Development of Rapid and Highly-Sensitive Biosensing Systems

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Received April 10, 2016

This article reviews the rapid detection of the biomarkers and discrimination of the specific surface antigen expressed on living cells based on the manipulation technique of particles and cells by dielectrophoresis and the sensitive detection of products generated by an enzyme reaction with chemical amplification system. The manipulation of particles and cells improves rapidity and simplicity for the immunosensing. The sensitivity (limit of detection) for immunoassay is enhanced by the chemical amplification systems.

Introduction

Immunoreactions are widely applied to detect target molecules such as tumor markers, hormones, and materials related to the environment. In the enzyme-linked immunosorbent assays (ELISAs) which generally used as a determination method of target based on the immunorecognition events, a target molecule in a sample is captured by an antibody immobilized on a solid substrate and labeled with an enzyme conjugated to an antibody by forming a sandwich type of an immunocomplex. The product generated by the captured enzyme is accumulated in a well and measured by spectrometry. However, conventional ELISAs require a relatively long reaction time for formation of the complexes due to the limitation of diffusion and several steps for the detection of immunocomplexes. Therefore, the development of a novel detection system with rapidity and simplicity has been desired. Micro- and nano-scale devices enable the diffusion-limited reaction to be rapid by the short diffusion distance.1)

Dielectrophoresis (DEP) is attractive for the manipulation of micro- and nano-objects including biological living cells and bacteria in a microfluidic device because of its non-contact nature.2) It has been used in a wide range of applications, such as separation and sorting,3) trapping,4) and patterning of cells or particles.5–7) When alternating current (ac) voltage is applied to the electrodes incorporated into the microfluidic devices, particles are manipulated based on the interaction between a nonuniform electric field and charge polarization induced on particles. Depending on the relative polarizability of the particles and suspending solution, particles direct to the region of higher electric field gradient by positive-DEP (p-DEP) or to the region of lower electric field gradient by negative-DEP (n-DEP). The particles can be rapidly accumulated by DEP force to form the pattern. However, the pattern fabricated by

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n-DEP are usually redispersed to bulk solution after switching off the applied voltage. The inhibition of the redispersion of particles by the immunoreaction was applied to develop the rapid and simple sensing system of the target analytes.

In the sandwich immunoassays, the number of the captured enzyme decreases with decreasing the concentration of the target analytes. Thus, the highly-sensitive detection system of the product generated by the captured label has been desired to improve the sensitivity of immunosensing. The recycling systems of the redox species by enzymatic, chemical, or electrochemical reactions were applied to improve the limit of the detectable concentration. Nanoparticles with catalytic properties have been utilized for signal amplification in sensitive immunosensing assays. The metal nanoparticle labels chemically catalyzed the deposition of silver to amplify the number of signal molecules, and subsequently, the deposited silver was electrochemically detected via stripping techniques. The collective stripping of the deposited silver allows the sensitive determination of the captured catalysts. The combination of two strategies for the signal amplification could improve the sensitivity (lower detection limit) for the electrochemical immunosensing.

In this review, we introduce the application of the particle and cell accumulation by DEP for the rapid and simple determination of the target analytes in the solution and discrimination of cells with the specific surface antigen, and a sensitive immunosensing procedure with signal amplification, which combines electrochemical-redox cycling and silver deposition using a galvanic cell.

**Sensors based on the dielectrophoresis**

*Rapid and simple sensors using n-DEP-based accumulation and redispersion of particles.* Interdigitated microband array (IDA) electrode has been used to fabricate the periodic particle or cell lines. The accumulation and redispersion behavior of particles based on n-DEP has been applied to develop a rapid and simple sandwich-type immunosensing system (Fig. 1). The mixture containing the analyte molecule and the microparticles modified with antibody was introduced into the microchannel fabricated between the upper substrate immobilized with antibody and the lower IDA electrode. When an ac voltage at an n-DEP frequency is applied to the IDA electrode to form nonuniform electric fields, particles are rapidly directed to the upper substrate to form the line pattern, thereby accelerating the reaction with the antibodies immobilized on the substrate.

In the absence of the analyte, the particles are redispersed into suspension from the substrate after switching off the voltage. On the other hand, the particles are reversibly captured on the substrate to form sandwich-type immunocomplexes in the presence of specific analytes because the particles modified with the antibody...
rapidly accumulated and pressed against the substrate modified with the antibody by n-DEP. Unreacted particles were automatically removed from the substrate by deregulation of DEP. Therefore, the concentration of analyte can be determined by the number of the particles remained on the upper substrate. The use of the fluorescent microparticles allows the selective measurement of the captured particles because the uncaptured particles automatically become out of focus in the fluorescent microscope detection. The present procedure with a simple device has substantial advantages of rapid and separation-free immunoreaction accelerated by n-DEP.

