Supplementary Material (ESI) for Chemical Communications
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Electronic Supplementary Information

A Disposable Paper-based Electrochemical Sensor with Addressable Electrode Array for Cancer Screening

Shenguang Ge,[a] Lei Ge,[b] Mei Yan,[a] Xianrang Song,[c] Jinghua Yu*[a] and Jiadong Huang[a]

[a]*Key Laboratory of Chemical Sensing & Analysis in Universities of Shandong, School of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, China.*
b Shandong Provincial Key Laboratory of Preparation and Measurement of Building Materials, University of Jinan, Jinan 250022, China.
[c]*Cancer Research Center, Shandong Tumor Hospital, Jinan 250117, China.*

Fax: +86-531-82765969; Tel: +86-531-82767161; E-mail: ujn.yujh@gmail.com

Photograph of Chip and Experimental Device

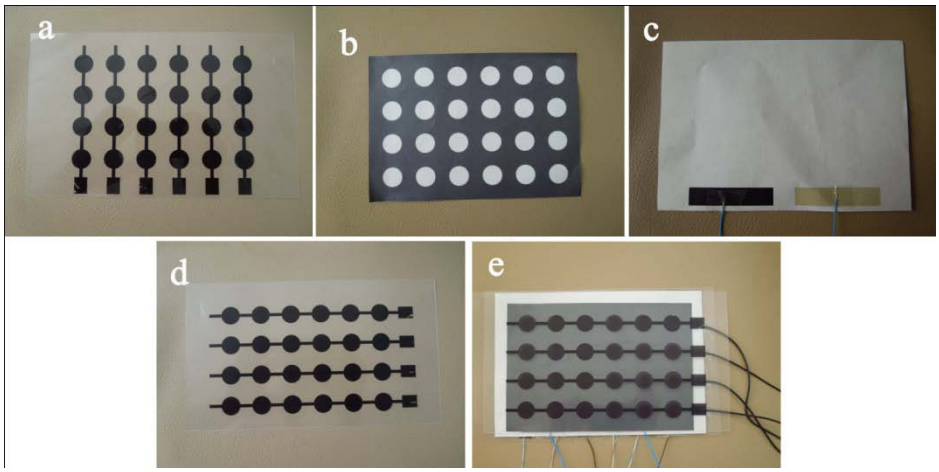

Figure S1 (a) column electrodes; (b) sensing sites; (c) reference electrode and counter electrode; (d) row electrodes; (e) immunosensor array device

Preparation of Microarray Immunosensor

The paper-based three-dimension addressable electrochemical immunodevice was prepared [Whatman chromatography paper 1# was obtained from GE Healthcare World-wide (Pudong Shanghai, China) and used with further adjustment of size. This type of Whatman paper was chosen because of its uniform composition (relative to other types of paper)]. The capture antibodies to AFP, CA 125, CA 153, and CEA were immobilized on each sensing sites in row. Each column could be used to simultaneously detect four tumor markers in a single sample; thus 6 columns could be simultaneously used to detect 6 samples.

The sensing sites were prepared by immobilizing the corresponding capture antibodies on the paper working zones of the wax-patterned paper through chitosan coating and glutaraldehyde cross-linking. First, MWCNTs were sonicated for 5 h at 300W in 3:1 H_2SO_4/HNO_3, and then washed repeatedly until pH 7.0. This procedure shortened MWCNTs, removed metallic and carbonaceous impurities, and generated carboxylate groups on the MWCNTs surface.[1] A portion of 4 μL carboxylated MWCNTs were applied to modify each sensing zone and dried at room temperature. Then, 3 μL of 0.25 mg/mL chitosan was coated on MWCNTs modified paper fibers and dried in the air. Next, 4 μL of 2.5% glutaraldehyde was dropped into the sensing sites and activated for 2 h, the electrode was washed with PBS, capture antibodies (5.0 μL) for AFP, CA

125, CA 153, CEA at 10 μg/mL and thionine (2.0 μL 3 mM) were individually dropped into the corresponding paper sensing sites exposed in four rows, respectively, and incubated at 4 °C for 30 min, subsequently, physically absorbed excess antibodies were rinsed with PBS and washing buffer, and 10 μL blocking buffer was applied into each paper sensing sites and incubated for 30 min at room temperature to block possible remaining active sites on paper fibers against nonspecific adsorption. After washing with PBS, the resulting array was obtained and stored at 4 ℃ in a dry environment prior to use.

