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Real-time monitoring of skin ethanol gas by a high-sensitivity gas phase biosensor (bio-sniffer) for the non-invasive evaluation of volatile blood compounds

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ABSTRACT

In this study, a highly sensitive and selective biochemical gas sensor (bio-sniffer) and real-time monitoring system with skin gas cell was constructed for the determination of ethanol gas concentration on human skin. This bio-sniffer measured the concentration of ethanol according to the change in fluorescence intensity of nicotinamide adenine dinucleotide (NADH), which is produced in an enzymatic reaction by alcohol dehydrogenase (ADH). The NADH detection system used an ultraviolet light emitting diode (UV–LED) as the excitation light, and a highly sensitive photomultiplier tube as a fluorescence intensity detector. The calibration range of the ethanol bio-sniffer was validated from 25 ppb to 128 ppm. To measure the concentration of ethanol within skin gas, subjects ingested an alcohol beverage, and the sensor output was monitored. We chose the central part of the palm, a back of the hand, and a wrist as targets. The real-time concentration of skin ethanol gas at each target was measured after drinking. The maximum output values were reached at approximately 70 min after drinking and then gradually decreased. We showed that ethanol release kinetics were different depending on the part of the hand measured with the developed monitoring system. Accordingly, this highly sensitive and selective bio-sniffer with a skin gas cell could be used to measure ethanol on the skin surface and could be applied for breath and skin gas research, as well as investigation of volatile blood compounds used as biomarkers for clinical diagnosis.

1. Introduction

Volatile organic compounds (VOCs) emitted from the human body reflect concentrations of blood constituents (Broza et al., 2015; de Lacy Costello et al., 2014). VOCs are expected to be applied to biological information monitoring and medical fields (Saalberg and Wolff, 2016). By non-invasively measuring biological gas concentration, simple disease screening and metabolic evaluation can be performed (Di Natale et al., 2014; Gallagher et al., 2008). The analysis of VOCs in blood provides more accurate results than those from exhaled breath (Wang et al., 2014). However, biological gases such as breath, transdermal gas, and skin gas still contain specific components related to metabolic products and diseases. Since skin gas is easier to collect continuously in comparison with breath, it is suitable for the monitoring of gas concentration (Dini et al., 2013; Ohira and Toda, 2008; Shimouchi et al., 2010; Yamada et al., 2013). Therefore, the method of skin gas measurement can be a simple evaluation for collecting important information such as that in observations of release dynamics and kinetics.

Biological gas is a mixture of more than 2000 gaseous components, and the concentration fluctuates greatly with time (Qin et al., 1997). In addition, the concentrations of the various gas components generated from the human skin are very low (ppb-level) in comparison with volatile components in breath and blood (Supplemental Table 1) (Anderson et al., 2003; Kaneko et al., 1994; Mochalski et al., 2014; Schmidt et al., 2013; Spaněl et al., 2007). Therefore, a measurement system having gas selectivity, sensitivity, responsiveness, and continuity is strongly required for skin gas measurement. Measurement methods for breath and skin gas using a gas chromatography mass

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spectrometer and a semiconductor gas sensor have already been reported (Deng et al., 2004; Liu et al., 2014). Although chromatography and mass spectrometry methods provide accurate determination and highly sensitive detection, they still have disadvantages in term of cost, measurement time, lack of continuity and procedures that require skills. Solid-state gas sensors have also been developed and commercialized for environmental monitoring and alcohol testing devices (Di Natale et al., 2014; Wen et al., 2007). They have advantages in terms of cost, continuity, simple operation, and reasonable sensitivity (Dubbe, 2003; Thungon et al., 2017). However, they are not suitable sensors for the monitoring of human breath and skin gas because of their sensitivity and selectivity.

A highly sensitive and selective gas measurement by a gas measurement method using enzymes has been reported (Asal et al., 2018; Azevedo et al., 2005). Enzyme-based biosensors are a promising approach. For example, there are several enzymes that catalyze the oxidation of various chemical components (Arakawa et al., 2015; Kudo et al., 2010b; Toma et al., 2016). The evaluation of continuous lipid metabolism by measuring breath acetone and the imaging of spatiotemporal changes in ethanol in skin gas after drinking have been reported (Arakawa et al., 2017; Chien et al., 2017). Particularly, nicotinamide adenine dinucleotide (NADH)-dependent dehydrogenase is suitable enzyme for using in biosensors. Optical detection methods were used for NADH determination. NADH can be excited by UV irradiation as well as fluorescence with an emission peak at 490 nm, whereas oxidized NAD (NAD^+) has no fluorescence (Davitt et al., 2005; Kudo et al., 2010a, 2009). In our previous works, we have developed several fiber-optic NADH sensors for the detection of various chemical components. For the sensor developed here, fluorescence intensity increases as NADH forms though an enzymatic reaction. A rapid, compact and highly selective biosensors for formaldehyde, acetaldehyde, isopropanol and acetone have been developed (Chien et al., 2017; Iitani et al., 2017; Kudo et al., 2010b; Mitsubayashi et al., 2005; Ye et al., 2015).

In this research, we aimed to perform non-invasive and high-sensitivity monitoring of human skin gas. We have constructed a biochemical gas sensor (termed bio-sniffer) for the highly sensitive measurement of ethanol using alcohol dehydrogenase (ADH). The sensor was then applied to the continuous monitoring of skin gas using a skin gas sampling cell with a perspiration meter. We investigated and verified this sensor as a possible real-time monitoring system for gas released through various parts of human skin.

