Carbon Felt-based Bioelectrocatalytic Flow Detectors: Highly Sensitive Amperometric Determination of Hydrogen Peroxide Using Adsorbed Peroxidase and Thionine

Yasushi HASEBE,[†] Ryosuke IMAI, Masaki HIRONO, and Shunichi UCHIYAMA

*Department of Applied Chemistry, Faculty of Engineering, Saitama Institute of Technology,
1690 Fusaiji, Fukaya, Saitama 369-0293, Japan*

Horseshoe peroxidase (HRP) and thionine (TN) were co-adsorbed onto a porous carbon felt (CF), and the resulting HRP and TN-adsorbed CF (HRP-TN-CF) was successfully used as a working electrode unit of a novel bioelectrocatalytic flow detector for a highly sensitive amperometric determination of hydrogen peroxide (H_2O_2). Co-adsorbed TN was essential to enhance the cathodic peak current of H_2O_2 , and the current responses of the HRP-TN-CF-based detector were much larger than those of the HRP-CF-based detector (without TN). When air-saturated 0.1 M phosphate buffer (pH 7.0) was used as a carrier at a flow rate of 3.9 ml/min, cathodic peak currents of H_2O_2 (sample injection volume, 200 μl) obtained at an applied potential of 0 V (vs. Ag/AgCl) increased linearly up to 50 μM with a detection limit of 0.1 μM . Repetitive 100 sample injection of 100 μM H_2O_2 induced no serious current decrease, and RSD was 0.41 to 1.21% ($n = 100$). The HRP-TN-CF retained 42% of its original activity after 8 days of storage in 0.1 M phosphate buffer at 4°C.

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Introduction

A three-dimensional porous macro-electrode is useful for a working electrode unit in electrochemical flow detectors.¹⁻⁵ A low diffusion barrier of porous electrode facilitates mass transport, and a high surface area enables a higher electrolytic efficiency, leading to a high sensitivity. Up to now, a reticulated vitreous carbon (RVC) has been successfully introduced to an electrochemical detector unit in a flow-injection analysis (FIA) system.¹⁻⁵

On the other hand, a carbon felt (CF) is a microelectrode ensemble of micro carbon fiber (*ca.* 7 μm diameter), and possesses a random three-dimensional structure. The CF has an extremely high surface area (estimated to be 0.3–30 $\text{m}^2 \text{g}^{-1}$), and shows a high conductivity and electrolytic efficiency.⁶⁻¹¹

As compared with RVC, the CF has the following advantages: 1) inexpensive; 2) physically stable; 3) easy handling; and 4) can be easily manufactured to arbitrary shapes. Thus, the CF is another attractive candidate for constructing an electrochemical flow-detector. From this context, we recently reported on a CF-based electrochemical flow detector for the amperometric and coulometric determination of NADH by using thionine-adsorbed CF.¹²

In general, immobilized enzyme electrodes are powerful and convenient tools for the highly selective detection of target species. Thus, enzyme-immobilized CF would allow the highly selective flow-detection of enzyme substrates. The main objective of this study was to construct a CF-based bioelectrocatalytic flow detector using enzyme-modified CF.

In this study, we selected horseradish peroxidase (HRP) as a

model enzyme, and the HRP and its electron-transfer mediator, thionine (TN), were co-adsorbed onto the CF surface simultaneously from a mixed solution containing HRP and TN.

Up to now, several HRP-based H_2O_2 -sensing electrodes have been fabricated by using TN as an electron-transfer mediator.¹³⁻¹⁵ In these cases, suitable coupling agents were employed for co-immobilization of the HRP and the TN on the electrode surface.¹³⁻¹⁵ In a previous study, we reported that the TN strongly adsorbed onto the CF surface, and that the adsorbed TN exhibited an efficient electrocatalytic activity for the oxidation of NADH.¹²

In this study, we succeeded to fabricate a highly sensitive bioelectrocatalytic flow detector for H_2O_2 by the simultaneous adsorption of the HRP and the TN onto the CF. Co-adsorbed TN was essential to obtain highly sensitive cathodic peak current responses of H_2O_2 . When 0.1 M phosphate buffer (pH 7.0) was used as a carrier at a flow rate of 3.9 ml/min, this HRP-TN-CF-based flow detector showed reproducible cathodic peak currents of H_2O_2 in the concentration range from 0.3 to 100 μM at an applied potential of 0 V vs. Ag/AgCl. The RSD was 0.41 to 1.21% in 100 successive assays at 100 μM . This would be a first example on the enzyme-modified CF-based bioelectrocatalytic flow detector, easily prepared by the co-adsorption of a redox enzyme and an electron mediator without any coupling reagent, surface treatment or special immobilization matrix.