HRP-Ab_2-AuNPs/MWCNTs bioconjugate was prepared. The carboxylated MWCNTs (0.5 mg/mL) was dispersed into a 0.20 % PDDA aqueous solution containing 0.5 M NaCl by 30 min sonication to generate a homogeneous black suspension. Residual PDDA polymer was left by high-speed centrifugation, and the complex was thrice washed with water to obtain PDDA functionalized MWCNTs.[2] Then, the PDDA functionalized MWCNTs (0.75 mg) were dispersed in 9.0 mL of as-prepared colloidal AuNPs of 13 nm in diameter according to the previous protocol[3] and stirred for 20 min. After centrifugation, light purple AuNPs/MWCNTs 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 ambient temperature, 375 μL of 2 mg/mL HRP and 15 μL of 0.5 mg/mL Ab_2 were added to 0.5 mL of the AuNPs/MWCNTs composites. The mixture were gently mixed for 3 h and centrifuged at 3500 rpm for 15 min at 4 ^{o}C. After centrifugation, the obtained bioconjugate was washed with washing buffer and resuspended in 100 μL of PBS containing 0.2% BSA as the assay solution. Prior to use, this solution was immediately 5-fold diluted with PBS.

The Ab_1 modified arrays were incubated with 10 μL of detecting Ag samples for 30 min at room temperature. The array was dropped into the 10 μL HRP-Ab_2-AuNPs@MWCNTs bioconjugate solution for an incubation time of 30 min. Finally, the array was washed thoroughly with PBS to remove nonspecifically bound conjugations, which could cause a background response before measurement.

The detailed washing steps were as follows: Due to the front and back surfaces of the wax-patterned paper electrochemical cell are open to atmosphere, thus the sensing sites can be washed by applying washing buffer. The washing buffer could go through the paper and migrate along the paper channels by the capillary and gravity action to wash the paper channels and paper sensing sites and carries the unbound reagents with it into the blotting paper. This effective

washing procedure was used in this work consistently and acquiescently. The washing process was important for preventing the nonspecific binding and for achieving the best possible signal-to-background ratio. Another purpose for this washing procedure was to stop the incubation reaction at exactly same time. The washing procedure was repeated twice to make sure the washing was performed completely. The finish of all sensing sites could be finished within 10 min.

Amperometric Scanning Procedure

The electrode arrays were assembled on the crossing points of the row/column electrodes to form a 4×6 array by a facile home-made device-holder. From up to bottom, the stacking order was row electrode (a), paper printed reference electrode and counter electrode (b), paper contained sensing sites (c), and column electrode (d), respectively. Layer "b" was soaked with buffer solution (pH 7.4 PBS) and the base solution (10.0 mM H_2O_2) when the current response was determined. The principle of multipoint detection of tumor marker was shown in Figure 1. The reduction and oxidation of the compound were carried out at the crossing points in the interspaces between the column and row electrodes, the amplification of the electrochemical signal occurred only at the crossing point of interest. The efficiency of the redox cycling was sensitive to the presence of thionine and HRP molecules at the electrode surface. The amperometric measurement procedure was shown in Figure 1 and a detailed description as follow. Before the amperometric was measured, a voltage (*V*C) was applied to all column electrodes through W1 of the potentiostat for the potential control for 5 s in order to stabilize the current on the electrodes. Then, the voltage of column electrode 1 (C1) was set at *V*C and the current responses was readout through W2, and the current data was transferred to a computer.

