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# A novel microfluidic biosensor based on an electrical detection system for alpha-fetoprotein

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**Abstract**

Conventional immunoassays are labor intensive, expensive and time consuming and require large pieces of equipment for detection. Therefore, we have developed and characterized a novel immunoassay methodology comprised of microbeads and microbiochips. In this method, microbeads are used to filter and immobilize antibodies and an immuno-gold silver staining (IGSS) method is then used to amplify electrical signals that correspond to the bound antibodies. The chip used for this system is composed of an inexpensive and biocompatible polydimethylsiloxane (PDMS) layer over a Pyrex glass substrate that contains a platinum (Pt) microelectrode, which is used to detect the electrical signal in this system, the microelectrode is fabricated on the substrate and a microchannel and pillar-type microfilter is formed in the PDMS layer. A sandwich immunoassay approach was applied to detect alpha-fetoprotein (AFP), a cancer biomarker, using this system. The results of this study showed that the time required for a complete assay was reduced by 1 h and a detection limit as low as 1 ng/mL was attained when this system used, which indicates that similar bead-based electrical detection systems could be used for the diagnosis of many forms of cancer.


*Keywords:* Immunoassay; Microbiochip; Immuno-gold silver staining; Microfilter; Microbead; Alpha-fetoprotein

## 1. Introduction

Alpha-fetoprotein (AFP) is a normal fetal serum protein synthesized by the liver, yolk sac, and gastrointestinal tract that is homologous with albumin. AFP is a glycoprotein that weighs approximately 70 kDa and contains an asparagine-binding double-chain sugar complex. The AFP gene is highly expressed in hepatocytes and endoderm cells of the yolk sac during fetal development, however, it is repressed after birth. AFP is a major component of fetal plasma and reaches peak concentrations of 3 mg/mL by 12 weeks of gestation. However, following birth, AFP is cleared rapidly from circulation, being reduced to half of its peak value within 3.5 days, and ultimately to less than 10 ng/mL. AFP is also a marker for hepatocellular and germ cell carcinoma, with elevated values being observed during normal pregnancy, or when benign liver disease (hepatitis, cirrhosis), and cancer (Bisceglie et al., 2005; Yuen and Lai, 2005) are present. Serum AFP levels are usually evaluated by an immunoassay (Ciana et al., 1996).

Immunoassay is one of the most powerful analytical tools due to the specificity and sensitivity of antigen–antibody interactions, therefore, it is used extensively for clinical diagnoses (Fu et al., 2006) and peptide screening (Liu et al., 2007). However, the conventional immunoassay is laborious, expensive, and requires a prolonged development time. In addition, because large-scale detection devices are also required, it is difficult to conduct immunoassays at Point Of Care Testing (POCT) facilities (Xue et al., 1996). In general, enzyme and fluorescence based optical detection systems require high cost microscopy equipment and a computer to analyze the signals, and in the

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case of radioimmunoassays there are radiation hazards to consider, including waste disposal. Therefore, many studies have been conducted in an attempt to develop alternative methods of immunoassay (Bange et al., 2005; Lin and Ju, 2005; Meyer et al., 2007; Wu et al., 2006; Xu et al., 2004). One possible solution involves the use of microbiochips that employ microfluidics, and many microfluidic devices that use MEMS technology have been developed in the last decade, including Lab-on-a-chip (Cho et al., 2006), biosensors (Lange et al., 2006; Zaytseva et al., 2005), and cell handling system (King et al., 2006). Based on the results of the studies conducted to date, microbiochip based microfluidics are likely to improve analytic efficiencies by reducing the required sample volume and time required for analysis while increasing sensitivity and enabling processing of multiple samples via automation (Kim and Park (2005); Tadic et al., 2004).

In this study, we describe a PDMS-glass microbiochip that can detect an antigen–antibody reaction through electrical signals. Platinum (Pt) electrodes were fabricated on a glass substrate to allow detection of electrical signals and a microchannel and pillar-type microfilter was formed in the PDMS layer. The microfluidic biosensor was then used to detect AFP to demonstrate that the microbead, microfilter and immuno-gold silver staining (IGSS) complex could be used for signal amplification (Baschong and Stierhof, 1998; Lackie, 1996; Weipoltshammer et al., 2000). In a previous study, we successfully used protein A as an antigen for detection via the system described here (Ko et al., 2006). Based on the results of our previous study, we attempted to use the system for the detection of AFP, a practical marker for cancer. Our results indicate that AFP was successfully assayed using our fabricated microbiochip, further demonstrating the utility of the system as a POCT diagnostic tool.

