

# Bioelectronic detector with monoamine oxidase for halitosis monitoring†

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Methyl mercaptan (MM) is known as one of the major chemicals of halitosis (bad breath). In this study, a bioelectronic gas sensor (bio-detector) for gaseous MM was developed and was applied to measure halitosis in breath. The bio-detector consisted of a Clark-type dissolved oxygen electrode, a monoamine oxidase type-A (MAO-A) immobilized membrane and a reaction unit that had liquid and gaseous compartments separated by a hydrophobic porous polytetrafluoroethylene (PTFE) diaphragm membrane. The tip of the electrode covered with MAO-A membrane was placed into the liquid compartment as touching to the PTFE diaphragm membrane. In order to amplify the bio-detector output, a substrate regeneration cycle caused by coupling the monooxygenase with L-ascorbic acid as reducing reaction with reagent system, was applied. The results of MM vapor measurements showed the calibration range of the bio-detector for MM vapor was from 0.087 to 11.5 ppm (correlation coefficient: 0.993) and included the human sense of smell level 5 (0.2 ppm). The bio-detector had good selectivity being attributed to enzyme specificity was obtained for several substances (trimethyl amine, ammonia, dimethyl sulfide, *etc.*). The bio-detector was applied for halitosis measurement. Expired gases in five subjects were sampled every hour and the concentrations of MM in the expired gases were monitored. The output of bio-detector showed behaviour of halitosis level changes in a day such as increasing with passage of time and decreasing after eating.

## Introduction

Humans are sensitive to halitosis (bad breath) in others but unable to assess the halitosis in their own breath. In clinical dentistry, halitosis is measured to diagnose dental hygiene. The main chemical constituents of oral malodor are volatile sulfides such as hydrogen sulfide (H<sub>2</sub>S) and methyl mercaptan (MM: CH<sub>3</sub>SH).<sup>1,2</sup> The American Conference of Governmental Industrial Hygienists (ACGIH) and the Environment Agency Government of Japan had specified the MM as a typical volatile organic compound (VOC) and malodorous substance. The maximum permissible concentration of gaseous MM in the work place is defined as 5.0 ppm (TLV-TWA: threshold limit value–time weighted average concentration).

However, there are no convenient approaches to measure the MM vapor for evaluating the halitosis. Many types of gas sensors have been investigated and developed. For example, semiconductor type gas sensors were improved the gas selectivity and sensitivity.<sup>3–5</sup> Nevertheless, semiconductor sensors are still inadequate to sense multiple substances as in expiratory gas, because the sensor outputs the change in electrical conductivity by adsorption of gaseous substances.<sup>3–7</sup>

On the other hand, a xenobiotic metabolizing enzyme has good selectivity to producing certain chemical changes in organic substances by catalytic action. For example, flavin-containing monooxygenase and monoamine oxidase type-A (MAO-A) are reported to catalyze the oxidation of nitrogen and sulfur compounds including the MM for a xenobiotic metabolism, and are a polymorphic family with several kinds of isoform with dissimilar specificities for the enzyme substrates.<sup>8–10</sup> A biosensor using the enzyme would achieve volatile vapor measurement with high selectivity.<sup>11–13</sup> A previous study considered a biochemical gas sensor (bioelectronic detector) using the FMO enzyme for MM measurement.<sup>14</sup> In this study, an MAO-A biochemical sensor for MM in the gas phase was developed and the characteristics were evaluated for halitosis monitoring.

## Experimental

### Construction of a bioelectronic detector

The MAO-A immobilized bio-detector for MM vapor was constructed using a commercially available Clark-type dissolved oxygen electrode (Model BO-P, ABLE Corp., Tokyo, Japan), a MAO-A immobilized membrane and a reaction unit. Fig. 1 shows the structure and the assembly parts of the bio-detector. The MAO-A solution (E.C.1.4.3.4., 142 nmol min<sup>-1</sup> (mg protein)<sup>-1</sup>, from adult human liver, Gentest corp., MA, USA) was mixed with PVA-SbQ (photocrosslinkable polyvinyl alcohol containing stilbazolium groups, Type: SPP-H-13 (Bio), Toyo Gosei Co., Ltd., Tokyo, Japan)<sup>15</sup> and was coated onto a dialysis membrane (part No. 157-0144-02, thickness: 15 μm, Technicon Chemicals Co., S.A., Oecq, Belgium). The MAO-A

