Ethanol sniffing sensor with alcohol oxidase as gas recognition material

*Kohji MITSUBAYASHI, Mitsuhiro OGAWA, Hirokazu SAITO and Kimio OTSUKA
Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University,
Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan
*TEL: x81-3-5280-8091    FAX: x81-3-5280-8094    e-mail: m.bdi@tmmd.ac.jp

Hiroyuki MATSUBAGA and Genki NISHIO
Graduate School of Engineering, Tokai University, 1117 Kitakaname, Hiratsuka, Kanagawa, 259-1292 Japan

Nicole JAFFREZIC-RENAULT
Ecole Centrale de Lyon, Bat. H9, 69134 Ecully Cedex, France
TELI. (33) (0)4 72 18 62 43    Fax (33) (0)4 78 43 37 17
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An alcohol oxidase (AOD) enzyme immobilized gas-sensor (bio-sniffer) with stick-type configuration was developed for convenient analysis of ethanol vapor. The stick-type sniffer device was constructed in a sandwich configuration with a filter paper placed between carbon- and Ag/AgCl electrodes. The electrode-coated paper was shaped by knife into 2mm-wide stick. In order to isolate a sensitive- (length: 5 mm) and terminal-areas, an epoxy-resin adhesive was applied to the middle part of the narrow stick paper. AOD was immobilized to the sensitive area with photocrosslinkable polymer. The characteristics of the stick-type sniffer moistened with phosphate buffer were assessed using standard ethanol vapor supplied from a gas generator. The oxidation current of hydrogen peroxide, produced by the AOD enzymatic reaction by applying ethanol vapor, was detected a computer-controlled potentiostat at a fixed voltage of +900 mV. The calibration range of the ethanol sniffer covered the concentration range encountered in breath after alcohol consumption including the permissible legal limit (130 ppm) for driving, and the sensing range of smell in humans. As the physiological application, the bio-sniffer was used to monitor the concentration change of breath ethanol after drinking.

Keywords: stick-type sniffer, alcohol sensor, breath air, alcohol, alcohol oxidase, ALDH, drinking

I. INTRODUCTION
Analysis of the volatile components in the expiratory gas of patients can help in the diagnosis of diseases such as oral ailments,1 hepatocirrhosis and cancer of the lung,2,3 head and neck regions. Expiratory gas analysis would provide a non-invasive, convenient and safe method of diagnosing and monitoring disease states. One of the major applications of the breath analysis is in the quantification and detection of ethanol in expiratory gas after alcohol consumption. Blood ethanol concentration can be determined from its concentration in breath,4 a blood-breath alcohol partition ratio of 2000 having been widely
adopted. The breath alcohol level can be related to the degree of intoxication (i.e. haziness: 0.5 to 1.0 g/l in blood (0.25 to 0.5 mg/l, 130 to 260 ppm in breath); slight drunkenness: 1.0 to 1.5 g/l (0.5 to 0.75 mg/l, 260 to 390 ppm); drunkenness: 1.5 to 2.5 g/l (0.75 to 1.25 mg/l, 390 to 650 ppm)) and used as a measure of drunk driving.

In certain industrial fields, such as fermentation and distillation, the ethanol vapor concentration can reach toxic levels, causing inflammation of the nasal mucous membrane and conjunctiva, irritation of the skin and, at high levels, even alcohol poisoning. The maximum permitted concentration of ethanol vapor in the work place as defined by ACGIH (the American Conference of Governmental Industrial Hygienists) is 1000 ppm. Since gaseous ethanol is mal- or odorous substance for human (the ethanol selection and detection limits for the human sense of smell have been reported to be 6.1 and 0.36 ppm respectively, the continuous monitoring of ethanol concentration in the gas phase is significant for assessing the human health and behavior related ethanol vapor from physiological point of view.

Many types of gas sensors have been investigated and developed. Considerable effort has gone into improving the ethanol selectivity and sensitivity of semiconductor type gas sensors. Semiconductors sensors are, however, still at present inadequate for sensing multianalyte samples such as exspiratory gas, because the sensor response is based on only changes in electrical conductivity of the device following adsorption of gaseous substances. Although an improvement in semiconductor sensors for gaseous ethanol has been reported, it is still far from the selectivity achievable using biological recognition systems such as enzymes.

For the measurement of chemical substances in the liquid phase, biosensors have been extensively researched. Biosensors use biologically derived materials such as enzymes, microorganisms, antigen & antibody and organelles, which possess high specificity for their substrates. An alcohol biosensor for the liquid phase has also been investigated and applied widely for the measurement of ethanol concentration in fermentation processes. Alcohol oxidase (AOD: EC 1.1.3.13) is commonly used in the construction of alcohol biosensors, catalyzing the oxidation of lower primary alcohols to produce acetaldheyde and hydrogen peroxide. We previously reported the gas-phase biosensor with a reaction system with a diaphragm membrane using a buffer circulation system.