2. Experimental

2.1. Fabrication and construction of the ethanol bio-sniffer

For ethanol gas measurement, alcohol dehydrogenase (ADH) was used. ADH oxidizes ethanol using oxidized NAD (NAD^+) as a coenzyme in the catalytic oxidation of ethanol, producing reduced NAD (NADH). In this reaction, acetaldehyde and NADH are produced through the following enzymatic reaction:

$$\text{ethanol} + NAD^+ \xrightarrow{ADH} \text{acetaldehyde} + NADH + H^+ \quad (1)$$

As NADH has fluorescence properties (ex. 340 nm, em. 491 nm), it is possible to quantify ethanol by detecting the fluorescence changes of NADH. We fabricated an optical fiber biosensor (bio-sniffer) using this principle. In order to continuously measure ethanol gas, a bio-sniffer for ethanol gas using a flow cell was constructed (Fig. 1(a)). For the ethanol bio-sniffer, a pump cell and a fluorescence detector are connected to a bifurcated optical fiber (BIF 600-UV/VIS, SMA-905, Ocean Optics), and a flow cell having an enzyme immobilized membrane as a gas–liquid barrier diaphragm. Excitation light generated from a UV-LED source (λ: 335 nm, UVTOP® BL 335, Sensor Electronic Technology) excites NADH from the end face of the optical fiber via a bandpass filter (BPF, λ: 340 ± 10 nm, Asahi Spectra). The emitted fluorescence was collected from the end face of the probe and detected with a photomultiplier tube (PMT, C 9692, Hamamatsu Photonics) via BPF (λ = 490 ± 10 nm, Asahi Spectra).

The flow cell attached to the end face of the optical fiber type probe was made by machine-processing a solution flow path of 1.0 mm diameter into the side surface, a sump with a diameter of 5.0 mm, and a depth of 0.5 mm at the end face of an acrylic pillar (diameter: 12 mm) (Fig. 1(b)). The bio-sniffer was constructed with the flow cell, fiber probe, enzyme membrane and O-ring. The position of the probe was adjusted so that the gap between the probe end face, and so that the enzyme membrane was 0.4 mm, and was attached to this flow cell. Finally, the ADH immobilized membrane was fixed to the end face of the flow cell with a silicone O-ring, to obtain a bio-sniffer for ethanol. A buffer solution containing 1.0 mmol/l NAD^+ was injected into the inside of the flow cell at a flow rate of 0.5 ml/min from a solution flow path provided on the side of the flow cell. A buffer solution containing NAD^+ was delivered to the interior of the flow cell, and when the ethanol molecule flowing through the cell underwent oxidation on the porous enzyme membrane, the coenzyme NADH formed. This change in the intensity of NADH fluorescence was detected using an optical fiber and was used for the evaluation of the concentration of ethanol gas. In addition, a buffer solution was injected into the flow cell to supply coenzyme NAD^+, to wash the reaction products, and to prevent deactivation of the enzyme and thus to enable continuous gas sensing. The enzyme membrane was prepared by adding 2-methacryloyloxyethyl phosphorylcholine (MPC) and 2-ethylhexyl methacrylate (EHMA) to a hydrophilic porous polytetrafluoroethylene (PTFE) membrane (pore size: 0.2 μm, t: 80 μm, Omnipore, Milli-pore). ADH was entrapped and immobilized with poly [MPC-*co*-EHMA] (PMEH) which is a copolymer of 2-ethylhexyl methacrylate (EHMA) (Chu et al., 2009). The advantage of using PMEH is its high biocompatibility. In the preparation of the ADH membrane, a mixture solution of 10 μl/cm^2 of an enzyme solution in which ADH (EC 1.1.1.1, 128 units/mg solid, Oriental Yeast) was dissolved in pure water was used in addition to 10 μl/cm^2 of 15% PMEH.

The response to an ethanol gas standard, quantitative characteristics, and gas selectivity were investigated for characteristics evaluations of the fabricated sensor. In the evaluation experiments, changes in fluorescence intensity were measured for ethanol gas at various concentrations (8 ppb to 128 ppm) from a standard gas generator, were measured. The system was evaluated for responsiveness and quantitative characteristics were examined.

2.2. Ethanol bio-sniffer for human skin gas measurement

We then applied the constructed sensor to evaluate ethanol concentrations in skin gas after human subjects had consumed alcohol. The approval of the Ethics Review Committee of the Tokyo Medical and Dental University Biological Materials Engineering Institute was received (approval number: 2012-6). Prior to alcohol intake, each healthy adult subject who gave consent was measured for body temperature, blood pressure, and pulse rate. The purpose of the experiment was explained, and the subject's physical condition was verified. A questionnaire on diet and lifestyle habits was also administered. An alcohol patch test containing ethanol was conducted to classify ALDH 2 [+] (active form) and ALDH 2 [−] (inactive form) in the subject.

