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Microbiochip for Electrical Detection of Carcinoembryonic Antigen Using Gold Nanoparticles and Silver Enhancement

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Abstract

The conventional immunoassay protocol for the detection of carcinoembryonic antigen (CEA) is expensive, laborious, and time-intensive. Additionally, it requires a very large detection device. In an effort to solve these problems, we have assessed the utility of a novel immunoassay technique based on microbeads and gold nanoparticles. In our microbiochip, we have adopted the filtration method to fix the microbeads, and used the immunogold silver staining (IGSS) method to amplify the electrical signal. The microbiochip was constructed of a Pyrex glass substrate and an inexpensive and biocompatible polydimethylsiloxane (PDMS) layer. The platinum (Pt) microelectrode for electrical signal detection was fabricated on the substrate. A microchannel and pillar-type microfilter was formed in the PDMS layer. The colon cancer marker, CEA, was assayed via a sandwich immunoassay. As a result, we obtained significant resistance signal as the difference in CEA concentration. The total analysis time was less than one hour. A bead-based electrical detection system, such as the one described in this study, can be applied to systems for cancer diagnosis.

Keywords: Carcinoembryonic antigen, Immunoassay, Microbiochip, Immunogold silver staining, Microfilter, Microbead

Introduction

Microbiochip systems, which integrate sample mixing, separation, and detection processes, are currently a matter of considerable interest. Over the past 10 years, microbiochips using MEMS technology have been developed for a variety of applications, including biosensors[1,2], Lab-on-a-chip systems[3], and cell handling systems[4]. Microbiochip-based microfluidic systems can improve analytic efficiency by reducing sample consumption and analysis time, increasing sensitivity, and allowing for multiple processes in an automation device. Owing to these advantages, these systems are thought to be quite useful in clinical immunoassays, as compared to the variety of other bio-applications for which they can be used.

Immunoassays are one of the most powerful analysis tools known, due principally to the specificity and sensitivity inherent to the antigen-antibody interaction. Due to their high specificity and sensitivity, immunoassays have been applied extensively to clinical diagnosis[5], as well as the detection of environmental toxicants[6]. However, conventional immunoassays are laborious, expensive, and require a great deal of analysis time. Due to the necessity of large-scale detection devices, immunoassays are difficult to conduct in Point-of-care (POC) applications[7]. Generally, enzyme and fluorescence-based optical detection requires expensive microscopy equipment and a computer for the analysis of the signal. Also, in the case of radioimmunoassays, radiation hazards also are relevant. Currently, however, in order to solve these problems, a variety of research has been conducted for improved immunoassay methods[8-12].

In our laboratory, we have developed a PDMS-glass microbiochip which can be used to detect a carcinoembryonic antigen (CEA) via electrical signals. Carcinoembryonic antigen (CEA) is a glycoprotein generated during fetal development, but the production of CEA stops before birth. The normal range is less than 2.5 ng/mL in a normal adult non-smoker, and less than 5.0 ng/mL in a normal adult smoker. CEA is a complex glycoprotein with a molecular weight of 20,000, which is associated with the plasma membranes of tumor cells, from which it may be secreted into the blood. Elevated CEA levels have been detected in a variety of cancers other than colonic, includ-

ing pancreatic, gastric, lung, and breast cancers. Such levels have also been detected under benign conditions, including cirrhosis, inflammatory bowel disease, chronic lung disease, and pancreatitis[13,14].

The detection of CEA was conducted with a microfluidic biosensor in order to demonstrate the efficacy of microbead, microfilter, and immunogold-silver staining (IGSS) for signal amplification. The fundamental principle underlying the functioning of the bead-based immunosensor is provided in Figure 1.

