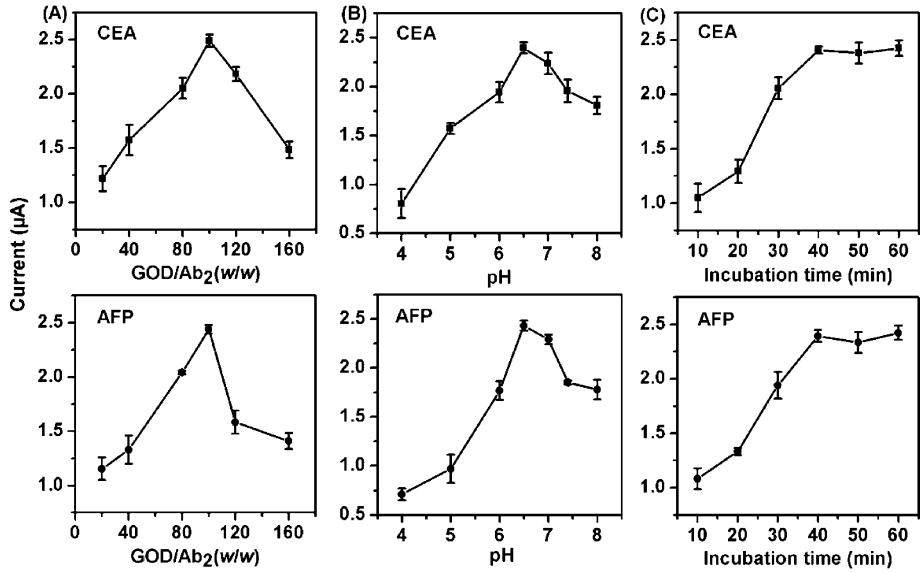

Figure 2. Effects of (A) weight ratio of GOD to Ab_2 for preparation of nanocomposite labeled antibody, (B) pH of detection solution, and (C) incubation time on amperometric responses of CEA and AFP at the immunosensor array.

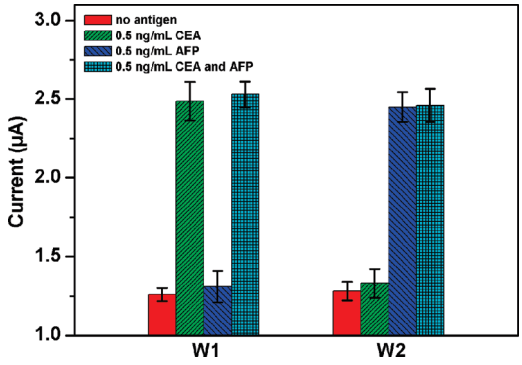

Figure 3. Amperometric responses of the immunosensor array to blank control, 0.5 ng/mL CEA, 0.5 ng/mL AFP, and the mixture of 0.5 ng/mL CEA and AFP.

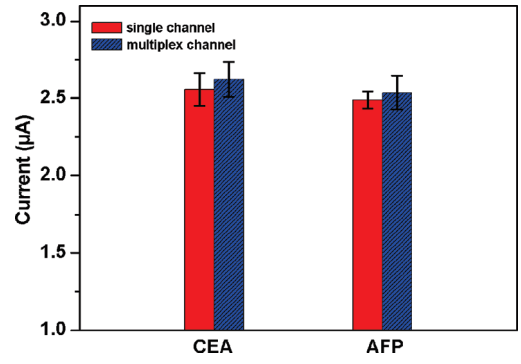

Figure 4. Amperometric responses of 0.5 ng/mL CEA and AFP in single and multiplex channel models.

shown). Upon the immobilization of the capture Ab_1, an obvious aggregation of the trapped biomolecules with a regular distribution could be observed on the surface (Figure 1f), indicative of the successful assembly of the capture Ab_1 on the SPCEs.

Immunoassay Using GOD-Au Nps/CNTs-Ab_2 Bioconjugate. HRP is a commonly used tracer enzyme in an electrochemical immunoassay, which requires deoxygenation and nitrogen atmosphere over the detection system. The use of GOD as a tracer enzyme avoided the effect of dissolved oxygen on detectable signal. After incubation of the immunosensor array with the standard antigen solutions or human serum sample for 40 min and then the 1:5 diluted GOD-Au Nps/CNTs-Ab_2 bioconjugate for another 40 min, the GOD-functionalized nanocomposites were attached to the working electrodes quantitatively by sandwich-type immunoreactions. The electrochemical response mechanism of the immunosensors was illustrated as Scheme 2. First, the GOD captured on the electrode surface could catalyze the oxidation of β-D-glucose substrate by dissolved oxygen to glucono-δ-lactone to produce H_2O_2. The PB film then acted as an electron transfer mediator to catalyze the reduction of the produced H_2O_2, leading to a current response at a low potential for immunoassay of analyte.[38] By combining the detection process with the immunosensor array, a simultaneous multiplexed immunoassay could be performed.

