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Sensitive and selective methanol biosensor using two-enzyme cascade reaction and fluorometry for non-invasive assessment of intestinal bacteria activity

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ABSTRACT

For understanding the status of intestinal flora non-invasively, methanol (MeOH) has been attracting the attention. In this study, we have developed and compared two different liquid-phase methanol biosensors. One, referred to as the AOD electrosensor, utilized alcohol oxidase (AOD) and an oxygen electrode. It electrochemically measured the decrease in oxygen through AOD-catalyzed oxidation of MeOH. The other, referred to as the AOD-FALDH fluorosensor, exploited a cascade reaction of AOD and formaldehyde dehydrogenase (FALDH) in conjunction with a fiber-optic sensor. It measured increase in the fluorescence from reduced form of β-nicotinamide adenine dinucleotide (NADH) that was a final product of the two-enzyme cascade reaction. Due to the cascade reaction, the AOD-FALDH fluorosensor showed 3 times better sensitivity along with 335 times wider dynamic range (494 nM–100 mM) than those of the AOD electrosensor (1.5–300 μM). The selectivity to MeOH was also improved by the cascade reaction with AOD-FALDH as no sensor output was observed from other aliphatic alcohols than MeOH in contrast to the AOD electrosensor. Although the use of FALDH resulted in the increase in the sensor output from aldehydes, such as acetaldehyde and formaldehyde, considering their concentrations in body fluids, the influence on the sensor output is limited. These results indicate that incorporating the cascade reaction into fluorometry enables enhanced biosensing of MeOH that will be useful for assessment of intestinal flora with little burden.

1. Introduction

In the intestine, more than 100 trillion bacteria are present and dominated by two species: the *Bacteroidetes* and the *Firmicutes* (Ley et al., 2006; Savage, 1977; Sekirov et al., 2016; Whitman et al., 1998). There were reports mentioning that the composition of these two types of intestinal bacteria and their metabolites affect the host's immune function, drug metabolism and absorption, and obesity (Achilefu et al., 2017; Hooper et al., 2003; Turnbaugh et al., 2006). Therefore, it is important to know the status of the intestinal microflora to maintain the health (Larsen et al., 2010; Ravcheev et al., 2013).

At present, the culture method is widely used to observe intestinal bacteria, in which the bacteria is evaluated from the morphology and colour tone of the obtained colonies (B.S.Drasar, 1967; Mata et al., 1969). Although this method is inexpensive, it requires a long time to obtain results and expertise in proper culture. Genome analysis by next-generation sequencers, which does not require the culturing, can obtain nucleotide sequence information in a short time with high-throughput, but the cost of the equipment, including the supercomputers for the data process, is a problem (Abubucker et al., 2012). In addition, both methods require stool collection, which is very burdensome for subjects.

Bacteroides in intestine consumes and decomposes dietary fiber, pectin, to produce methanol (MeOH). This metabolic MeOH is transferred to the bloodstream and released from biological gases such as exhaled breath. For example, Dorokhov et al. reported that MeOH concentration in blood increased up to 1.5 times after intaking vegetables (Dorokhov et al., 2012). A study by Lindinger et al. also reported

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that not only directly having pectin but also eating fruits containing pectin, such as apples, resulted in the increase in MeOH concentration in breath (Lindinger et al., 1997).

According to other reports, not only biological gases but also some noninvasively available body fluids contain MeOH. For example, the studies by Leaf et al., Kawai et al. and Shindyapina et al. reported that MeOH concentrations in blood and urine, or saliva are correlated (Kawai et al., 1991; Leaf and Zatman, 1952; Shindyapina et al., 2014). MeOH is a toxic substance but, even it is produced in a body, the concentration in blood is 0.20–5.37 mg/L, which is 400–1000 times less than the hazardous level. The MeOH concentrations in saliva and urine are lower than that in blood, and they are about 1/3 and 1/1.3 of the blood, respectively (Leaf and Zatman, 1952; Shindyapina et al., 2014). These reports suggest the potential for non-invasive assessment of intestinal bacteria activity by measuring MeOH in breath, saliva, or urine.