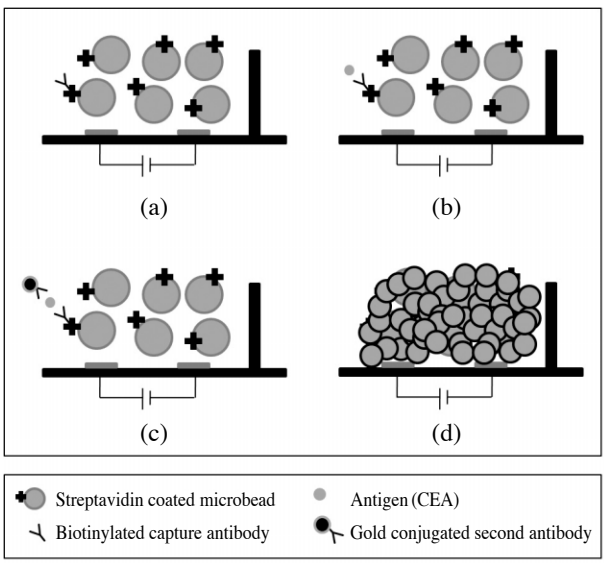

Figure 1. The principle of electrical immunoassay with microbead which coupled the IGSS.

Streptavidin-coated microbeads were selected for the solid phase. The microbeads were coated with biotinylated capture antibody using the ABC (avidin-biotin coupling) method in tubes, then introduced into microbiochip with a syringe pump (Figure 1(a)). They were then coated onto a Pt electrode by the microfilter in the microbiochip. Next, a sample containing CEA antigen was allowed to react with the beads, after which gold nano-particle conjugated second antibody was introduced into the microchannel (Figure 1(b, c)). Prior to the injection of a silver enhancer, unbound antibody and antigen were washed out. In order to amplify the electrical signal, the silver enhancer was injected. Gold particles in the presence of silver ions and a reducing agent function as catalysts for the reduction of silver ions to metallic silver. The silver is deposited onto the gold, enlarging the particles to between 30 and 100 nm in diameter. Finally, washing buffer was injected. If a sandwich immunecomplex was specifically formed, the formation of a silver bridge was also detected between the Pt electrodes (Figure 1(d)). Thus, it is possible for the Pt electrode to be charged with electric current. At this time, we assessed the electric resistance using a multimeter.

In our previous research, we successfully detected protein A using this detection system[15]. Based on the methods and results of previous research, we conducted an experiment that utilized CEA as a practical tumor marker. As a result, CEA was successfully assayed in our fabricated microbiochip, and the results showed the utility of the system as a POC diagnostic