This readout process was sequentially repeated from C1 to C6 with an interval of 1 s for the readout time in each step, and the other column electrodes not used for the readout and all row electrodes were set at *VC* through W1. Since all the column and row electrodes were set at *V*C, no redox cycling happened at the crossing points. The sequential data from C1 to C6 through the above process were used as the background signals. Next, a voltage (*V*R) was applied to row electrode 1 (R1) through W3 of the potentiostat for 5 s for preconditioning purposes to stabilize the current, while the other row electrodes (R2-R4) were set at *V*C through W1. Then, the currents at C1-C6 were detected sequentially one by one (1 s for readout time). The change in the current

compared with the background signal at the same column electrode was considered as the current response at the crossing point between the column electrode and R1. The same measurements were sequentially repeated for the other row electrodes (R2-R4) to address and acquire all the responses at every crossing point. By sequentially changing the potential applied to the column and row electrodes and the readout column electrode, we collected the electrochemical responses at the entire crossing points, allowing the system to be addressable. Therefore, a highly integrated device with massive electrochemical addressable points could be realized on the basis of the present architecture.

UV-vis spectroscopy of HRP-Ab_2-AuNPs/MWCNTs

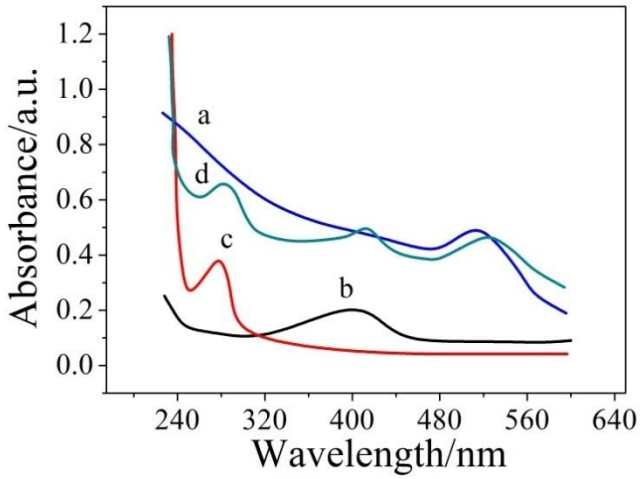

Figure S2 UV-vis spectra of Au NPs (a), HRP (b), Ab_2(AFP) (c), and HRP-Au NPs/MWCNTs-Ab_2 bioconjugate (d) using anti-AFP as model.

The resulting HRP-Ab_2-AuNPs/MWCNTs bioconjugate was further characterized by UV-vis spectroscopy. As shown in Figure S2, an obvious absorption peak at 514 nm (curve a) was observed, which was the typical resonance band of AuNPs. Pure HRP, Ab_2 displayed absorption peaks at 404 nm (curve b) and 282 nm (curves c), respectively. When Ab_2 together with HRP were bound to AuNPs/MWCNTs, three obvious absorption peaks were observed on the resulting HRP-Ab_2-AuNPs/MWCNTs (curve d), indicating successful binding of Ab_2 and HRP onto AuNPs/MWCNTs.

Electrochemical Characteristics

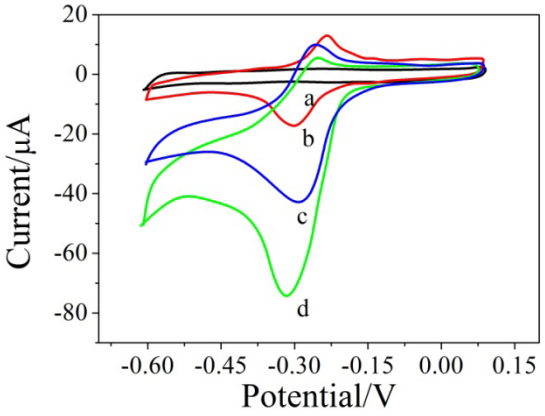

Figure S3 Cyclic voltammograms of bare paper (a), thionine-Ab_1/MWCNTs(b), HRP-Au NPs/MWCNTs-Ab_2/Ag /thionine-Ab_1/MWCNTs (c-d), Ag 0.1ng/mL(c); 50ng/mL(d) using anti-AFP as model. Notes: in pH 5.5 ABS and 10.0 mM H_2O_2.