Conventionally, gas-liquid chromatography is used to detect MeOH in blood, but the system is large, and it requires trained personnel. In contrast to analytical devices, there are many expectations for sensors as simpler and more compact detection methods. For such a sensor to be useful for methanol measurement, it must have high sensitivity and selectivity. In particular, it is difficult but important to be able to distinguish between MeOH and aliphatic alcohols, which have similar physical and chemical properties. As the state-of-the-art research, various approaches have been introduced to selectively detect MeOH. For example, Zou et al. used alcohol responsive polymer, poly(N-isopropylacrylamide-*co*-N,N-dimethylacrylamide) (poly(NIPAM-*co*-DMAA)), which formed the shrinking state in ethanol and stretching state in methanol. Therefore, the methanol concentration could be measured separately from ethanol by optical transmittance through this polymer solution (Zou et al., 2014). Acharyya et al. made three dimensional nanoflowers of ZnO on which nanoparticles of NiO and PdO were dispersed. This nanostructure allowed to selectively measure methanol at low temperature of 150 °C (Acharyya et al., 2016). Wang et al. reported a metal organic framework which showed more enhanced fluorescence to methanol out of ethanol and n-propanol (J. J. Wang et al., 2016). There are also approaches using microelectromechanical systems. Wang et al. developed a microfluidic distillation chip that allowed for separation of MeOH from water and ethanol using differences in the vapor points of each substance (Y. N. Y.N. Wang et al., 2016).

We have developed biosensors that utilized enzymes to selectively measure volatile organic compounds both in the liquid and gas phases, such as formaldehyde, ethanol, acetaldehyde, acetone, and isopropanol for the non-invasive metabolism assessment and disease screening (Arakawa et al., 2019; Chien et al., 2017; Iitani et al., 2018; Toma et al., 2016; Ye et al., 2015). In this study, we present two different liquid phase methanol biosensors for evaluation of intestinal bacteria activity and compare their characteristics. The first biosensor, referred to as the AOD electrosensor, utilized an alcohol oxidase (AOD) that catalyzed MeOH oxidation. MeOH was measured by oxygen consumption by a Clark oxygen electrode. The other biosensor, referred to as the AOD-FALDH fluorosensor, utilized two enzymes, AOD and formaldehyde dehydrogenase (FALDH), for a cascade reaction. MeOH was firstly oxidised by AOD, then produced formaldehyde was oxidised by FALDH together with reduction of a coenzyme, oxidised form of β-nicotinamide adenine dinucleotide (NAD^+). The resultant reduced form of NAD (NADH) was then detected by its autofluorescence.

2. Experimental

2.1. Materials

AOD (A2404-1KU, from *Pichia pastoris*) was purchased from Sigma-Aldrich (Japan). FALDH (from *Pseudomonas* sp.) was purchased from Funakoshi (Japan). NAD^+ was purchased from Oriental Yeast (Japan). A hydrophilic polytetrafluoroethylene (H-PTFE) membrane (thickness of 80 μm, porosity of 80%, pore size of 0.2 μm) used for an enzyme-immobilized membrane was from Millipore (USA). Poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-2-ethylhexyl methacrylate (EHMA)] (PMEH) was synthesized in house by free radical-polymerization method (Kudo et al., 2008). Polyethyleneimine (PEI, average molecular weight of 10000, 164-17821) and glutaraldehyde (GA, 079-00533) were from Wako (Japan). Polyvinyl alcohol bearing styryl pyridinium groups (PVA-SbQ) was from Toyo Gosei (Japan).

All the chemicals to prepare the following two different buffer solutions were from Fujifilm Wako Pure Chemical (Japan). Phosphate buffer (PB, 100 mM, pH 7.5) solution was prepared by adding potassium dihydrogen phosphate solution (100 mM in ultrapure water) to disodium hydrogen phosphate solution (100 mM in ultrapure water) to buffer the solution pH to 7.5. Tris-HCl solution (100 mM) was prepared to add HCl solution to 100 mM trimethylolaminomethane solution to buffer the pH to 8.5.

2.2. Construction of AOD electrosensor

In the AOD electrosensor, MeOH was measured by the decrease in oxygen concentration at a platinum (Pt) electrode of the Clark oxygen electrode (Fig. 1a). The Pt electrode catalytically reduces oxygen when the voltage of −600 mV is applied between the Pt electrode and an Ag electrode. Therefore, the AOD electrosensor outputs the increase in the current with increasing MeOH concentration.

The AOD electrosensor comprised of the Clark oxygen electrode (SBO-00P0.5, Able, Japan) and an AOD membrane, and the sensor was connected to a pontentiostat (MODEL1112, Husou seisaku, Japan). The data was acquired from an analogue digital converter (ADC-16, Pico Technology, UK) which was connected to the potentiostat. The AOD membrane was attached on the tip of the Clark oxygen electrode and fixed together with a nylon mesh by an o-ring.