2.3. Measurement of skin ethanol gas from the whole hand

In order to confirm the existence of ethanol in the skin gas and to estimate the gas concentration, ethanol gas released from the whole hand after alcohol consumption was measured (Supplemental Fig. 1). A Teflon sample bag was prepared as the skin gas collection bag containing an opening for hand insertion, a carrier gas introduction portion, and a skin gas discharge portion. The prepared skin gas sampling

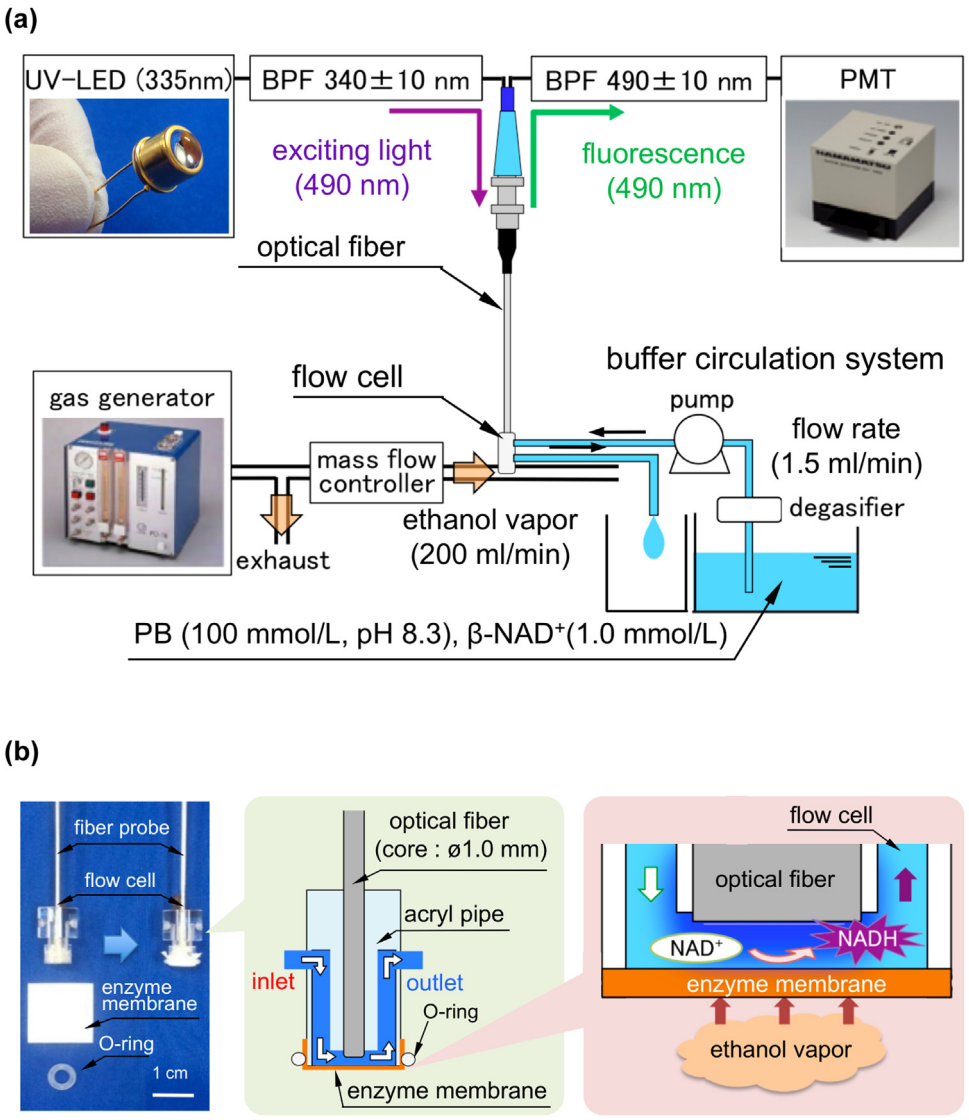


Fig. 1. Schematic illustration of ADH-immobilized bio-sniffer for the detection of ethanol gas. (a) Experimental setup of ethanol bio-sniffer using UV-LED as excitation light source and (b) photograph of a bio-sniffer and structural drawing of a flow cell (including enzyme membrane and optical fiber probe).

bag was placed on the subjects and sealed at the radiocarpal joints. Next, the carrier gas, clean air, was introduced from the gas inlet of the sampling bag, and the skin gas was collected (60 and 200 ml/min) from the outlet of the other end to the sensor for measurement.

2.4. Skin ethanol gas measurement using sampling cell

In order to investigate the release kinetics of skin gas in detail, skin gas measurement using the skin gas sampling cell was performed (Fig. 2). In the skin gas cell, a cylindrical PTFE material (ø20 mm) was machined, grooves with a diameter of 11.3 mm (skin gathering area 1.0 cm^2) and a depth of 2.5 mm were machined out in the center part, and then the carrier gas inlet and fittings (for ø4 mm) were installed as gas outlets (Fig. 2(a)). Similarly, we prepared two other skin gas collection cells with collection areas of 4.0 cm^2 (diameter 22.3 mm) and 10.0 cm^2 (diameter 35.7 mm), and the optimal skin gas cell size was evaluated. For skin gas collection by skin gas cell, double-sided tape was cut into a circle having the same size as the cell collection area, attached to the skin-contact side of the skin gas cell, and fixed to the skin site tightly. Clean air was introduced through the gas inlet of the skin gas cell and released (60 ml/min) through the outlet of the cell, and this was applied to the sensor sensitive part as skin gas. Three skin targets were evaluated: the central part of the palm, the back of the hand, and the wrist. The change over time of the ethanol concentration in skin gas at the time of drinking was measured (Fig. 2(b)). By simultaneously performing skin gas measurement and perspiration measurement, the effect of perspiration on skin gas measurement was also investigated. For perspiration measurement, a ventilated capsule-type perspiration meter (Digital perspiration meter, SKN-2000, Nishizawa Electric Meters Manufacturing Co., Ltd.) quantifying the difference relative to a reference was used. In the perspiration measurement, sweating capsules were attached in the vicinity of the skin gas cell, and perspiration was measured simultaneously with skin gas (Fig. 2(c)).

