Disposable Reagentless Electrochemical Immunosensor Array Based on a Biopolymer/Sol-Gel Membrane for Simultaneous Measurement of Several Tumor Markers

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BACKGROUND: A reagentless sensor array for simultaneous multianalyte testing (SMAT) may enable accurate diagnosis and be applicable for point-of-care testing. We developed a disposable reagentless immunosensor array for simple immunoassay of panels of tumor markers.

METHODS: We carried out SMAT with a direct capture format, in which colloidal gold nanoparticles with bound horseradish peroxidase (HRP)-labeled antibodies were immobilized on screen-printed carbon electrodes with biopolymer/sol-gel to trap their corresponding antigens from sample solution. Upon formation of immunocomplex, the direct electrochemical signal of the HRP decreased owing to increasing spatial blocking, and the analytes could be simultaneously determined by monitoring the signal changes.

RESULTS: The proposed reagentless immunosensor array allowed simultaneous detection of carcinoma antigen 153, carcinoma antigen 125, carbohydrate antigen 199, and carcinoembryonic antigen in clinical serum samples in the ranges of 0.4–140 kU/L, 0.5–330 kU/L, 0.8–190 kU/L, and 0.1–44 μg/L, respectively, with detection limits of 0.2 kU/L, 0.5 kU/L, 0.3 kU/L, and 0.1 μg/L corresponding to the signals 3 SD above the mean of a zero standard. The interassay imprecision of the arrays was <9.5%, and they were stable for 35 days. The positivity detection rate of panels of tumor markers was >95.5% for 95 cases of cancer-positive sera.

CONCLUSIONS: The immunosensor array provides a SMAT with short analytical time, small sampling volume, no need for substrate, and, no between-electrode cross-talk. This method not only proved the capability of the array in point-of-care testing, but also allowed simultaneous testing of several tumor markers.

Cancer is one of the leading causes of mortality, and early clinical diagnosis is crucial for successful treatment of the disease. Many immunosensors and immunoassay methods have been developed for the determination of a single tumor marker, whose concentration in human serum is associated with the stages of tumors (1–4). Because many cancers express 1 marker [e.g., breast cancer is associated with carcinoma antigens 153 and 125 (CA 153 and CA 125)4 and carcinoembryonic antigen (CEA)], and concentrations of several tumor markers often increase in the serum of a patient, accurate simultaneous multianalyte test (SMAT) of combinations of tumor markers may improve the diagnosis of certain types of tumor (5–8).

SMAT may offer a shorter analytical time, higher sample throughput, lower sampling volume, and lower cost per assay compared with traditional single-analyte tests (9, 10). Thus, multilabel assays and spatially resolved assay systems have been developed as the main modes to perform SMAT (11–25). Application of the multilabel assays has been limited by difficulty in accurate quantification due to different optimal assay conditions and the signal overlap of different labels (11–13). Although a set of substrate zone-resolved techniques have been proposed to overcome these...
drawbacks (14, 15), the restriction in the number of available labels still greatly limits their application.

Spatially resolved assays with a single label seem well suited for performing SMAT, and optical SMAT, relying on fluorescence emission and optical reflectance, has been developed into mature technology. Optical SMAT, however, often needs an expensive array detector, such as a charge-coupled device camera (16, 17). Electrochemical array, which is distinguished by its convenient miniaturization for high-throughput systems, low assay cost, and absence of sophisticated and expensive array detectors, shows promising application in cancer screening (18, 19). Electrochemical cross-talk caused by diffusion of the detectable enzymatic products is the main problem in the fabrication of electrochemical array. To solve this problem, many approaches have been followed. One approach, for example, is to ensure that the distance between adjacent electrodes is larger than the diffusion distance of enzymatic product (20–23), but such an approach conflicts with the goal of miniaturization. Another simple method to completely avoid the electrochemical cross-talk can be achieved by immobilizing the electron-transfer mediator on an individual immunosensor to shuttle electrons (24, 25), but this approach requires the addition of hydrogen peroxide, leading to limited practical application.