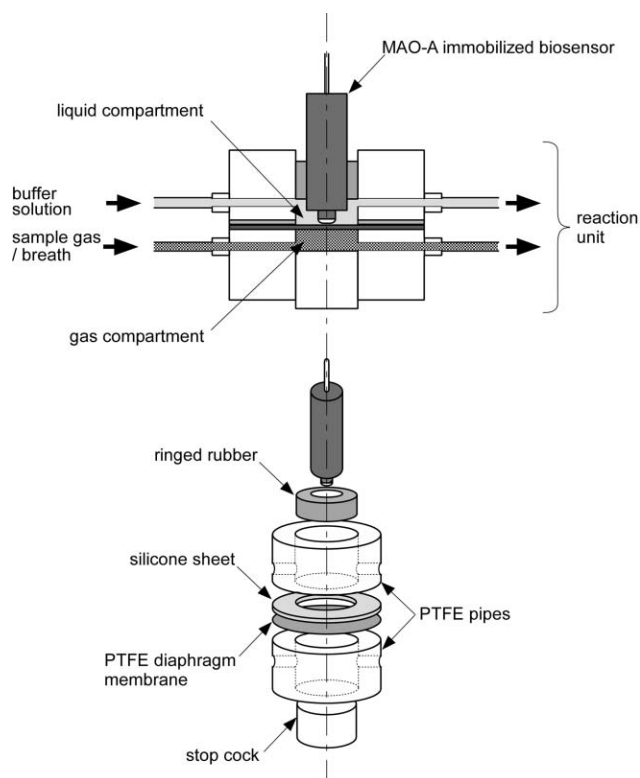
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**Fig. 1** Bioelectronic detector with the Clark-type oxygen electrode immobilized MAO-A (MM biosensor) and a reaction cell with liquid and gas phase compartments separated by a porous PTFE membrane.

membrane was desiccated in the dark below 2 °C for 1 h, and then was irradiated with a fluorescent light for 30 min. The MAO-A membrane was cut into 7 mm square, and was placed onto the sensing area of the Clark-type dissolved oxygen electrode covered with a supporting nylon mesh net, and was secured with a silicone O-ring.

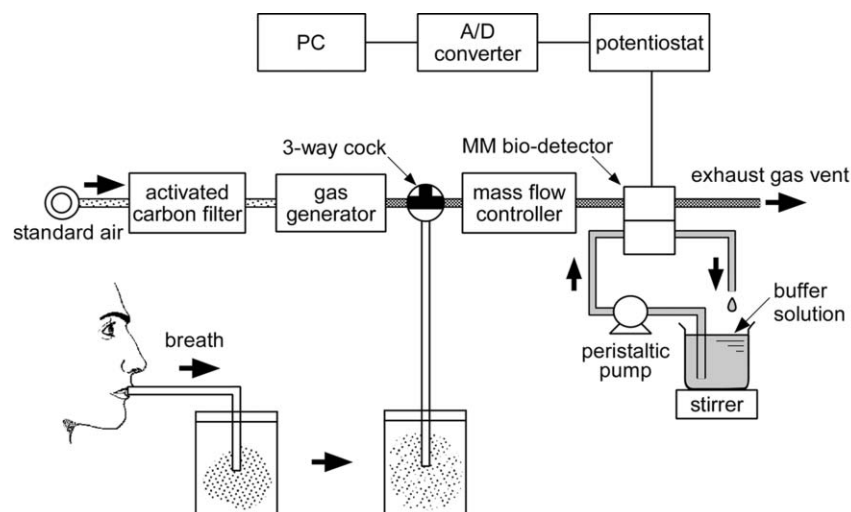
The oxygen electrode with the enzyme membrane was built into a reaction unit with liquid and gaseous compartments separated by a hydrophobic porous polytetrafluoroethylene

(PTFE) diaphragm membrane (Type A-105, pore size: 30–60  $\mu\text{m}$ , thickness: 0.20 mm, ZITEX, NORTON KK, Nagano, Japan). The tip of the enzyme biosensor was placed into the liquid compartment as touching to the PTFE diaphragm membrane. The phosphate buffer solution and the MM vapor were individually flowed to the liquid- and gas-compartments of the reaction unit through the each inlet, respectively. The buffer solution was regulated at pH 8.5 by mixing of disodium hydrogenphosphate and sodium dihydrogenphosphate. The gaseous MM permeated through the micropores of the diaphragm membrane would be detected by the MAO-A immobilized bio-detector.

