Determination of Hydrogen Peroxide Based on the Charge Accumulation and Electrochemical Reduction at an Osmium Complex/Peroxidase-coated Electrode

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Enhanced sensitivity of hydrogen peroxide was performed by accumulating the charge through oxidation of $[Os(bpy)_2]^{2+}$ (bpy = bipyridyl) into $[Os(bpy)]^{3+}$ in a peroxidase-containing polymer layer on an electrode under the open-circuit condition and then reduced by applying negative potential. The accumulation resulted in a large current response, and the lower detection limit for hydrogen peroxide and that for glucose oxidase, a hydrogen peroxide-producing enzyme, were 0.1 nM and 2×10^{-4} U L^{-1}, respectively.

The simple and sensitive determination of hydrogen peroxide is essential for the realization of oxidase-based biosensors and, especially of oxidase-labeled immunoassay system. Poly(vinylpyridine) containing $[Os(bpy)_2]^{2+}$ and horseradish peroxidase (Os^{II}/HRP-polymer) has been widely used for the amperometric determination of hydrogen peroxide in the concentration range from 10^{-7} to 10^{-4} M.[1]

Iwasaki et al.[2] have applied the Os^{II}/HRP-polymer to detect enzymatic reactions by using a surface plasmon resonance (SPR) sensor system: $[Os(bpy)_2]^{2+}$ in the polymer was oxidized by hydrogen peroxide in a test solution to produce $[Os(bpy)_2]^{3+}$, which followed the transport of counter anion from the solution into the polymer layer. The anion accumulated was detected by the layer-attached SPR sensor.[2,3] The SPR-based detection system is of interest because it can provides the real time visualization of the two-dimensional concentration distribution for enzymatically produced hydrogen peroxide.[3] However, the sensitivity for the hydrogen peroxide detection was rather low (detection limit: ca. 10^{-4} M), owing to the small change in the refractive index of the layer upon the incorporation of the anionic species.

On the other hand, the accumulation process is inherently advantageous for increasing the sensitivity for the analyte.[4] Instead of the insensitive SPR measurement of the counter anion, we have coulometrically determined the $[Os(bpy)_2]^{3+}$ accumulated, i.e., an Os^{II}/HRP-polymer-coated electrode was immersed in a test solution containing hydrogen peroxide under the open circuit condition for several minutes, then the reduction of the $[Os(bpy)_2]^{3+}$ on the electrode surface was conducted by applying a negative potential. We have successfully determined hydrogen peroxide at sub-nanomolar levels.

A glassy carbon electrode of 3.0-mm diameter (Bioanalytical Systems, West Lafyette, IN) was polished with aqueous slurry containing successively finer aluminum powder (down to 1 μm) and sonicated for 2 min in water. An aqueous solution of Os^{II}/HRP-polymer (Bioanalytical Systems) was placed on the electrode and the electrode was allowed to dry for 24 h. The surface concentration of the osmium complex on the electrode, which could easily be determined electrochemically, was ca. 1.74×10^{-9} mol cm^{-2}. Potential-step chronoamperometric (PSCA) measurements were carried out by using an electrochemical analyzer (HZ-5000; Hokuto Denko, Tokyo). The Os^{II}/HRP-polymer-coated electrode, an Ag/AgCl electrode (saturated with KCl) and a platinum wire were employed as the working, reference and auxiliary electrodes, respectively. The test solution used was 0.1 M phosphate buffer (pH 7.0, 25 ± 1 °C). The Os^{II}/HRP-polymer-coated electrode was immersed in the buffer solution containing hydrogen peroxide for 10 min, where the electrode was set at the open-circuit condition (potential, ca. 0.26 V vs. Ag/AgCl) and the solution was stirred with a magnetic bar. Then the stirring of the solution was stopped and the electrode potential was set at −0.1 V vs. Ag/AgCl to reduce the $[Os(bpy)_2]^{3+}$ accumulated during the above immersing period. The charge passed for the reduction of $[Os(bpy)_2]^{3+}$, which was measured by subtracting the charge obtained for the solution without hydrogen peroxide from that for the solution containing the analyte, was recorded.

Figure 1 shows the current–time curves for the potential step to −0.1 V vs. Ag/AgCl obtained after soaking the solutions containing different concentrations of hydrogen peroxide. In association with the increase in the hydrogen peroxide concentration, the cathodic current increased. A linear relationship was obtained between the charge for the reduction of $[Os(bpy)_2]^{3+}$ and the hydrogen peroxide concentration up to 20 nM (Figure 2). The charge increased upon the addition of 20 nM

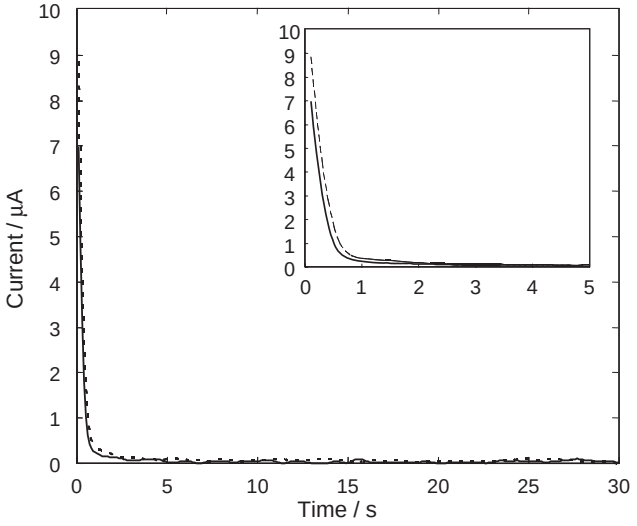

Figure 1. Current–time curves for the potential step from open-circuit potential to −0.1 V vs. Ag/AgCl after soaking 0.1 M phosphate buffer (pH 7.0) solutions containing 10 (—) and 20 (- - -) nM hydrogen peroxide.

