Magnetic Control of an Electrochemical Microfluidic Device with an Arrayed Immunosensor for Simultaneous Multiple Immunoassays

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Background: Methods based on magnetic bead probes have been developed for immunoassay, but most involve complicated labeling or stripping procedures and are unsuitable for routine use.

Methods: We synthesized magnet core/shell NiFe$_2$O$_4$/SiO$_2$ nanoparticles and fabricated an electrochemical magnetic controlled microfluidic device for the detection of 4 tumor markers. The immunoassay system consisted of 5 working electrodes and an Ag/AgCl reference electrode integrated on a glass substrate. Each working electrode contained a different antibody immobilized on the NiFe$_2$O$_4$/SiO$_2$ nanoparticle surface and was capable of measuring a specific tumor marker using noncompetitive electrochemical immunoassay.

Results: Under optimal conditions, the multiplex immunoassay enabled the simultaneous detection of 4 tumor markers. The sensor detection limit was $<0.5$ µg/L (or $<0.5$ kunits/L) for most analytes. Intra- and interassay imprecisions (CVs) were $<4.5\%$ and $8.7\%$ for analyte concentrations $>5$ µg/L (or $>5$ kunits/L), respectively. No nonspecific adsorption was observed during a series of procedures to detect target proteins, and electrochemical cross-talk (CV) between neighboring sites was $<10\%$.

Conclusion: This immunoassay system offers promise for label-free, rapid, simple, cost-effective analysis of biological samples. Importantly, the chip-based immunosensor could be suitable for use in the mass production of miniaturized lab-on-a-chip devices and open new opportunities for protein diagnostics and biosecurity.

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Tumor markers, such as α-fetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen 125 (CA 125), and CA 15-3, can be found in the body (usually blood or urine) when cancer is present (1). Tumor markers are mainly used for evaluating therapy and detecting recurrence or metastasis (2). Immunological methods have become the predominant analytical techniques for quantitative detection of tumor markers (3, 4). Despite the high sensitivity of conventional immunoassay methods such as RIA and ELISA, they have some limitations, such as the short shelf life of $^{125}$I-labeled antibody, radiation hazards, a complicated wash procedure, and a relatively long analysis time (5, 6). An alternative immunosensing strategy that is based on an electrochemical principle and does not require an antibody-labeling reaction would be advantageous because of simple instrumentation and easy signal quantification. A simultaneous multianalyte immunoassay, in which 2 or more analytes are measured in a single assay, would be advantageous in the clinical laboratory to simplify testing, throughout, and reduce overall cost per test. Various assay formats have been devised to realize simultaneous multianalyte analysis, using either multiple labels or spatial resolution to discriminate between the different analytes (7, 8). Although modification of the individual electrodes with different biological recognition elements would enable the construction of miniaturized biosensor arrays, the directed immobilization of functional proteins on individual microscopic regions is still a challenge (9, 10).

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1 Nonstandard abbreviations: AFP, α-fetoprotein; CEA, carcinoembryonic antigen; CA, cancer antigen; TEOS, tetraethoxysilane; RMS, root mean square.
Magnetic controlled molecular electronics and bioelectronics examine the effect of an external magnetic field on electrochemical signals of functionalized magnetic particles associated with electrodes (11). Magnetic sorting protein assay systems of various throughput have been built and used for clinical applications (12–16). On a single chip, batch-type magnetic separators have been fabricated that trap magnetic particles in flowing fluids by use of an external magnetic field (17). The loading capacity of these devices is limited, however, because accumulation of the collected particles can restrict fluid flow or lead to irreversible entrapment of samples, and their use is hampered by the need to disrupt continuous operation for sample elution. We used the physical characteristics of magnetic nanocore and the good biocompatibility of silica shell to construct a nontoxic biomimetic interface for the immobilization of proteins. Attraction of the functionalized magnetic nanoparticles to the probe surface with an external magnet activates the electrical contact between the immobilized proteins and the base electrode, and the sensor’s circuit is switched on. Positioning the magnet above the cell retracts the magnetic nanoparticles from the probe surface, and the electrochemical activity of the functional magnetic particles is switched off.