## 2. Experimental

### 2.1. Detection principle

The working concept of the bead-based immunosensor is shown in Fig. 1. For this system, streptavidin coated microbeads, which were selected for the solid phase, were coated with biotinylated antibody (capture antibody) using the ABC (avidin–biotin coupling in a separate tube) method. The complexes were then introduced to the microbiochip by a syringe pump (Fig. 1(a)), after which they were applied to a Pt electrode using a microfilter in the microbiochip. Next, a sample containing AFP as an antigen was reacted with the beads, and the gold nano-particle conjugated antibody (second antibody) was introduced into the microchannel (Fig. 1(b, c)). Unbound antibody and antigen were then removed by washing buffer, after which a silver enhancer was injected into the chip to amplify the electrical signal. Finally, washing buffer was injected, which resulted in a silver bridge being formed between the Pt electrodes if a sandwich immune-complex had been specifically formed (Fig. 1(d)). This allowed the Pt electrodes to be charged with electric current, after which electrical resistance was then measured using a multimeter.

### 2.2. Materials and reagents

AFP (100 μg/mL), AFP Ab-1 (1.0 mL, mouse monoclonal antibody) and AFP Ab-2 (1.0 mL, rabbit polyclonal antibody)

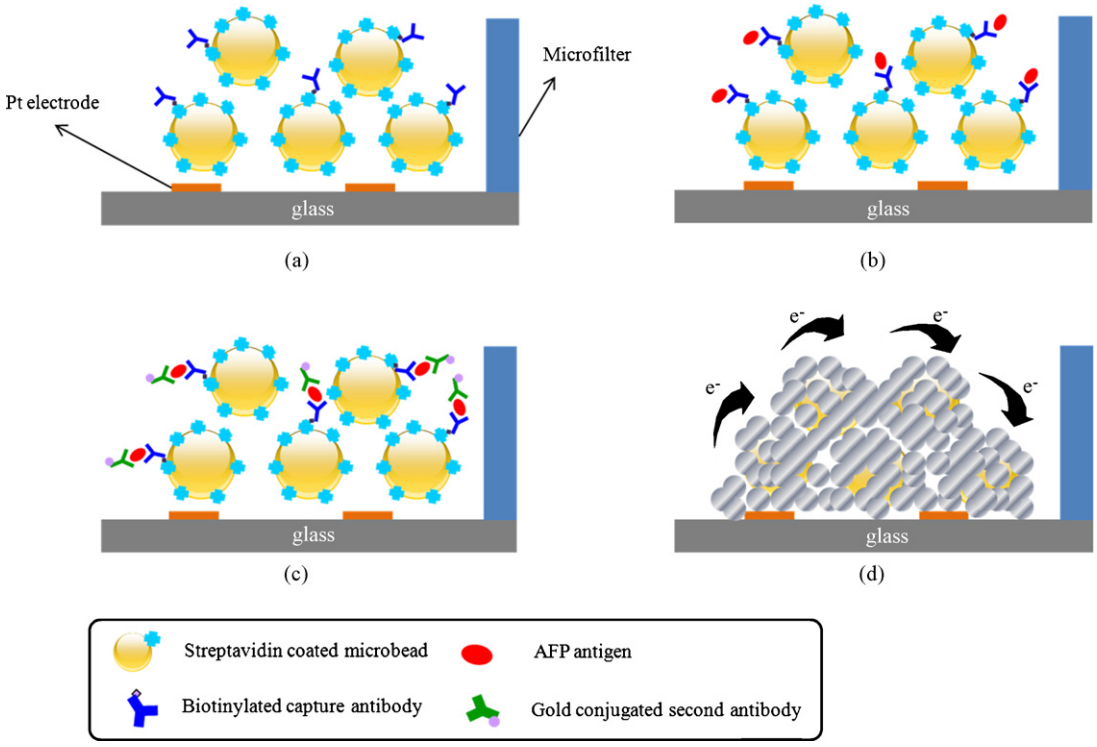


Fig. 1. Analytic concept for the electrical immunoassay using microbeads to facilitate the IGSS.