A reagentless electrochemical immunosensor is an attractive strategy (26). In our previous work, we prepared several reagentless immunosensors by using sol-gel matrix to immobilize immunoreagents and detected the direct electron transfer of labeled enzyme, horseradish peroxidase (HRP) (27–31). To achieve SMAT, this work further fabricated a reagentless immunosensor array by individually embedding 4 kinds of HRP-labeled antibody-modified gold nanoparticles in a newly designed biopolymer/sol-gel matrix formed on screen-printed carbon electrodes (SPCEs), where the HRP-Ab-Au nanoparticles were limited in the holes of the biopolymer/sol-gel film. Chitosan, a biopolymer with excellent film-forming ability, biocompatibility, nontoxicity, and high mechanical strength, acted as the adhesion frame in the synthesis of the sol-gel and made the electrical communication between redox sites of the enzyme and sensing surface easier due to the cooperative effort of chitosan and sol-gel matrix. The presence of gold nanoparticles accelerated the electron transfer between immobilized HRP and the electrode and increased the hole size for improving the permeability of the sol-gel matrix so that the antigens in solution could easily penetrate into the sol-gel film for immunoreaction. Upon formation of immunocomplexes, the electrochemical responses decreased due to increasing spatial blocking, leading to the reagentless immunosensing to corresponding antigens without cross-talk. The proposed electrochemical immunosensor array had high analyte throughput, showed acceptable comparability to conventional methods for measuring several tumor markers, could be fabricated with mass production techniques, and thus provided the potential for application in point-of-care testing (POCT).

Materials and Methods

REAGENTS

We purchased CA 153, CA 125, carbohydrate antigen 199 (CA 199), and CEA ELISA kits from CanAg Diagnostics AB. They consisted of a series of CA 153, CA 125, CA 199, and CEA standard solutions with concentrations from 0–250 kU/L, 0–500 kU/L, 0–240 kU/L, and 0–75 μg/L, respectively, and the stock solutions of HRP-labeled CA 153, CA 125, CA 199, and CEA monoclonal antibodies with concentrations of 50, 30, 40, and 60 mg/L. We purchased bovine serum albumin, chitosan (molecular weight approximately 1 × 10^6 Da; approximately 85% deacetylation), and (3-aminopropyl)triethoxysilane from Sigma-Aldrich Chemical Co. Tetraethoxysilane (analytical reagent grade) was from Shanghai Chemical Company. 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 1% bovine serum albumin blocking buffer. By mixing the stock solutions of NaH2PO4 and Na2HPO4 and adjusting the pH with 0.2 mol/L NaOH and H3PO4, we prepared 0.2 mol/L PBS of various pHs. Doubly distilled water was used throughout the experiments. Serum specimens from Jiangsu Institute of Cancer Prevention and Cure were stored at 4 °C.

APPARATUS

We performed electrochemical measurements on an Epsilon electrochemical analyzer (Bioanalytical Systems Inc.) with a 3-electrode configuration, in which 4 working electrodes were connected with a switch. We obtained scanning electron micrographs (SEM) of the working electrode surfaces using a JEOL JSM-5610LV scanning electron microscope (JEOL), and we measured concentrations of the tumor markers in sera with an electrochemiluminescent analyzer used for routine clinical laboratory testing (Elecsys 2010; Roche).

FABRICATION OF IMMUNOSEROS ARRAY

Construction of the multianalyte immunosensor array is shown in Fig. 1. The 6-electrode array was fabricated as reported (32), and it contained 4 graphite working electrodes (2 mm in diameter, 1 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. The insulating...
layer printed around the working area constituted a reservoir of the electrochemical microcell.