Figure S3 displayed the cyclic voltammograms of variously modified electrodes at 50 mV/s in pH 5.5 ABS and 10.0 mM H_2O_2. No peak was observed for the bare paper (Figure S3 a). When thionine-Ab_1/MWCNTs was modified on the surface of sensing site, a couple of redox peaks were obtained (Figure S3 b), indicating that the immobilized thionine could act as a good electron mediator for electron transfer. To further verify the feasibility of the sandwich-type electrochemical immunoassay, the immunosensor was used for the detection of tumor markers using 0.1 ng/mL and 50 ng/mL AFP as model analytes. The formed sandwich type immunocomplex were investigated in pH 5.5 ABS and 10.0 mM H_2O_2. As seen in Figure S3c and d, an obvious redox reaction appeared with a distinct increase of the oxidation current and a decrease of the reduction current upon the addition of H_2O_2 in pH 5.5 ABS. The current mainly derived from the immobilized HRP toward the reaction of H_2O_2 relative to the thionine system. Thus, we might quantitatively evaluate the concentration of cancer marker according to the relationship between the current and Ag concentration.

Comparison of Different Types of Signal Tags

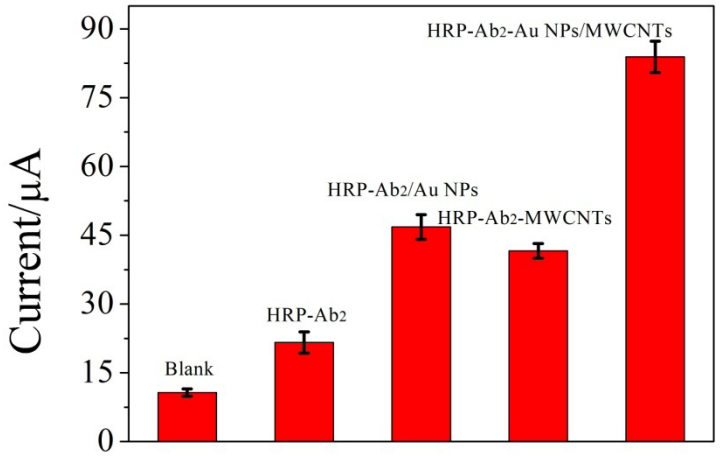

Figure S4 Performance of different bionanolabels on the sensing array using anti-AFP as model in the presence and absence of 50 ng/mL CEA, pH 5.5 ABS containing 10.0 mM H_2O_2, scan rate 50 mV/s. Error bars represent standard deviations (SD).

A comparative study of the amperometric responses of immunoreaction was carried out by HRP directly labeled with Ab_2 conjugates (HRP-Ab_2), AuNPs labeled with HRP and Ab_2 (HRP-Ab_2/AuNPs), MWCNTs labeled with HRP and Ab_2 (HRP-Ab_2-MWCNTs), HRP-Ab_2-AuNPs/MWCNTs in the sandwich immunoassay on the proposed array with the same detection conditions, at 50 mV/s in pH 5.5 ABS, respectively. The comparison was based on the shift in the cathodic current relative to zero analyte (0 ng/mL antigen). The results were showed in Figure S4. As seen from Figure S4, the blank current peaks were weak (a). The shift in the cathodic current by HRP-Ab_2-AuNPs@MWCNTs as signal tags was significantly larger than those of other signal tags. Some possible reasons may contribute to these observations: (i) the MWCNTs can provide a large room for the immobilization of nanogold and biomolecules, and MWCNTs could catalyze the reduction of H_2O_2 to some extent; (ii) AuNPs are coated on the MWCNTs or into the nanotube, which greatly improved the conductivity of the MWCNTs; and (iii) the presence of many Ab_1 molecules on the MWCNTs can increase the possibility of antigen-antibody interaction. When one Ab_1 on the MWCNTs reacts with one Ag on the electrode, the whole bionanolabel can participate in the electrochemical reaction.

