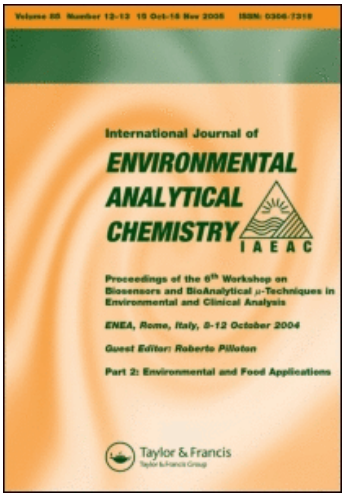

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Ammonia sensor with flavin-containing monooxygenase

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A biosensor for ammonia solution was developed with a flavin-containing monooxygenase-3 (FMO3). The biosensor consisted of a Clark-type dissolved oxygen electrode and an FMO3 immobilized membrane. In order to amplify the biosensor output, a substrate regeneration cycle obtained by coupling the monooxygenase with L-ascorbic acid (AsA) as the reducing reagent system, was applied. The AsA 10.0 mmol L^{-1} concentration was able to optimally amplify the sensor output 11 times greater. The FMO3 biosensor was used with AsA to measure ammonia solution from 3.20 to 14.29 mmol L^{-1} and from 0.09 to 21.7 mmol L^{-1} with 5.0 and 10.0 mmol L^{-1} AsA, respectively. The FMO biosensor also had a good reproducibility such as a 2.5% coefficient of variation in eight multiple measurements, and the output current was maintained over a few hours. The selectivity of the FMO biosensor being attributed to enzyme specificity was obtained for several chemical substances (trimethyl amine, methyl mercaptan, dimethyl sulphide, and so on).

Keywords: Biosensor; Enzyme; Flavin-containing monooxygenase-3; L-Ascorbic acid; Reducing reagent system

1. Introduction

Ammonia is produced in the human body and is commonly found in nature [1–5]. Ammonia is also generated in pharmacy and food industrial processes, and causes a burning sensation in eyes, nose and throat. The severity of health effects depends on the concentration and duration of exposure [6]. The American Conference of Governmental Industrial Hygienists (ACGIH) and the Environment Agency Government of Japan had specified ammonia as a typical volatile organic compound (VOC) and malodorous substance. The maximum permissible ammonia concentration in the work place is defined as 25 ppm (threshold limit value–time-weighted average concentration) and the maximum dissolved ammonia concentration in freshwater as 34 mmol L^{-1}. There are several methods to measure ammonia using absorption photometry and an ion electrode, but these have complications and low selectivity

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problems, respectively [7–13]. Therefore, the development of a convenient and highly selective ammonia sensor is expected.

On the other hand, a xenobiotic metabolizing enzyme has a good selectivity for producing certain chemical changes in organic substances by catalytic action. For example, flavin-containing monooxygenases (FMOs) have been reported to catalyse the oxidation of nitrogen- and sulphur-compounds for xenobiotic metabolism, and are a polymorphic family with several kinds of isoform, with a dissimilar specificity for enzyme substrates [14–16], thus a biosensor using an enzyme would measure a chemical substance with high selectivity [17–22]. In this study, an FMO biochemical sensor for ammonia in the liquid phase was developed, and the characteristics were evaluated.

2. Experimental

2.1 *Materials*

An ammonia biosensor was constructed as described in the next section 2.2, using a commercially available Clark-type dissolved oxygen electrode (Model BO-P, ABLE Corp., Tokyo) and an enzyme membrane. The enzyme membrane was prepared from flavin-containing monooxygenase-3 (FMO3: EC 1.14.13.8, 30200 pmol min^{-1} mg^{-1}, from adult human liver, Gentest Corp., Woburn, MA), photocrosslinkable polyvinyl alcohol containing stilbazolium groups (PVA-SbQ: Type: SPP-H-13 (Bio), Toyo Gosei Co., Ltd, Tokyo) [23], and a dialysis membrane (part no. 157-0144-02, thickness: 15 μm, Technicon Chemicals Co., S.A., Oecq, Belgium).