Experimental

Reagents and chemicals

Horseshoe peroxidase (HRP, EC 1.11.1.7., 100 > units mg^{-1}) and 30% hydrogen peroxide were purchased from Wako Pure Chemicals, and used as received. Thionine chloride (TN;

[†] To whom correspondence should be addressed.
E-mail: hasebe@sit.ac.jp

Fig. 1 (A) Schematic illustration of CF-based flow analysis system. P, Pump; S, sample injector (injection volume, 20 or 200 μ l); D, CF-based bioelectrocatalytic detector; EA, electrochemical analyzer; W, waste. (B) Sectional view of the CF-based flow detector. a, O-ring; b, electrode holder; c, Ag/AgCl reference electrode; d, PTFE tube; e, joint; f, silicon spacer; g, auxiliary electrode (Pt wire, 1 mm diameter); h, HRP-TN-CF; i, Pt lead wire (0.5 mm diameter).

Lauth's Violet) was purchased from Tokyo Kasei Kogyo. A carbon felt (CF; GF-20-3F which was prepared by pyrolysis of polyacrylonitrile at 2000°C) was obtained from Nippon Carbon Ltd. All other chemicals were of the highest grade available. A phosphate buffer (0.1 M, pH 7.0) was used for the carrier solution. The standard solution of H_2O_2 was prepared daily by the dilution of 30% H_2O_2 solution with a buffer used as a carrier. All of the solutions were prepared with double-distilled water.

Preparation of HRP and TN co-adsorbed CF

The CF was cut into a certain size ($10 \times 3 \times 3$ mm; density, 1.4 g/cm³; porosity, more than 90%), and was used as an immobilization matrix of the HRP and the TN. Prior to the modification protocol, the CF was thoroughly washed with distilled water by ultra-sonication, and was dried at 120°C for 12 h. Next, the CF was immersed in a mixed aqueous solution containing HRP (0.1 mg/ml) and TN (0.5 mM) for 24 h at room temperature under a dark condition. For control experiments, HRP-adsorbed CF (HRP-CF) and TN-adsorbed CF (TN-CF) were separately prepared in a similar manner. Each CF was thoroughly washed with water and a buffer solution, and was set in the electrochemical flow detector. When not in use, the HRP-TN-CF was stored in 0.1 M phosphate buffer (pH 7.0) at 4°C in a refrigerator.

To estimate a surface density of an adsorbed TN on the HRP-

Fig. 2 Dependency of the applied potential on the peak-currents of 100 μ M H_2O_2 by using the HRP-TN-CF (●), HRP-CF (□). An air-saturated 0.1 M phosphate buffer (pH 7.0) was used as a carrier. The carrier flow rate was 3.9 ml/min. The injection volume of H_2O_2 was 200 μ l.

TN-CF, cyclic voltammetry (CV) was carried out in 15 ml of deoxygenated phosphate buffer (0.1 M, pH 7.0) with a conventional three-electrode system. The potential scan rate was 5 mV/s. Nitrogen gas was introduced into the buffer for at least 30 min to remove dissolved oxygen. The HRP-TN-CF (with platinum lead wire), Pt wire (1 mm diameter) and an Ag/AgCl electrode (BAS RE-1B, sat. NaCl) were used as working, auxiliary and reference electrodes, respectively.

Flow-determination of H_2O_2

Figure 1(A) illustrates the flow injection analysis (FIA) system. The system is composed of a double-plunger pump (DMX 2000T, SNK) with a six-way injection valve (SVM-6M2, SNK) and an electrochemical flow detector, which is essentially the same as that of a previously reported system.¹² The electrochemical flow detector was connected to an electrochemical analyzer (ALS 6122A).