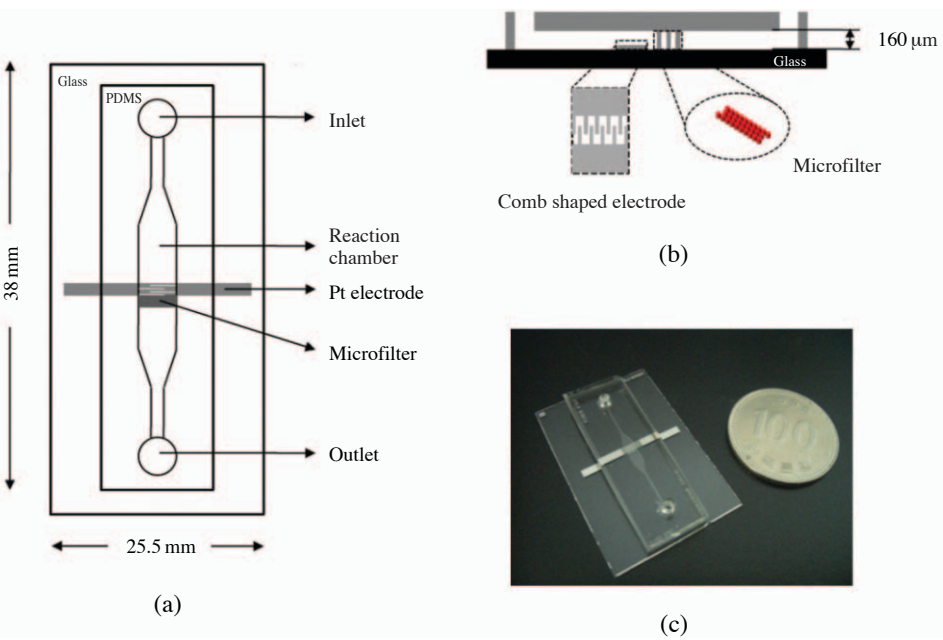

Figure 2. Layout and images of the microfluidic immunosensor for electrical signal detection. (a) Top view, (b) Cross section of the microbiochip, (c) Photograph of the fabricated microbiochip.

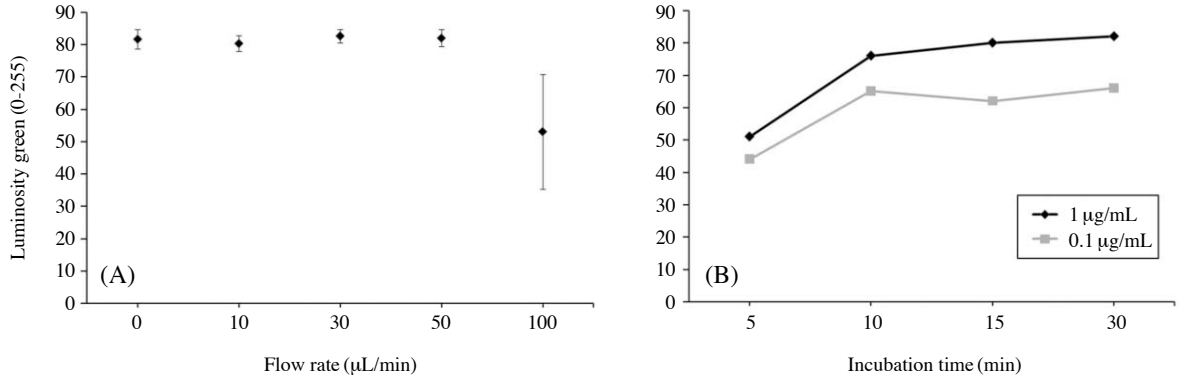

Figure 3. Optimization of the flow rate and incubation time using fluorescence tagged second antibody. At low flow rates, the results evidenced no difference. However, non-specific adsorption was observed. Above a flow rate of 100 μL/min, the microbeads were not filtered by high pressure. Optimal incubation time in the microbiochip was 10 minutes.

tool.

Results and Discussion

Microfluidic Chip Fabrication

A schematic diagram of the microchip is provided in Figure 2. The inlet and outlet channels, pillar-type microfilter, and reaction chamber are formed in the PDMS layer. The electrodes are formed in a Pyrex glass substrate. The electrodes are located in a reaction chamber located in front of the microfilter. Therefore, the microbeads injected from the inlet are collected on the electrode by the microfilter. The diameter and space of the filter were 150 μm and 40 μm, respectively. The width and height of the microchannel were 300 μm and 160 μm, respectively. The width of the electrode was 20 μm and the gap between electrodes was 20 μm. The width and volume of the chamber were 2 mm and approximately 7 μL, respectively.