The focus of this report is the fabrication of a novel bead-bed immunoassay system on a microchip for multiplex measurement of 4 tumor markers, AFP, CEA, CA 125, and CA 15-3, with the switching and controlling of electrochemical signals by means of an external magnet. Monitoring the changes in the potential signals before and after the antigen–antibody interaction provides the basis for the immunoassay.

**Materials and Methods**

**MATERIALS**

CEA, AFP, CA 125, and CA 15-3 antigens and mouse monoclonal anti-CEA, anti-AFP, anti–CA 125, and anti–CA 15-3 antibodies (clone no. 10) were purchased from Zhengzhou Bioell Institute. Tetraethoxysilane (TEOS) and BSA (96%–99%) were obtained from Sigma. Bis-(2-ethylhexyl)sodium sulfosuccinate was the product of Tiantai Fine Chemical. All other reagents were of analytical grade and were used without further purification, unless specified otherwise. Deionized and distilled water was used throughout the study. Clinical serum samples were gifts from Chongqing Institute of Cancer Prevention and Cure, China. PBS (pH 7.4) containing 0.1 mol/L NaH2PO4 and 0.1 mol/L K2HPO4 was used as supporting electrolyte.

**PREPARATION OF NiFe2O4 AND NiFe2O4/SiO2 NANOPARTICLES**

We dissolved Fe(NO3)3·9H2O, Ni(NO3)2·6H2O, and glycine in distilled water (note: Fe3+/Ni2+ = 2:1, glycine/nitrate = 4:1, in molar ratio). After filtration, we heated the transparent brown precursor solutions until a combustion reaction rapidly diffused. After combustion for several seconds, the loose pink powders formed were rinsed with deionized water and ethanol, filtered, dried at 150 °C for 2 h, and calcinated at 800 °C for 4 h to prepare the NiFe2O4 nanoparticles.

We diluted 0.5 g NiFe2O4 with 10 mL ethanol and added 50 mL deionized water, surfactant [bis-(2-ethylhexyl)sodium sulfosuccinate], and 30 mL aqueous ammonia (250 g/L) into the suspension in turn. After stirring the mixture for 20 min, we added 4.5 g TEOS and stirred the suspension for another 4 h at room temperature. To investigate the effect of the TEOS amount on the performance of the NiFe2O4/SiO2 nanoparticles, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 g TEOS was used to prepare the composite. The mixture was then filtered, rinsed with deionized water and ethanol, dried at 150 °C for 2 h, and calcinated at 800 °C for 6 h to prepare the core-shell NiFe2O4/SiO2 nanoparticles.

We characterized the as-synthesized nickel ferrite particles by use of tunnel electron microscopy (H600, Hitachi Instrument). We used atomic emission spectroscopy (ICP) to qualitatively confirm that silica shell formed on the NiFe2O4 surface.

**FABRICATION OF THE IMMUNOASSAY SYSTEM**

Scheme 1 shows the structure of the microfluidic device and the fabrication, measurement, and regeneration procedures of the immunosensor. It consists of 5 gold disk working electrodes (3.0-mm diameter) and an Ag/AgCl reference electrode, which were integrated around a laboratory-made detection vessel. The external magnet was set to the back. Before the experiment, 1.5 g NiFe2O4/SiO2 nanoparticles were stirred in 5 mL PBS (pH 7.4) for 1 h. We added 500 μL anti-CEA (300 μg/L), anti-AFP (300 μg/L), anti–CA 125 (200 units·mL−1), and anti–CA 15-3 (200 kunits/L) to the resulting solution and incubated for 12 h at 4 °C with frequent stirring. The unbound antibodies were removed via magnetic separation. After that, the assay system was cleaned in 1.0 mol/L NaOH for 10 min and 1.0 mol/L HCl for 10 min, washed with alcohol and deionized water, and dried with nitrogen gas in sequence.
To avoid the nonspecific absorption, we injected a 10 g/L BSA solution into the detection vessel and incubated it for 1 h at room temperature to block the nonspecific binding sites of the electrode surface. We then dropped 100 μL of the antibody-functionalized magnetic nanoparticles (bionanoparticles) onto the surface of 4 BSA-functionalized gold electrodes (Scheme 1, working electrodes 1–4; electrode 5 is a control drying in air). With an external magnet, the magnetic bionanoparticles could be attached to the electrode surface.