As shown in Fig. 1(B), the HRP-TN-CF (working electrode) with a platinum lead wire was placed in the flow-path (net volume, 1.8 cm³; half space, 0.9 cm³ filled by CF), and a platinum wire auxiliary electrode was positioned behind the working electrode. An Ag/AgCl (sat. NaCl) reference electrode (RE-1B; 70 $\times$ 6 mm) with an electrode holder was placed in a separate space down-stream of the flow cell. PTFE tubing (0.5 mm i.d.) was used throughout the FIA system.

All FIA experiments were carried out at room temperature. An air-saturated 0.1 M phosphate buffer was used as the carrier. The storage stability was checked by measuring continuous (100 times) measurements of 100 μ M H_2O_2 every 2 or 3 days for 8 days.

Results and Discussion

Since the rate of direct electron transfer from the electrode to the redox active center of HRP is generally slow, various electron-transfer mediators, such as phenothiazine derivatives including TN, were employed to fabricate HRP-based H_2O_2 -sensing electrodes.¹³⁻¹⁵ Figure 2 shows the dependency of the applied potential on the cathodic peak currents for 100 μ M H_2O_2 obtained by HRP-TN-CF and the HRP-CF electrodes-based

Fig. 3 Effects of the carrier flow rate on the electric efficiency (A) and the cathodic peak-current (B) of 100 μM H_2O_2 at an applied potential of 0 V vs. Ag/AgCl. The sample injection volume: $\bullet$, 20 μl ; $\square$, 200 μl .

flow detectors. Compared with HRP-CF, the HRP-TN-CF induced a larger response over a wide potential region from +0.4 to -0.5 V. In contrast, TN-CF and bare-CF showed no apparent peak-current responses under the same experimental conditions (data not shown). These results clearly indicate that co-adsorbed TN is essential to increase the peak-current responses. In other words, the co-adsorbed TN together with HRP on the CF effectively facilitates electron shuttling between the CF and the HRP. The surface density of an adsorbed TN (Γ_{TN}) was estimated to be $ca. 2.12 \times 10^{-11} \text{ mol cm}^{-2}$, based on an equation; $Q = nFA\Gamma$, here, n is the charge of the redox reaction (in this case, $n = 2$), Q is the quantity of electricity of the cathodic wave of TN on the CV response, F is the Faraday constant and A is the effective surface area of the CF (in this case, $A = 135 \text{ cm}^2$).¹² A current decrease in the negative potential region of less than -0.4 V would be attributed to an increase in the background current originating from the reduction of dissolved oxygen in the carrier solution. Since a low background current is preferable for highly sensitive determination, we selected 0 V as the optimum working potential.

The adsorption conditions (*e.g.*, adsorption time, temperature, concentration of HRP and TN, ionic strength and pH of the HRP-TN-mixed solution) would directly influence the performance characteristics of this biosensing system. In addition, how co-adsorbed TN facilitates the electron transfer between the HRP and the CF surface is an interesting topic. Optimization of the adsorption conditions and a detailed

Fig. 4 Typical peak-current responses of the HRP-TN-CF-based flow-biosensor obtained at a carrier flow rate of 3.9 ml/min. The applied potential was 0 V vs. Ag/AgCl. The sample injection volume was 200 μl .

systematic study on the electron-transfer mechanism are both now under way.

The effect of the carrier pH on the peak current responses was evaluated from pH 5.0 to 8.0 using 100 μM H_2O_2 . The HRP-TN-CF showed a broad pH optimum, with the maximum response at pH 6.0 and 7.0; the response was maintained at about 88 and 76% of the maximum even at pH 5.0 and 8.0, respectively. Such a broad pH dependence of the response might be caused by several factors; *i.e.* a pH dependency on the activity of the HRP, and the electrochemical activity of the TN. We selected pH 7.0 for the following experiments.