were obtained from LAB VISION Co., Inc. (Fremont, CA, USA). Anti-mouse IgG (FITC conjugate) was obtained from Sigma–Aldrich (St. Louis, MO, USA). All antibodies were used without further purification. Polystyrene beads (UltraLink immobilized streptavidin, Ø50 μm) and biotin powder (EZ-Link Sulfo-NHS-Biotin, M.W. 556.59; 100 mg) were obtained from PIERCE (Rockford, USA). For the detection of electrical signals, gold nano-particles (Ø 10 nm) and silver enhancer were obtained from Sigma–Aldrich (St. Louis, MO, USA). Phosphate buffer was prepared from Phosphate Buffered Saline Pack (0.1 M, pH 7.2, PIERCE). To prevent non-specific binding to the microchannels and microbeads, a 0.2% BSA (Sigma–Aldrich) preparation in phosphate buffer was used as a blocking reagent. Ultrapure water was filtered through a 0.2 μm membrane filter before use. All experiments were conducted at room temperature.

### 2.3. Microbiochip fabrication and preparation

A schematic diagram of the PDMS-glass hybrid immunoassay microchip is shown in Fig. 2. The inlet and outlet channels, pillar-type microfilter, and reaction chamber were formed in the PDMS layer, whereas the electrodes were formed in a Pyrex glass substrate that was then positioned in a reaction chamber in front of the microfilter. This allows microbeads that are injected through the inlet to be collected on the electrode by the microfilter. The diameter and space between the microfilter were maintained at 150 and 40 μm, respectively, and the width and the height of the microchannel were maintained at 300 and 160 μm, respectively. The width of the electrode was 20 μm, as was the gap between the electrodes. In addition, the width and volume of the chamber were 2 mm and approximately 7 μL, respectively.

The Pt microelectrode was formed on a glass wafer using a lift-off process as follows: a photoresist (AZ1512) was spincoated onto a glass wafer and then patterned by photolithography. Next, Pt/Cr (700 Å/100 Å) was deposited onto the patterned glass wafer by PVD (physical vapor deposition), after which the electrode on the glass substrate was completed by removing the photoresist from underneath the deposited metal using a solvent.

The PDMS molding technique was used to fabricate the microchannel and microfilter. Briefly, a negative photoresist (SU-8) mold was made on the silicon wafer by photolithography. Next, a 10:1 mixture of PDMS polymer and curing agent (Sylgard 184, Dow Corning) was poured into the mold and hardened in an oven at 65 °C for 4 h. The cured PDMS layers were then allowed to cool for a few hours, after which they were separated from the mold. The inlet and outlet were then made in the PDMS layer using a mechanical punching technique, after which the PDMS layer and the glass substrate were bonded by oxidizing the PDMS via $O_2$ plasma treatment, which allowed it to bond to the glass. The time and power of the plasma treatment was 15 s and 18 W, respectively.

### 2.4. Preparation of the capture antibody coated microbeads

Before initiating the experiment, the microbeads were coated with AFP Ab-2 using a streptavidin–biotin coupling method to

