Clinical Chemistry 53:8
1495–1502 (2007)

Automation and
Analytical Techniques

A Disposable Multianalyte Electrochemical Immunosensor Array for Automated Simultaneous Determination of Tumor Markers

Jie Wu,[1] Feng Yan,[2] Jinhai Tang,[2] Chun Zhai,[1] and Huangxian Ju[1*]

Background: **Automated and convenient multianalyte detection with high throughput is increasingly needed in clinical diagnosis. We developed a disposable 4-by-2 array for programmed simultaneous amperometric immunoassay of 4 tumor markers.**

Methods: **We used a screen-printed technique, 1-step immobilization method, and flow injection technique. We immobilized carcinoembryonic antigen, α-fetoprotein, β-human choriogonadotropin, and carcinoma antigen 125 as model analytes in a redox mediator–grafted, biopolymer-modified, screen-printed carbon electrode array to capture corresponding horseradish peroxidase-labeled antibodies in competitive immunoreactions. The simultaneous multianalyte immunoassay was automatically carried out to amperometrically monitor the mediator-catalyzed enzymatic response to hydrogen peroxide, which decreased in proportion to the concentrations of analytes in samples.**

Results: **The multianalyte immunosensor array had a throughput of 60 samples/h and allowed simultaneous detection of carcinoembryonic antigen, α-fetoprotein, β-human choriogonadotropin, and carcinoma antigen 125 in clinical serum samples with concentrations up to 188 μg/L, 250 μg/L, 266 IU/L, and 334 kIU/L, respectively. The detection limits (limits of the blank, mean of blank plus 3 SD) were 1.1 μg/L, 1.7 μg/L, 1.2 IU/L, and 1.7 kIU/L. The inter- and intraassay imprecision (CVs) of the immunosensor arrays were <7.8% and <9.0%, respectively. The immunosensor arrays were stable for 28 days.**

Conclusions: **This newly constructed immunosensor array provides a simple, automated, simultaneous multianalyte immunoassay with high throughput, short analytical time, and sufficiently low detection limits for clinical application. This method offers the capability of miniaturizing the multianalyte detection device.**
© 2007 American Association for Clinical Chemistry

Recently, immunosensors and immunoassay methods for single-tumor markers have been well developed for cancer screening *(1, 2)*. Their applications are facing great challenges, because most markers are not specific to a particular tumor. Panels of tumor markers can improve their diagnostic value *(3–5)*. Thus a simultaneous multianalyte immunoassay with short analytical time, high throughput, small sampling volume, and low cost possesses considerable importance for cancer screening *(6, 7)*.

Various approaches have been proposed to perform simultaneous multianalyte immunoassays *(8–12)*, including label-free and labeled probe methods *(13)*. Label-free immunoassays are difficult to develop because of the lack of a detectable protein signal or a signal too weak to quantify the trace amount of analytes *(14)*. Labeled probe methods such as multilabel and spatially resolved assays can offer amplified detection signals for multianalyte immunoassays. Multilabel methods need several labels, such as radioisotopes *(15)*, fluorescence dyes *(16)*, enzymes *(17, 18)*, metal ions *(12, 14, 19)*, or quantum dots *(20, 21)*, which limits their application. Spatially resolved array assays with a single label can perform simultaneous detection of analytes by both optical *(22–24)* and electrochemical *(25)* responses. Although the optical multianalyte immunoassay has been well developed, electrochemical arrays have the advantage of not needing a sophisticated and expensive array detector, and thus show promise *(7, 26)*.

The electrochemical immunoassay has the advantages of low detection limit, small analyte volume, simple instrumentation, and minimal manipulation, and the assay system can easily be miniaturized *(27, 28)*. Many

[1] Key Laboratory of Analytical Chemistry for Life Science (Ministry of Education of China), Department of Chemistry, Nanjing University, Nanjing 210093, Peoples Republic of China.
[2] Jiangsu Institute of Cancer Prevention and Cure, Nanjing 210009, Peoples Republic of China.
* Address correspondence to this author at: Key Laboratory of Analytical Chemistry for Life Science (Ministry of Education of China), Department of Chemistry, Nanjing University, Nanjing 210093, Peoples Republic of China. Fax 86-25-83593593; e-mail hxju@nju.edu.cn.
Received February 6, 2007; accepted May 31, 2007.
Previously published online at DOI: 10.1373/clinchem.2007.086975

attempts have been made toward the development of electrochemical array-sensors focusing on immobilizing receptors at sensitive transducers, minimizing cross-talk, and maximizing performance. A proposed method for creating a protein microchip involves immobilization of capture antibodies in siloxane layers to retard the diffusion of enzyme-generated product *(29)*. Wilson et al. *(7, 25, 26)* reported a series of electrochemical arrays generated by covalent binding or physical adsorbtion of analytes on iridium oxide electrodes to recognize alkaline phosphatase-labeled antibodies. They avoided electrochemical cross-talk by making the distance between adjacent electrodes larger than the diffusion distance of the enzymatic product; this procedure, however, was disadvantageous to the further miniaturization of the electrochemical sensor array.

Chitosan (CHIT)[3], a kind of natural biopolymer, has been widely used as an immobilization matrix because of its excellent film-forming ability, high permeability toward water, good adhesion, biocompatibility, nontoxicity, and high mechanical strength *(30)*. Recently, CHIT has been increasingly used for biofabrication because of the presence of reactive amino and hydroxyl functional groups *(31)*. CHIT grafted with mediators has also been shown to have attractive properties of enzyme immobilization and activity maintenance *(32–35)*. For these reasons, we used grafted CHIT in the present studies. Our goal was to develop a simple and low-cost method for diagnosis of cancer with a combination of multitumor markers.

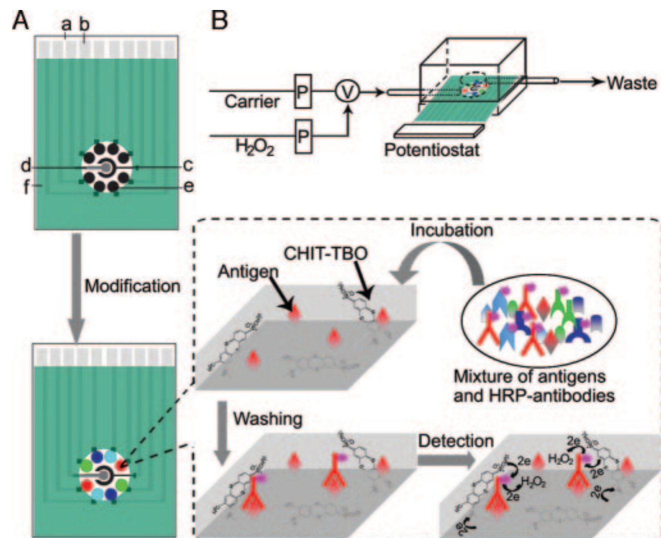


Fig. 1. Schematic diagrams of the immunosensor array (*A*) and automated multianalyte electrochemical immunoassay system (*B*).