An external voltage of  $-700$  mV was maintained on Pt working electrode versus Ag/AgCl counter electrode by a computer-controlled potentiostat (Model 1112, BAS Inc., Tokyo, Japan). The sensor output of oxygen consumption induced by MAO-A enzymatic reaction was monitored by a personal computer (PCG-FX11V, SONY, Tokyo, Japan) via the potentiostat and analogue to digital converter (ADC-16, Pico Technology Co., Ltd., Cambridgeshire, UK).

### Gas flow measurement using bio-detector

The MAO-A immobilized bio-detector was applied to a gas flow measurement for evaluating the characteristics of MM detection. Fig. 2 shows a schematic diagram of a gas flow measurement setup for the bio-detector. Standard gaseous substances were supplied from a gas generator (PERMEATER PD-1B-2, Gastec Corp., Yokohama, Japan). The flow rate of the standard gaseous substances was regulated to  $100$  ml  $\text{min}^{-1}$  using a mass flow controller with a needle valve (RK1200, Koflok, Tokyo, Japan). A peristaltic pump (Type: MP-3N, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) circulated phosphate buffer solution ( $100$  mmol  $\text{l}^{-1}$ , pH 8.5) between a carrier reservoir and the liquid compartment of the reaction unit. The flow rate of the buffer circulation was set to  $1.68$  ml  $\text{min}^{-1}$ . The circulation of phosphate buffer solution was applied to achieve continuous measurement of the gaseous substances by supplying the dissolved oxygen, and by removing the enzyme products and surplus gaseous substances



**Fig. 2** Schematic diagram of a gas-flow and breath measurement setup for the bioelectronic detector.

that were diffused through the diaphragm membrane into the liquid compartment and the enzyme membrane at the tip of the biosensor.

In order to amplify the biosensor output, a substrate regeneration cycle initiated by L-ascorbic acid (AsA; 196-01252, Wako pure Chemical Industries, Ltd., Osaka, Japan) by coupling the monooxygenase as reducing reagent system,<sup>14,16–20</sup> was applied for MM measurement. In our previous study,<sup>16</sup> the outputs of a biosensor that had a bio-detector with a similar structure were increased by addition of AsA into the buffer solution. Further, the maximum output of the biosensor with MAO-A was obtained at 10 mmol l<sup>-1</sup> of AsA concentration to MM solution. Therefore AsA concentration of 10 mmol l<sup>-1</sup> was generally used in all experiments with buffer solution. The bio-detector output was monitored by a personal computer *via* the potentiostat and the A/D converter.

The selectivity of the bio-detector for several volatile substances (2 mmol l<sup>-1</sup> concentration of trimethyl amine, ammonia, dimethyl sulfide, ethanol, acetaldehyde, acetone and hexane) was then evaluated and was compared with a semiconductor gas sensor (TGS822, FIGARO Eng. Inc., Osaka, Japan).

#### Application of the bio-detector for halitosis monitoring

The bio-detector was applied for monitoring of halitosis. As shown as Fig. 2, the expired breaths of seven healthy male volunteers (age 26.4 ± 7.3 years) were sampled to 800 ml volume polyethylene bags every hour from 0800 to 1600 over the course of a day. A single bio-detector was used in all measurements. All subjects had neither oral disease nor bad breath. The subjects took no food before 30 min of breath sampling, and neither smoked nor chewed gum during the experiments. The flow rate of the sampled breath was regulated to 100 ml min<sup>-1</sup> using the mass flow controller and was led to the bio-detector.

## Results and discussion

#### Measurements of gaseous MM

The performance of the bio-detector for MM gas was assessed with the batch flow measurement system. Fig. 3 shows the calibration curve of the MAO-A immobilized bio-detector for MM. As Fig. 3 illustrates, the changes in output of the bio-detector were found to be related to the MM concentrations in the gas phase, since gaseous chemicals that diffused through the enzyme membrane were oxidized by MAO-A using oxygen as electron acceptor, causing a decrease in the concentration of dissolved oxygen. The calibration range of the bio-detector for MM vapor was from 0.087 to 11.5 ppm (correlation coefficient: 0.993) and included the human sense of smell level 5 (0.2 ppm). The sensor output deduced from logarithmic regression analysis of the semi-logarithmic plot by the least-squares method was according to the following equation:

$$\text{sensor output/nA} = 14.05 + 3.99 \log ([\text{MM}]/\text{ppm})$$

(with 10.0 mmol l<sup>-1</sup> AsA)

In the measurement of MM vapor using the MAO-A immobilized bio-detector, buffer circulation (flow rate = 1.68 ml min<sup>-1</sup>) was