Fig. 2. (a) Particles with diameter of 2 µm manipulated by n-DEP under 10 Vpp voltage at a frequency of 2 MHz, an electrode width of 20 µm. Fluorescence microscopic images of (b) the re-dispersed microparticles 60 s after turning off the voltage and (c) the particles captured by immunoreactions. Reprinted with permission from Ref. 13 (Copyright 2008, Elsevier)

Capture of HL60 cells with CD33 antigen by n-DEP. DEP has also been used to pattern cells. Generally, p-DEP patterning is utilized to attract in array formats with the pair of electrodes at every elements modified with cell adhesive layers to produce cell patterns. The strategy for n-DEP cell patterning is the construction of the localized position enclosed with strong electric fields and allows to cell trapping by the repulsion force balanced from every direction. We have also previously contributed to the fabrication of periodic and alternate cell lines incorporating two cell types of cells using n-DEP.17) Furthermore, by using both p-DEP and n-DEP, individual cell types with different dielectrophoretic properties were separately patterned at the different positions.18,19) Two different patterns with particles can be easily and reversibly fabricated by controlling the direction of the DEP force with applied frequency. However, one feature was only used in most cases of cell patterning.

DEP manipulation was applied for the rapid and simple discrimination of cells which were expressed a specific surface antigen.20,21) Surface antigen patterns expressed on living cells depend on a lineage, differentiation and maturation. Phenotyping, which can be identified the presence and proportion of pathogenic cell populations, are useful for early medical diagnosis and prognosis prediction. A common approach is to use fluorescence labeling via antigen-binding to relieve specific antigens at cell surfaces; however, the methods are often qualitative and low throughput, and involve several complex steps for a modification and washing. The cell binding assay to the immobilized antibody was accelerated by n-DEP cell accumulation and the discrimination of the unbound cells was performed with spatial separation by switching the formation of electric field. Trapping of human promyelocytic leukemia (HL60) cells with surface antigen, CD33 on the gap region modified with anti-CD33 antibody was introduced.

The number of cells accumulated in the gap region based on n-DEP was estimated. DEP device was fabricated by the upper indium tin oxide (ITO) electrode and the lower ITO-IDA electrode which modified with anti-CD33 antibody (Fig. 4). The ac voltage at the
frequency for n-DEP force was applied between the upper and lower electrodes after the suspension of HL60 cells was introduced into the channel between the upper and lower electrodes. Randomly dispersed cells were forced to move toward the gap regions between the bands and captured with the antibodies via immunoreactions, because the relatively lower electric field was formed at the gap region on the lower substrate. Uncaptured cells were removed and separated from the gap region to the band B by switching the applied voltage for band B to zero. The strong electric field disappeared after the voltage for band B was turned off, thereby a weak electric field appeared on the band B. As a result, the ratio of captured cell density can be easily calculated from the images of the line structure of cells in the gap region.

Figure 4 shows the optical images of cells accumulated in the gap region (Fig. 4A) and captured cells after separating the unbound cells (Fig. 4B). Cells were accumulated in the gap region with n-DEP for 60 s and then the voltage for the band B was switched off to remove the cells from the gap region. Again, the uniformly dispersed cells initially start to move toward the gap region due to the strong repulsive force for n-DEP to form clear line patterns (Fig. 4A). After the voltage of band B was switched to zero, some cells accumulated in the gap region modified with anti-CD33 antibody were remained on the gap region even after the voltage was switched off, while the other moved on the band B (Fig. 4B). In contrast, almost all cells moved on band B after switching off the voltage to the band B when cells were accumulated in that with anti-mouse IgG antibody (Figs. 4C and 4D). The results suggested that the cells with CD33 surface antigens were reacted with the antibodies immobilized in the gap region and irreversibly captured at that position. The number of cells in the gap region quickly decreased in 30 s after switching off the voltage, and then reached at steady-state value. The cell binding efficiency is estimated from the steady-state value and found to be 68.3 ± 3.2%. The time as short as 30 s was required for removing unbound cells. Therefore, the cells with CD33 cell surface antigen can be rapidly identified from the cell suspension by the spatial separation based on the immunoreactions and DEP manipulation. After the cells accumulated in the gap, the intensity of the applied voltage to band B was successively switched from 20 V_{pp} to 18 or 15 V_{pp} at intervals of 5 s to swing the accumulated cells. Cell binding efficiency improved to 83.9 ± 1.4%
using the cell swing method, because the slight swing increased the opportunity to contact the surface antigen with the antibody immobilized on the substrate.