3. Results and discussion

3.1. Characteristics of the ethanol bio-sniffer

The response and quantitative characteristics of an ethanol standard measured with prepared bio-sniffer to standard ethanol gas are shown in Fig. 3. We confirmed a steady increase in measured output accompanying gas load and a stable concentration (Fig. 3(a)). Recovery to the initial value after stopping the gas supply was observed. The change in fluorescence intensity due to ethanol oxidation on the ADH-

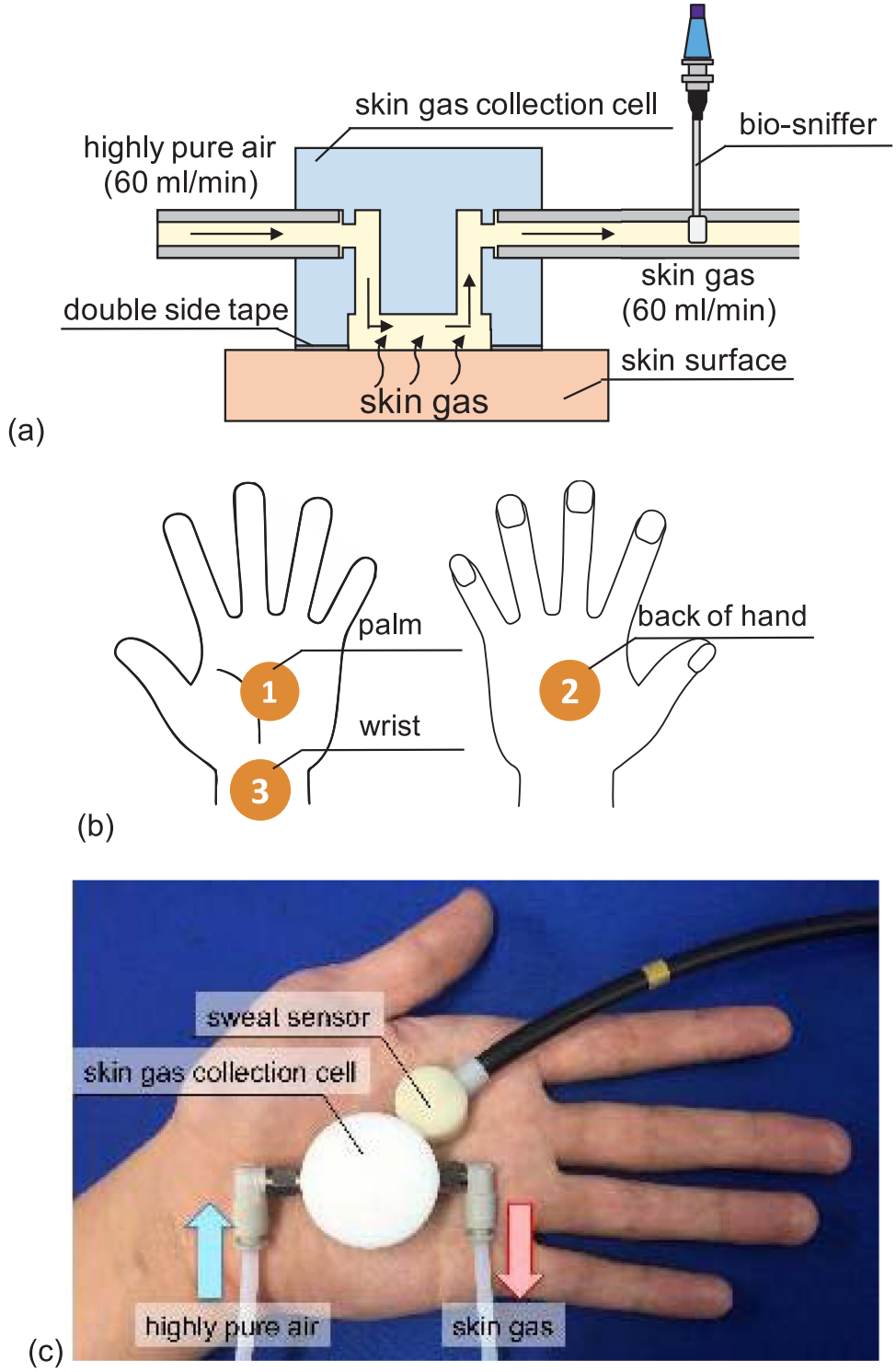
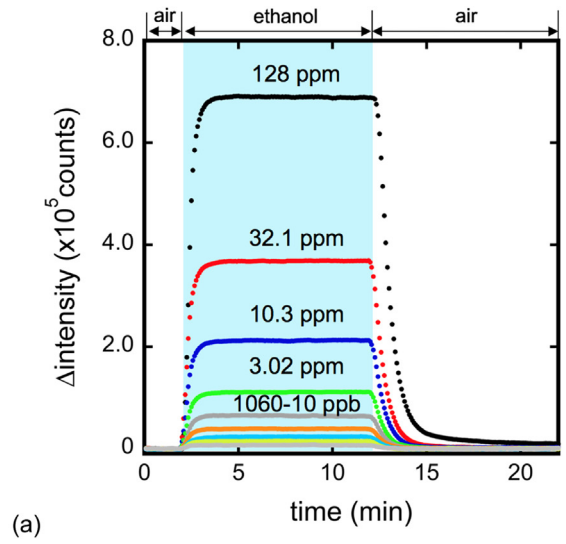