Due to the intrinsic property of high surface-to-volume ratio, the carboxylated CNTs could load numerous GOD molecules with a relatively low content of Ab_2 by controlling the weight ratio of GOD to Ab_2 for preparation of nanocomposite labeled antibody. As shown in Figure 2A, the amperometric responses of the immunosensors for CEA and AFP all increased with the increasing weight ratio of GOD to Ab_2 with the maximum responses at the ratio of 100, which was selected as an optimal condition for preparation the bioconjugate. The decrease of the responses was due to the low binding ability of the bioconjugate at the low amount of antibody in the sandwich-type immunoreactions. The excessive GOD molecules limited the binding sites of the CNTs with Ab_2. From the optimal ratio, several hundred enzyme labels per binding event occurred at W1 and W2, which led to dual signal amplification of both highly loading enzyme amount and enzymatic cycle for the ultrasensitive immunoassay.

Optimization of Detection Conditions. The pH of detection solution was an important factor in the enzymatic response. The effects of solution pH on the amperometric responses for CEA

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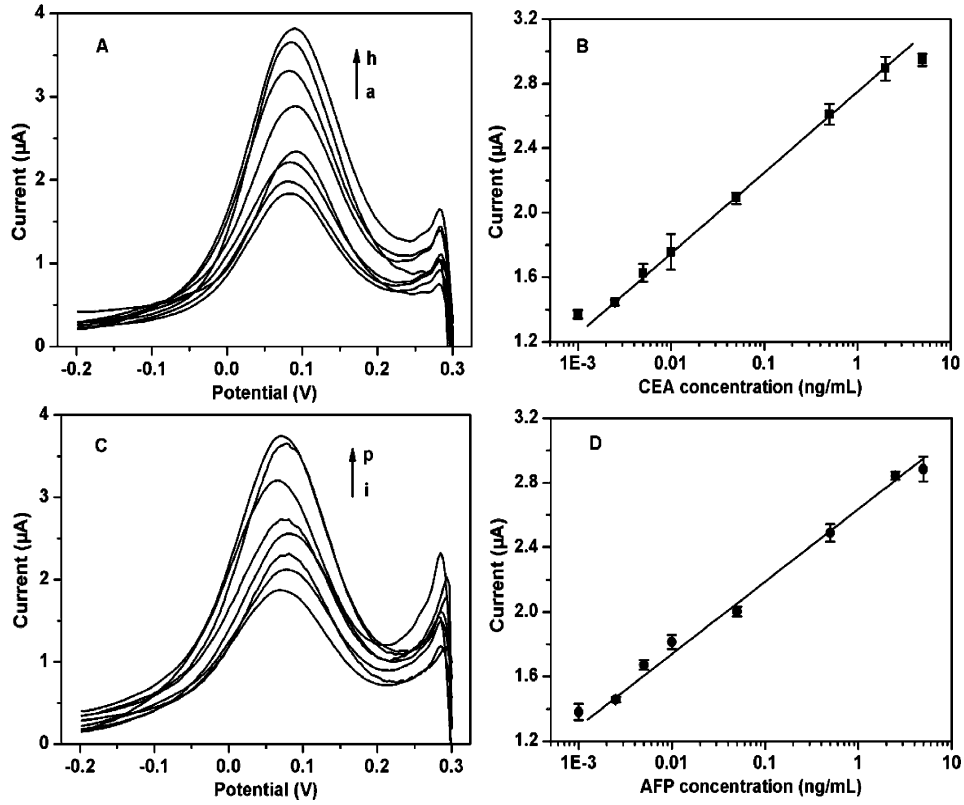

Figure 5. DPV responses (A, C) and calibration curves (B, D) for simultaneous multiplexed detection of CEA (A, B) and AFP (C, D) using the tracer and immunosensor array. Curves a−h and i−p are from W1 and W2 with increasing CEA and AFP concentration from 1.0 pg/mL to 5.0 ng/mL, respectively.

Table 1. Assay Results of Clinical Serum Samples Using the Proposed and Reference Methods (in ng/mL)

analyte	CEA			AFP		
sample no.	1	2	3	1	2	3
proposed method	1.83	1.02	2.81	2.02	2.12	1.88
reference method	1.98	1.00	3.12	1.96	2.31	2.14
relative error (%)	−7.6	2.0	−9.9	3.1	−8.2	−12.1

and AFP are shown in Figure 2B. The responses increased steeply with the increasing pH value from 4.0 to 6.5 and then decreased gently when the pH was over 6.5. Thus, 0.05 M pH 6.5 PBS containing 0.1 M KCl was selected as the detection solution for simultaneous multiplexed detection of CEA and AFP.