2.3. Construction of AOD-FALDH fluorosensor

To develop the AOD-FALDH fluorosensor, a cascade reaction with AOD and FALDH was employed (Fig. 1b). In the reaction, initially MeOH is oxidised by AOD that catalyses oxidation of primary alcohols to produce formaldehyde. Then, the produced formaldehyde is oxidised by FALDH along with the reduction of a coenzyme, NAD^+, to produce formic acid and NADH. The biosensor utilized NADH autofluorescence, emitting the fluorescence (λ_{em} = 490 nm) under the excitation of ultraviolet light (λ_{ex} = 340 nm), which enabled to detect increase in the MeOH concentration by the increase in the fluorescence intensity from NADH.

The setup comprised of a bifurcated optical fiber (BIF600-UV/VIS, Ocean Optics, USA), an optical fiber probe (F1000-900, Ocean Optics, USA), an UV-light emitting diode (UV-LED, UF4LU-0GD01, DOWA, Japan), a photomultiplier tube (PMT, C9692-11, Hamamatsu Photonics, Japan). The AOD-FALDH membrane was attached on the end of the optical fiber probe with a silicone tube. Two bandpass filters (BPFs, λ = 340 ± 10 nm and 490 ± 10 nm, Asahi Spectra, Japan) were placed in the light pathways to eliminate unwanted light for the excitation and fluorescence light.

2.4. Preparation of enzyme membranes

First, an AOD membrane was prepared using three different materials to find the suitable material for the enzyme membrane: PEI and GA; PVA-SbQ; PMEH (Fig. 2). The immobilization with PEI and GA relied on the chemical bonding between PEI, GA and AOD, whereas that with PVA-SbQ or PMEH was physical entrapment in a polymer matrix.

The preparation of the AOD membrane with PEI and GA was carried out by chemical bonding of AOD through crosslinking of GA to PEI at a H-PTFE membrane as follows: 300 μL PEI solution (1w/w% in PB) was added dropwise to the 2 × 2 cm^2 H-PTFE membrane and gently shaken

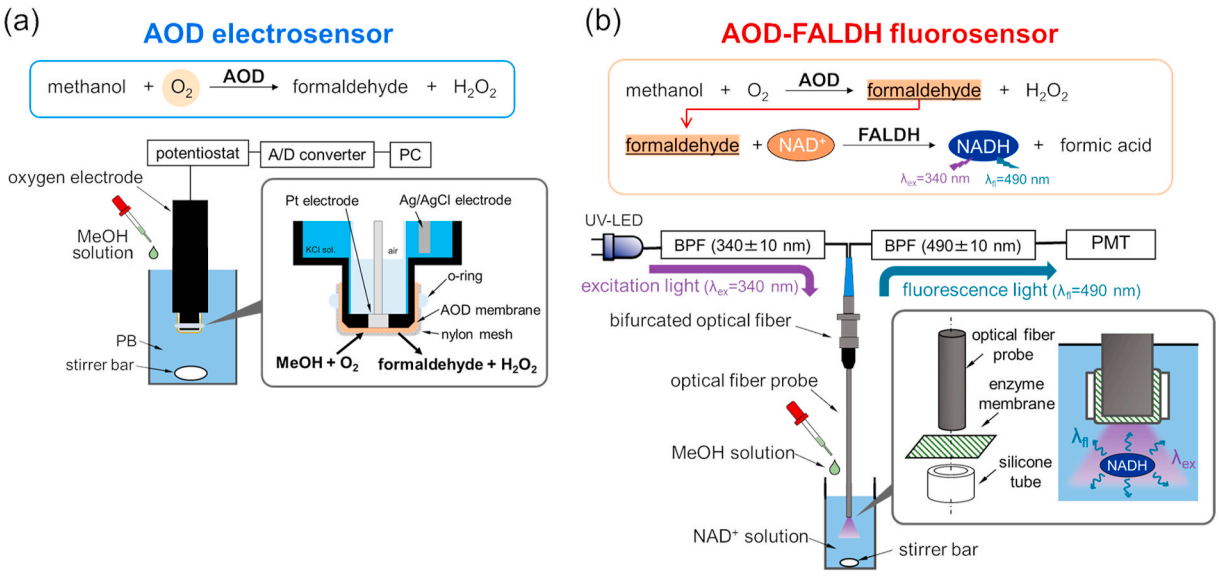

Fig. 1. Measurement principles (upper) and schematic illustrations (lower) of (a) AOD electrosensor and (b) AOD-FALDH fluorosensor.