A phosphate buffer solution was adjusted to pH 8.5 by mixing disodium hydrogenphosphate and potassium dihydrogenphosphate (197-02865 and 9169-04245, Wako Pure Chemical Industries, Ltd, Osaka, Japan), and was mixed with 3.3 mmol L^{-1} of magnesium chloride hexahydrate (131-00162, Wako) and 0.065 mmol L^{-1} of nicotinamide adenine dinucleotide phosphate reduced form (305-50473, Oriental Yeast Co. Ltd, Tokyo). L-Ascorbic acid (AsA; 196-01252, Wako pure Chemical Industries, Ltd, Osaka, Japan) was used to amplify the biosensor output caused by substrate regeneration. Then, several substances such as trimethyl amine, methyl mercaptan, dimethyl sulphide, 1-propanol, 1-butanol, acetone, hexane, 2-butanone, benzene, methanol, and ethanol were used to evaluate the reproducibility and selectivity of the biosensor.

A batch-flow measurement system was constructed using a personal computer (PCG-FX11V, Sony, Tokyo), a computer-controlled potentiostat (Model 1112, BAS Inc., Tokyo), and an analogue-to-digital converter (ADC-16, Pico Technology Co., Ltd, St Neots, UK).

2.2 *Methods*

The ammonia biosensor was constructed using the Clark-type dissolved oxygen electrode and the FMO3 immobilized membrane. Figure 1 shows the process of immobilizing the enzyme to the dialysis membrane and a schematic of the ammonia biosensor. The FMO3 solution was mixed with PVA-SbQ, coated onto the

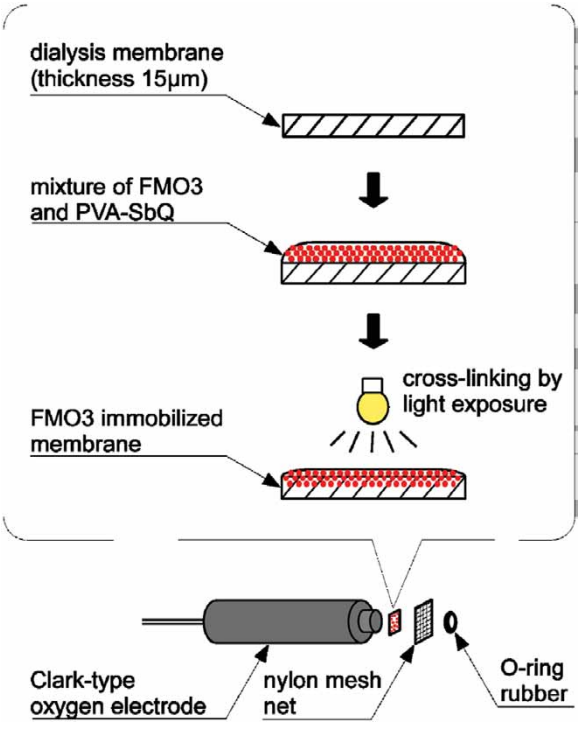

Figure 1. Construction process of an FMO3 immobilized membrane and biosensor for ammonia solution.

dialysis membrane, and desiccated in the dark below 2°C for 1 h. Then, the membrane was irradiated with a fluorescent light for 30 min. The FMO3 immobilized membrane was cut and placed onto the sensing area of the oxygen electrode covered with a supporting nylon mesh net, and secured with a silicone O-ring. The FMO3 immobilized biosensor was kept at 2°C in a refrigerator when not in use. The tip of the enzyme electrode was immersed in phosphate buffer solution regulated to pH 8.5.

The ammonia biosensor with immobilized FMO3 was evaluated using ammonia solutions in the batch flow measurement system. The tip of the biosensor was dipped into the buffer solution. Then 100 mmol L^{-1} ammonia solution were added to the buffer solution. During the measurement, the buffer solution was agitated using a stirrer. An external voltage of −600 mV was maintained on a Pt working electrode by the potentiostat vs. Ag/AgCl counter electrode. The sensor output of oxygen consumption induced by the FMO3 enzymatic reaction was monitored using the personal computer via the potentiostat and the analogue-to-digital converter.