In this work, we have constructed a stick-type bio-sniffer using the enzyme catalytic reaction for convenient measurement of gaseous ethanol. The performance of the sniffer device is evaluated, such as sensitivity, calibration behavior and selectivity, and compared with the performance of a commercially available semi-conductor gas sensor. As the physiological application, the sniffer device was also used for monitoring a breath ethanol after drinking for some subjects.

II. EXPERIMENTAL SECTION

The photograph of the stick-type bioelectronic sniffer is shown in Fig. 1. Carbon and Ag/AgCl electrodes were formed by printing their paste (carbon paste: Electrodag505SS, LOT.0029; Ag/AgCl paste: Electrodag6037SS, LOT.0015, Acheson Japan Co., Ltd., Kobe, Japan) onto each side of a filter membrane (No.2, thickness:0.25 mm, ADVANTEC TOYO Co., Ltd., Tokyo, Japan), respectively.

![Fig. 1. Photograph of stick type bio-sniffer for ethanol.](image)

The electrode-coated membrane was cut using a scalpel into 2.0-mm wide and 70-mm length sticks. In order to isolate a sensitive area (length = 5 mm), a
cyanoacrylate adhesive (Aron Alpha, Toa gosei Co., Ltd., Tokyo, Japan) was applied to the middle part of the electrode membrane and dried at room temperature for 24 hour. Because the liquid cyanoacrylate adhesive can infiltrate from side face, the filter membrane and both electrode layers were coated with cyanoacrylate resin, with the effect that the adhesive coated region acts as an insulated electrical lead. The stick membrane was thus separated into three discrete areas: sensitive area, lead area and electrical terminal area.

A stick-type biosensor was constructed by immobilizing a alcohol oxidase (AOD:A2404, EC1.1.3.13, 10-40 units/mg protein, from Pichia pastoris, SIGMA-ALDRICH JAPAN K.K., Tokyo, Japan) into the sensitive region of the stick membrane. AOD was mixed with photocrosslinkable poly (vinyl alcohol) having stilbazolium groups (PVA-SbQ, Type: SPP-H-13 (Bio), Toyo Gosei Kogyo Co., Ltd., Tokyo, Japan) in a weight ratio of 1:1. The enzyme/PVA-SbQ mixture was placed onto the sensitive area and spread over the surface and side face of the membrane. The membrane stick were then placed in the dark at room temperature for 1 hr to allow for complete permeation, and then irradiated with a fluorescent light for 30 min. The device was immediately rinsed in phosphate buffer solution (100 mmol/L, pH 8.0) and any mixture on the surface of the electrode was removed. Because the mixture swells when wetted, it peels away from the surface and could be easily removed by gentle rubbing. After inspection for electrical shorts between the two electrodes, the stick type biosensor was stored below 10 °C until required.

The stick-type biosensor was calibrated using standard ethanol solutions in a batch measurement system (Fig. 2), by placing it into a 50 ml measuring cell filled with phosphate buffer (100 mmol/L, pH 8.0) at 25 °C. A two-electrode electrochemical method was employed, whereby the carbon layer was used as the working electrode and the Ag/AgCl layer was used as the counter/reference electrode. The stick-type biosensor was connected to the measurement system using a grip type connector.

Fig. 2. Experimental set-up of ethanol measurement.

Aliquots of standard ethanol solution (057-00451, Wako Pure Chemical Industries, Ltd., Osaka, Japan.) were added to the reaction cell and mixed using a magnetic stirrer (Pasorina mini-stirrer, TR-100, LOT.2048-807, As One Corp., Osaka, Japan). A computer controlled potentiostat (Model 1112, BAS Inc., Tokyo, Japan) was used to measure the oxidation current of hydrogen peroxide, produced by the enzymatic reaction of alcohol oxidase, at a fixed voltage of +900 mV which was the reduction peak potential of a cyclic voltammogram for hydrogen peroxide. The output current was continuously monitored with analog-to-digital converter (ADC-16, Pico Technology Co., Ltd., Cambridge, UK) on a computer graphic display and saved on hard disk for later analysis.

Next, the stick-type sensor was used as the sniffer device in a gas measurement system. A standard substance in the gas phase was supplied from a gas generator (PERMEATER, TYPE: PD-1B, GASTEC CORP., Kanagawa, JAPAN) which is a standardized machine approved by the Ministry for Labor and the Environmental Agency in Japan and by the Environmental Protection Agency (EPA) and the National Bureau of Standards (NBS) in the USA for gas calibration purposes. A gas sampling bag (880 ml, SAN GIP:G-4, 200×140×0.04 mm, C. I. Sanplus Co., Ltd.) was filled with various concentrations of gaseous substances supplied the gas generator. The sensitive area of the stick-type sniffer wetted with 10 μL of phosphate buffer (pH 8.0, 100mM) was quickly inserted into the opening mouth of the sampling bag with gaseous substance. The sensor current was measured by the computer-controlled potentiostat as noted above. A commercially available semi-conductor gas sensor used for alcohol testing (TYPE: TGS822, FIGARO ENGINEERING INC.,
OSAKA, JAPAN) was also used simultaneously in the measurement system and the characteristics of the two devices were compared. Other gaseous substances (n-pentane [160-00605], benzene [024-00706], methylethylketone [130-02496], n-hexane [085-00416], acetone [01200343], Wako Pure Chemical Industries, Ltd., Osaka, Japan), both individually and in a blended form, were also analyzed in order to investigate the selectivity of the sensors.