Fig. 2. (a) Experimental setup of skin gas measurement using a skin gas cell, (b) Schematic drawing of the measurement sites (1. palm, 2. back of hand, 3. wrist), (c) Photograph of the experimental setup for simultaneous measurement of sweating and skin gas.

immobilized membrane was measured with the loading of ethanol, and NADH was generated and increased. Quantitative characteristics of ethanol gas were investigated using the average value for 2 min, during which the fluorescence output of the sensor was stable as the output value. As a result, we could quantify in the concentration range of 25 ppb to 128 ppm, including the ethanol concentration at rest (37–207 ppb), the exhaled breath concentration at drinking alcohol regulation value (78.2 ppm or more), and the maximum skin ethanol gas concentration (73.9–112.1 ppb/cm^2). The sensor was able to sufficiently and quantitatively measure ethanol in both the breath and skin gas after alcohol consumption. The correlation between ethanol concentration and fluorescence output is given by the Eq. (2) (r = 0.999).

$$\Delta\text{intensity (counts)} = -4.04 \times 10^3 + \frac{7.91 \times 10^5 + 4.04 \times 10^3}{\left(1 + \left(\frac{\text{ethanol conc [ppb]}}{9.97 \times 10^4}\right)^{-1.06}\right)^{0.52}} \quad (2)$$



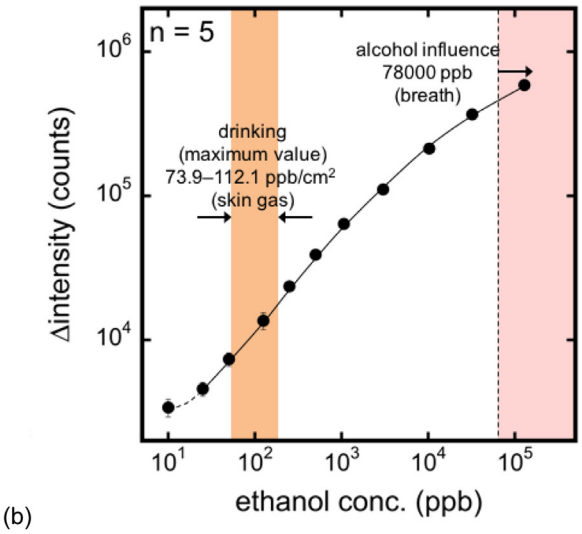


Fig. 3. (a) Changes in various concentrations of standard ethanol gas over time. The steady-state value was determined as the balance production rates of NADH. (b) Calibration curve for the ethanol bio-sniffer using ethanol gas. The fluorescence intensity relates to the concentration of ethanol gas from 25 ppb to 128 ppm. (C.V = 3.62%, 1060 ppb ethanol gas).

The gas selectivity of the prepared bio-sniffer is shown in Fig. 4. In the evaluation of gas selectivity, outputs were compared on the basis of representative expiratory components contained in human breath. Exhaled breath concentrations have been reported for ethanol (10.0 ppb), methanol (142.0 ppb), 1-propanol (8.10 ppb), 2-propanol (3.21 ppb), 1-butanol (1.32 ppb), formaldehyde (4.30 ppb), acetaldehyde (7.00 ppb), acetone (628 ppb), 2-butanone (0.38 ppb) (Konvalina and Haick, 2014). The output to ethanol is assumed to be 100%, and the output is compared with other gas species (1000 ppb). As shown in Fig. 4, the highest output of ethanol was confirmed. An output of 32.3% for 1-propanol was confirmed, but almost no output was shown for other components. Thus, gas selectivity based on the substrate specificity of the enzyme was obtained. The concentration of 1-propanol contained in human breath was approximately 8.1 ppb, which is extremely low compared with the ethanol concentration of exhaled breath after drinking. Therefore, the influence on ethanol gas measurement associated with drinking alcohol was small.

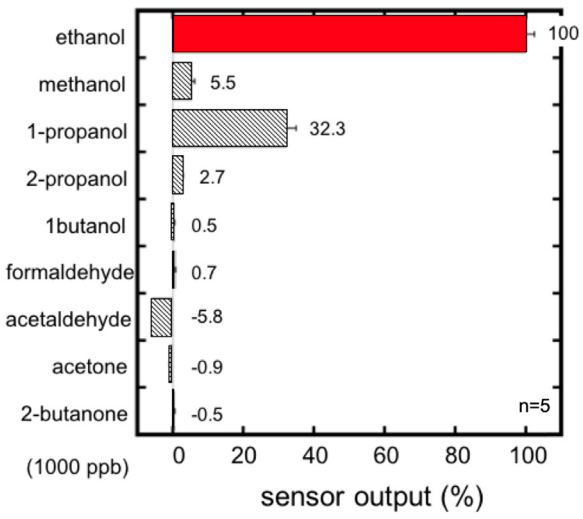


Fig. 4. Gas selectivity of the ADH bio-sniffer. The bio-sniffer response to 1 propanol and methanol were 32.3% and 5.5%, respectively. A smaller response was detected for other chemical substances.