Optimization of Flow Rate and Incubation Time

In our microbiochip, all reactions and washing processing were conducted using a syringe pump. The handling of the syringe pump was very simple and allowed for sufficient precision. Microfluidic control using a syringe pump was also far easier than pipetting in the conventional immunoassay technique. The flow rate of the sample and reagent has an effect on the antigen-antibody reaction efficiency. Unlike conventional immune-reactions in tubes or 96-well plates, sample and reagent flow continuously in the microbiochip. Thus, we believe that the flow rate is one of the important factors to be considered. The optimal flow rate required to bind to the antigen was determined via the treatment of 0.1 μg/mL of CEA solution. Then, fluorescence intensity during the reaction between capture antibody and antigen, which is the most critical process for the determination, was assessed via CLSM. As shown in Figure 3(a), the flow rate from 0 to 50 μL/min had only minimal effects on the antigen-antibody reaction. However, we observed non-specific adsorption on the microchannel surface when the flow rate was 0 and 10 μL/min. At flow rates over 100 μL/min, we were able to observe microbeads passing through the microfilter with high fluid flow pressure. Therefore, a 30 μL/min rate for sample injection and a 50 μL/min rate for washing buffer injection were utilized in our bead-based immunoassay.

Incubation time, like flow rate, is a critical factor. The determination of the minimum time required for antigen binding is crucial, as there is a clear need to decrease the total analysis time. In order to determine the minimum reaction time, we conducted assays with 1 and 0.1 μg/mL of CEA solution. Incubation time was determined as 5, 10, 15 and 20 minutes. As seen in Figure 3(b), the fluorescence intensity of each CEA concentration was saturated in 10 minutes. The result indicates that this time is sufficient for the reaction of antigen and antibody. Therefore, 10 minutes was determined as the optimal incubation time.

Determination of Silver Enhancer Treatment Time

As the result of the immune-complex, the microbead surface was coated with gold nanoparticles, which functioned as nucleation sites for the catalysis of silver ion reduction. In order to optimize the signal and to reduce the silver staining background, the microbeads were rinsed after the sandwich reactions using deionized water in order to remove all salt content, thus preventing the self-nucleation of the silver.

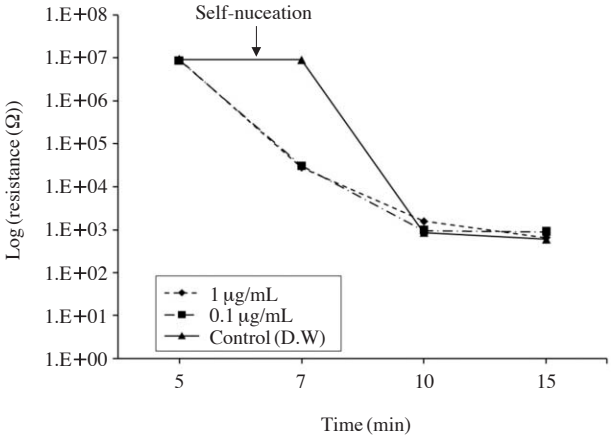

Figure 4. The effect of silver enhancement as time flow. After 7 minutes, control was saturated because of self-nucleation.

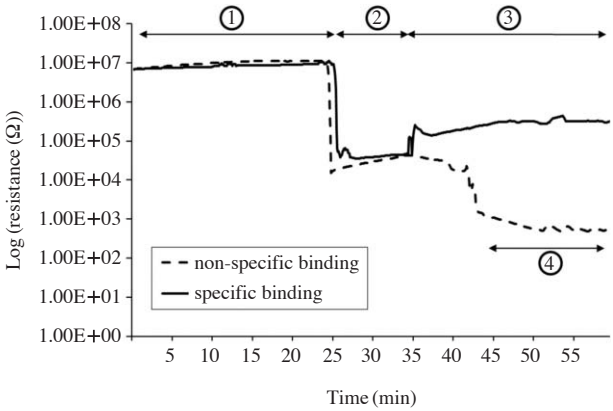

Figure 5. The electrical signal pattern of specific and non-specific samples. Total assay time was less than one hour. The result showed that electrical signal was dynamically changed in each step (region 1: microbead, sample, second antibody; region 2: silver enhancer; region 3: washing buffer; region 4: detection point).