Chitosan solution (1% by weight) was prepared by ultrasonically dissolving chitosan powder in 1% acetic acid. We prepared the biopolymer/sol-gel by mixing 50 µL (3-aminopropyl)triethoxysilane, 25 µL tetraethoxysilane, 10 µL of 10 mmol/L HCl as catalyst, and 500 µL of 1% acetic acid and 200 µL of 1% (wt) chitosan solution in a small test tube under stirring for 5–6 min at room temperature. We optimized these ratios of (3-aminopropyl)triethoxysilane, tetraethoxysilane to chitosan according to the direct electrochemical signal of the labeled HRP. We prepared 24-nm diameter colloidal gold nanoparticles as described (33) and HRP-labeled CA 153, CA 125, CA 199, and CEA monoclonal antibody–modified gold nanoparticles by mixing the as-prepared nanoparticles with the HRP-labeled antibodies at the same volume ratio of 1:1. After these mixtures were stored at 4 °C for 12 h, they were mixed with biopolymer/sol-gel at the volume ratio of 2:1. We then dropped 0.5 µL of these mixtures individually on the working electrodes and dried them under ambient conditions for 2–3 h to form an immunosensor array. The immunosensor array was incubated with blocking buffer for 20 min to block the sites against nonspecific adsorption (which has been accepted as a common blocking method in immunosensor fabrication), thoroughly rinsed with doubly distilled water, and stored in air before use.

ASSAY PROTOCOL
We carried out SMAT with an assay format of direct capture, in which the HRP-labeled antibodies immobilized on working electrodes reacted with the antigens in sample solution, respectively (Fig. 1). The electrochemical response was derived from the direct reduction of immobilized HRP from its resting state [Fe(III)]. Formation of the HRP-Ab/Ag complex blocked electron transfer between HRP and electrode, leading to signal reduction, which was proportional to the amount of formed immunocomplex. The amounts of immunocomplexes produced on immunosensor surfaces depended on the concentration of antigens in standard or sample solutions applied to the electrode. The incubation step of 40 min could be performed in batch by dropping 20-µL mixtures of standard CA 153, CA 125, CA 199, and CEA solutions or samples on the entire immunosensor array at room temperature and a relative humidity of 100% to avoid evaporation of solvent. After the residual of the incubation solution was removed with doubly distilled water, the immunosensor arrays were detected in 50 µL anaerobic 0.2 mol/L PBS, pH 6.9, as supporting electrolyte with differential pulse voltammetry (DPV) from −100 to −800 mV (vs Ag/AgCl) at pulse amplitude of 50 mV and pulse width of 50 ms in nitrogen atmosphere. The dissolved oxygen could be reduced on the underlying carbon electrode in the potential window used, and this effect was precluded by placing the system in a nitrogen atmosphere. When performing the POCT, the reduction of dissolved oxygen could be precluded by adding Na2SO3 into the detection solution to replace the nitrogen atmosphere.

Results and Discussion

CHARACTERIZATION OF SOL-GEL MEMBRANES ON SPCEs
SPCEs are one of the most desirable techniques for the development of POCT. Immobilization of proteins on SPCEs, however, is more difficult than on graphite
electrodes owing to the insulating polymers mixed in printing inks. Furthermore, the randomly distributed carbon particles produce inhomogeneous and uneven surface of SPCE (Fig. 2A), leading to irreproducibility of sensor fabrication. The chemical modification of the SPCE surface with different biocompatible membranes provides a way to prepare immunosensors with good reproducibility (34). As shown in Fig. 2B, however, when the organically modified silicate sol-gel membrane, a commonly used matrix for protein immobilization on graphite electrode (30, 31), was formed on the SPCE surface, the membrane was unstable and showed some fissures, which resulted in membrane falling off from the SPCE surface. For preparing an entirely uniform and stable membrane, chitosan was introduced into the sol-gel matrix as the adhesion frame, and the formed biopolymer/sol-gel membrane on the SPCE surface showed homogeneous porous structure (Fig. 2C). It provided good biocompatibility and high capability for protein loading.