3.2. Human breath and skin gas measurements

Measurement of breath ethanol gas at rest and after drinking was conducted in multiple human subjects. As with the gas standard, a stable value was obtained according to the increase in output and the concentration with breath. The calibration formula was determined by the breath ethanol gas concentration average upon drinking, resting and maximum values were calculated to be 57.9 ± 9.3 ppb (n = 10) and 47.1 ± 8.2 ppm (n = 10), respectively. Breath ethanol concentration of healthy volunteers at rest was 37–207 ppb. A breath ethanol concentration of a similar degree of alcohol consumption was reported. A breath ethanol concentration of similar degree of alcohol consumption was reported as 45.8 ± 6.0 ppm (Jones and Andersson, 2003). Since the skin gas concentration upon drinking was 73.9–112.1 ppb/cm^2, the bio-sniffer measurements were sufficient and sensitive.

Next, the ethanol gas contained in the skin gas released from the whole hand (from the fingertip to the wrist) was measured with an improved sample bag (200 ml/min). As shown in Supplemental Fig. 2, the concentration of ethanol gas contained in the skin gas increased approximately 7 min after drinking, and the maximum concentration reached 14.3 ppm at about 23 min, and then gradually decreased. At the same time, breath measurement was carried out, and the concentration over the same period was similar to the reported values.

Since the area of the whole hand from the fingertip to the wrist is about 300 cm^2, the skin gas concentration after drinking was estimated to be 46 ppb/cm^2. The reported ethanol concentration in skin gas after drinking was 73.9–112.1 ppb (Jones and Andersson, 2003). This result was lower than the reported value. A part of the skin gas seemed to be dissolved in moisture condensed in the sample bag due to sweating. The influence of perspiration as a result of sealing the sample bag was considered. As mentioned above, it was possible to measure skin ethanol gas with the constructed bio-sniffer, and the possibility of noninvasive measurement of the ethanol concentration in blood after drinking could be confirmed.

However, due to the influence of sweating and the necessity of using a sampling bag, we decided to investigate the effects of wearing the skin gas cell on its use and selected the best measurement area to suppress the effect of perspiration. In addition, the reduction of gas concentration in the skin gas cell was taken into consideration. The influence of the carrier gas flow rate on the output was investigated by the method using the above sampling bag. By reducing the flow rate of 200–60 ml/min, it was possible to increase the output by 3.25 times on the average.