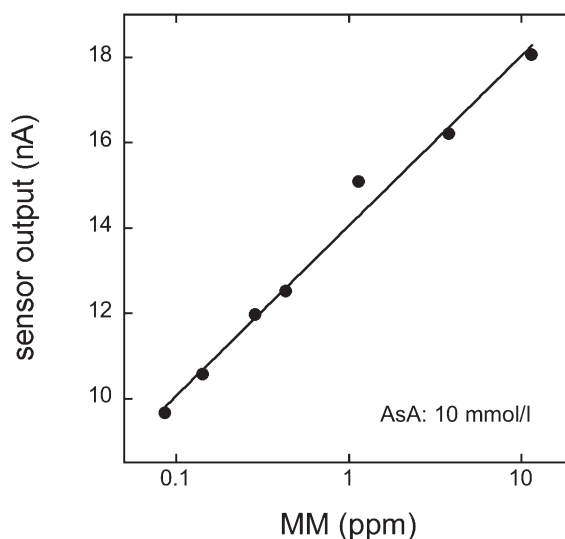


Fig. 3 Calibration curve of the bio-detector for MM vapor.

applied to achieve continuous measurement. That is to say, the buffer flow removed gaseous substances that diffused through the diaphragm membrane and the enzyme membrane in the liquid compartment, thus restoring the sensor output to the initial current. As mentioned above, MAO-A is one of the xenobiotic metabolizing enzymes in living organism-catalyzed oxidation of MM, and is possible to use as a recognition material for the measurement of the malodorous substances.

#### Reproducibility and selectivity of the bio-detector output

The reproducibility of the bio-detector was evaluated at 5.7 ppm concentration of the MM vapor. The sensor performance was reproducible over five multiple measurements at 30 min intervals, showing a mean standard deviation, and a coefficient of variation as 19.6 ± 1.47 nA and 7.48%, respectively (Fig. 4). The sensitivity of biosensor would decrease by deactivation of enzyme during long-term measurement. In the

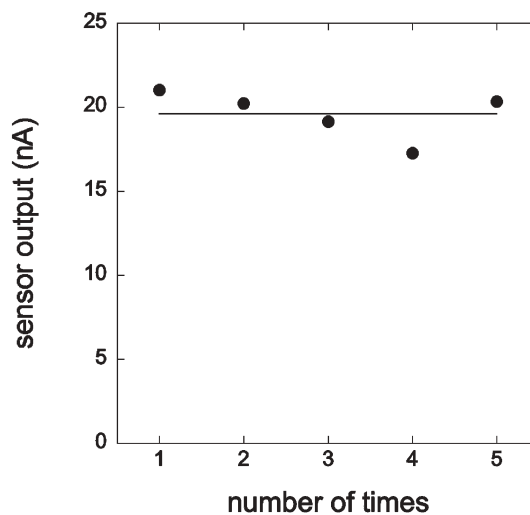
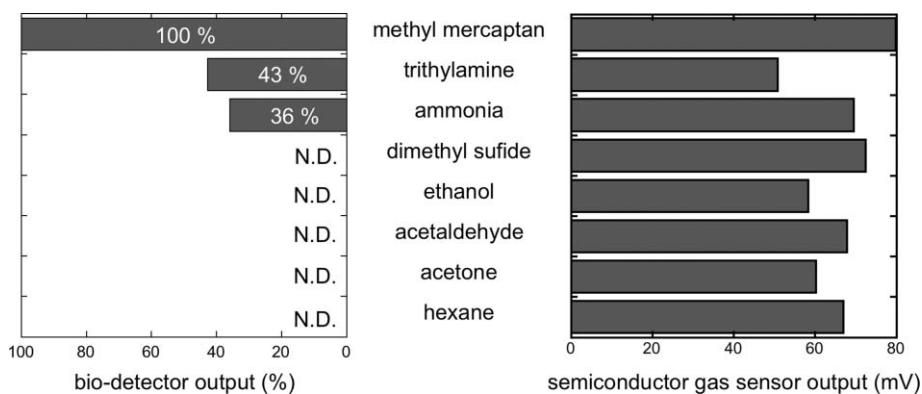


Fig. 4 Reproducibility of the bio-detector output for MM vapor. The bio-detector was evaluated by five multiple measurements at 30 min intervals at 5.7 ppm concentration of the MM vapor.