ELECTROCHEMICAL BEHAVIOR OF Labeled HRP IN BIO POLYMER/SOL-GEL

The DPV curves of both the bare and biopolymer/sol-gel modified SPCEs in 0.2 mol/L PBS, pH 6.9, did not show any detectable signal in the applied potential window (Fig. 3). After we embedded 0.17 μL of 50 mg/L HRP-anti-CA 153 in the biopolymer/sol-gel, the modified SPCEs displayed a sensitive peak around −540 mV (vs Ag/AgCl) (curve d, Fig. 3), which was close to the reduction peak potential of HRP/biopolymer/sol-gel prepared with 0.17 μL of 2.0 mg/L HRP (curve c, Fig. 3), indicating the direct electron transfer between electrode and the labeled HRP with regard to Fe(III)–Fe(II) conversion. The small difference of peak potentials between HRP-anti-CA 153 and HRP resulted from the change of microenvironment around HRP molecules because of the presence of antibody. In the presence of gold nanoparticles in the biopolymer/sol-gel, the reduction peak of the equal amount of HRP-antibody conjugate increased 2.1-fold (curve e, Fig. 3). The cyclic voltammetric experiments at different gold electrodes showed the same appearance, and upon incorporation of gold nanoparticles into the biopolymer/sol-gel at SPCEs, the reduction peak current at the same scan rate increased 1.98-fold (see Supplemental Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue9). Thus the Au nanoparticles could accelerate the direct electrochemistry of HRP to further amplify the detectable signal. This peak was also 2.7 times higher than that of HRP-anti-CA 153–Au nanoparticles/biopolymer/sol-gel modified SPCE (curve f, Fig. 3), indicating the positive effects of chitosan with good biocompatibility and hydrophilicity (35), which
enhanced water uptake and swelling of the film and led to better permeability of the film for the transfer of counter ions to neutralize the charge change during the redox process and a favorable microenvironment for electron hopping or electron self-exchange between immobilized HRP molecules \((36)\). Thus electron transfer kinetics and direct electrochemical signal increased. After the modified SPCE was incubated with CA 153, the direct electrochemical signal decreased markedly due to the increased barrier that resulted from the formation of immunocomplex (curve g, Fig. 3), leading to a reagentless immunosensing method for antigen detection.

**OPTIMIZATION OF IMMUNOSensor PERFORMANCE**

The formation of immunocomplex depended on the incubation temperature and time. For the sake of convenient manipulation, the incubation step was performed with 20 \(\mu\)L antigen solution or the mixture of antigens for SMAT at room temperature, after which the DPV response of the labeled HRP decreased with increasing incubation time and reached a relatively stable value at 30–40 min (Fig. 4A), indicating saturated formation of immunocomplex in the membrane. Thus, 40 min was chosen as the optimal incubation time for SMAT.

The acidity of detection solution also exerted a marked effect on the direct electrochemical signal of the immobilized HRP. The maximum responses for CA 153, CA 125, and CEA occurred at pH 6.9, whereas the optimal response for CA 199 occurred at pH 7.3 (Fig. 4B). Thus pH 6.9 was selected as the optimal pH value for the SMAT.

**EVALUATION OF CROSS-REACTIVITY**

The immunosensing method was based on monitoring the direct electrochemical signal of the labeled HRP, and no substrate or peroxide was added; thus electrochemical cross-talk that generally comes from the diffusion overlap of signal molecule on 1 electrode to neighboring electrodes was not seen in the proposed system. The lack of cross-talk was an advantage for further miniaturization of the array, as the distance between neighboring electrodes could be reduced.

The cross-reactivity between the HRP-labeled antibody immobilized on the SPCE and other noncorresponding tumor markers at 1 immunosensor or between 1 tumor marker and other noncorresponding HRP-labeled antibodies at the other 3 immunosensors was another factor affecting the performance of the immunosensor array for SMAT. The experimental results indicated that only the immunosensor corresponding to the tumor marker in the incubation solution showed a sharp decrease of direct electrochemical signal, and others could retain at least 90% of their original responses in the detectable concentration ranges. Thus the cross-reactivity at the array was negligible, suggesting that the 4 tumor markers could be assayed individually in a single run without interference from each other.