**MEASUREMENT METHOD**

The manifold consists of a syringe pump directly connected to a distribution valve system with 8 ports. All basic operations, including the antigen-antibody reaction, immunocomplex capture, and washing, were carried out automatically. PBS or sample was injected at 0.25 mL/min through the reactor, and each sample to be analyzed was introduced into the detection vessel after stabilization of response (shift <1 mV/min) by a digital ion analyzer (model PHS-3C, Dazhong Instruments). The detection principle is based on the shift in the potential before and after the antigen–antibody interaction. To avoid possible error resulting from different additions of samples and deduct the responses induced by nonspecific adsorption, the response of each sensor was recorded as the immunoreaction from 30 s (after the addition of samples) until equilibrium was reached, ~5 min. The control tests with normal (negative) samples and the evaluations for clinical specimens were performed accordingly. After each immunoassay, we regenerated the contaminated probes by pulling out the permanent magnet and washing with pH 7.4 PBS.

**ELISA FOR CEA, AFP, CA 125, AND CA 15-3**

In sandwich ELISA with standard polystyrene 96-well plates (Kehua), 50 μL serum sample suspension was incubated at 37 °C for 30 min, and the wells were rinsed 3 times (3 min each) with 0.1 mol/L PBS (pH 7.4) containing 0.5 mol/L NaCl and 1 mL/L Tween 20. Then we added 50 μL conjugate solution and incubation continued for 1 h. The wells were again rinsed and 50 μL dye-reagent was added and incubated at 37 °C for 10 min. The enzymatic reaction was stopped by adding 50 μL 2.0 mol/L H2SO4 to each well. The results of ELISA were measured by a spectrophotometric ELISA reader at a wavelength of 450 nm.

**Results and Discussion**

**CONSTRUCTION AND ANALYSIS OF THE MICROFLUIDIC DEVICE**

The tunnel electron microscopy results showed that the particles did not have a narrow size distribution. The mean sizes of purified NiFe2O4 nanoparticles and the core-shell NiFe2O4/SiO2 nanoparticles were 20 and 25 nm, respectively. Atomic emission spectroscopy showed that the prepared composite had the characteristic peaks at 232.003, 283.204, and 212.412 nm, demonstrating the existence of Ni, Fe, and Si. Furthermore, Fourier-transformed infrared spectroscopy of the antibody-functionalized NiFe2O4/SiO2 nanoparticles showed 2 adsorption bands around 1650 and 1540 cm⁻¹ for amide I and amide II, indicating that the immunoproteins adsorbed on the surface of NiFe2O4/SiO2 nanoparticles could retain their native structure.

To further monitor the formation of bionanoparticles on the probe surface, we used atomic force microscopy (AFM) to investigate the topography of different modifications (images not shown). The root mean square (RMS) roughness values of bare gold surface and BSA-modified surface were 1.47 and 4.56 nm, respectively, because BSA protein could physically hold the surface of the bare gold bulk. The RMS value of NiFe2O4/SiO2-BSA-modified gold surface was 6.93 nm, and the RMS of bionanoparticle-functionalized gold surface was 5.76 nm, because the interstitial places between nanoparticles were blocked by the smaller sizes of antibody molecules. These results revealed that the magnetic bionanoparticles could be attached to the base surface with an external magnet.

To verify the reproducibility of the magnetic NiFe2O4/SiO2 nanoparticles for protein adsorption, we used a quartz crystal microbalance (Pico Balance) to evaluate the immobilized amount of anti–CA 15-3, as a model protein, on the quartz crystal surface according to the Sauerbrey equation (18):

\[
\Delta F_x = -2.3 \times 10^{-6} F \Delta M / A,
\]

where \( \Delta F_x \) is the change in frequency (Hz) arising from a change in mass (ΔM), \( F \) is the basic resonant frequency of the crystal (MHz), \( ΔM \) is the mass accumulation on the crystal surface (g), and \( A \) is the deposited electrode area (cm²). According to the Sauerbrey equation, the coverage of anti–CA 15-3 molecules on the quartz crystal surface was ~260 ng/cm².