Electrochemical Impedance Spectroscopy

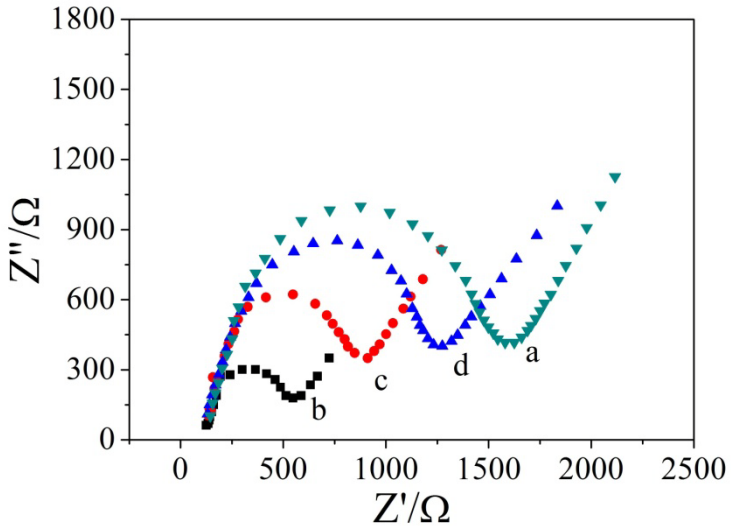

Figure S5. EIS of a) bare sensing sites; b) MWCNTs-modified sensing sites; c) Ab_1/GA/chitosan/ MWCNTs modified sensing sites; and d) signal tags/AFP/ /Ab_1/GA/chitosan/MWCNTs modified sensing sites in 0.1M KCl with 5 mM $[Fe(CN)_6]^{3-/4-}$.

Electrochemical impedance spectroscopy (EIS) gave further information about the modification process (Figure S5). EIS were carried out in a solution of 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1M KCl. The frequency range was from 100 MHz to 10 KHz. Figure S5 showed the EIS of electrode array at different stages of treatment. The electron-transfer of $[Fe(CN)_6]^{3-/4-}$ on each sensing site was blocked by the formation of BSA/Ab_1/chitosan films, which resulted in an increase in electron-transfer resistance (Ret). The EIS of bare paper showed a relatively big Ret value (Figure S5, curve a). After MWCNTs was modified, the EIS showed a decrease in Ret (Figure S5, curve b), and the reason may be that the MWCNTs can facilitate the interfacial electron-transfer. An increase of the Ret value (Figure S5, curve c) was observed after the Ab_1 was immobilized with GA, because of the association of GA and Ab_1 blocked many of the amino groups of chitosan. Similarly, the immobilization of BSA, antigens, and signal tags all independently slowed down the electron-transfer kinetics of the redox probe on each sensing site, thereby resulting in the increasing impedance of the sensing site (Figure S5, curve d), which testified to the immobilization of these substances.

Optimization of the Detection Conditions

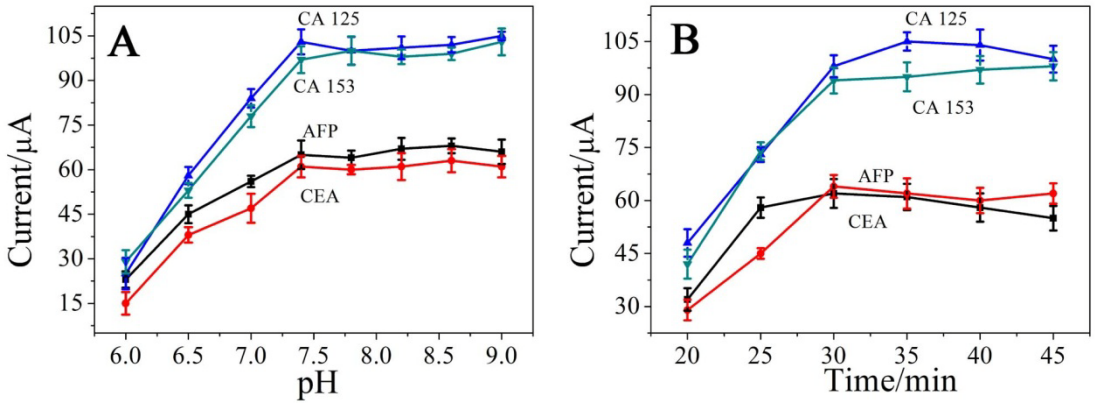

Figure S6 Effect of pH(A) and incubation time(B) for 10.0 ng/mL AFP and CEA, and 10.0 U/mL CA 125 and CA 153.

The electrochemical response of this immunoarray sensor showed great dependence on the pH value of the solution. The influence of the pH value on electrochemical response was investigated from pH 6.0 to 9.0, and the optimal range was observed at pH 6.8–8.0. Since the optimal pH value for biological systems was 7.4, electrochemical responses was performed in PBS (pH=7.4)(See Figure S6 A).