**SMAT OF 4 TUMOR MARKERS**

After the immunocomplexes were formed on the immunosensor array with an incubation step, the SMAT was performed on a single-channel potentiostat with a sequential detection format without interval between 2 assays. Under optimal conditions, with the increasing concentrations of tumor markers, the DPV peak currents of the immunosensors decreased (Fig. 5), which resulted from the increasing amount of immunocomplexes formed and the increasing barrier to the electron transfer. The calibration curves were obtained with several sensor arrays to measure the detection signals corresponding to a series of mixtures of the 4 antigens at different concentrations. The calibration curves showed linear relationships in the concentration ranges of 0.4–140 (CA 153, \(R^2 = 0.9916\)), 0.5–330 (CA 125, \(R^2 = 0.9959\)), 0.8–190 kU/L (CA 199, \(R^2 = 0.9936\)), and 0.1–44 \(\mu\)g/L (CEA, \(R^2 = 0.9930\)), respectively, in the incubation solution with the slopes of \(-1.63, -1.03, \) and \(-1.95\) nA/(kU/L) and \(-3.81\) nA/\((\mu\)g/L). The detection limits corresponding to the signals 3 SD above the mean for a zero standard for the 4 analytes were 0.2, 0.5, and 0.3 kU/L and 0.1 \(\mu\)g/L, respectively. The proposed detection ranges were sufficient for detection of CA 153, CA 125, CA 199, and CEA concentrations in clinical samples with cutoff values of 25, 35, and 37 kU/L and 3 \(\mu\)g/L for a positive result.

**APPLICATION IN DETECTION OF SERUM TUMOR MARKERS**

The 4 tumor markers in human serum samples were detected after the immunosensor array was incubated...
with 20 μL serum samples. When the decrease of peak current went beyond the linear range shown in Fig. 5, appropriate dilution of the serum samples was necessary. The concentrations of the 4 tumor markers were measured by this approach in serum specimens of 95 patients with pathologically diagnosed cancers, including 53 specimens with colorectal or gastric cancer, 22 with epithelial ovarian cancer, 8 with breast cancer, and 12 with lung cancer, and 20 serum specimens from healthy volunteers. The SMAT results were compared with results produced by a commercial electrochemiluminescent single-analyte measurement system. Only 6 of a total 215 results (2.8%) showed some difference from the results of the commercial assay. When considering the other 209 results using orthogonal regression analysis, the plots of the SMAT results vs the control results for CA 153, CA 125, CA 199, and CEA gave the slopes of 0.99, 0.97, 1.04, and 0.99, intercepts of 0.42 kU/L, 6.1 kU/L, 12.6 kU/L, and 0.55 μg/L, S_{lim} of 3.88 kU/L, 21.2 kU/L, 83.7 kU/L, and 23.6 μg/L, and R^2 values of 0.990, 0.998, 0.997, and 0.999, respectively (see Supplemental Fig. 2 in the online Data Supplement). The regression parameters indicated acceptable agreement of this proposed method with an established clinical laboratory method.

When the SMAT was used for POCT, nitrogen atmosphere was inconvenient. We used a detection solution of 0.2 mol/L PBS, pH 6.9, containing 0.1 mol/L Na₂SO₃ without presence of nitrogen atmosphere for POCT. We examined the relative errors of the obtained antigen concentrations in 20 standard solutions with different concentrations from those using 0.2 mol/L PBS, pH 6.9, as detection solution in the presence of nitrogen atmosphere, and the maximum value was 6.0%. Thus, this SMAT system could be used for POCT by using 0.2 mol/L PBS, pH 6.9, containing 0.1 mol/L Na₂SO₃ as detection solution.