Scheme 1 illustrates the reversible magnetic field–induced contacting and retraction of magnetic NiFe2O4/SiO2 nanoparticles functionalized with anti–CA 15-3 (as a model). Fig. 1 shows the potentiometric responses of the immunoassay system toward the same concentration of bionanoparticles and CA 15-3 before and after regeneration. Compared with curves a and a’ in Fig. 1, the CV at the same concentration of bionanoparticles was 6.3%. Similarly, the CV at 50 kunit/L CA 15-3 was 8.1%, demonstrating acceptable regeneration.

**OPTIMIZATION OF ANALYTICAL CONDITIONS**

In the flow-injection immunoassay system, a flow rate that was too high would damage the protein immobilized on the magnetic nanoparticle surface and produce unstable detection signal, whereas at a slow flow rate, the detection signal would suffer from tailing. Thus, a flow rate of 0.25 mL/min was used for the detection of tumor markers in the following experiments.
To arrive at the optimum conditions for antigen detection, we optimized both the preparation of magnetic nanoparticles and the fabrication of sensors. The SiO$_2$ provides a good surface for subsequent functionalization with protein. The SiO$_2$ coating is not sufficiently thick to keep particles from aggregating, however. Because ionic capping ligands, which bind to the particles’ surface, must be added during nanoparticle synthesis, the ligands’ electrostatic charge causes the particles to repel, countering the magnetic attraction pulling them together. When the SiO$_2$ coating is too thin, the repelled ligand–ligand force is less than the magnetic attraction force. As a result, the bionanoparticles were also aggregated. According to the experimental results, 4.5 g TEOS was chosen for the preparation of the NiFe$_2$O$_4$/SiO$_2$ nanoparticles (data not shown).

We investigated the effect of magnetic intensity at the probe surface on the potentiometric responses of the immunoassay system. The highest signal-to-background response was obtained at 0.25 T. When the magnetic intensity was larger than 0.25 T, a higher signal was achieved, but the background signal also increased. Therefore, we selected 0.25 T for the potentiometric measurements of the immunoassay system.

**PRINCIPLE AND CHARACTERISTICS OF THE IMMUNOASSAY SYSTEM**

Either antibodies or antigens in aqueous solution have a net electrical charge polarity, which is associated with the isoelectric points of the species and the ionic composition of the solution. If antibodies are immobilized on the electrode, the surface charge of the electrode will rely on the net charge of the immobilized antibody. When antigen molecules are present in the solution, the immunochemi-
cal reaction will take place at the interface with a resulting change of the surface charge. This change can be measured potentiometrically against the reference electrode immersed in the same solution (19–21).

We investigated the response characteristics of the immunoassay system. Fig. 2 shows the typical responses monitored in situ for evaluating 4 tumor markers. To further relegate the differences in response between probes to interference degree or crossing recognition level, 4 types of antigen were injected into the detection vessel. The potential changes to each antigen between probes were recorded, and the results are described in Table 1. The interference degree of variability between lineage-different immunological markers was <10%, indicating acceptable cross-recognition of the integrated microdevices. Moreover, the signals for the antibody-defined probes are much higher for the antigen/antibody–free electrode (electrode 5 in Table 1). The steady-state response time was also observed to be ~5 min (Fig. 2). When normal (negative) serum samples were analyzed using the integrated immunosensor, all probes showed substantially low signals (△E <10 mV, Table 1), in contrast to the results obtained when the corresponding positive serum was assayed, revealing a significant difference between the lineage-specific recognition and the nonspecific adsorption.

