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Highly Sensitive Detection of N^1,N^{12}-Diacetylspermine Based on Electrochemical Charge Accumulation

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A novel immunosensor is presented based on a charge accumulation system using an electrode modified with a polymer containing [osmium(4,4′-dimethyl-2,2′-bipyridine)$_2$-chloride]$^{+/2+}$ and horseradish peroxidase (Os/HRP polymer) to detect N^1,N^{12}-diacetylspermine (DiAcSpm). Glucose oxidase (GOx)-labeled antibody captured with immunoreaction on Os/HRP polymer catalyzed the production of hydrogen peroxide, which oxidizes $[Os(bpy)_2Cl]^+$ in polymer with HRP. $[Os(bpy)_2Cl]^{2+}$ was gradually accumulated in the polymer and reduced back to $[Os(bpy)_2Cl]^+$ by applying -0.1 V. This accumulation system of redox species allows enhancement of the response.

The development of a rapid, simple, and sensitive detection system for biomarkers is of overriding importance to high-throughput screening of a disease at an early stage and monitoring the recurrence of the disease following medical treatment. Highly-sensitive electrochemical detection techniques have been focused on and developed to detect trace amounts of targets. 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.[1–5]

Recently, novel amplification methods were proposed based on signal source storage on the electrode surface. Insoluble products were accumulated by chemical and biocatalytic reactions triggered by the trapped label.[6–10] Another approach is the combination of silver metalization connected with enzyme reaction with anodic stripping.[11–13] 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.[14]

In a previous contribution, we reported the development of a sensitive detection system for hydrogen peroxide with charge accumulation using an electrode modified with a poly(vinylpyridine) containing [osmium(4,4′-dimethyl-2,2′-bipyridine)$_2$-chloride]$^{+/2+}$ ($[Os(bpy)_2Cl]^{+/2+}$) and horseradish peroxide (Os/HRP polymer),[15] which was developed by Heller's group.[16,17] The $[Os(bpy)_2Cl]^{2+}$ generated by the enzyme reaction of HRP in the presence of hydrogen peroxide is accumulated in the polymer, and the charge for the accumulated $[Os(bpy)_2Cl]^{2+}$ measured by potential stepping is enhanced to give detection limit as low as <1 nM. Most recently, we applied this method to a competitive immunosensing for insulin using glucose oxidase (GOx) as a labeled enzyme.[18] However, immuno-complexes were formed to measure antigen at a place other than the electrode modified with Os/HRP polymer. In this report, we describe the development of a simple and highly-sensitive immunoassay platform coupling the antigen–antibody recognition events on Os/HRP polymer. N^1,N^{12}-Diacetylspermine (DiAcSpm) was chosen as the model target to illustrate the proposed system. Urinary diacetylpolyamines serve as a reliable tumor marker in diagnosis.[19] The urinary marker is extremely valuable because urine samples can be collected easily, freely, and noninvasively from patients.

Figure 1 illustrates the procedure to form immuno-complexes on the polymer layer and the electrochemical detection of the $[Os(bpy)_2Cl]^{2+}$ produced with enzyme reactions of GOx trapped by immunoreaction and HRP in the polymer. An aliquot of Os/HRP polymer (Bioanalytical Systems) (1.5 μL) was placed on indium–tin–oxide (ITO, Sanyo Vacuum Industries Co., Ltd.) electrodes (diameter, 1.6 mm). The surface concentration of the osmium complexes was determined from electric charges in cyclic voltammetry and found to be 15.8 ± 0.8 nmol cm^{-2}. Os/HRP polymer layer was treated with 0.1 M phosphate buffer saline (PBS, pH 7.0) containing 2.0 mM *N*-acetylspermine hydrochloride (AcSpm), 2.5% (v/v) glutaraldehyde (GA) and 1.0 mg mL^{-1} casein for 60 min (Figure 1a). GA was used as a cross-linker in order to immobilize AcSpm and casein, acting as both a competitor for immuno-sensing and blocking agent to prevent nonspecific binding of antibodies, respectively. 0.1 μg mL^{-1} mouse monoclonal anti-DiAcSpm antibody was added to various concentrations of N^1,N^{12}-diacetylspermine hydrochloride (DiAcSpm) and reacted for 40 min. The polymer layer was then treated with the mixture for 20 min to capture unreacted