The carrier flow rate is an important factor that influences the electric efficiency of an electrochemical flow detector. Figure 3(A) shows the dependency of the carrier flow rate on the electric efficiency of 100 μM H_2O_2 (sample injection volumes were 20 and 200 μl). It is clear that a slower flow rate and a smaller sample volume were preferable to enhance the electric efficiency. These results are essentially similar to our previous work on a coulometric flow detector of NADH using TN-adsorbed CF.¹² Almost complete electrolysis (95.5%) was obtained for 20 μl of 100 μM H_2O_2 only at a carrier flow rate of 0.5 ml/min. However, in this case, more than 250 s was required for one sample measurement. A detailed study on the coulometric determination of H_2O_2 using the HRP-TN-CF system is now under way, and will be reported in another paper.

Figure 3(B) shows the effect of the carrier flow rate on the peak heights for 100 μM H_2O_2 . Although the peak currents of 20 μl samples gradually increased with increasing flow rate, the peak currents of 200 μl samples were scarcely influenced by the flow rate. These results indicate that the rate of the bioelectrocatalysis by the HRP-TN-CF system is sufficiently fast, and the mass-transfer process would be the limiting step. The time to baseline reversion at a flow-rate of 3.9 ml/min was $ca. 30 \text{ s}$, permitting continuous analysis of 60 samples/h. The typical peak-current responses of 100 μM H_2O_2 (sample injection volume, 200 μl) are shown in Fig. 4.

Figure 5 shows the calibration curves of H_2O_2 , plotting the peak current against the H_2O_2 concentrations. The value of the peak current is the average of 3 measurements. The relative standard deviation (RSD) was found to be 0.4 to 1.7. The peak currents increased linearly with increasing the concentration of H_2O_2 over the concentration range from 0.3 to 50 μM . The

Fig. 5 Calibration curve of the HRP-TN-CF-based amperometric bioelectrocatalytic flow detector. The operational conditions were the same as in Fig. 4.

sensitivity, *i.e.*, the slope of the linear portion of the calibration graph, was $0.720 \mu\text{A}/\mu\text{M}$ and the correlation coefficient was 0.997. The detection limit was found to be $0.1 \mu\text{M}$ based on the signal-to-noise characteristics ($S/N = 3$). The fabrication reproducibility of the HRP-TN-CF prepared independently revealed acceptable reproducibility with a variation coefficient of 4.8% ($n = 5$) for the determination of $100 \mu\text{M H}_2\text{O}_2$.

To check the operational stability and storage stability, we measured $100 \mu\text{M H}_2\text{O}_2$ (100 times) every 2 or 3 days, and the HRP-TN-CF was stored in 0.1 M phosphate buffer at 4°C in a refrigerator, when not in use (Fig. 6). During 100 repetitive sample measurements (experimental period for 2 h), no serious decrease in the peak current was observed ($\text{RSD} = 0.41$ to 1.21%), indicating that serious inactivation and leaking of HRP and TN from the CF surface was negligible. However, during storage in a buffer, the responses gradually decreased. After 8 days of storage, the peak currents decreased to 42% activity at the beginning of the experimental period. From these results, it can be safe to say that the HRP-TN-CF based flow-detector exhibited an excellent reproducibility and acceptable storage stability.

Conclusion

We have developed a novel bioelectrocatalytic flow detector for the amperometric determination of H_2O_2 by using HRP and TN co-adsorbed CF. The resulting HRP-TN-CF-based flow detector exhibited highly sensitive cathodic peak currents to H_2O_2 in the concentration range from 0.3 to $100 \mu\text{M}$. This system exhibited excellent reproducibility and acceptable storage stability. The dissolved oxygen in the carrier scarcely influenced the sensitivity of the response at an applied potential of 0 V vs. Ag/AgCl . Thus, this system would be applicable for a flow biosensing system for oxidase substrates, by coupling to corresponding oxidases. Furthermore, the coulometric determination of H_2O_2 would also be possible by using this flow biosensing system. These features are now being developed in our laboratory.

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Fig. 6 Operational and storage stabilities of the HRP-TN-CF-based bioelectrocatalytic flow detector. The stability was checked by 100 repetitive sample injections. Sample: $100 \mu\text{M H}_2\text{O}_2$ ($200 \mu\text{l}$); a, first day; b, 4th day; c, 5th day; d, 6th day; e, 7th day; f, 8th day. The HRP-TN-CF was stored in 0.1 M phosphate buffer ($\text{pH } 7.0$) at 4°C , when not in use.

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