![Fig. 4. Optical images of cells patterned by n-DEP.](image)

Fig. 4. Optical images of cells patterned by n-DEP. (A) Cells accumulated in the gap region modified with the anti-CD 33 antibody by applying ac voltage of same intensity (20 Vpp) and frequency (100 kHz) as bands A and B. (B) Cell pattern captured in the gap region modified with the anti-CD 33 antibody after separating unbound cells by switching off the band B voltage. (C) Cells accumulated in the gap region modified with the anti-mouse IgG antibody. (D) Cells removed from the gap region modified with the anti-mouse IgG antibody after the band B voltage was turned off. Duration of voltage application for accumulation and removal with n-DEP: 60 and 60 s. Reprinted with permission from Ref. 21 (Copyright 2012, American Chemical Society)

Non-specific cells used as a CD33 negative cell was prepared by treating HL-60 cells with anti-CD33 antibody. Cells were also labeled with fluorescent molecules (CFDA SE) to distinguish the antibody-treated non-specific cells. The suspensions of each specific and non-specific cell were mixed at different ratios to study the relationship between the content of CD33 positive cells in suspensions. The mixtures were introduced into the device and accumulated in the gap region by n-DEP for 60 s. The binding efficiency of the cells was calculated after switching off the voltage for band B for 60 s. The efficiency was defined as a ratio of average density of cells in the gap region immediately before and 60 s after ac voltage for band B was switched off to eliminate the unbound cells.

![Fig. 5. Cells captured in the gap region from mixed suspensions of specific and nonspecific HL-60 cells.](image)

Fig. 5. Cells captured in the gap region from mixed suspensions of specific and nonspecific HL-60 cells. (A) Photographs were obtained by combining the optical and fluorescent images. Initial ratios in the original suspensions were set to 50% of specific cells. (B) Ratio of cells captured in the gap. Reprinted with permission from Ref. 21 (Copyright 2012, American Chemical Society)

Mixtures of HL-60 cells specific and nonspecific to anti-CD33 were used to determine the number of cells captured in the gap area modified with anti-CD33. Fig. 5A shows the image obtained by combining optical and fluorescent images, which were obtained 60 s after the applied voltage to band B was switched to zero. The initial concentration ratio of specific cells was set to 50%. Almost all nonspecific cells with a fluorescent signal moved to band B, whereas the specific cells were captured in the gap between the band electrodes, even in the presence of treated nonspecific cells (Fig. 5A). Fig. 5B shows the ratio of cells captured in the gap between the band electrodes. The ratio of captured cells increased linearly with the increasing ratio of specific cells in the prepared mixture suspension. These results indicate that the presence of cells without the target antigen did not obstruct specific cell binding for detecting cells with
surface antigens. The binding efficiency obtained in the present study improved compared to that obtained in our previous study using a combination of p- and n-DEP.

**Sensitive immunosensing procedure with signal amplification**

*Immunosensor based on electrochemical charge accumulation system.* Highly-sensitive detection techniques are important to detect trace amounts of biomarkers and for screening of a disease at an early stage and monitoring the recurrence of the disease following medical treatment. Electrochemical detection for biomarkers has been accomplished by coupling a catalytic reaction of labeled enzyme to antigen-antibody recognition events performed on or near electric transducers. Amplification methods based on signal source storage on the electrode were proposed to enhance the sensitivity. Insoluble products were accumulated by chemical and biocatalytic reactions triggered by the trapped label.22) Another approach is the combination of silver metalization connected with enzyme reaction with anodic stripping.23) Moreover, the system was improved in a galvanic cell with an anode for the oxidation of reducing agent generated by enzyme reaction and with a cathode for the deposition of silver.24)

A simple and highly-sensitive immunoassay platform was developed by combining the antigen-antibody recognition events on a poly(vinylpyridine) containing [osmium(4,4'-dimethyl-2,2'-pyridine)$_2$Cl]$^{2+}$ ([Os(bpy)$_2$Cl]$^{2+}$) and horseradish peroxide (Os/HRP polymer)25, which immobilized the electrodes.26, N$^1$,N$^{12}$, Diacetylspermine (DiAcSpm) was used as the model target to illustrate the proposed system. Urinary diacetylpolyamines serve as a reliable tumor marker in diagnosis. The urinary marker is extremely valuable because urine samples can be collected easily, freely and noninvasively from patients.

