Development of Highly-sensitive Electrochemical Biosensors

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Abstract

This article describes the development of biosensors with electrochemically-amplified responses. In general, chemical amplification involves a reaction sequence for a substrate to generate a relatively large amount of the product. Thus a trace concentration of analyte can be caused to yield orders of magnitude higher product concentration which may be more easily be measured than the analyte itself. For electrochemical biosensors that detect biochemical reactions on the electrode surfaces, chemical amplification procedures suitable for concentrating the electroactive species near the electrode/test solution interface should be utilized to enhance the sensor response effectively. Thus the use of combination of bio-electrochemical processes such as catalytic reaction to regenerate electroactive species and adsorptive stripping procedure to accumulate electroactive species on electrode surfaces can be useful for realizing highly-sensitive biosensors. Actually, immunoassay protocol combined with such electrochemical amplification techniques has enabled us to determine biomarkers with picomolar-levels. Recently we have successfully applied particle manipulation based on dielectrophoresis to develop rapid and separation-free immunoensing systems. The union of highly-sensitive electrochemical detection and dielectrophoretic particle manipulation (or accumulation) would provide biosensor systems suitable for the purpose of the point-of-care testing of biomarkers.

Keywords: electrochemical biosensor, chemical amplification, dielectrophoresis, biomarkers

Introduction

Analytical devises combining the high specificity of biochemical reaction with the simplicity of electrochemical signal transduction have attracted increasing interest. The market of the electrochemical biosensor for self-monitoring blood glucose has been expected to reach 1 trillion yen in the world [1], which clearly demonstrate the usefulness of biosensor technologies and stimulated us to develop electrochemical biosensors for the point-of-care testing (POCT) of various biomarkers to diagnose diseases such as cancers and heart failures. For example, there exist 1 million Japanese with severe congestive heart failure (CHF).
A useful information for the diagnosis of CHF can be given by determining a cardiac biomarker, B-type natriuretic peptide (BNP), in blood [2]. However, the concentrations of such biomarkers are usually extremely low: the normal blood BNP concentrations are picomolar levels.

The lower detection limit is, of course, determined by the signal-to-noise ratio in measuring the analyte. For the simple and inexpensive POCT sensors, the incorporation of sufficient noise reduction system is usually difficult. Therefore, methods for enhancing signal output should be employed so as to measure extremely low concentration of analyte with simple POCT sensors. Chemical amplification is a suitable way for increasing signal levels. In general, chemical amplification involves a reaction sequence that produces a relatively large amount of product from a substrate [3]. Hence a trace concentration of analyte can give order of magnitude higher concentration of product that may be more easily be measured than the analyte itself.

For electrochemical biosensors to detect biochemical reactions on the electrode surfaces, chemical amplification procedures that concentrate the product at the electrode/test solution interface should be utilized for enhancing the sensor response effectively. Previous works teach us that some electrochemical processes such as catalytic reaction to regenerate electroactive species, and adsorptive stripping procedure to accumulate electroactive species on electrode surfaces seem to be suitable for the purpose [4]. Thus we have developed bio-electrochemical amplification procedures based on catalytic reactions at the enzyme-modified electrodes [5-7] and also on the accumulation of biochemical reaction products on the electrode surfaces [8-10], for realizing highly-sensitive biosensors. Thus we have successfully determined A-type natriuretic peptide (ANP), a cardiac marker, at picomolar level by utilizing a catalytic reaction system [11], and BNP at the same concentration level based on the accumulation/detection procedure [12].

This article describes our research results for preparing biosensors with chemically amplified responses for that provide simple and highly-sensitive assays [13,14].

**Catalytic reactions by using a couple of enzymes**

By using an appropriate enzyme couple, we can cast an analyte into a cycling role to cause a relatively large concentration change of measureable species:

\[
\text{enzyme 1} \quad \text{enzyme 2} \\
S + R_1 \rightarrow P + P_1; \quad P + R_2 \rightarrow S + P_2
\]

If only enzyme 1 is used for the determination of the substrate S, the concentration change of the reactant R_1 or the product P_1 is stoichiometrically limited by the concentration of S. On the other hand, the additional use of enzyme 2 allows the enhanced concentration change of R_1 or P_1 beyond the stoichiometric limitation. An L-lactate-sensing electrode with the detection limit of 1 nM could be prepared by using a Clark-type oxygen electrode with a bi-enzyme layer containing lactate oxidase to oxidize the analyte with consumption of oxygen and lactate dehydrogenase to regenerate the analyte in the presence of NADH. The current change for L-lactic acid was amplified ca. 200 times by introducing the LDH-catalyzed analyte regeneration [6]. The determination of NAD(P)H in nanomolar level could be achieved by using the dehydrogenase/NAD(P)H oxidase-couple [15,16]. The high selectivity of enzyme is useful for casting the analyte into the cyclic reaction sequence without the loss by the side reactions.