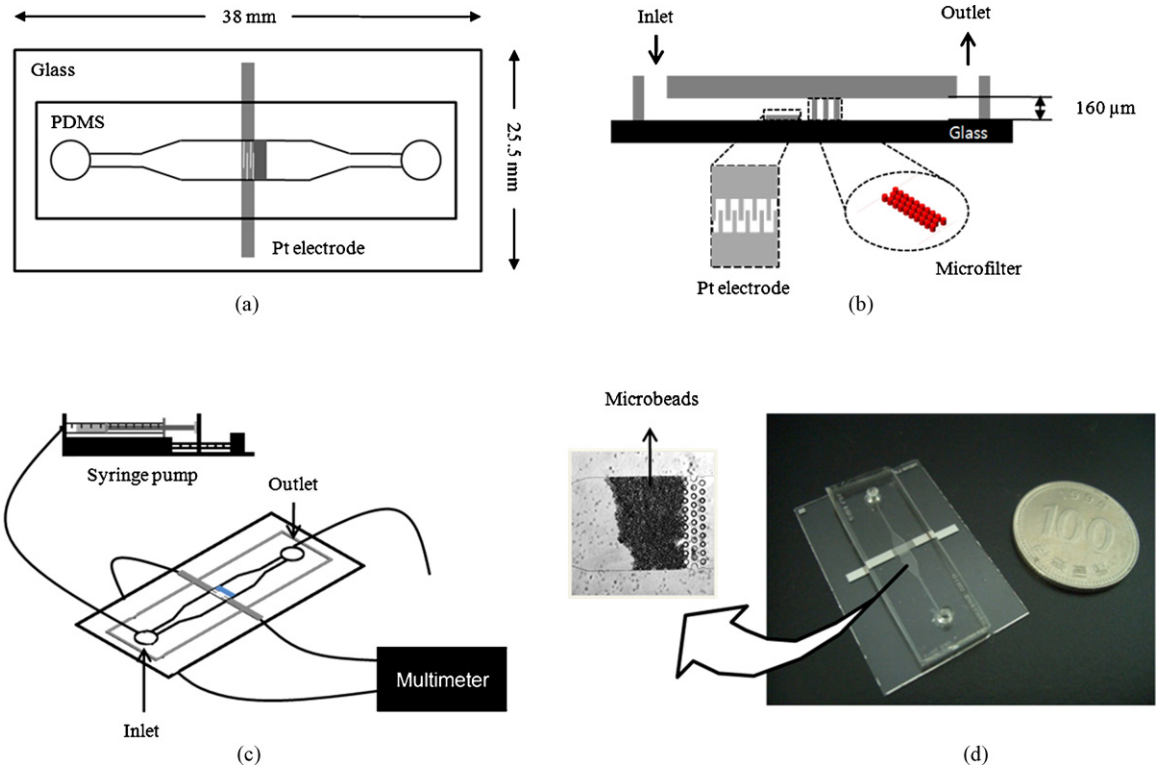


Fig. 2. Schematics and images of the microfluidic immunosensor used for electrical signal detection. (a) Top view; (b) cross-section of the microbiochip; (c) schematic of the experimental setup; and (d) photograph of the fabricated microbiochip and an enlarged photograph of the microbeads and microfilter.

immobilize the capture antibody. Briefly, 100 μL of streptavidin coated polystyrene beads were mixed with 15 μL of 1 mg/mL biotinylated AFP Ab-2 in a test tube and then gently agitated in a vortex mixer for 30 min at room temperature. The supernatant was then discarded, thereby removing excess antibody. Next, the microbeads were washed five times with phosphate buffer and then resuspended in 100 μL of phosphate buffer.

### 2.5. Preparation of colloidal gold-antibody conjugates

The second antibody-colloidal gold conjugates were prepared according to the following method (Xiulan et al., 2005). Briefly, the AFP Ab-1 (10% excess) was added to 1 mL of pH-adjusted colloidal gold suspension and then incubated at room temperature for 1 h. The conjugate was then centrifuged at 45,000 g for 30–60 min, after which the soft sediment was resuspended in 0.01 M Tris-buffered saline. Glycerol was then added to a final concentration of 50% to allow storage of the colloidal gold second antibody conjugate at −20 °C for several months.

### 2.6. Apparatus

The flow of the liquid solution was controlled using a syringe pump (KD Scientific, Boston, USA) in conjunction with a microtube (Ø 0.7 mm, Tygon, USA) and syringe (Korea vaccine, Korea). An inlet in the microbiochip was connected to a syringe pump through a microtube, and a Leica TCS SL confocal laser scanning microscope (CLSM, Leica Microsystems Inc., Germany) was then used to measure the fluorescence intensity profile. Next, the images were analyzed using a Leica LCS software (Leica Microsystems) system. In addition, the electrical signal was measured using a multimeter (Fluke-189, Fluke, USA) and the signal processing was then performed using Fluke View Forms software (Fluke, USA).

## 3. Results and discussion

Bead-based immunoassays, which are very easy to use in conjunction with microchips, provide several advantages over conventional techniques, such as ease of handing and high reaction efficiencies when both molecules of various dimensions (DNA and protein) and cells are assayed (Bienvenue et al., 2006; Choi et al., 2002; Lim and Zhang, 2007; Sato et al., 2002). Nevertheless, various factors that affect the biochemical reaction must be considered because the reaction conditions in the microbiochip are different than those of conventional microtubes or well plates. In this study, we found that when a florescence tagged second antibody and a silver enhancer were used, the most important factor was the time for silver enhancer treatment, however flow rate and incubation time were also found to be important.

### 3.1. The effects of flow rate and incubation times

Microfluidic control systems are essential for the control of a minute volume of fluid because of their rapid and precise control features, therefore, in our microbiochip, all reactions and washing procedures were performed using a syringe pump. The flow rates of the sample and reagent have an effect on the reaction efficiencies of the antigen–antibody interactions, and unlike conventional immunoassays, samples and reagents in our system are continuously flowing through the microbiochips. Therefore, it is very important to consider flow rate when designing microfluidic biosensors.