The electrode coated with thin layer of Os/HRP polymer was modified with acetylspermine (Fig. 6a). The layer was treated with the mixture of the anti-AcSpm antibody and target AcSpm molecules. The antibody captured to acetylspermine immobilized on the polymer which is unreacted to the AcSpm in the mixture was labeled with the anit-mouse IgG antibody conjugated with glucose oxidase (GOx) (Figs. 6b and 6c). The oxidized form of Os complex was produced by the enzyme reactions of HRP and GOx and accumulated in the polymer (Fig. 6d). Finally, accumulated [Os(bpy)$_2$Cl]$^{2+}$ was collectively reduced back to [Os(bpy)$_2$Cl]$^{+}$ at the electrode (Fig. 6e).

![Fig. 6](image-url) Procedure to form immuno-complexes on the polymer layer and the electrochemical detection of [Os(bpy)$_2$Cl]$^{2+}$ produced with enzyme reactions of GOx trapped by immuno-reaction and HRP in the polymer. Reprinted with permission from Ref. 26 (Copyright 2010, The Chemical Society of Japan)

This accumulation system of redox species allows for response enhancement compared to the response based on the continuous reduction of [Os(bpy)$_2$Cl]$^{+}$ generated by enzyme reactions.
The enhanced responses increased with decreasing DiAcSpm concentration, since the amount of unreacted anti-DiAcSpm antibodies and GOx-conjugated antibodies captured to the polymer by immuno-reactions increased with decreasing DiAcSpm concentration Therefore, we can quantify the target marker molecule by the magnitude of current responses.

**Dual signal amplification with redox cycling and coulometric signal transduction.** An electrochemical immunosensing method have been developed by applying an electrochemical signal amplification system that combines electrochemical-redox cycling coupled with accumulation of silver deposited on the electrode surface (Fig. 7).27) In this study, target molecule (carcinoembryonic antigen, CEA) was captured on the microparticles and labeled with hydrolase enzyme, β-galactosidase (β-gal). The oxidation of 4-aminophenol (PAP) generated from the labeled β-gal at the anode (W1) occurs concurrently with the reduction of silver ions and deposition of metal silver at the cathode, and hence converting and concentrating the signal material. Moreover, the comb of the IDA electrode (W2) is used to re-produce PAP from its oxidized form, quinoneimine (QI), facilitating coulometric signal transduction. The dual amplification system based on electrochemical-redox cycling and signal accumulation enables sensitive and facile determination of immunocomplexes by stripping measurements.

The detection limit of 10 nM obtained by the proposed method was more than two orders of magnitude lower than that obtained for the conventional voltammetric method. The sensitivity was improved by one order of magnitude by charge accumulation and a further order of magnitude by redox cycling. The sensitivity of the CEA assay using this combined technique for signal enhancement is one order of magnitude higher than that of the conventional enzyme linked-immunosorbent assay using microtiter plates. The detectable concentration is competitive with the other electrochemical signal amplification, e.g. an electrochemical metalloimmunoassay11,28). The accumulation of silver and electrochemical recycling of the enzyme-catalyzed product produce a highly amplified stripping signal, because the silver accumulated on the GC electrode was converted to an electrochemical response in a significantly short period. Therefore, the system presented here is a highly sensitive method for detecting immunorecognition events.

**Fig. 7.** Representation of the formation of immunocomplexes on the microparticles and the electrochemical detection of PAP produced by the enzyme reaction of β-gal trapped by immunoreaction. Reprinted with permission from Ref. 27 (Copyright 2012, Elsevier)

**Conclusions**

DEP has been proved to be a powerful tool for rapid and simple detection of analyte and discrimination of surface antigen expressed on cells. The DEP patterning with particles or cells allows to the rapid capturing by immuno-
recognition events with specific antibody immobilized on the surface. Moreover, unreacted particles and cells can be rapidly and simply removed from the accumulated position by controlling the direction of DEP force. Thus, immunoassay and cell binding assay can be rapidly and simply performed based on the manipulation of particles and cells by DEP.

Highly-sensitive immunosensing method has been developed by applying an electrochemical signal amplification system that combines electrochemical-redox cycling coupled with accumulation of silver deposited on the electrode surface. The recognition event was finally converted to metal silver on the electrode. The accumulated silver was converted to an electrochemical response in a significantly short period. Therefore, the system is a highly sensitive method for detecting immunorecognition events.

The combination of DEP manipulation and signal amplification system will be performed to achieve the rapid, simple and sensitive assay for the point-of-care testing.

Acknowledgements

The author would like to thank to Prof. Mizutani (University of Hyogo) and Prof. Matsue (Tohoku University) for their supports.

References