(*a*) Nylon sheet, (*b*) silver ink, (*c*) graphite auxiliary electrode, (*d*) Ag/AgCl reference electrode, (*e*) graphite working electrode, and (*f*) insulating dielectric. P, peristaltic pump; V, injection valve.

Materials and Methods

OVERVIEW

We used grated CHIT to immobilize 4 tumor markers at screen-printed carbon electrodes (SPCE) for preparation of a novel immunosensor array. The redox mediator, toluidine blue O (TBO) to CHIT using glutaric dialdehyde (GDI) as a molecular tether *(35)*, accelerated the electrochemical reduction of hydrogen peroxide catalyzed by horseradish peroxidase (HRP)-labeled monoclonal antibodies; this increased the sensitivity of the immunoassay. The immobilized TBO and the captured HRP-labeled antibodies avoided cross-talk between immunosensors.

APPARATUS

The disposable immunosensor array was connected to a flow injection system as illustrated in Fig. 1. Briefly, this system comprised 2 peristaltic pumps (Baoding Longer Precision Pump Co.), an 8-port rotary injection valve, and a homemade flow cell, which were connected using polytetrafluorethylene tubes (0.8 mm internal diameter). We performed electrochemical measurements on a 4-channel potentiostat (eDAQ Co.) equipped with an e-corder system. We used an electronic switch coupled with the potentiostat for simultaneous 8-analyte detection of 2 groups of a 6-electrode system. We obtained scanning electron micrographs with a JSM-5610LV scanning electron microscope (JEOL), infrared spectra with a VECTOR 22 spectrometer by preparing the samples on KBr tablets, and UV-Vis spectra with a Shimadzu UV-3600 UV-Vis Spectrophotometer. We detected control levels of the tumor markers in sera with an automated electrochemiluminescence (ECL) analyzer (Elecsys 2010; Roche).

REAGENTS

We purchased carcinoembryonic antigen (CEA), α-fetoprotein (α-AFP), and carcinoma antigen 125 (CA 125) ELISA reagent sets from CanAg Diagnostics. They consisted of a series of CEA, α-AFP, and CA 125 solutions with concentrations from 0 to 75 μg/L, 0 to 500 μg/L, and 0 to 500 kIU/L, respectively, and the stock solutions of HRP-labeled CEA, α-AFP, and CA125 monoclonal antibodies. The β-human choriogonadotropin (β-hCG) ELISA reagent set (Biocell Biotechnology) consisted of a series of β-hCG solutions from 0 to 400 IU/L and a stock solution of HRP-labeled β-hCG monoclonal antibody. We purchased BSA, CHIT (molecular weight $\sim 1 \times 10^6$ Da; ~85% deacetylation), and TBO from Sigma-Aldrich Chemical. All other reagents were of analytical reagent grade and used without further purification. We used 0.1 mol/L Tris-HCl (pH 7.2) containing 10 g/L BSA as blocking buffer and dilution solution of the enzyme conjugates. We prepared 0.2 mol/L PBS of various pHs by mixing the

[3] Nonstandard abbreviations: CHIT, chitosan; SPCE, screen-printed carbon electrode; TBO, toluidine blue O; GDI, glutaric dialdehyde; HRP, horseradish peroxidase; ECL, electrochemiluminescence; CEA, carcinoembryonic antigen; α-AFP, α-fetoprotein; CA 125, carcinoma antigen 125; β-hCG, β-human choriogonadotropin.

stock solutions of NaH_2PO_4 and Na_2HPO_4 and adjusting the pH with 0.2 mol/L NaOH and H_3PO_4. Doubly distilled water was used throughout the experiments. Serum specimens from Jiangsu Institute of Cancer Prevention and Cure were stored at 4 °C.

Synthesis of TBO-Grafted CHIT Biopolymer

The covalent attachment of TBO molecules to CHIT biopolymer relied on the formation of Schiff bases by reacting amino groups of TBO and CHIT with GDI *(35)*. First, we prepared CHIT stock solution (10 g/L) by ultrasonically dissolving CHIT powder in 1% acetic acid and adjusting the pH to 5.0. We mixed the CHIT solution with an excess of GDI and allowed them to react for 48 h with constant stirring at room temperature. A high molar ratio of GDI to CHIT glucosamine units (200:1) was adopted to ensure that only 1 aldehyde group of GDI reacted with the amino groups of CHIT. The free GDI was removed from the mixture by dialyzing it against 1% acetic acid, which was monitored by UV-Vis spectrophotometry. Next, the CHIT-GDI solution was reacted with an excess of TBO for 48 h with constant stirring at room temperature. Again, dialysis was used to remove free TBO from the mixture. Finally, the Schiff bases in the product were reduced to more stable secondary amines using sodium cyanoborohydride to obtain the CHIT-TBO solution with a concentration corresponding to 1 g/L CHIT. We removed the remaining sodium cyanoborohydride and its oxidized product from the formed films by simple washing.

Fabrication of Immunosensor Array

Construction of the multianalyte immunosensor array is shown in Fig. 1A. It contained 8 (4-by-2) graphite working electrodes (2 mm diameter, 0.5 mm edge-to-edge separation), 1 graphite auxiliary electrode, and 1 Ag/AgCl reference electrode. All working electrodes shared the same reference and auxiliary electrodes. We fabricated the 10-electrode array according to a previous report *(36)*. The insulating layer printed around the working area constituted a reservoir of the electrochemical microcell.

CEA (75 μg/L), α-AFP (500 μg/L), CA 125 (500 kIU/L), and β-hCG (400 IU/L) were mixed with the CHIT-TBO solution at the same volume ratio of 1:3. After the mixtures were placed at 4 °C for 12 h, 1 μL of each was individually dropped on the working electrodes (Fig. 1). After drying at room temperature for 2–3 h, the formed films were incubated with blocking buffer for 30 min to block the sites against nonspecific adsorption and thoroughly rinsed with water to obtain a multianalyte immunosensor array, which was stored in air before use.