The performance of electrochemical immunoassay depended on the formation of sandwich immunocomplex, which was related to the incubation temperature and time. For convenient manipulation, the incubation steps were performed at room temperature. The effect of incubation time was examined using 10.0 ng/mL AFP and CEA, and 10.0 U/mL CA 125 and CA 153. The electrochemical responses to four tumor markers increased with increasing incubation time, and all trended to their maximum values at 25-40 min, respectively, indicating the saturated binding of antigen to Ab_1 and Ab_2-HRP-AuNPs/MWCNTs complex on the sensing sites. Thus, 30 min was chosen as the incubation time for the two incubation steps in the sandwich immunoassay (See Figure S6 B).

Cross-reactivity of the Immunosensor Array

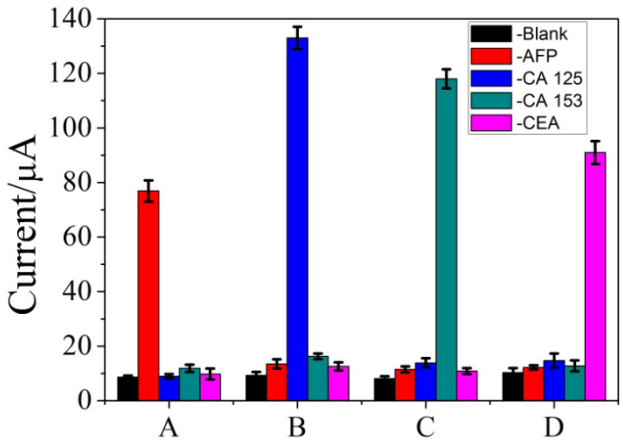

Figure S7 Electrochemical response for different antigens on different sensing site. A) AFP; B) CA125; C) CA153; D) CEA. Error bars represent standard deviations (SD).

Cancer Screening

To improve the accuracy of diagnosis, the panel of tumor markers was very significant,[4] such as AFP−CEA panel for liver cancer, CA 125−CEA−AFP panel for ovarian cancer, and CA 153−CA 125−CEA panel for breast cancer. Hence, the electrochemical response of the sensing site for these markers showed the highest current in the marker panel corresponding to liver, breast, and ovarian cancers, respectively. The proposed method may be used to screen the cancer. To evaluate a potential application of this microarray immunosensor, different cancer serum samples obtained from Shandong Cancer Hospital were assayed using the proposed method in this work. When the levels of tumor markers were higher than the upper limit of calibration ranges, serum samples were serially diluted with 0.01 M PBS (pH 7.4) prior to the assay. The results were listed in Table S1. The RSD was 1.4-7.3 %, indicated that the proposed method had good precision. The obtained results were compared with those of commercially available Electrochemiluminescent (ECL, Roche E601, Switzerland) Analyzer as a reference method (Table S1). The results of the proposed method were in agreement with the results of the ECL method. A t-test indicated no significant difference between the results obtained using the proposed method and the ECL method at a confidence level of 95%. Therefore, the addressable microarray immunosensor fabricated in this work can potentially be applied in clinic tests.

Table S1 Assay results of real human serum by the proposed and reference method.

Samples	AFP			CA 125		
	Found	RSD/%	Ref.	Found	RSD/%	Ref.
1	87.5±2.6	3.0	85.4±1.1	63.2±3.7	5.9	65.3±2.0
2	50.7±2.6	5.1	48.9±1.3	146.3±3.7	2.5	150.5±5.1
3	10.5±0.4	3.8	11.2±0.6	107.2±3.8	5.4	104.8±2.6
4	8.2±0.6	7.3	7.8±1.1	28.9±1.3	4.5	29.1±0.8
Samples	CA 153			CEA		
	Found	RSD/%	Ref.	Found	RSD/%	Ref.
1	48.3±2.9	6.0	47.7±3.1	75.8±4.1	5.4	72.1±1.5
2	32.1±1.1	3.4	30.3±1.7	64.9±0.9	1.4	65.0±1.5
3	204.5±4.1	2.0	200.7±3.9	71.9±1.8	2.5	73.5±2.4
4	34.8±1.7	4.9	35.9±2.0	4.8±0.3	6.3	4.7±0.2

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