Self-nucleation allows for the silver to be spontaneously precipitated after a certain time beyond the enhancement time[16,17]. In this case, a high background signal was detected. Therefore, under optimal conditions, we conducted an experiment for the determination of silver enhancer treatment time. Silver enhancer treatment time was measured as 5, 7, 10, and 15 minutes. Antigen concentration was measured as 1, 0.1 μg/mL and deionized water (control). As is shown in Figure 4, because silver enhancement occurs in a time-dependent manner, the resistance decreased gradually with time. However, in the case of the control, we identified that resistance decreased after 7 minutes. From this result, we concluded that silver enhancement time can significantly affect the sensitivity of the detection system.

The Detection Region and Analytic Procedure

Based on the results of a previous experiment, we measured an electrical signal of CEA reaction from microbead injection to silver enhancement. Figure 5 shows the signal pattern of resistance corresponding to time. Resistance, which is measured from the injection of microbeads to the second antibody treatment is approximately 8-10 MΩ. The resistance remains almost constant during each step. Next, when silver enhancer was injected for signal amplification, resistance decreased dramatically, to approximately 20-80 kΩ. However, the resistance for specific and non-specific binding decreased similarly during silver enhancer treatment, and was not distinguishable, because silver solution by itself is an electrolyte. Thus, we added a washing step to the detection region, after which the resistances of specific and non-specific binding were assessed with a multimeter in our microbiochip. In this step, the resistance was differently measured as the concentration of CEA. In fact, significant differences between the resistance of specific and non-specific binding could be observed as 10^3 orders of magnitude. In our experimental results, the resistance to be detected during the last washing step was determined. In addition, we obtained an unstable signal due to the remaining silver enhancer, as soon as the final washing buffer was injected. In order to acquire a reliable resistance value, we determined the mean value of measured resistance for 10 minutes over the next 5 minutes of adding washing buffer. The analytical procedures required for CEA immuno-

Table 1. Sequence for bead based immunoassay.

Sequence	Condition	Time
Capture antibody coated microbeads	Polystyrene bead (Ø50 μm)	20 sec
Antigen	CEA	10 min
Washing buffer	Flow rate: 50 μL/min (PBS, containing BSA)	30 sec
Second antibody	10 nm gold particle conjugated	10 min
Washing buffer	Flow rate: 50 μL/min (deionized water)	30 sec
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

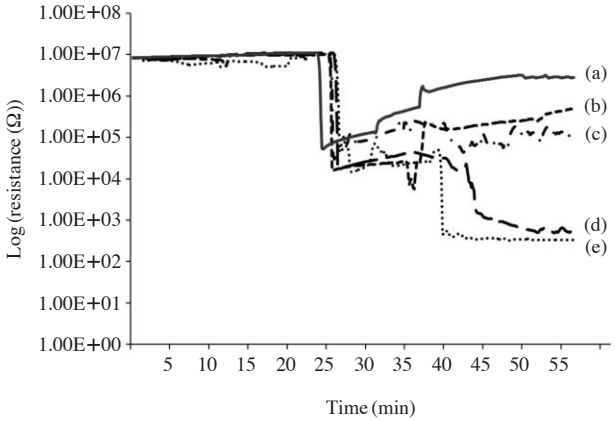

Figure 6. Immunoassay results measured by electrical detection. Each CEA concentration has different resistance range. (a) 0, (b) 10^{-1}, (c) 1, (d) 10, (e) 10^{2} ng/mL. When silver enhancer or washing buffer is injected, an unstable signal is sometimes detected.

assay are shown in Table 1. The required total immunoassay time was approximately 50-60 minutes.

Electrical Signal Detection in the Microfluidic Chip

Under optimal conditions, we measured an electrical signal with differing CEA concentrations. The immunoassay results for different CEA concentrations are shown in terms of resistance-time signals in Figure 6. A blank signal is caused by non-specific binding of the second antibody. The total assay time was less than one hour, including all incubation and detection steps. As the concentration of CEA was reduced, electrical resistance was increased gradually. At low concentrations, resistance was relatively unstable. It would appear that the detection range was from 10^{-1} to 10^{2} ng/mL. These results revealed that all steps required for a complete immunoassay could be achieved with a microfluidic and electrical detection system.