Figure 2 shows the principle of ammonia measurement using the FMO3 enzymatic reaction and substance regeneration [22, 24–27]. In order to amplify the biosensor output, the substrate regeneration cycle obtained with AsA by coupling the monooxygenase as the reducing reagent system was used for ammonia measurement. The ammonia would then be oxidized to hydroxylamine by the FMO3 and then be reproduced by the AsA. The AsA would be oxidized to dehydroascorbic acid. The AsA

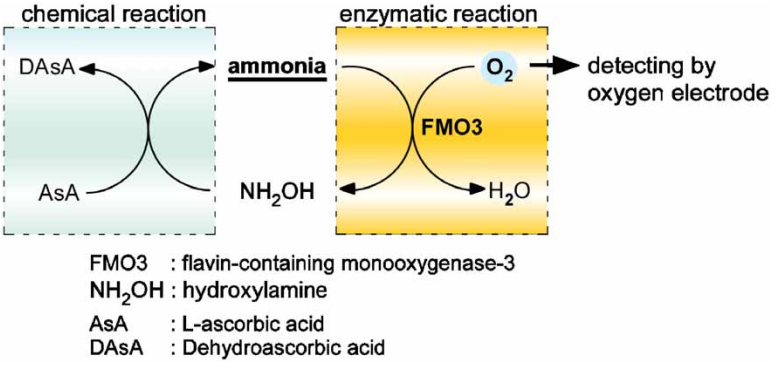

Figure 2. Principle of ammonia measurement using the FMO3 enzymatic reaction and substance regeneration.

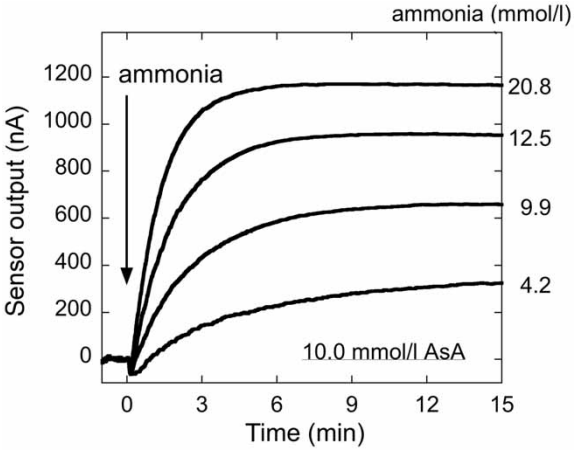

Figure 3. Typical responses of the ammonia biosensor with 10.0 mmol L^{-1} of of L-ascorbic acid (AsA). Ammonia solutions were injected at the point shown by the arrow on the graph.

should increase the ammonia concentration by reducing the hydroxylamine generated by FMO3 oxidation.

And then, reproducibility and selectivity of the biosensor were evaluated by multiple measurements for the ammonia solutions and using solutions of several chemical substances.

3. Results and discussion

3.1 *Evaluation of the biosensor for the ammonia measurement*

The performance of the biosensor in the liquid phase was assessed with the batch flow measurement system. Figure 3 shows typical response curves of the FMO3 biosensor for several concentrations of ammonia solution with 10.0 mmol L^{-1} of AsA.

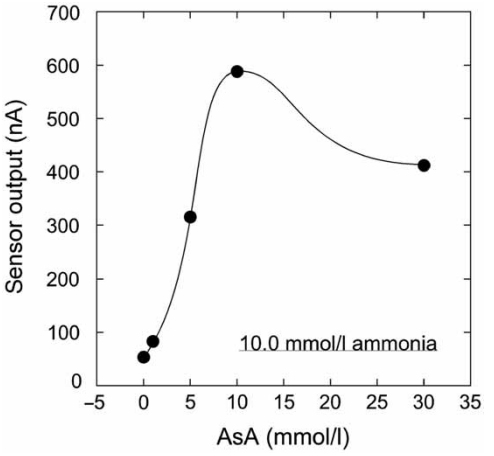

Figure 4. Effect of AsA concentration on the buffer solution. The AsA amplified the sensor output by a reducing reagent system. The maximum output was obtained at an AsA concentration of 10.0 mmol L^{-1}.