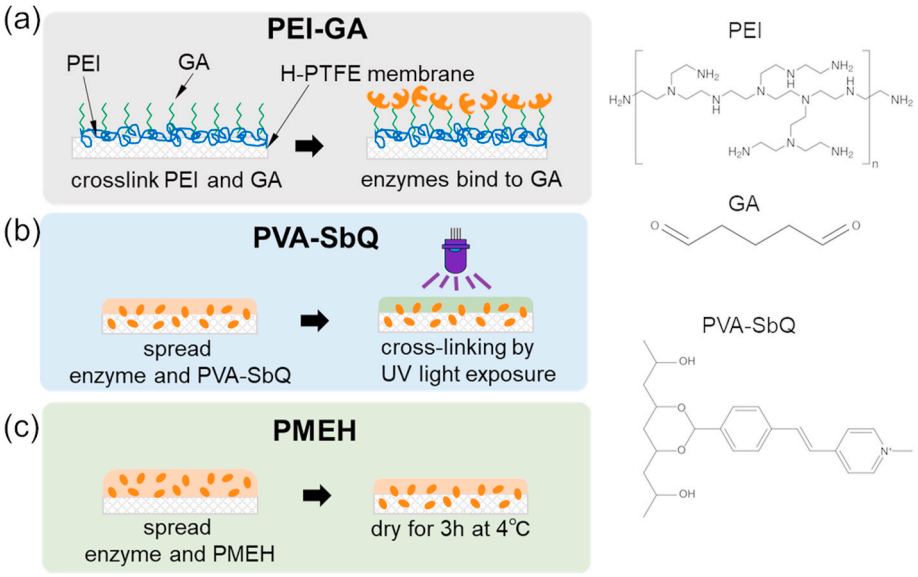

Fig. 2. Preparation processes of AOD membranes using (a) PEI-GA, (b) PVA-SbQ, and (c) PMEH.

for 5 min; the membrane was rinsed with 300 μL PB for three times to remove excessive PEI; 300 μL GA solution (2.5v/v% in PB) was added to the membrane dropwise and gently shaken for 5 min to crosslink PEI and GA; the membrane was rinsed with PB for three times to remove excessive GA; AOD at a certain volume was applied to the membrane and left for 15 min at a dark cold place (4 °C) to react with GA for being immobilized at the membrane.

The AOD membrane with UV-curable polymer, PVA-SbQ, was prepared as follows: A mixture of 5.67 μL AOD (2.0 U/cm^2) and 120 mg PVA-SbQ was homogeneously spread onto a 3 × 3 cm^2 H-PTFE membrane; after drying the membrane at a dark cold place (4 °C) for 1 h, the membrane was exposed to UV-light (λ = 352 nm, 6.4 μW/cm2) for 5 min to cure PVA-SbQ for entrapping AOD in the polymer matrix.

The AOD membrane with a biocompatible polymer, PMEH, was prepared similarly to the membrane with PVA-SbQ: A mixture of 5.67 μL AOD (2.0 U/cm^2) and 40 μL PMEH (10w/w% in ethanol) was homogeneously spread onto a 3 × 3 cm^2 H-PTFE membrane, then the membrane was dried at a dark cold place (4 °C) for 3 h.

An AOD-FALDH membrane was prepared in a similar way to the AOD membrane using PEI and GA. Briefly, after crosslinking PEI and GA and rinsing excessive GA with PB for three times, a mixed solution of AOD and FALDH was coated dropwise to the membrane and left for 15 min at a dark cold place (4 °C). Finally, Tris-HCl solution was dropped to the membrane to passivate residual aldehyde group of GA in order to suppress non-specific binding of NADH to GA because NADH also has an amino group.

3. Results and discussion

3.1. Investigation of a suitable material for enzyme membranes

First, the immobilization material for an enzyme membrane was selected by preparing an AOD membrane with three different materials. Fig. 3a shows the sensor responses of the differently prepared AOD electrosensor to 100 μM MeOH solution. The output currents from the sensor using the PEI-GA showed the largest increase in the output