At room temperature, the amperometric responses for CEA and AFP increased with the increasing incubation time used in sandwich-type immunoassay and then tended to constant values after 40 min (Figure 2C), which showed a saturated binding between the analyte and the capture antibody on electrode surface. Therefore, 40 min of incubation time was selected for the sandwich-type immunoassay.

Evaluation of Cross-Reactivity and Cross Talk. An excellent immunosensor array must exclude cross-reactivity between analytes and noncognate antibodies and the cross-talk problem between neighboring electrodes.[24] This work immobilized two different capture Ab_1 for CEA and AFP on W1 and W2 separately. The cross-reactivity was evaluated by comparing the amperometric responses at W1 and W2 to the mixture of two analytes to those containing only one of the two analytes. As expected, the amperometric responses showed minimal difference when the incubation solution contained one or two kinds of analytes (Figure 3). The small responses for blank control at both W1 and W2 resulted from the redox of the immobilized PB. Obviously, the cross-reactivity between the two analytes and noncognate antibodies was negligible.

The electrochemical cross talk in immunosensor array generally results from the diffusion of electroactive product of the enzymatic reaction on one electrode to neighboring electrodes. In this work, the amperometric responses for quantitive analysis were obtained via PB-mediated reduction of H_2O_2. The PB film was immobilized on the surface of independent working electrodes. Once the H_2O_2 was produced in a GOD-enzymatic cycle, it would be quickly reduced at the working electrode. The cross talk could be well avoided. As shown in Figure 4, the amperometric responses for CEA and AFP with multiplex channel mode were almost the same as those with single channel mode. Moreover, the peak currents for five times successively simultaneous DPV measurements at this immunosensor array showed small standard deviation. Thus, ultrasensitive simultaneous multiplexed immunoassay of tumor markers could be performed in a single run using the designed disposable immunosensor array.

Analytical Performance. Under the optimum conditions, the peak currents of the immunosensor array for simultaneous detection of CEA and AFP increased with the increasing concentration of analytes (Figure 5). The calibration plots showed good linear relationships between the peak currents and the logarithm of the analyte concentrations in the ranges from 2.5 pg/mL to 2.0 ng/mL with a correlation coefficient of 0.9995 ($n = 6$) for CEA

and 2.5 pg/mL to 2.5 ng/mL with a correlation coefficient of 0.9970 ($n = 6$) for AFP, respectively. The limits of detection for CEA and AFP were 1.4 and 2.2 pg/mL at a signal-to-noise ratio of 3, respectively, which were not only much lower than those reported previously in a multiplexed immunoassay[21,23,26,39] but also lower than those in a single-analyte assay with amplification strategies.[13,16,19,40]

The interassay precision of the immunosensor array was examined with two panels of tumor markers at different concentrations. Each panel was measured for five times using five arrays. The coefficients of variation were 2.5% and 3.2% for 0.05 ng/mL CEA and AFP, and 3.8% and 3.2% for 0.5 ng/mL CEA and AFP, respectively. In addition, the immunosensor array could be stored dry at 4 °C. In this way, over 93% of the initial responses remained after one week and 82% of the initial responses remained after one month for both CEA and AFP. These results indicated the immunosensor array had acceptable stability and reproducibility.

Application in Detection of Serum Tumor Markers. To evaluate the analytical reliability and application potential of this multiplexed immunosensor array, the assay results of clinical serum samples using the proposed method were compared with reference values obtained by commercial electrochemiluminescent single-analyte tests. When the levels of tumor markers were over the calibration ranges, serum samples were appropriately diluted with 0.02 M pH 7.2 Tris−HCl prior to the assay. The results were listed in Table 1, which showed an acceptable agreement with relative errors less than 12.1%. Wilcoxon signed-rank test indicated no significant difference between the results obtained using the proposed method and the reference method.

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CONCLUSION

A novel nanoparticle probe-labeled signal antibody bioconjugate with high ratio of tracer GOD to antibody was designed for dual signal amplification. The bioconjugate could conveniently be used for ultrasensitive multiplexed immunoassay by the use of a newly proposed immunosensor array to combine a PB-mediated electron transfer process. Both the bioconjugate and the array could be simply prepared with a layer-by-layer technique. The cross-reactivity between analytes and the bioconjugates and the cross talk between different immunosensors on the same array could be completely excluded. The proposed method avoided the need of deoxygenation and nitrogen atmosphere for electrochemical immunoassay and showed excellent performance for simultaneous detection of CEA and AFP with wide linear ranges and low detection limits and acceptable stability, reproducibility, and accuracy. The convenient operation and ultrasensitivity of the proposed method provided a promising potential in clinical applications.

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