Conclusion

The electrical detection of carcinoembryonic antigen within short time was successfully accomplished herein. The integrated microbiochip, which includes a Pt electrode and a microfilter was fabricated. The microbead was efficiently filtered by a microfilter and the dynamic electrical signal could be continuously monitored. Also, its potential for miniaturization and immunoassay results render it very appealing. However, an unstable signal was measured due to bubbles. This problem must be addressed for the desired high sensitivity and selectivity. Currently, our research group is working toward the further optimization of various conditions, as well as a novel sample injection method. We are also applying a variety of biomarkers (CA125, PSA and so on) and high-throughput systems for the detection of such biomarkers.

Materials and Method

Materials

CEA (100 μg/mL), CEA Ab-1 (1.0 mL, mouse monoclonal antibody) and CEA Ab-2 (1.0 mL, rabbit polyclonal antibody) were obtained from LAB VISION Co., Inc. (Fremont, USA). All antibodies were utilized without further purification. Polystyrene beads (UltraLink immobilized Streptavidin, Ø50 μm, 50% aqueous slurry containing 0.02% sodium azide) and biotin powder (EZ-Link Sulfo-NHS-Biotin, M.W. 556.59, 100 mg) were obtained from PIERCE (Rockford, USA). For electrical signal detection, gold nanoparticles (Ø10 nm) and silver enhancer were obtained from SIGMA (USA). Phosphate buffer was prepared from BupH™ Phosphate Buffered Saline Pack (0.1 M, pH 7.2, PIERCE). Ultrapure water was filtered through a 0.2 μm membrane filter prior to use. In order to prevent nonspecific binding to a microchannel and microbeads, 0.2% BSA (SIGMA, USA) in phosphate buffer was utilized as a blocking reagent. All experiments were conducted at room temperature.

Preparation of Capture Antibody-coated Microbeads

Prior to the microbiochip experiment, the microbeads were coated with CEA Ab-2. We employed a streptavidin-biotin coupling technique to immobilize the capture antibody. In the first step, 100 μL of streptavidin-coated polystyrene beads were mixed with 15 μL of 1 mg/mL biotinylated CEA Ab-2 in a test tube, with gentle agitation in a vortex mixer for 30 minutes at room temperature. To remove excess antibody, the supernatant was discarded. The microbeads were then washed in phosphate buffer. After the fifth rinse, the microbeads were resuspended in 100 μL of phosphate buffer.

Preparation of Colloidal Gold-antibody Conjugate

Second antibody-colloidal gold conjugates were prepared in accordance with the modifications described in the literature[18]. In brief, the CEA Ab-1 (10% more than the minimum amount) was added to 1 mL of pH-adjusted colloidal Au suspension, followed by

one hour of incubation at room temperature. The conjugate was then centrifuged at 45,000 g for 30-60 minutes, and the soft sediment was resuspended in 0.01 mol/L Tris-buffer saline. The addition of glycerol to a final concentration of 50% allowed the storage of the colloidal Au second antibody conjugate at −20˚C for several months.

Apparatus

The flow of liquid solution was controlled with a syringe pump (KD Scientific, Boston, USA). A microtube (Ø0.7 mm, Tygon®, USA) and syringe (Korea Vaccine, Korea) were used for sample flow. An inlet in the microbiochip was connected to a syringe pump via a microtube. In order to measure the fluorescence intensity profile, a Leica TCS SL confocal laser scanning microscope (CLSM, Leica Microsystems Inc., Germany) was utilized. In order to analyze the images, Leica LCS software (Leica Microsystems) was used. The electrical signal was measured with a multimeter (Fluke-189, Fluke, USA) and signal processing was done with Fluke View Forms software (Fluke, USA).

Acknowledgements

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