**Electrocatalytic reactions using a single enzyme**

The use of an electrochemical reaction for replacing an enzymatic reaction results in a single enzyme-based system with electrochemical current...
measurement and enzymatic analyte regeneration. We have determined catechols by using a glucose oxidase (GOD)-modified electrode [7]:

\[
\text{catechol} \rightarrow o\text{-quinone} + 2\text{H}^+ + 2e
\]

(electrochemical reaction)

\[
o\text{-quinone} + \text{glucose} \rightarrow \text{catechol} + \text{gluconolactone} \text{ (enzymatic reaction)}
\]

The regeneration of the analyte by the GOD-catalyzed reaction provided an amplified response (amplification factor, 60) for catechol (and dopamine).

For the electrochemical determination of dopamine in biological samples, there is a serious problem with interference by oxidizable species, such as L-ascorbic acid and uric acid, coexisted in the samples. This could be solved by performing the catalytic reactions in a rapid coulometric cell developed by Uchiyama et al. [17] using a porous carbon felt electrode impregnated with a GOD-containing electrolytic solution. The current for oxidizing the interferants were diminished within 1 min after the addition of sample because they were consumed through the electrooxidation. On the other hand, the current for oxidizing dopamine remained unchanged owing to the regeneration by the enzyme. From the steady-state current obtained 1 min after the addition of the sample, the dopamine concentration (> 5 nM) could exactly be determined even when a high concentration (ca. 1 μM) of L-ascorbic acid (and uric acid) was coexisted [18,19].

The application of catalytic procedure to various biosensing systems

By introducing a catalytic procedure into an enzyme immunoassay protocol, we have determined ANP in picomolar level [11] with alkaline phosphatase-labeled anti-ANP antibody (ALP-Ab), as illustrated in Fig. 1. A certain amount of ANP-containing sample was added to an ALP-Ab solution to undergo the immunological reaction. Then an ANP-modified gold disc was dipped into the immune-reaction mixture in order to attach unreacted ALP-Ab on the disc. The disc with ALP-Ab was soaked in a p-aminophenylphosphate (PAPP) solution to generate p-aminophenol (PAP) through the enzymatic reaction. PAP was oxidized on the GOD-modified electrode but regenerated through the GOD-catalyzed reaction, which resulted in the highly-sensitive determination of the ALP activity on the disc. As the result, tiny amount of ANP could be determined: the detection limit of ANP, 1.5 pM, was low enough for the determination of blood ANP levels.

Another type of the application of catalytic system was the determination of DNA. A redox-active ligand for DNA, methylene blue (MB), has been employed with a peroxidase (HRP) -modified electrode [20]. MB is reduced on the electrode but regenerated by HRP-catalyzed reaction in the presence of hydrogen peroxide. The current response for MB was remarkably reduced by the complexation with DNA: the steric hindrance of MB in the complexes (and the lowering of the diffusion coefficient after the complexation) brought about the decreases in both electrochemical and enzymatic reaction rates. The amplification of the electrode response for MB resulted in the highly-sensitive measurement of the
decrease of the both reaction rates by the complexation with DNA, and that of the DNA concentration. The detection limit of DNA (from salmon testes) was 10 ng/mL. Such a simple and sensitive method would be useful for monitoring biopharmaceutical production process using continuous cell lines [21].

The similar methodology could be applied to the detection of lipopolysaccharide (LPS) by using a ferrocene-attached ligand for LPS, a ferrocene boronic acid derivative [22] (Fig. 2) or a ferrocene-attached polymixin B [23].

**Accumulation of the enzymatic reaction product**

When a chemical species is readily accumulated on the electrode surface, it can be measured electrochemically with enhanced sensitivity. Thiols are chemisorbed on metal (such as, gold, silver and copper) electrodes and desorbed through one-electron path [24]. Thus the chemisorption (accumulation)/desorption (detection) process can be applied to the highly-sensitive determination of thiols and thiol-producing enzymes (Fig. 3). Actually, 6-mercaptopurin [9], a drug of antimetabolite, and acetylcholinesterase (AChE) [25,26] that produced thiocholine through the hydrolysis of acethythiocholine, could be determined with the detection limits of 10 nM and 10 mU/L, respectively. The procedure for measuring the AChE activity has successfully been applied to immunoassay systems by employing AChE as a marker enzyme: BNP from 15 to 150 pM could be determined within an hour [12, 27].

More sensitive and rapid determination of BNP has been achieved by the combination of the accumulation-based assay with a microfluidic system. BNP as low as 1.5 pM could be detected within 30 min by using a microfluidic device [26]. In general, (bio-)chemical reactions occurred on substrate surfaces proceed quickly in miniaturized systems, since a small diffusion length and a high ratio of the surface area to sample volume in the system readily increase in the number of collision between molecules in the sample solution and the surface [29].

The accumulation-based AChE assay system was applied to the detection of organophosphorus pesticides [30]. The inhibition of AChE activity by organophosphorus pesticides (diazinon and ethylthiometon) was monitored from the desorption current of thiocholine.