Assay Protocol

The simultaneous multianalyte immunoassay included an incubation step, in which the antigens immobilized on working electrodes and the antigens in sample solution competitively bound a limited amount of HRP-labeled antibodies, and an automated detection step to amperometrically monitor the enzymatic reduction of H_2O_2 by the labeled HRP in the presence of the immobilized TBO as mediator to shuttle electrons between the HRP and the electrodes (Fig. 1). The 40-min incubation step could be performed in batches with different immunosensor arrays, using the mixture solutions of diluted HRP-labeled anti-CEA, anti–α-AFP, anti-CA 125, and anti–β-hCG and samples of CEA, α-AFP, CA 125, and β-hCG solutions at room temperature and 100% relative humidity to avoid evaporation of solvent. After the residual was removed with water, the arrays were sequentially fixed in the flow system with deoxygenated 0.2 mol/L PBS (pH 6.5) as carrier at a flow rate of 3.6 mL/min. We then applied −0.5 V (vs Ag/AgCl) to each immunosensor until a stable chronoamperometric baseline was obtained. Finally, we injected 120 μL of 10.7 mmol/L H_2O_2 into the flow microcell (420 μL) to record the amperometric signals. Before injection, H_2O_2 solution was deoxygenated with nitrogen. The cyclic voltammetry measurements were performed with a stop flow mode. All manipulations except the fixing of array could be automatically performed with a computer.

Results and Discussion

Characterization of CHIT-TBO

We verified the grafting of CHIT with TBO molecules with infrared spectra (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue8). The CHIT showed a strong adsorption of $–NH_3^+$ deformation around 1572 cm^{-1} *(37)*, which covered the characteristic absorption of amide I (C=O in O=C–NH) and amide II (–NH in O=C–NH) of CHIT at pH 5.0 *(38)*. Although we observed a very weak absorption of O=C– at 1714 cm^{-1}, it was greatly strengthened after the reaction of CHIT with GDI, indicating the presence of –CHO group in CHIT-GDI. Thus the GDI molecules were attached to CHIT with 1 end. This reaction greatly decreased the adsorption of $–NH_3^+$ deformation, and we observed a weak adsorption peak for amide I (C=O in O=C–NH) of CHIT at 1640 cm^{-1}. After grafting of CHIT with TBO molecules and reduction of the formed Schiff bases, the peak at 1714 cm^{-1} decreased again, and we observed the same strength as that for CHIT. At the same time, we observed the adsorption ascribed to the TBO unit at 1640 cm^{-1}, indicating the formation of CHIT-TBO.

The grafting of TBO molecules to CHIT matrix could further be supported by UV-Vis spectra (see Fig. 2 in the online Data Supplement). The UV-Vis spectrum of CHIT-TBO solution displayed the absorption band of free TBO with a peak at 625 nm, which did not occur on the UV-Vis spectra of CHIT or CHIT-GDI solutions.

The formed CHIT-TBO film showed an even surface, which made up the surface defects of bare SPCE (see Fig. 3 in the online Data Supplement) and greatly improved the repeatability for electrochemical measurement. After

antigen molecules were entrapped in the grafted biopolymer, the resulting modified SPCE showed a uniform porous structure. Thus the HRP-labeled antibody molecules could be easily accessible to the immobilized antigen molecules to form immunocomplexes.

Optimization of detection system

We performed the simultaneous multianalyte immunoassay of 4 tumor markers with a flow injection system at an optimized applied potential of −0.5 V (see Fig. 4 in the online Data Supplement). The flow rate of the carrier solution should be considered: high flow rates would produce unstable detection signals, whereas detection signals suffer from tailing at a slow flow rates. Here, we used a flow rate of 3.6 mL/min for the simultaneous multianalyte immunoassay, at which only the concentration of H_2O_2 and the pH of the detection solution affected the amperometric response. Fig. 2 shows their effect on the responses of the immunosensor array.

With the increasing concentration of H_2O_2, all immunosensors for the 4 tumor markers showed increasing responses, which reached maximum values at the H_2O_2 concentration of 10.7 mmol/L. The enzymatic reaction depended on the solution pH, and the effects of pH on the responses of these immunosensors also showed the same tendency due to the same response mechanism. The maximum responses occurred around pH 6.5. Thus, we used pH 6.5 PBS with an injection of 10.7 mmol/L H_2O_2 for the simultaneous multianalyte immunoassay.

Optimization of immunoreactions

Under optimal detection conditions, the amperometric responses of the immunosensor array depended on the formation of immunocomplexes, which was related to the incubation temperature, incubation time, and working concentration of HRP-labeled antibodies. For convenient manipulation, the incubation step was performed at room temperature, at which the amperometric responses increased with the increasing incubation time and trended to the constant values at 40 min, except that for CEA at 30 min (Fig. 3, A and B), indicating a saturated binding between the immobilized antigens and HRP-labeled antibodies. Thus we chose 40 min as the optimal incubation time for the simultaneous multianalyte immunoassay.

The amount of HRP-labeled antibodies in the incubation solution was a key in the competitive immunoassay format. The effects of dilutions of HRP-labeled antibodies in the incubation solution on the amperometric responses are shown in Fig. 3, C and D. At high concentrations, the immunosensors showed the maximum responses due to the saturated binding, which persisted to dilutions of 1:70 for HRP-labeled CEA antibody, 1:70 for HRP-labeled α-AFP antibody, 1:80 for HRP-labeled CA 125 antibody, and 1:50 for HRP-labeled β-hCG antibody. Further dilutions resulted in the decrease of amperometric responses of these immunosensors. Thus, the incubation solution was prepared according to the optimal dilutions.