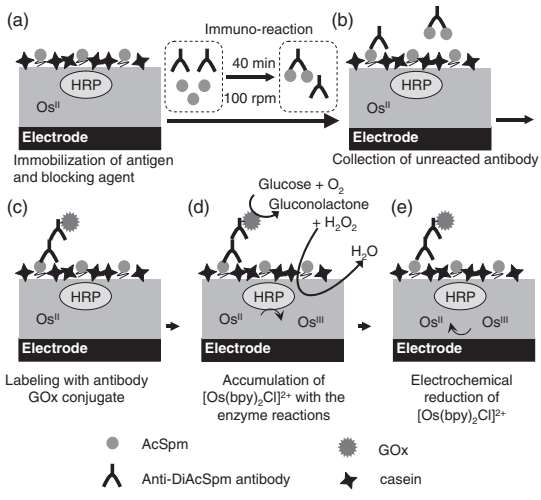

Figure 1. Procedure to form immuno-complexes on the polymer layer and the electrochemical detection of $[Os(bpy)_2Cl]^{2+}$ produced with enzyme reactions of GOx trapped by immunoreaction and HRP in the polymer.

anti-DiAcSpm antibodies (Figure 1b). Anti-DiAcSpm antibody was prepared by a previously reported method.[20] After washing with PBS, the polymer layer was treated with 1.0% solution of the anti-mouse IgG GOx conjugate for 20 min (Figure 1c).

Electrochemical measurements were carried out using a potentiostat (HZ-5000, Hokuto Denko Corp.). An Ag/AgCl electrode and platinum wire were used as a reference electrode and an auxiliary electrode, respectively. We applied −0.1 V to ITO in order to reduce the osmium complexes. The polymer layer was immersed in PBS with 10 mM glucose under open-circuit conditions for 5 min (Figure 1d). ITO electrode potentials were stepped to −0.1 V from the open-circuit condition to reduce $[Os(bpy)_2Cl]^{2+}$ accumulated by enzymatic reaction of HRP and labeled GOx (Figure 1e). Electric charges were obtained from reduction current responses up to 5 s after application of the potential.

The immunoassay components yielded the accumulations of $[Os(bpy)_2Cl]^{2+}$ and the responses of the reduction current in the Os/HRP polymer. Hydrogen peroxide formed by the enzyme reaction of GOx diffused to HRP to oxidize $[Os(bpy)_2Cl]^{+}$. $[Os(bpy)_2Cl]^{2+}$ generated by GOx and HRP gradually accumulated during the residence time of the glucose solution. Finally, accumulated $[Os(bpy)_2Cl]^{2+}$ was collectively reduced back to $[Os(bpy)_2Cl]^{+}$ at the electrode by applying −0.1 V. This accumulation system of redox species allows for response enhancement compared to the response based on the continuous reduction of $[Os(bpy)_2Cl]^{+}$ 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 immunoreactions increased with decreasing DiAcSpm concentration, resulting in an increase of electrochemical response. Therefore, we can quantify the target marker molecule by the magnitude of current responses.

DiAcSpm detection was demonstrated using the charge accumulation system described above. Figure 2A shows examples of reduction current responses obtained after the potential was stepped to −0.1 V. The spike-like responses of the capacitive currents were observed immediately after the potential step; subsequently, reduction currents decreased gradually to almost zero in 5 s. The charges passed up to 5 s were used as responses for the calibration curve.

Figure 2B shows the plot of charges obtained from reduction currents as a function of the concentration of DiAcSpm. The response decreased with increasing concentrations of DiAcSpm. Good correlation between DiAcSpm concentration and charge response was obtained in the range 1.0 pM–1.0 nM DiAcSpm. The charge accumulation technique is 2 orders of magnitude more sensitive than conventional enzyme linked-immunosorbent assay (ELISA) using microtiter plates. However, a high background signal was observed for 10 nM DiAcSpm. It is suggested that GOx-labeled antibody adsorbed on Os/HRP polymer nonspecifically. However, the sensitivity of the present assay protocol is 2–3 orders of magnitude higher than that required for the concentration level in urine samples. The surface modification of the Os/HRP polymer on the electrode provides a sensitive and simple detection platform for competitive immunoassay. For reducing the total assay time, we will combine this accumulation technique with the particle manipulation based on the dielectrophoresis.[21] We will also attempt to detect DiAcSpm and creatinine simultaneously by combining the highly sensitive charge accumulation system and an enzyme biosensor incorporated with multienzymes to correct target concentration by urinary creatinine concentrations.

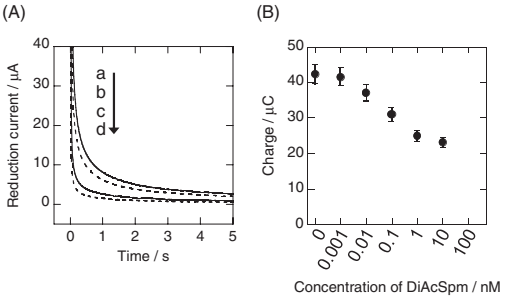

Figure 2. (A) Reduction current responses obtained after stepping the voltage to −0.1 V. Concentration of added DiAcSpm, (a) 0, (b) 0.01, (c) 1, and (d) 10 ng mL^{-1}. (B) Calibration curves for DiAcSpm by using 0.1 μg mL^{-1} anti-DiAcSpm antibody.

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