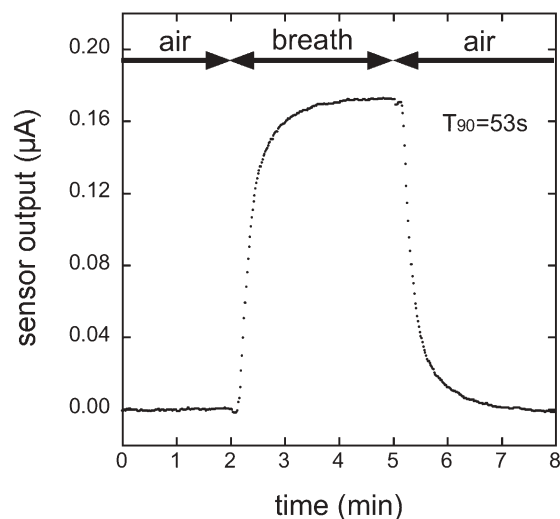
**Fig. 5** Gas-selectivity of the bio-detector and the semiconductor gas sensor for some substances (2.0 ppm concentration). A sensing element of the semiconductor gas sensor (type TGS822, FIGARO Eng. Inc.) that was made of tin dioxide (SnO<sub>2</sub>) had low conductivity in clean air. The sensor's conductivity increases depending on the gas concentration in the air.

experiments for a few hours, the output of the bio-detector would be maintained by circulation of the phosphate buffer solution to the sensing area. This result showed that the bio-detector would be applicable for continuous measurement of MM vapor.

The selectivity of the bio-detector to several gaseous substances at 2 mmol l<sup>-1</sup> concentration is shown in Fig. 5. The MAO-A bio-detector indicated good selectivity. This is attributed to the enzyme specificity for chemical substrates because the MAO-A could not catalyze the oxidation of the organic compounds that lacked thiol and amino groups, such as dimethyl sulfide, acetaldehyde, acetone, ethanol and hexane.<sup>2-4</sup> However, the semiconductor gas sensor responded to the applied volatile substances.

#### Halitosis monitoring using the bio-detector

Fig. 6 shows a typical response of the bio-detector to the sampled breath. As Fig. 6 indicates, output of the bio-detector increased following application of the sampled breath. Thus, the MAO-A bio-detector responded to chemical substrates



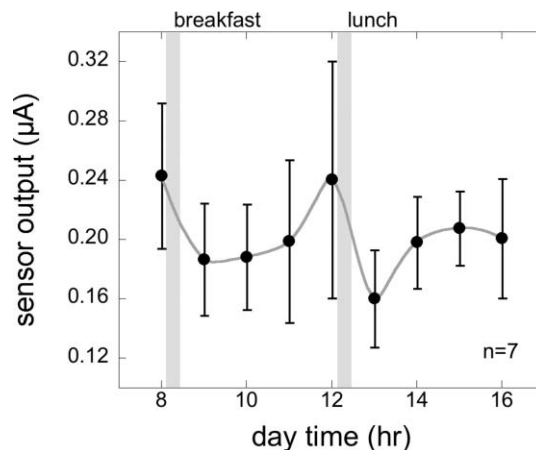
**Fig. 6** Typical response of the MM bio-detector for sampled breath with 10 mmol of AsA.

including thiol and amino in the breath. A 90% full-scale response time was 53 s.

Figure 7 shows the average and the standard deviation of changes in the bio-detector output responded to sampled breath of the subjects during the daytime. Human halitosis changes throughout a day, such as decreasing after food consumption and increasing over the course of time relating to saliva flow.<sup>21,22</sup> The high value of standard deviations of the bio-detector output showed individual differences of halitosis intensities were wide. The bio-detector output decreased significantly after breakfast and lunch, and then increased. In this study, halitosis was not quantitatively estimated. However, these results indicate the changes in the sensor output reflect the halitosis level, the MAO-A bio-detector would be capable to use for halitosis diagnosis.

#### Conclusions

The bio-detector for gaseous methyl mercaptan (MM) was constructed of Clark-type dissolved oxygen electrode, a



**Fig. 7** Changes in the bio-detector output to sampled breath of the seven healthy male volunteers (age  $26.4 \pm 7.3$  years) during daytime. The high value of standard deviations of the bio-detector output showed wide individual differences of halitosis intensity. However, the bio-detector output and the standard deviation both reduced after eating.

monoamine oxidase type-A (MAO-A) immobilized membrane and a reaction unit with liquid-gaseous compartments. The MAO-A bio-detector was used with AsA to measure gaseous MM from 0.087 to 11.5 ppm (correlation coefficient: 0.993) and included the human sense of smell level 5 (0.2 ppm). The bio-detector had good selectivity being attributed to enzyme specificity was obtained for several substances. The bio-detector would provide the application including not only environmental assessment but also diagnosis of halitosis caused in metabolic diseases such as fish odor syndrome.

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