Evaluation of cross-reactivity and cross-talk behavior

An excellent immunosensor array must exclude cross-reactivity between analytes and noncognate antibodies and cross-talk between neighboring electrodes *(26)*. We examined cross-reactivity between the tumor marker and

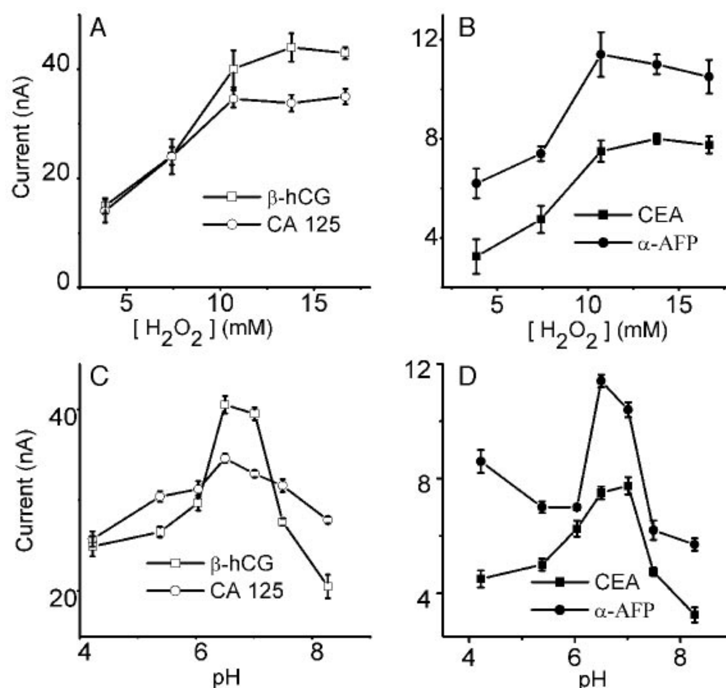


Fig. 2. Effects of H_2O_2 concentration (*A* and *B*) and pH of detection solution (*C* and *D*) on amperometric response of enzymatic activity after incubating the immunosensor array under other optimal conditions at room temperature.

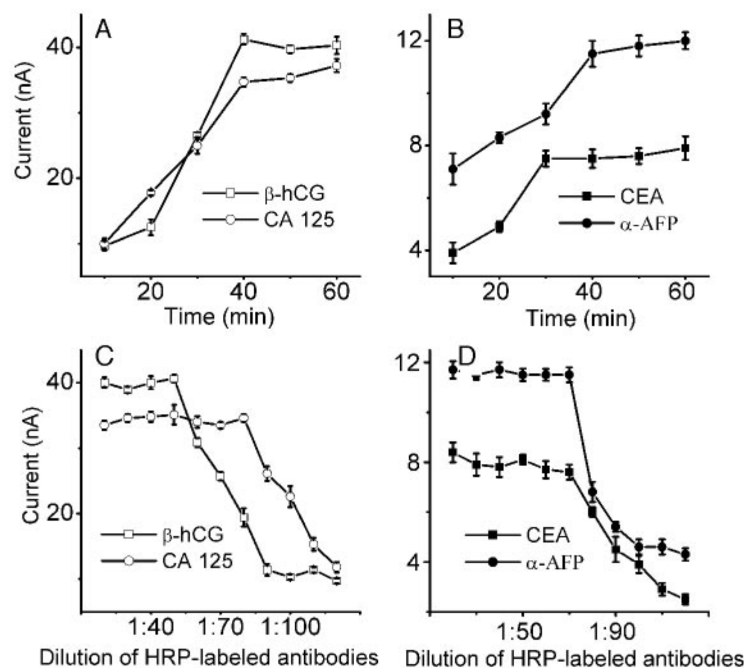


Fig. 3. Effects of incubation time (*A* and *B*) and working concentration of HRP-labeled antibodies in incubation solution (*C* and *D*) on amperometric response of the immunosensor array under other optimal conditions at room temperature.

other 3 noncorresponding antibodies using cyclic voltammetry and incubation in the solution containing 1 HRP-labeled antibody. Although all immunosensors showed the electrocatalytic response to the reduction of H_2O_2 catalyzed by the HRP, only the immunosensor incubated with its corresponding HRP-labeled antibody solution showed the electrocatalytic response (see Fig. 4 in the online Data Supplement). Thus the cross-reactivity and nonspecific binding were negligible, suggesting that the 4 tumor markers could be assayed individually in a single run without interference.

Generally, electrochemical cross-talk in the sensor array results from the diffusion of electroactive product of the enzymatic reaction on 1 electrode to neighboring electrodes. To our best knowledge, its elimination mainly focuses on making sure the distance between adjacent electrodes is larger than the diffusion distance of enzymatic product *(7, 25, 26, 29)*. In this work, the electron-transfer mediator TBO was covalently immobilized on the immunosensor surfaces during the fabrication of the immunosensor array instead of in the detection solution; no diffusion of electroactive product occurred, and the electrochemical cross-talk between the electrodes could be completely avoided. It was unnecessary to consider the separate distance between neighboring electrodes, enhancing further miniaturization of the array. Using CEA immunosensor as an example, the results are shown in Fig. 4. After an array containing both CEA immunosensor and CHIT-TBO film-modified electrode was incubated in a diluted HRP-labeled antibody solution, upon injection of H_2O_2 the modified electrode showed a very small response from the direct reduction of H_2O_2 at −0.5 V, whereas the CEA immunosensor produced a prominent amperometric response due to the enzymatic reaction (Fig. 4, curve b vs a). The response of the CEA immunosensor in a multichannel format was the same as in a single-channel format (Fig. 4, curve a vs c).

SAMPLE THROUGHPUT AND ASSAY PERFORMANCE

The amperometric response of H_2O_2 at the array showed a half-width of 4 s with a base-width of 15 s (Fig. 4). Thus the simultaneous multianalyte detection of 4 tumor markers using 1 array could be accomplished within 60 s, containing a period of 15 s for the fixing of the incubated

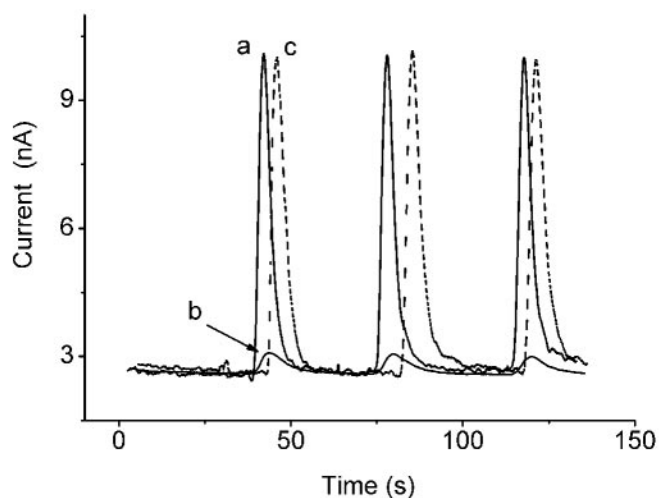


Fig. 4. Amperometric responses of CEA/CHIT-TBO (*a*) and CHIT-TBO (*b*) modified electrodes in multianalyte simultaneous determination format and CEA/CHIT-TBO modified electrode in single channel format (*c*) after incubation in the mixture of 1:70 diluted HRP-labeled CEA antibody, 1:70 diluted HRP-labeled α-AFP antibody, 1:80 diluted HRP-labeled CA 125 antibody, and 1:50 diluted HRP-labeled β-hCG antibody to injection of 120 μL 10.7 mmol/L H_2O_2 in pH 6.5 PBS at −0.5 V.