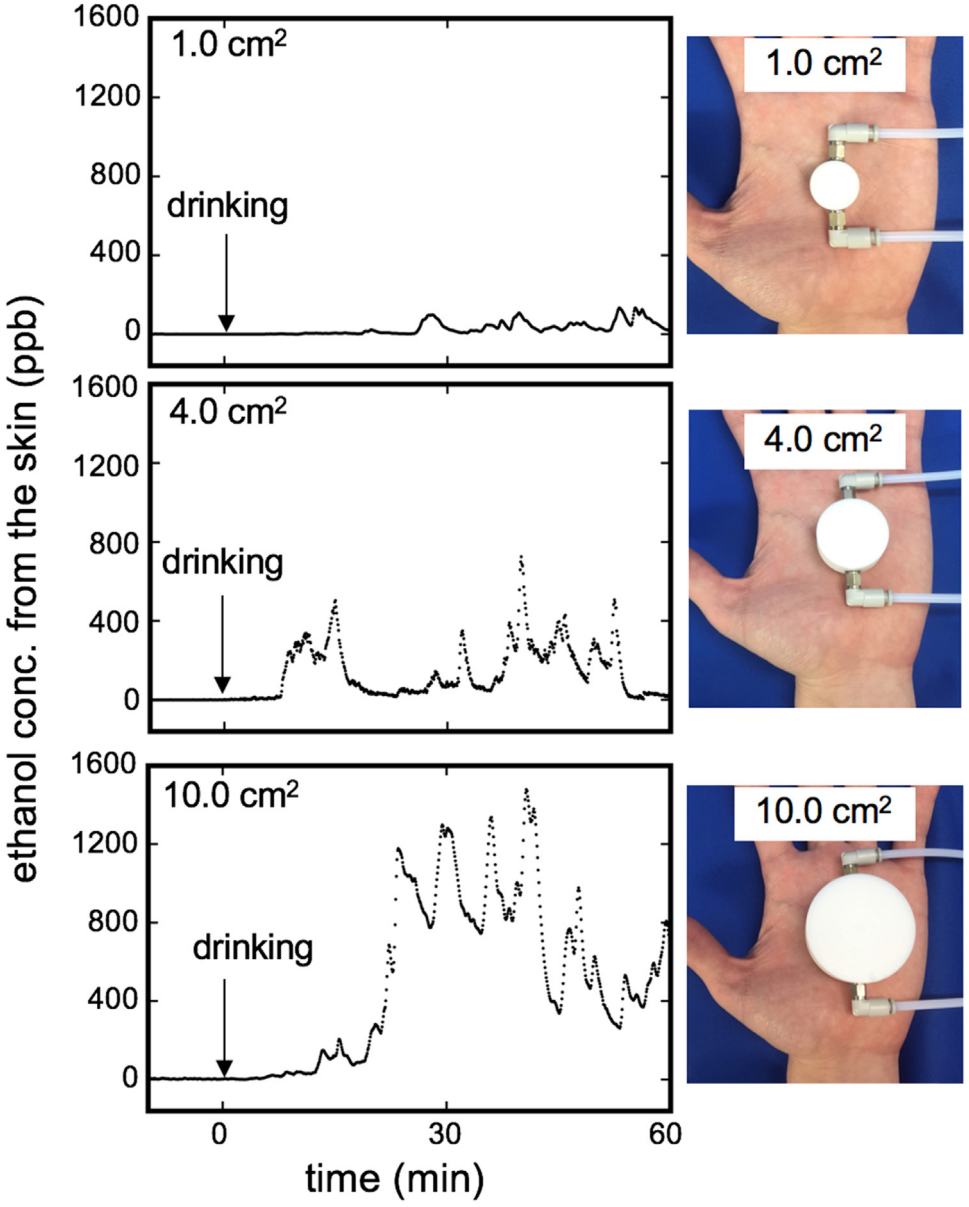


Fig. 5. Output response in various skin gas cells (1.0, 4.0, and 10.0 cm^2). The skin gas cell with a collection area of 10.0 cm^2 have stable and accurate skin gas measurement by reducing the influence of sweating.

In subsequent experiments on skin gas cells, the flow rate was set at 60 ml/min.

3.3. Skin gas cell for ethanol gas measurement

Skin gas measurements were conducted at three sites (the palm, the back of the hand, and the wrist) using a skin gas cell with a skin gas sampling area of 1.0 cm^2 after alcohol consumption. As shown in Supplemental Fig. 3, a high concentration of ethanol gas was observed as a spike in the output of the palm area. This is based on intermittent perspiration of the palm, which is not suitable for noninvasive measurement of ethanol. However, comparatively stable change in ethanol concentration with time were observed in skin gas measurements at the back of the hand and the wrist, but the outputs were low. The ethanol concentrations of the wrist and the back of the hand were estimated to be around 15–50 ppb. The concentration of the ethanol gas that could be collected was low, and accurate measurement was difficult using the skin gas cell with an area of 1.0 cm^2. Therefore, cells with larger collection areas were examined in order to stably measure the gas. We compared the ethanol concentration in the skin gas using three types of skin gas cells (collection areas of 1.0, 4.0 and 10.0 cm^2), as shown in Fig. 5. The total amount (mg) of ethanol collected in 60 min for each cell size was determined. High output values were obtained in skin gas cells with a collection area of 10.0 cm^2, in addition to stable and accurate skin ethanol gas measurements. Thus, the skin gas cell with collection area of 10.0 cm^2 was applied to skin gas measurement.

3.4. Evaluation of breath and skin gas measurement

The measurement of ethanol in the skin gas at a skin gas using a cell with a collection area of 10.0 cm^2 and flow rate of 60 ml/min was carried out on the palm, the back of hand and the wrist. Fig. 6 shows the measurement results of the skin gas, the concomitant results of the breath ethanol in the exhaled gas, and the perspiration measurement. The upper graph shows perspiration rate, the lower graph shows the ethanol concentration in the skin gas using bio-sniffer, and the plot shows the ethanol concentration in the breath. Fig. 6(a) shows the results of the palm area. Similar to the skin gas cell with an area of 10.0