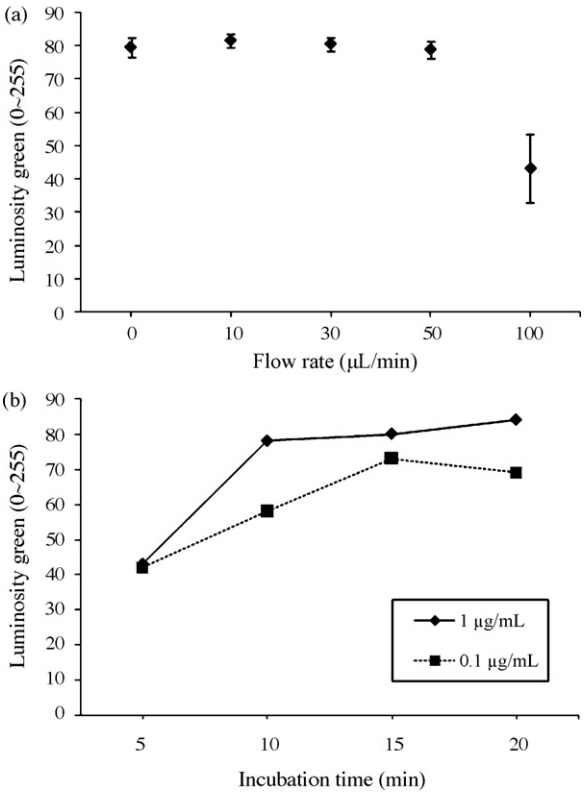


Fig. 3. Optimization of the flow rates and incubation times using fluorescence tagged second antibody. (a) For the low flow rate, the results presented no apparent difference, however, non-specific adsorption was observed. Additionally, when a flow rate greater than 100 μL/min was used the microbeads were not filtered by high pressure, but instead passed through the microfilter. (b) The optimal incubation time determined for the microbiochip was 15 min.

The optimal flow rate was determined by treating a 0.1 μg/mL solution of AFP and evaluating the fluorescence intensity generated during the reaction between the capture antibodies and the antigen (the most critical process for the determination) using CLSM. As shown in Fig. 3(a), flow rates from 0 to 50 μL/min had little effect on the antigen–antibody reaction, however, we observed non-specific adsorption onto the surface of the microchannel when the flow rate was between 0 and 10 μL/min. Conversely, when the flow rate exceeded 100 μL/min the microbeads began passing through the microfilter. Therefore, a flow rate of 30 and 50 μL/min was used for the sample injection and the washing buffer injection, respectively.

Incubation time is also a critical assay factor, and the minimum time required for antigen binding is very important when minimizing the total analysis time. Therefore, in order to determine the minimum reaction time required for our assay system, we evaluated its performance using concentrations of AFP that ranged from 1 to 0.1 μg/mL and incubation times of 5, 10, 15 and

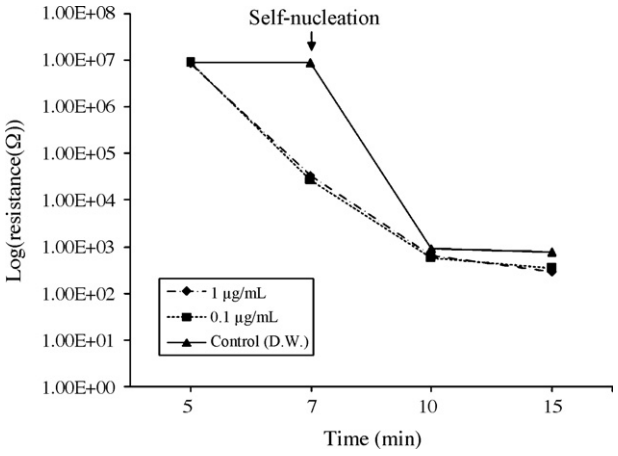


Fig. 4. The effects of silver enhancement as time increases. After 7 min, the control became saturated due to self-nucleation.