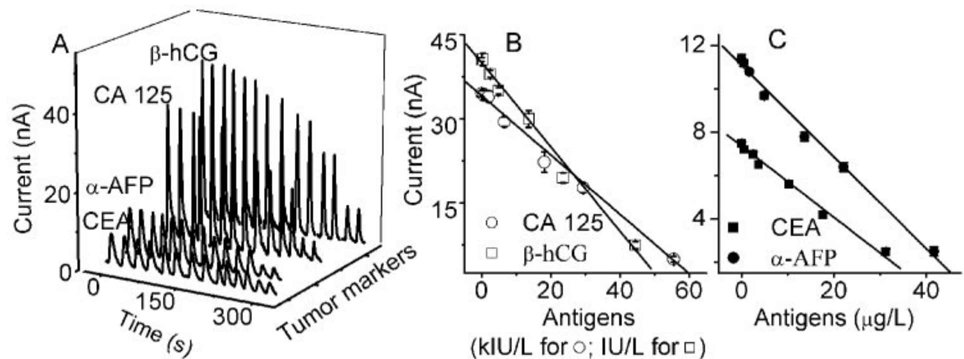


Fig. 5. (*A*), amperometric responses for multianalyte simultaneous immunoassay of 0, 0.5, 2.5, 3.7, 10.2, 17.6, and 31.3 μg/L CEA; 0, 0.4, 1.7, 4.9, 13.6, 22.1, and 41.7 μg/L α-AFP; 0, 0.5, 2, 6.6, 18.2, 29.4, and 55.6 kIU/L CA 125; and 0, 0.4, 2.5, 4.9, 13.6, 23.5, and 44.4 IU/L β-hCG to injection of 120 μL 10.7 mmol/L H_2O_2 in pH 6.5 PBS at −0.5 V. (*B* and *C*), dose–response curves.

array to the flow system and a period of 30 s for obtaining the stable baseline. Thus the sample throughput was 60 samples/h after the low-cost immunosensor arrays were incubated in batches for 40 min with the incubation solutions containing different serum samples. The dose–response and calibration curves for these tumor markers are shown in Fig. 5. The linear regression equations for CEA, α-AFP, CA 125, and β-hCG were $I = 7.28 - 0.16$ [CEA] (μg/L; $R^2 = 0.9947$), $I = 11.08 - 0.21$ [α-AFP] (μg/L; $R^2 = 0.9962$), $I = 33.94 - 0.54$ [CA 125] (kIU/L; $R^2 = 0.9950$), and $I = 39.85 - 0.76$ [β-hCG] (IU/L; $R^2 = 0.9939$) over the concentration intervals of 0.5–31.3 μg/L for CEA, 0.4−41.7 μg/L for α-AFP, 0.5–55.6 kIU/L for CA 125, and 0.4−44.4 IU/L for β-hCG. The detection limits corresponding to the signals of 3 SD for the 4 analytes were 0.19 μg/L, 0.28 μg/L, 0.28 kIU/L, and 0.20 IU/L, respectively. It can be seen from Fig. 5 that 2 couples of immunosensors for the same markers at 1 array possessed good repeatability.

Application in simultaneous detection of serum multitumor markers

After 10 μL serum sample was mixed with 50 μL mixture solution of diluted HRP-labeled antibodies with the optimized amount in the resulting incubation solution, we could simultaneously detect the concentrations of 4 tumor markers. With the calibration curves obtained above, the practical detectable linear ranges for CEA, α-AFP, CA 125, and β-hCG in serum samples were up to 188 μg/L, 250 μg/L, 334 kIU/L, and 266 IU/L with the detection limits of 1.1 μg/L, 1.7 μg/L, 1.7 kIU/L, and 1.2 IU/L, respectively. The results of the simultaneous multianalyte detection with the proposed arrays for 34 serum samples are shown in Fig. 6, compared with those obtained using the