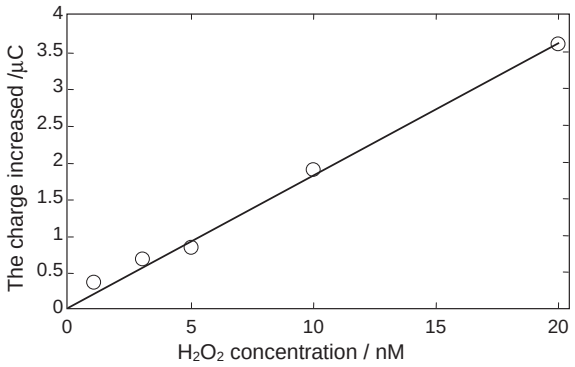

Figure 2. The relationship between the charge increased upon the addition of the hydrogen peroxide and the hydrogen peroxide concentration.

hydrogen peroxide was ca. 3.6 μC, indicating that about 60% of $[Os(bpy)_2]^{2+}$ on the electrode surface was oxidized during the incubation period. This means that the rate for the oxidation of $[Os(bpy)_2]^{2+}$ is independent of its surface concentration in rather wide range, which may be resulted from a low Michaelis constant of the enzyme for $[Os(bpy)_2]^{2+}$. Iwasaki et al.[2] have also obtained the similar result on their SPR sensor responses. The calibration graph was not linear for >20 nM. However, our method is particularly advantageous to detect trace amount of hydrogen peroxide, and it will usually used when rather small amount of $[Os(bpy)_2]^{2+}$ is oxidized with the analyte.

The relative standard deviation for 5 measurements of 2.5 nM hydrogen peroxide was 9.3%. The detection limit, 0.1 nM (signal-to-noise ratio = 3), was lower by three orders of magnitude than that obtained for conventional amperometric method using the same polymer-coated electrode.[1] The dynamic range for the determination of the hydrogen peroxide concentration can be shifted by varying the soaking time into the analyte-containing solution. When the soaking time was shorter (e.g. 2.5 min), the hydrogen peroxide with higher concentration range (up to 40 nM with the detection limit of 0.4 nM) could be measured.

The present method for measuring hydrogen peroxide was applied to the determination of glucose oxidase (GOD, EC. 1.1.3.4). In this case, the Os^{II}/HRP-polymer-coated electrode was immersed in the buffer solution containing glucose (10 mM) and GOD for 10 min. The current response for the reduction of $[Os(bpy)_2]^{3+}$ was proportional to the GOD activity up to 2×10^{-2} U L^{-1} with the detection limit being 2×10^{-4} U L^{-1}. The charge for the reduction of $[Os(bpy)_2]^{3+}$ obtained for 5×10^{-4} U L^{-1} GOD was 0.431 μC, which almost coincided with the charge obtained upon the addition of 2.5 nM hydrogen peroxide, 0.440 μC. This is reasonable because the GOD activity of 5×10^{-4} U L^{-1} corresponds to the catalytic activity to produce hydrogen peroxide with the rate of 0.5 nM min^{-1}, and the average concentration of hydrogen peroxide in the enzymatic reaction mixture for soaking time of 10 min is 2.5 nM.

The stability of the Os^{II}/HRP-polymer-coated electrode was examined by determining 10 nM hydrogen peroxide 5 times a day each day for two weeks. The average value of the electrode response for the 5 measurement did not decrease for 10 days. The electrode responses decreased after 10 days, but they are still usable until the end of two weeks. The charge response on the 14th day was ca. 70% of the initial value. The Os^{II}/HRP-polymer-coated electrode thus showed an adequate stability.

The present method for measuring the GOD activity was 1000 times more sensitive than conventional fluorometric[5] and electrochemical[6] methods. Such a high sensitivity could be obtained in a standard solution containing glucose and a tiny amount of GOD. The addition of real samples (e.g., blood) may cause the adsorption of protein molecules on the electrode surface, which follows the change of non-faradaic current at the Os^{II}/HRP-polymer-coated electrode. This would cause a serious error for determining the hydrogen peroxide concentration. However, by applying an appropriate enzyme immunoassay procedure,[4] we can prevent the sample solution from flowing over the sensing electrode surface so as to be able to determine the enzyme activity in a simple substrate-containing buffer system. The preparation of GOD-labeled immunoassay system for insulin based on the proposed sensitive and simple measuring procedure is now in progress.

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