The negative currents of the biosensor output decreased, since ammonia was oxidized by the FMO3 reaction and induced the dissolved oxygen consumption, and then reached 90% of the steady-state level within 3.0 min for 20.8 mmol L^{-1} ammonia solution. In this paper, the changes in negative current of the biosensor were illustrated.

The effect of AsA in the buffer solution on the steady-state currents for ammonia measurement is shown in figure 4. The experimental conditions of AsA concentration for the batch flow measurement were evaluated by injection of 10.0 mmol L^{-1} ammonia solutions. The sensor outputs were increased by addition of the AsA into the buffer solution, and the maximum output was obtained at an AsA concentration of 10.0 mmol L^{-1}. Therefore, the AsA concentration of 10.0 mmol L^{-1} was generally used in all experiments with buffer solution. At high concentrations, higher than 10.0 mmol L^{-1}, the output was decreased from the maximum. The behaviour of the biosensor output shown in figure 4, suggested that the dissolved oxygen concentration was increased since the ammonia regeneration by AsA exceeded the oxidation by FMO3 and then balanced in a condition depend on a thickness, an area and permeability of the enzyme membrane. The reason for the decreased sensor output under higher concentrations of AsA seemed to be the decrease in dissolved oxygen by auto-oxidation of the AsA by oxygen.

Figure 5 shows the calibration curves of the biosensor with the FMO3 immobilized membrane to ammonia in the liquid phase with 5.0 and 10.0 mmol L^{-1} of AsA. The changes in output current of the biosensor related to the concentration of the ammonia over the range of 3.20–14.29 mmol L^{-1} and 0.09–21.7 mmol L^{-1} with correlation coefficients of 0.995 and 0.994 with 5.0 and 10.0 mmol L^{-1} of AsA, respectively, were deduced from exponential regression analysis of the log–log plot by a least squares method, according to the following equations:

$$\text{sensor output } (\mu\text{A}) = 19.0\,[\text{ammonia}(\text{mmol L}^{-1})]^{1.39} (\text{with 5.0 mmol L}^{-1}\text{ AsA})$$

$$\text{sensor output } (\mu\text{A}) = 264.1\,[\text{ammonia}(\text{mmol L}^{-1})]^{0.423} (\text{with 10.0 mmol L}^{-1}\text{ AsA}).$$

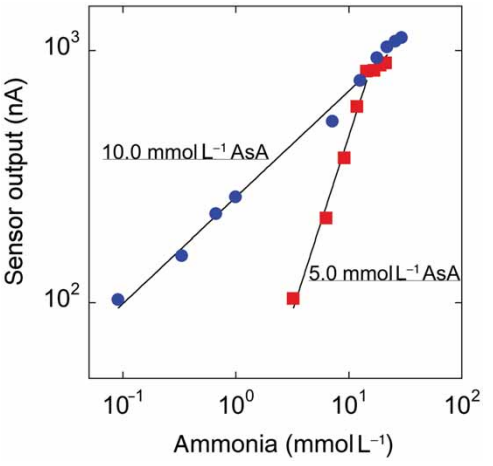

Figure 5. Calibration curves of the biosensor for ammonia solutions with 5.0 and 10.0 mmol L^{-1} of AsA.

The steady-state output signals over 15 mmol L^{-1} (5.0 mmol L^{-1} AsA) and 22 mmol L^{-1} (10.0 mmol L^{-1} AsA) of ammonia were represented for saturated dissolved oxygen solution. Comparing the two regression curves with 5.0 and 10.0 mmol L^{-1} AsA, the calibration range could be translated to a lower concentration level with a change in slope for the regression curves with the concentration of AsA, thus improving the lower detection limit from 3.2 to 0.09 mmol L^{-1} of ammonia solution. This result shows that the sensitivity of the biosensor depends on the AsA concentration. A sensitive measurement and a high resolution measurement for low and high concentrations of ammonia can be obtained by regulating the AsA concentration.