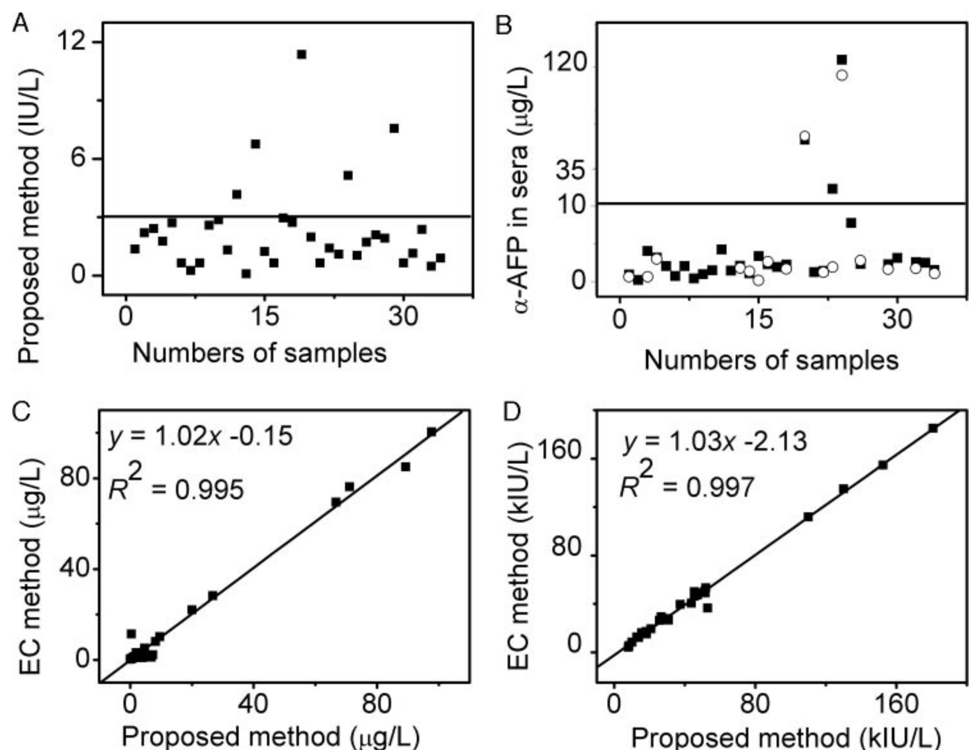


Fig. 6. Results of multianalyte simultaneous immunoassay of β-hCG (*A*), α-AFP (*B*), CEA (*C*), and CA 125 (*D*) in human sera compared with (*A*) β-hCG threshold value (—), (*B*) α-AFP threshold value (—) and control results (○), (*C*) CEA control results, and (*D*) CA 125 control results.

commercial ECL single-analyte testing. The commercial ECL assay showed that all β-hCG concentrations in these samples were lower than the threshold value, whereas our proposed method showed 5 samples that gave slightly higher results than those obtained from the commercial assay, indicating acceptable reliability for β-hCG. The commercial testing of α-AFP showed that 28 of 34 samples were clinically negative and 6 samples were clinically positive, of which 4 samples showed concentrations >2000 μg/L beyond the linear detection range of the proposed method. Fig. 6B shows the control results of 16 samples. The results obtained with our proposed method were in good agreement with the control results except for sample 23, indicating acceptable accuracy for α-AFP. The orthogonal regression analyses of the obtained results vs the control results for CEA and CA 125 in the 34 samples gave the equations of $Y = -0.149 + 1.02\ X$ ($R^2 = 0.9945$) and $Y = -2.13 + 1.03\ X$ ($R^2 = 0.9969$), respectively (Fig. 6, C and D). Both the good correlation coefficients and the slopes close to 1 indicated acceptable accuracy of this proposed method.

REPEATABILITY, REPRODUCIBILITY, AND STABILITY OF THE ARRAY

We evaluated the interassay imprecision of the immunosensor arrays at different concentrations of the 4 tumor markers using 5 arrays. The CVs were 4.3% and 3.2% for 10.2 and 17.6 g/L CEA, 3.4% and 4.2% for 4.9 and 22.1 g/L α-AFP, 7.8% and 4.3% for 6.6 and 29.4 kIU/L CA 125, and 5.5% and 7.2% for 4.9 and 23.5 IU/L β-hCG, indicating acceptable imprecision and fabrication reproducibility. The intraassay CVs of this method examined with 5 injections of H_2O_2 at 1 array were <2.2%, 5.4%, 9.0%, and 3.7% for CEA, α-AFP, CA 125, and β-hCG, respectively, showing acceptable repeatability. When the immunosensor array was stored in air at room temperature, it could keep the initial responses of these immunosensors for 28 days. After a storage period of 40 days, the amperometric responses of the CEA, α-AFP, CA 125, and β-hCG immunosensors were 85.7%, 92.3%, 93.7%, and 85.5% of their initial responses, respectively. Thus the immunosensor array had acceptable storage stability and is suitable for clinical diagnosis.

In conclusion, the proposed automated method coupled with the immunosensor arrays is simple, convenient, rapid, high-throughput, and low-cost. For widening the linear ranges of the simultaneous multianalyte detection further, work based on a sandwich format is going on in this laboratory.

Grant/funding support: This research was financially supported by the National Science Funds for Distinguished Young Scholars (20325518) and Creative Research Groups (20521503), the Key Program (20535010) from the National Natural Science Foundation of China, and the Science Foundation of Jiangsu (BS2006006, BS2006074).
Financial disclosures: None declared.

References

1. Ghindilis AL, Atanasov P, Wilkins M, Wilkins E. Immunosensors: electrochemical sensing and other engineering approaches. Biosens Bioelectron 1998;13:113–31.
2. Lin JH, Ju HX. Electrochemical and chemiluminescent immunosensors for tumor markers. Biosens Bioelectron 2005;20:1461–70.
3. Louhimo J, Finne P, Alfthan H, Stenman UH, Haglund C. Combination of HCG beta, CA 19-9 and CEA with logistic regression improves accuracy in gastrointestinal malignancies. Anticancer Res 2002;22:1759–64.
4. Tsao KC, Wu TL, Chang PY, Hong JH, Wu JT. Detection of carcinomas in an asymptomatic Chinese population: advantage of screening with multiple tumor markers. J Clin Lab Anal 2006;20: 42–6.
5. Hayakawa T, Naruse S, Kitagawa M, Ishiguro H, Kondo T, Kurimoto K, et al. A prospective multicenter trial evaluating diagnostic validity of multivariate analysis and individual serum marker in differential diagnosis of pancreatic cancer from benign pancreatic diseases. Int J Pancreatol 1999;25:23–9.
6. Ito K, Oda M, Tsuji A, Maeda M. Simultaneous determination of alpha-fetoprotein, human chorionic gonadotropin and estriol in serum of pregnant women by time-resolved fluoroimmunoassay. J Pharm Biomed 1999;20:169–78.
7. Wilson MS, Nie WY. Multiplex measurement of seven tumor markers using an electrochemical protein chip. Anal Chem 2006; 78:6476–83.
8. Kricka LJ. Multianalyte testing [Editorial]. Clin Chem 1992;38: 327–8.
9. Binder SR. Autoantibody detection using multiplex technologies. Lupus 2006;15:412–21.
10. Espina V, Woodhouse EC, Wulfkuhle J, Asmussen HD, Petricoin EF, Liotta LA. Protein microarray detection strategies: focus on direct detection technologies. J Immunol Methods 2004;290: 121–33.
11. Wilson R, Cossins AR, Spiller DG. Encoded microcarriers for high-throughput multiplexed detection. Angew Chem Int Edit 2006;45:6104–17.
12. Zhang SC, Zhang C, Xing Z. Simultaneous determination of alpha-fetoprotein and free beta-human chorionic gonadotropin by element-tagged immunoassay with detection by inductively coupled plasma mass spectrometry. Clin Chem 2004;50:1214–21.
13. Hoheisel JD. Microarray technology: beyond transcript profiling and genotype analysis. Nat Rev Genet 2006;7:200–10.
14. Hu SH, Zhang SC, Hu ZC, Xing Z, Zhang XR. Detection of multiple proteins on one spot by laser ablation inductively coupled plasma mass spectrometry and application to immuno-microarray with element-tagged antibodies. Anal Chem 2007;79:923–9.
15. Wlans FH, Dev J Jr, Powell MM, Heald JI. Evaluation of simultaneous measurement of lutropin and follitropin with the simulTROPIN™ radioimmunoassay kit. Clin Chem 1986;32:887–90.
16. Swartzman EE, Miraglia SJ, Mellentin-Michelotti J, Evangelista L, Yuan PM. A homogeneous and multiplexed immunoassay for high-throughput screening using fluorometric microvolume assay technology. Anal Biochem 1999;271:143–51.
17. Choi MJ, Choe IS, Kang HK, Lee JS, Chung TW. Simple enzyme immunoassay for the simultaneous measurement of whole choriogonadotropin molecules and free beta-subunits in sera of women with abnormal pregnancies or tumors of the reproductive system. Clin Chem 1991;37:673–7.