**Table 1. Interference degree or crossing recognition level.**

<table>
<thead>
<tr>
<th>Crossing reagent</th>
<th>1 (with anti-AFP)</th>
<th>2 (with anti-CEA)</th>
<th>3 (with anti–CA 125)</th>
<th>4 (with anti–CA 15-3)</th>
<th>5 (control test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>36.7</td>
<td>1.3</td>
<td>0.9</td>
<td>2.4</td>
<td>2.3</td>
</tr>
<tr>
<td>CEA</td>
<td>2.1</td>
<td>46.3</td>
<td>2.3</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>CA125</td>
<td>2.5</td>
<td>1.5</td>
<td>1.8</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>CA15–3</td>
<td>1.0</td>
<td>6.3</td>
<td>7.8</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Negative sample</td>
<td>3.5</td>
<td>2.7</td>
<td>5.4</td>
<td>6.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*a Concentration of crossing reagent is 50 μg/L or kunits/L.

To investigate the reproducibility of the newly prepared immunoassay protocol, we repeatedly assayed 20 times for different concentrations of 4 tumor markers. The CVs among 20 runs were 8.6%, 7.5%, and 4.3% for 2.5, 80, and 160 μg/L AFP; 6.3%, 5.4%, and 3.7% for 2.0, 100, and 180 μg/L CEA; 6.1%, 7.4%, 4.9% for 3.5, 30, and 50 kunits/L CA 125; and 3.5%, 7.8%, and 9.2% for 3.0, 120, and 220 kunits/L CA 15-3. The prepared biomagnetic nanoparticles dispersed uniformly, resulting in relatively large variation. Considering this factor, these CV values were acceptable. The low CV values indicated that the proposed immunosensor could be regenerated and used repeatedly, and further verified the possibility of batch preparation of biomagnetic nanoparticles. When the biomagnetic nanoparticles were not in use, they were stored in PBS (pH 7.4) at 4 °C. No obvious change was observed after storage for 7 days. Thus, the magnetic NiFe2O4/SiO2 nanoparticles could efficiently immobilize various proteins.

**PRELIMINARY APPLICATION OF THE IMMUNOASSAY SYSTEM**

To monitor the effect of composite substrate on the immunoassay system, routine samples of various concent-

<table>
<thead>
<tr>
<th>Concentration of AFP (μg/L)</th>
<th>Potential shift (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig. 3. Calibration plots of potentiometric shifts vs AFP concentrations as a function of target protein concentration in pH 7.4 PBS. Each data point represents the mean of the potential shift of triplicate measurements. Dynamic concentration range of 2.5–300 μg/L with detection limit of 0.5 μg/L was observed for standard AFP samples. Inset, calibration plots in low concentration.
trations of 4 tumor markers were added into normal lipemia and hemolysis solutions and analyzed. The recovery was from 90.1% to 116.3%. To further investigate the technique’s application for clinical analysis, we examined 38 serum specimens on the immunoassay and ELISA. The results are described in Fig. 4. The regression equations (linear) for these data are as follows (x axis, immunosensor; y axis, ELISA):

\[
y = 1.2721 + 0.9793x \quad (r^2 = 0.986) \text{ for AFP};
\]

\[
y = 0.4591 + 0.9782x \quad (r^2 = 0.978) \text{ for CEA};
\]

\[
y = 0.369 + 0.983x \quad (r^2 = 0.985) \text{ for CA 125};
\]

\[
y = -1.0743 + 1.0137x \quad (r^2 = 0.995) \text{ for CA 15-3}.
\]

These data show no significant difference between the results of the 2 methods.

We describe a miniaturized, integrated, electrochemical immunosensor that pulls antibodies bound to magnetic nanoparticles from one laminar flow path to another by applying a local magnetic field gradient and selectively removes them from flowing biological fluids without any wash steps. The main strength of the new assay is a label-free approach that measures the change in electrode potential upon binding of antigen to antibody. Moreover, the system can be regenerated and the suspected sample can be analyzed once with simultaneous output of multiple factors for phenotype decision-making, thus allowing rapid (~50 min) immunophenotyping. Importantly, this approach does not require sophisticated fabrication and is well suited for high-throughput biomedical sensing and application in both clinical and biodefense areas.

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