**Accumulation of [Os(bpy)$_2$]$_3$$^{3+}$ on enzyme/polymer coated electrode**

Serum samples contain cholinesterase (ChE; acylcholine acylhydrolase) with high activities (ca. 400 U/L). Since ChE also catalyzes the hydrolysis of acethythiocholine, the nonspecific adsorption of serum ChE in the thiocholine-based assay device produce of excessive thiocholine moieties from acethythiocholine to cause a serious error for measuring the analyte. Hence we have used heat-treated, ChE-inactivated serum as the sample for the exact determination of BNP [28].

To avoid such a troublesome pretreatment of the
sample, the use an enzyme modifier whose activity in the sample is extremely low is a suitable approach. Thus we have begun to use GOD as the modifier for antibodies. GOD is not contained in human body and shows high stability and specific activity.

For the sensitive measurement of hydrogen peroxide, the product of GOD-catalyzed reaction, poly(vinylpyridine) containing $[\text{Os(bpy)}_2]^2+$ and HRP (Os$^{III}$/HRP-polymer) was employed. The polymer has widely been used for the amperometric determination of hydrogen peroxide from 0.1 to 100 μM [31]. On the other hand, Iwasaki et al. [32] has applied the polymer for the detection of enzymatic reactions with a surface plasmon resonance (SPR) sensor: $[\text{Os(bpy)}_2]^2+$ in the polymer was oxidized by hydrogen peroxide to accumulate $[\text{Os(bpy)}_2]^3+$, which brought about some SPR signal change. Although the sensitivity of the SPR detection method for hydrogen peroxide was low (detection limit, 0.1 mM), the application of coulometric detection of the $[\text{Os(bpy)}_2]^3+$ accumulated was considered to be carried out with much enhanced sensitivity. A glassy carbon electrode coated with Os$^{III}$/HRP-polymer was immersed in a test solution containing hydrogen peroxide (or glucose and GOD) for 10 min under the open circuit condition, then the reduction of the $[\text{Os(bpy)}_2]^3+$ accumulated was conducted by applying the potential of −0.1 V vs. Ag/AgCl. The detection limit for the hydrogen peroxide concentration and GOD activity were 0.1 nM and 0.1 mU/L, respectively [10]. We have applied the polymer-based $[\text{Os(bpy)}_2]^3+$-accumulation method for the determination of insulin, a peptide hormone to control blood glucose levels, at pM levels [33].

$N^1,N^2$-Diacetylspermine, an urinary tumor marker, could also be determined by applying an Os$^{III}$/HRP-polymer system to an immunoassay protocol with the detection limit of 1 pM. This enabled the simultaneous measurement of the analyte and creatinine in diluted urin samples by using a dual sensor system [34].

**Dual amplification system**

Recently we have prepared an immunosensing system for carcinoembryonic antigen (CEA), a tumor marker, by applying a dual-amplification technique consisting of an electrochemical redox cycling and coulometric signal transduction [35]. 4-Aminophenol produced through the β-galactosidase-catalyzed reaction was oxidized on a comb of an interdigitated array (IDA) electrode by the coupled reduction of silver ions at a glassy carbon electrode. Another comb of the IDA electrode was used to regenerate 4-aminophenol. The deposited silver was converted to a signal by anodic stripping voltammetry (Fig. 3). The detection limit of CEA was 10 pg/mL.

**Towards the realization of rapid, simple and highly-sensitive immunosensing systems**

We have recently developed rapid and simple immunosensing methods by applying dielectrophoretic (DEP) manipulation of microparticles.
For example, the assay of alpha-fetoprotein (AFP), a tumor maker, was carried out as follows [36]. Microparticles modified with an anti-AFP antibody that had introduced in a microfluidic cell were accumulated on designated areas of the cell modified with the secondary antibody molecules within 1 min by DEP upon the application of AC voltage upon an IDA electrode placed in the microfluidic cell. The presence of AFP permitted the irreversible capture of microparticles via the formation of immunocomplex. On the other hand, uncaptured microparticles redispersed after switching off of the AC voltage. The signal from the irreversibly captured microparticles (fluorescent light in this case) allowed us to determine the concentration of AFP.

The rapid manipulation of microparticles was useful to shorten the assay time (within 5 min), and the separation of captured and uncaptured particles could be achieved only by switching off the AC voltage, which significantly simplify the immunoassay procedure. Furthermore, the accumulation of microparticles was effective for enhancing the sensitivity in the analyte determination. The range for AFP assays was 0.1-100 mg/mL, which was sufficient to cover the concentration range required for the medical use.

The DEP techniques combined with immunoreactions could successfully be applied to the recognition of the surface antigens of living cells [38,39].

The introduction of chemical amplification techniques to the DEP-based sensing system would provide simple, rapid and highly-sensitive biosensing systems. Studies for the realization of such systems are now in progress.

Conclusions
The combination of chemical amplification methods with biosensing technologies have proved to be suitable approach for preparing simple and highly-sensitive biosensing systems involving immunoassay systems for detecting biomarkers.

DEP manipulation of microparticles has provided an excellent technique for the accumulation and separation of the particles. The combination of DPE manipulation of microparticles with appropriate biofunction and chemically-amplified signal transduction would be useful for realizing rapid, simple and highly-sensitive POCT systems.

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References