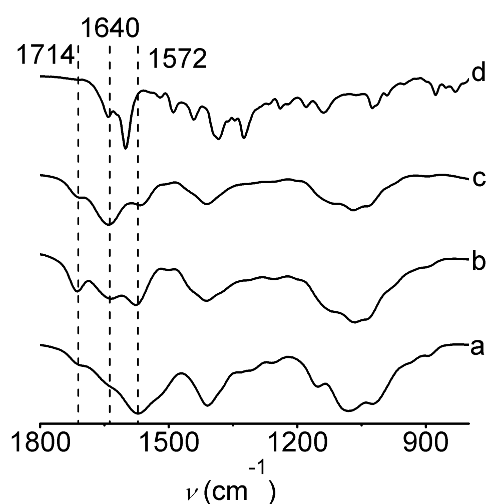
18. Fu ZF, Liu H, Ju HX. Flow-through multianalyte chemiluminescent immunosensing system with designed substrate zone-resolved technique for sequential detection of tumor markers. Anal Chem 2006;78:6999–7005.
19. Hayes FJ, Halsall HB, Heineman WR. Simultaneous immunoassay using electrochemical detection of metal ion labels. Anal Chem 1994;66:1860–5.
20. Goldman ER, Balighian ED, Mattoussi H, Kuno MK, Mauro JM, Tran PT, et al. Avidin: a natural bridge for quantum dot-antibody conjugates. J Am Chem Soc 2002;124:6378–82.
21. Liu G, Wang J, Kim J, Jan MR, Collins GE. Electrochemical coding for multiplexed immunoassays of proteins. Anal Chem 2004;76: 7126–30.
22. Rowe CA, Tender LM, Feldstein MJ, Golden JP, Scruggs SB, MacCraith BD, et al. Array biosensor for simultaneous identification of bacterial, viral, and protein analytes. Anal Chem 1999;71: 3846–52.
23. Delehanty JB, Ligler FS. A microarray immunoassay for simultaneous detection of proteins and bacteria. Anal Chem 2002;74: 5681–7.
24. Knecht BG, Strasser A, Dietrich R, Märtlbauer E, Niessner R, Weller MG. Automated microarray system for the simultaneous detection of antibiotics in milk. Anal Chem 2004;76:646–54.
25. Wilson MS. Electrochemical immunosensors for the simultaneous detection of two tumor markers. Anal Chem 2005;77:1496–1502.
26. Wilson MS, Nie WY. Electrochemical multianalyte immunoassays using an array-based sensor. Anal Chem 2006;78:2507–13.
27. Yu H, Yan F, Dai Z, Ju HX. A disposable amperometric immunosensor for alpha-1-fetoprotein based on enzyme labeled antibody/ chitosan membrane modified screen-printed carbon electrode. Anal Biochem 2004;331:98–105.
28. Yao H, Halsall HB, Heineman WR, Jenkins SH. Electrochemical dehydrogenase-based homogeneous assays in whole blood. Clin Chem 1995;41:591–8.
29. Kojima K, Hiratsuka A, Suzuki H, Yano K, Ikebukuro K, Karube I. Electrochemical protein chip with arrayed immunosensors with antibodies immobilized in a plasma-polymerized film. Anal Chem 2003;75:1116–22.
30. Luo XL, Xu JJ, Du Y, Chen HY. A glucose biosensor based on chitosan-glucose oxidase-gold nanoparticles biocomposite formed by one-step electrodeposition. Anal Biochem 2004; 334:284–9.
31. Yi HM, Wu LQ, Bentley WE, Ghodssi R, Rubloff GW, Culver JN, et al. Biofabrication with chitosan. Biomacromolecules 2005;6: 2881–94.
32. Cruz J, Kawasaki M, Gorski W. Electrode coatings based on chitosan scaffolds. Anal Chem 2000;72:680–6.
33. Wei X, Cruz J, Gorski W. Integration of enzymes and electrodes: spectroscopic and electrochemical studies of chitosan-enzyme films. Anal Chem 2002;74:5039–46.
34. Zhang MG, Gorski W. Electrochemical sensing based on redox mediation at carbon nanotubes. Anal Chem 2005;77:3960–5.
35. Zhang MG, Gorski W. Electrochemical sensing platform based on the carbon nanotubes/redox mediators-biopolymer system. J Am Chem Soc 2005;127:2058–9.
36. Wu J, Tang JH, Dai Z, Yan F, Ju HX, Murrc NE. A disposable electrochemical immunosensor for flow injection immunoassay of carcinoembryonic antigen. Biosens Bioelectron 2006;22:102–8.
37. Lin YH, Chung CK, Chen CT, Liang HF, Chen SC, Sung HW. Preparation of nanoparticles composed of chitosan/poly-γ-glutamic acid and evaluation of their permeability through Caco-2 cells. Biomacromolecules 2005;6:1104–12.
38. Liao JD, Lin SP, Wu YT. Dual properties of the deacetylated sites in chitosan for molecular immobilization and biofunctional effects. Biomacromolecules 2005;6:392–9.