The positive detection rates in 115 serum samples using the SMAT method are shown in Table 1. According to the panels of tumor markers for combination diagnosis, CA 199–CEA panel for 53 cases of colorectal and gastric cancer, CA 153–CA 125–CEA panel for 8 cases of breast cancer, and CA 199–CEA panel for 12 cases of lung cancer showed positive detection rates of 100%, whereas CA 125–CA 199–CEA panel for 22 cases of epithelial ovarian cancer showed positive detection rates of 95.45%. As controls, 20 healthy volunteers showed 2 positive results. Thus, the present immunosensor array appears promising for SMAT of CA 153, CA 125, CA 199, and CEA concentrations in clinical applications.

**REPRODUCIBILITY AND STABILITY OF THE ARRAYS**

Because 0.5 μL sol-gel matrix was used to fabricate the sensor, the fabrication reproducibility was first examined by measuring the direct electrochemical signals in
and 11.3 kU/L CA 153, 24.9 kU/L CA 125, 32.0 kU/L CA 199, and CEA antibody in 10 immunosensor arrays were HRP labeled to CA 153, CA 125, CA 199, and CEA in the absence of analyte. The CVs for the responses of HRP labeled to CA 153, CA 125, CA 199, and CEA antibody in 10 immunosensor arrays were <5.1%, 2.5%, 5.6%, and 5.1%, respectively (see Supplemental Fig. 3 in the online Data Supplement), indicating acceptable fabrication reproducibility. We examined the interassay imprecision of the immunosensor arrays with 2 panels of tumor markers at various concentrations. Each panel was measured 5 times using 5 arrays. The CVs were 4.6%, 7.8%, 9.5%, and 7.1% for 13.5 kU/L CA 153, 6.7 kU/L CA 125, 8.0 kU/L CA 199, and 5.0 µg/L CEA, and 3.3%, 5.6%, 7.5%, and 3.7% for 20.9 kU/L CA 153, 24.9 kU/L CA 125, 32.0 kU/L CA 199, and 11.3 µg/L CEA. These results indicated acceptable imprecision and fabrication reproducibility.

The immunosensor arrays could be stored in dry air at room temperature. The DPV responses were 87.9%, 89.6%, 97.6%, and 94.7% of initial responses for CA 153, CA 125, CA 199, and CEA after a storage period of 35 days. Thus, the storage stability of the immunosensor arrays was acceptable, and the proposed array was suitable for clinical diagnostics.

In comparison with previous reports (24, 25), this array avoids the addition of mediator to shuttle electrons, and thus can exclude the electrochemical cross-talk at the electrode dimensions used here. Furthermore, the measurement of the direct electrochemical signal of HRP labeled to immunoreagents also avoids the need for other reagents in the detection process. Although the measurements show acceptable results, adding sulfite in the detection solution is not the best solution for the removal of oxygen. Thus, a system has been developed for POCT to exclude oxygen from the detection solution (see Supplemental Fig. 3 in the online Data Supplement).

### Table 1. Positivity detection rates of clinical sera.

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Associated tumor markers</th>
<th>Positive cases, n</th>
<th>Positivity detection rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal or gastric cancer</td>
<td>53</td>
<td>CA 199, CEA</td>
<td>53*</td>
<td>100</td>
</tr>
<tr>
<td>Epithelial ovarian cancer</td>
<td>22</td>
<td>CA 125, CA 199, CEA</td>
<td>21*</td>
<td>95.5</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>8</td>
<td>CA 153, CA 125, CEA</td>
<td>8*</td>
<td>100</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>12</td>
<td>CA 199, CEA</td>
<td>12*</td>
<td>100</td>
</tr>
<tr>
<td>Normal serum</td>
<td>20</td>
<td>CA 153, CA 125, CA 199, CEA</td>
<td>2*</td>
<td>10</td>
</tr>
</tbody>
</table>

* All concentrations obtained with the proposed method are higher than the clinical cutoff values of individual tumor markers.

# References


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