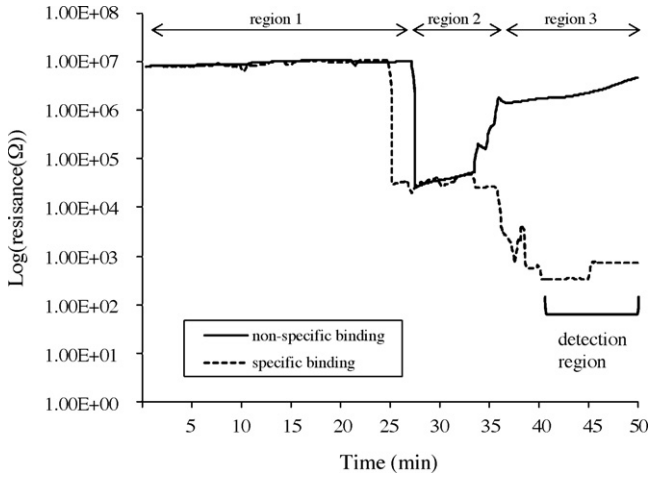


Fig. 5. The electrical signal curves of specific and non-specific test samples. The total assay time was less than 1 h. The results indicate that the electrical signal curve was dynamically changed in each step (region 1: microbead, sample, second antibody; region 2: silver enhancer; region 3: washing buffer).

20 min. As seen in Fig. 3(b), the fluorescence intensity of each AFP concentration was saturated at 15 min, which demonstrates that 15 min is sufficient for the antigen–antibody reaction.

### 3.2. Determination of the required treatment time of the silver enhancer

The immune-complex formations caused the microbead surface to be coated with gold nano-particles, which serve as nucleation sites that catalyze silver ion reduction. However, self-nucleation, which is a process whereby silver precipitates spontaneously beyond the enhancement time (Liang et al., 2004; Su et al., 2001; Xue et al., 2002), thereby leading to an elevated background signal that obscures the final result, can occur in these types of systems. Therefore, the microbead surfaces were treated with a silver enhancer, which did not allow the self-nucleation to be observed.

To optimize the signal and reduce silver staining of the background, the microbeads were rinsed with deionized water after the sandwich reactions to remove all of the salt content and to avoid self-nucleation of the silver. This silver enhancement method was measured at 5, 7, 10 and 15 min using 1 and 0.1 μg/mL AFP antigen and deionized water as a control to determine its optimum time. Fig. 4 shows that silver enhancement is time-dependent, with resistance gradually decreasing as the treatment time increases. However, in the case of the control, a decrease in resistance was observed after 7 min. Taken together, these results indicate that silver enhancement can affect the sensitivity of the detection system and that a silver enhancer treatment time of 7 min was optimal.

### 3.3. The detection region and the analytic procedure

Based on our previous experimental results, we measured an electrical signal for the AFP reaction from the microbead injection and silver enhancement. The signal curves of resistance corresponding to time are shown in Fig. 5. The resistance, which was measured from when the pre-buffer (containing microbead) injection was administered until after the second antibody was injected, was approximately 8–10 MΩ. In addition, the resistance was nearly constant during each step. The resistance at the time of the pre-buffer injection is very important for calibration of the detection system, and if the resistance value is not normal at this time the prepared microbiochip must be replaced. In this study, when the silver enhancer was injected, the resistance decreased dramatically to approximately 1–10 kΩ. However, when the silver enhancer was used, the resistance of specific and non-specific binding also decreased. Furthermore, due to the electrolyte character of the silver solution, the two signal curves could not be distinguished. Therefore, we added a washing step, after which the resistance of both the specific and non-specific binding was measured using a multimeter at an interval of 5 s. This resulted in a significant difference of approximately 5000 times being observed between the resistance of the specific and non-specific binding.

In addition, immediately after the final washing buffer was administered, an unstable signal that persisted for 5 min was observed due to the presence of residual silver enhancer. Therefore, to obtain a reliable resistance value, we determined the mean value of resistance based on the resistance that was measured for 10 min after the signal stabilized. As shown in Table 1, which summarizes the complete analytical procedure required for the AFP immunoassay using our system, the total time

Table 1
Sequences required for the bead-based immunoassay

| Sequence | Condition | Time |
|---|---|---|
| Pre-buffer | Capture antibody coated microbeads (Ø 50 μm) | 20 s |
| Antigen | AFP | 15 min |
| Washing buffer | Flow rate: 50 μL/min (PBS, containing BSA) | 30 s |
| Second antibody | 10 nm gold particle conjugated | 15 min |
| Washing buffer | Flow rate: 50 μL/min (deionized water) | 30 s |
| Silver enhancer | $Ag^+$ + hydroquinone (reducing agent) | 7 min |
| Washing buffer | Flow rate: 50 μL/min (deionized water) | 15 min |
| Signal analysis | Fluke View Form (analysis software) | 2 min |