3.2 *Reproducibility and selectivity of the ammonia biosensor*

The reproducibility of the biosensor was evaluated at an ammonia solution concentration of 10.0 mmol L^{-1}. The sensor performance was reproducible over eight multiple measurements, showing a mean ± standard deviation, and a coefficient of variation of 251.5 ± 6.4 (nA) and 2.5%, respectively, as shown in figure 6. The biosensor sensitivity would decrease as a result of enzyme deactivation during long-term measurement. During the experiments, over a few hours, the output of the biosensor would be maintained by circulation of the phosphate buffer solution to the sensing area. This result showed that the biosensor would be applicable for continuous measurement of ammonia solution.

The selectivity of the biosensor among solutions of several substances (10.0 mmol L^{-1} trimethyl amine, methyl mercaptan, dimethyl sulphide, 1-propanol, 1-butanol, acetone, hexane, 2-butanone, benzene, methanol, and ethanol) is shown in figure 7. The FMO3 biosensor indicated a good selectivity attributed to enzyme specificity for chemical substrates because the FMO3 could not catalyse the oxidation of the organic compounds, which did not have thiol and amino such as dimethyl sulphide, 1-propanol, 1-butanol, acetone, hexane, 2-butanone, benzene, methanol, and ethanol [28, 29]. As mentioned above, FMO3, one of the xenobiotic metabolizing enzymes in living

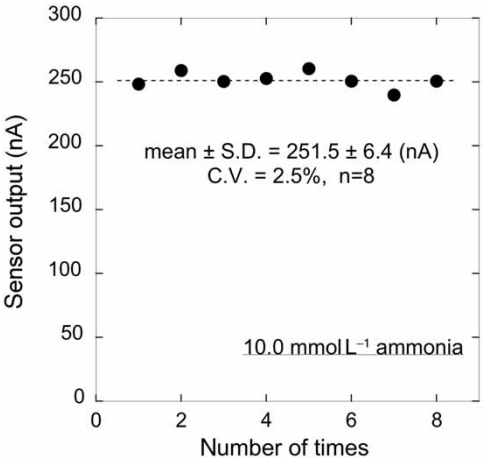

Figure 6. Reproducibility of the biosensor to 10.0 mmol L^{-1} of ammonia solutions.

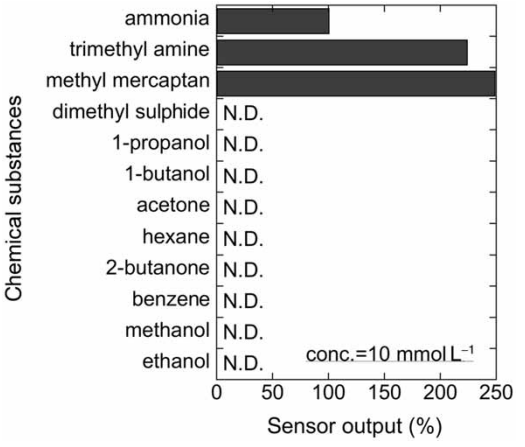

Figure 7. Selectivity of the ammonia biosensor to various substance solutions.

organisms, catalysed oxidation of the ammonia, and the FMO biosensor indicated good selectivity for ammonia.

4. Conclusions

The biosensor for ammonia solution was constructed using a Clark-type dissolved-oxygen electrode and a flavin-containing monooxygenase-3 (FMO3) immobilized membrane. In order to amplify the biosensor output, a substrate regeneration cycle obtained by coupling the monooxygenase with L-ascorbic acid (AsA) as a reducing reagent system, was used. The AsA 10 mmol L^{-1} concentration could optimally amplify the sensor output 11 times greater. The ammonia biosensor was used with AsA to measure the ammonia solution from 0.09 to 21.7 mmol L^{-1}, and the sensitivity of the

biosensor could be regulated by the AsA concentration. The ammonia biosensor had a good reproducibility (e.g. a 2.5% coefficient of variation) and had a good selectivity attributed to the enzyme specificity for several chemical substances.

Potential applications of ammonia biosensors include not only environmental assessment but also medical diagnosis of *Helicobacter pylori* and evaluation of soil fertility in the agricultural industry.

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