Clin. Chem. 53, 1495-1502 (2007) Supplemental Data clinchem.2007.086975-1

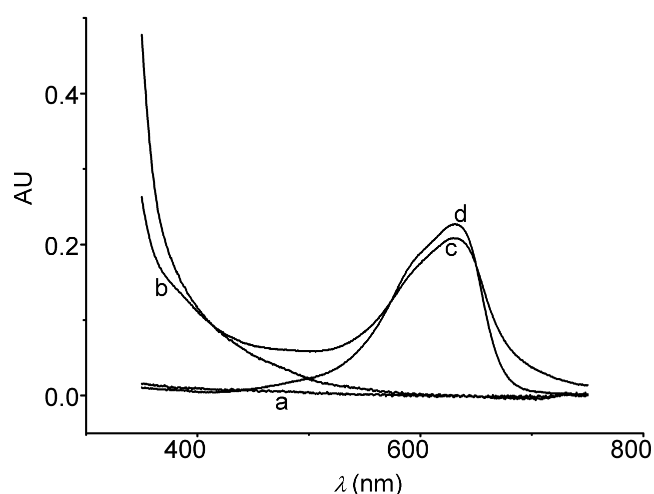
A Disposable Multianalyte Electrochemical Immunosensors Array for Automated Simultaneous Determination of Tumor Markers

JIE WU[1], FENG YAN[2], JINHAI TANG[2], CHUN ZHAI[1] and HUANGXIAN JU[1*]

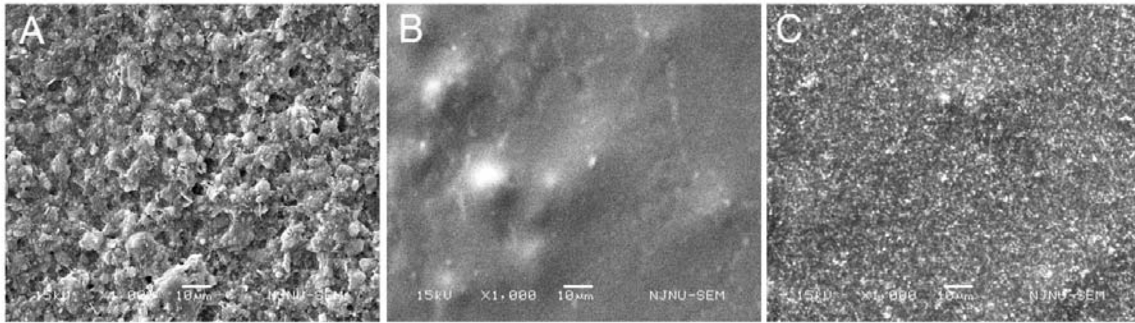
Supplemental Data



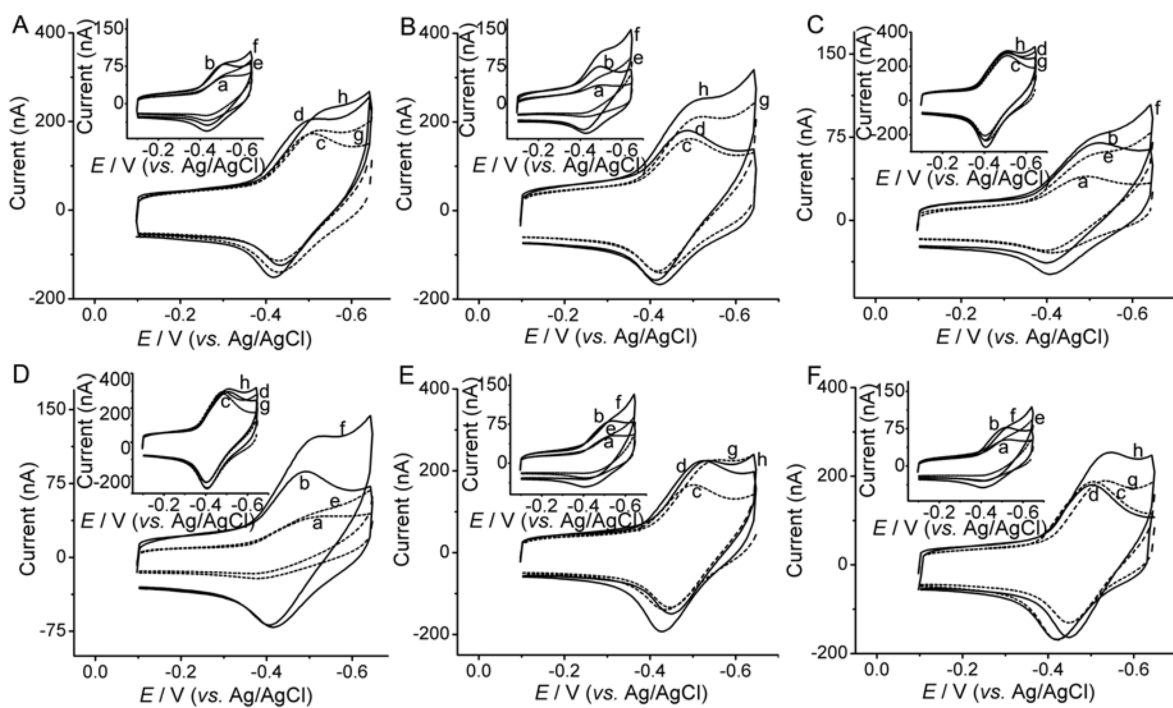
Supplemental Data Figure 1 Infrared spectra of (a) CHIT, (b) CHIT-GDI film, (c) CHIT-TBO film and (d) free TBO.



Supplemental Data Figure 2 UV/Vis spectra of (a) CHIT, (b) CHIT-GDI, (c) CHIT-TBO and (d) TBO solutions.



Supplemental Data Figure 3 SEM images of (A) bare SPCE, (B) CHIT-TBO film and (C) antigen/CHIT-TBO film modified SPCEs



Supplemental Data Figure 4 Simultaneous cyclic voltammetric responses of immunosensors array for CEA (a, e), α-AFP (b, f), CA 125 (c, g) and β-hCG (d, h) in pH 6.5 PBS with (a, b, c, d) and without (e, f, g, h) injection of 120 μL 10.7 mmol/L H_2O_2 after incubated in (A) 60 μL solution of blocking buffer, (B) mixture of diluted HRP-labeled antibodies, (C) 1:70 diluted HRP-labeled CEA antibody, (D) 1:70 diluted HRP-labeled α-AFP antibody, (E) 1:80 diluted HRP-labeled CA 125 antibody, and (F) 1:50 diluted HRP-labeled β-hCG antibody.