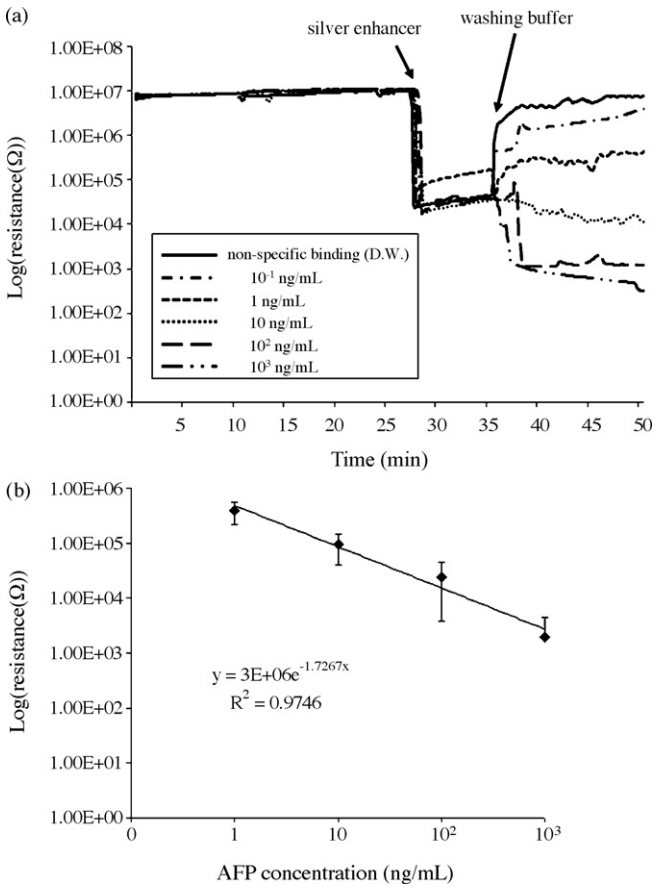


Fig. 6. Immunoassay results measured in our microfluidic biosensor chip and a calibration curve for AFP. (a) The signal was varied against the AFP concentration and each concentration showed a different resistance range. A blank signal represents non-specific binding of second antibody. (b) The signal was then linearized so that it could be used to determine the concentration of unknown samples.

required for the immunoassay was found to be approximately 50–60 min.

### 3.4. Electrical signal detection in the microfluidic chip

Under the optimal conditions we were able to measure an electrical signal when various concentrations of AFP were used (Fig. 6(a)). The total assay time, including all incubation and detection steps for the AFP measurements, was less than 1 h, and as the concentration of AFP decreased, the electrical resistance gradually increased. However, the resistance was unstable when a low concentration of AFP was used, which indicates that the detection range is from 1 to $10^3$ ng/mL. Taken together, these results indicate that a complete immunoassay could be achieved using the microfluidic and electrical detection system.

We also developed a calibration curve to predict the concentration of AFP within the range of 1–$10^3$ ng/mL (Fig. 6(b)). The error bars represent the standard deviation of 10 replicate measurements. The correlation coefficient and detection limit of AFP were 0.9746 and 1 ng/mL, respectively. These values demonstrate that our microfluidic biosensor can be used to quantify the amount of AFP in unknown samples.

## 4. Conclusion

In conclusion, we have developed a microbiochip that is based on an electrical detection system that can be used for the detection of AFP antigen. In this system, a Pt electrode is used to measure an electrical signal and a microfilter is used to fix the microbeads. Despite the simple structure of the microfilter, the microbeads were efficiently fixed, and AFP could be detected at concentrations as low as 1 ng/mL using this system. These results indicate that this system would be adequate for use in clinical diagnosis. Moreover, if certain design aspects are addressed (e.g. bubbles, dead volumes) this system can be further miniaturized and integrated with microfluidic devices to provide a detection system with high sensitivity and selectivity for commercial and academic use. In future studies, we will focus on optimizing the operating conditions and expanding the applications of the system to other important biomarkers of disease (CEA, CA125 and PSA), as well as developing an even higher throughput system for the simultaneous detection of multiple biomarkers.

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