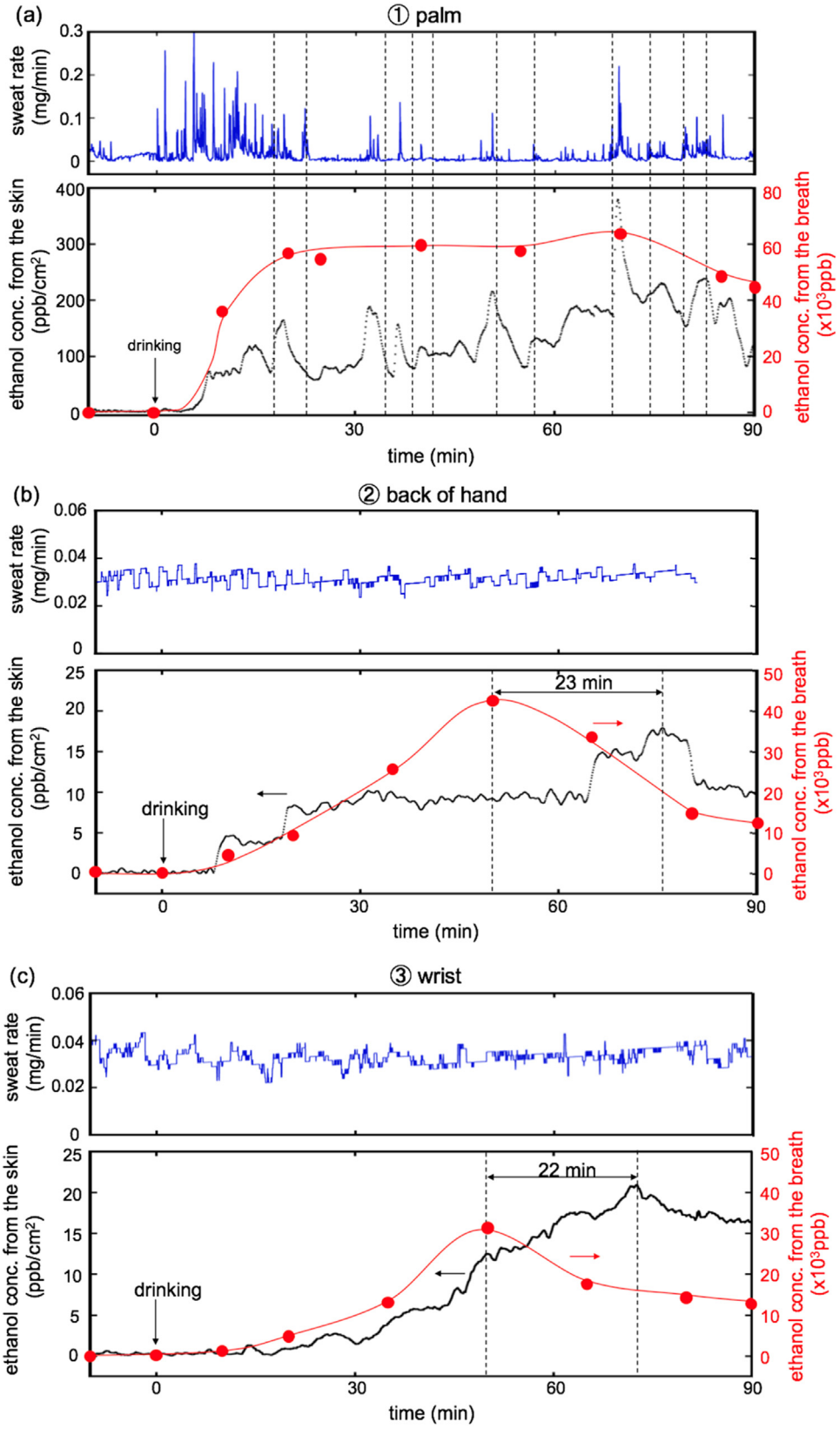


Fig. 6. Simultaneous measurement of concentration of skin ethanol gas and sweating amount in skin gas (a) Sweat rate, (b) ethanol concentration on skin, (1. palm, 2. back of hand, 3. wrist).

cm^2, the skin ethanol gas in the palm area had a concentration higher than those of the other parts and produced a spike-like output, and the concentration fluctuation was large. In the perspiration measured at the same time, a strong spike-like perspiration was observed shortly after drinking. After that, it gradually decreased and returned to the value at rest. When comparing the results of perspiration with ethanol

concentration in skin gas, the tendency of the output of both was consistent. The density of the sweat gland in the palm (approximately 620 ± 120/cm^2) was higher than that in other parts (forearm 225 ± 25/cm^2), this perspiration phenomenon was considered to be largely influence skin ethanol gas concentration (Wilke et al., 2007). As the sweating phenomenon depends on temperature, humidity, and psychological conditions, we considered it to be unsuitable as a stable evaluation site of blood ethanol concentration.

Fig. 6(b) shows the back of the hand, and Fig. 6(c) shows the experimental results for the wrist. As can be seen from the figure, the time course of the ethanol concentration in the skin gas showed a relatively smooth output change. In addition, the spike-shaped output and spike-like perspiration as seen in the palm were not observed in perspiration measurements for these other areas; in other words, there was a smaller influence of perspiration. We also compared the changes over time of ethanol concentration in breath gas and skin gas. Ethanol concentrations in the skin gas of both the wrist and back of the hand and breath gas showed the similar concentration changes with a delay of about 20 min. Especially in the wrist, it was possible to measure ethanol in skin gas with good correlation with breath concentration. The maximum concentration of ethanol in the skin gas from the back of the hand was 17.5 ppb/cm^2, and the wrist was 20.9 ppb/cm^2, which is slightly lower than the reported value, but it is a reasonable result.

Using the developed ethanol bio-sniffer, we carried out measurements of ethanol in skin gas at three sites: the palm, back of the hand, and wrist, under the optimal conditions of the skin gas cell (collection area of 10.0 cm^2 and a flow rate of 60 ml/min). Correlating the measurement of skin ethanol gas with breath concentration after drinking was possible because skin gas measured at the wrist had little dependence on perspiration. The wrist is thin in the stratum corneum and the skin layer; desirable results have been reported in the measurement of arterial blood gas. Thus, the wrist is a suitable part of the human body for measuring skin gas derived from blood ethanol.

4. Conclusion

In conclusion, a highly sensitive bio-sniffer using ADH was constructed, and skin gas measurement after drinking alcohol was carried out. We constructed an ADH-immobilized bio-sniffer, and selective measurement of ethanol gas, including skin gas during breathing and ethanol concentration during exhalation, was possible within the concentration range of 25 ppb to 128 ppm. In order to continuously measure ethanol in the skin gas using a bio-sniffer, the skin gas measurement device composed of a skin gas cell was applied to three parts of the hand: the palm, back of the hand, and wrist.

Continuous measurement of the ethanol in skin gas was possible without the effect of perspiration at the wrist. This result correlates with simultaneous exhalation measurement, which suggests that blood ethanol can be evaluated noninvasively on the skin surface. In the future, it is expected that volatile components in the blood including acetone, which is an indicator of lipid metabolism, can be noninvasively evaluated through the skin by increasing the sensitivity of various types of bio-sniffers

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2018.09.070.

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