The ethanol concentration in expired gas after drinking was measured for healthy male subjects. The subjects were instructed before hand as to how determination was to be performed and asked to behave naturally. Fig. 3 illustrates the measurement processes of the ethanol vapor in breath air after drinking by the bio-sniffer with AOD and a detector tube for ethanol. The expired gas was collected into the sampling bag at 15 minute intervals after the subjects took 350 ml of beer (5.5 % alcohol), and then applied for the measurement of gaseous ethanol with the same method as described above. The sniffer device was used repeatedly after rinsed with phosphate buffer solution.

Fig. 3. Experimental set-up of both breath analyses with the bio-sniffer and the commercialized detector tube after drinking.

An ethanol detector tube (detective range 50-2000 ppm, Gastec Corp., Kanagawa, JAPAN) was also used simultaneously for the gas analysis of breath ethanol, and the characteristics of the two devices were compared. Prior to examine, an alcohol patch test with ethanol solution was applied to the subject’s forearm skin for evaluating their alcohol metabolic activity (ALDH2), and then the concentration change of breath air with time for some subjects with different ALDH activities was compared.

III. RESULTS AND DISCUSSION

As the results in the liquid phase, the current increased rapidly following addition of standard ethanol solution to give a steady state output. The response time to reach 90% of the steady current after applying ethanol solution was approx. 15 sec. The output current of the biosensor was linearly related to the ethanol concentration.

Next, the stick-type biosensor was applied for the gas measurement. The sensor current also increased rapidly following addition of standard ethanol vapor to give a steady state output which related to the applied ethanol concentration. The response time to reach 90% of the steady current after applying ethanol vapor was approx. 40 sec, which is slow than that in the liquid phase. The steady output was related to the concentration of ethanol in the gas phase. This calibration range covers the alcohol levels encountered in breath (over 130 ppm) after drinking. The detection limit is lower than the ethanol selective detection limit for the human sense of smell (6.1 ppm).

The selectivity of both the stick-type bio-sniffer and the commercially available semi-conductor gas sensor was examined with several kinds of gases and mixed gas. The semi-conductor gas sensor responded to all of the applied gases, showing the extremely poor selectivity of such devices as generally-known fact. The stick-type bio-sniffer using AOD, however, gave negligible responses to all the chemicals other than ethanol. Even by applying the mixed gas with ethanol and some solvent vapor, the bio-sniffer indicated the correct value without the influence from the solvent vapor. Consequently, the sniffer device possessed much greater selectivity and accuracy than the semi-conductor gas sensor (same selectivity as for the enzyme electrode).

Fig. 4 illustrates the comparison of the average time-variations of ethanol concentration in the expired air between ALDH2 (-) and ALDH (+) subjects. (As the results of the patch test, 40 % of the
Japanese subjects were determined to be negative of ALDH2. As the figure indicates, the sensor current increased following alcohol drinking with the peak value for both subjects 30 minutes after drinking. Then, the ethanol concentration in the expired air decreased gradually as the result of the metabolic response in the body. The ethanol values are consistent with that of the commercial available ethanol detector tube. The sniffer device is convenient to use for the non-invasive analysis of ethanol metabolism condition using the expired air.

Fig. 4. Comparison of ethanol concentration change in the expired air between ALDH2 (-) [filled circle] and ALDH (+) [open circle] subjects.

By comparison between both curves in the Fig. 4, the breath ethanol concentration in the ALDH (-) subjects was extremely higher than that in the ALDH (+) subjects from beginning to end of the examination. As the previous paper reported, the lower activity of ALDH2 induces the adverse affect to the ethanol metabolism, thus remaining ethanol and acetaldehyde molecule in the human body for a long time. The bio-sniffer is effective and convenient non-invasive approach to evaluate the ethanol metabolism condition using the expired air.

V. CONCLUSION

The stick-type bioelectronic sniffer (length: 70 mm, width: 2.0 mm) was constructed in a sandwich configuration with a filter paper placed between carbon- and Ag/AgCl electrodes by immobilizing AOD. The calibration range for ethanol vapor covered the concentration range encountered in breath after alcohol consumption including the permissible legal limit (130 ppm) for driving, and the sensing range of smell in humans. The bio-sniffer was used to monitor the concentration change of breath ethanol after drinking. Potential application of expiratory gas analysis with the enzyme device includes not only the ethanol metabolism condition, but also a non-invasive, convenient and safe method of diagnosing and monitoring disease states at a respiratory and digestive system.

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