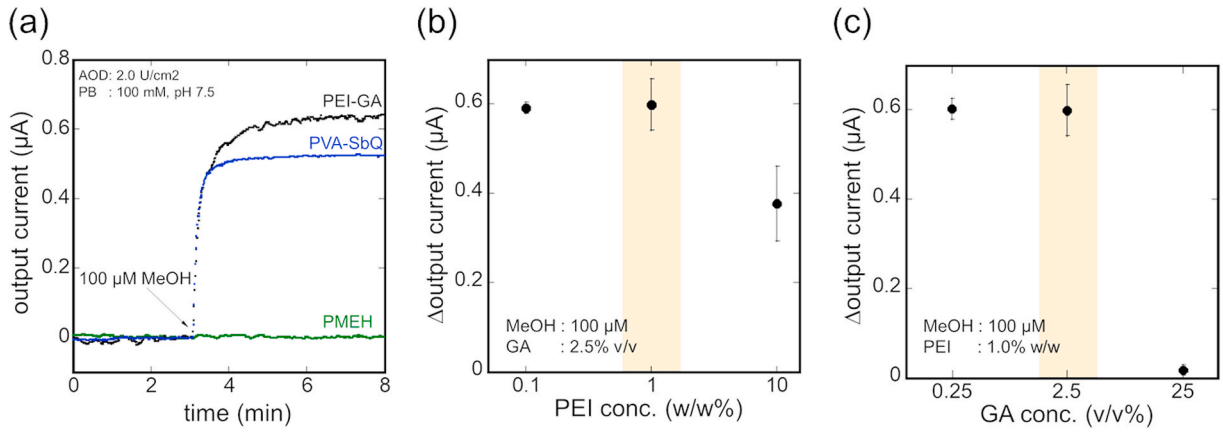

Fig. 3. (a) Typical sensor responses to MeOH solution by the AOD electrosensor using three differently-prepared AOD membranes. Influences of (b) PEI and (c) GA concentrations on the output current.

current when MeOH solution was added, whereas that of the PMEH did not change. This no signal change when using PMEH might be attributed to that hydrogen peroxide, which was produced through AOD-catalyzed oxidation of EtOH, oxidizes essential –SH group of AOD and inactivated the enzyme (Baratti et al., 1980). Based on these results, we chose PEI-GA for immobilization of AOD in the membrane preparation.

Next, the influences of PEI and GA concentrations were investigated using the AOD electrosensor. Fig. 3b and c shows the output currents to 100 μM MeOH solution with various concentrations of PEI or GA for the AOD membrane preparation. For the PEI, the output current was stable with the PEI concentrations of 0.1–1.0w/w% and decreased with the concentration of 10w/w%. This decrease may be because the amino groups of excess PEI had already reacted with both aldehyde ends of GA and thus less AOD could react with GA to be immobilized.

For the GA, the output current was stable with the GA concentrations of 0.25–2.5v/v%, but it dropped close to zero with the concentration of 25v/v%. This may be attributed to the inactivation of AOD by the unrinsed free GA during the AOD membrane preparation. Based on these results, the enzyme membrane was prepared with 1.0w/w% PEI and 2.5w/w% GA subsequently.

3.2. Optimization of AOD-FALDH membrane

The optimum amount of two kinds of enzymes for the AOD-FALDH membrane was investigated. First, the amount of AOD was varied to compare the output current of the AOD electrosensor to 100 μM MeOH solution. The result presented in Fig. 4a shows the increase in the output current until the AOD amount is less than 2.0 unit/cm^2. As the larger amount of AOD did not influence on the output current, 2.0 unit/cm^2 of AOD was selected. Next, the amount of FALDH was optimized by comparing the fluorescence signal change from the AOD-FALDH fluorosensor when measuring 1 mM MeOH. The change in the fluorescence signal increased with increasing the FALDH amount, but it turned into a decrease after reaching the peak at 1 unit/cm^2. This decrease in the output may be attributed to decrease in the activity of AOD and FALDH as a result of large amount of formic acid produced, which caused a local decrease in pH. According to the above results, the AOD-FALDH membrane prepared with 2.0 unit/cm^2 of AOD and 1 unit/cm^2 of FALDH was used in subsequent experiments.

3.3. Sensitivity and selectivity to MeOH

The sensitivity and selectivity of the AOD electrosensor and the AOD-FALDH fluorosensor were investigated. Fig. 5a and b shows the sensor responses to various concentrations of MeOH solution. In the both cases, increase in the sensor signal upon application of MeOH solution and concentration-dependent saturation values were observed. The relation between the sensor output of each sensor and MeOH concentration is plotted in Fig. 5c, and these plots were fitted by the following equations:

$$\text{AOD: } \Delta\text{current } (\mu\text{A}) = A1 + (B1 - A1) / \{1 + (C1 / [\text{MeOH } (\mu\text{M})])^{D1}\}^{E1}, \quad (1)$$

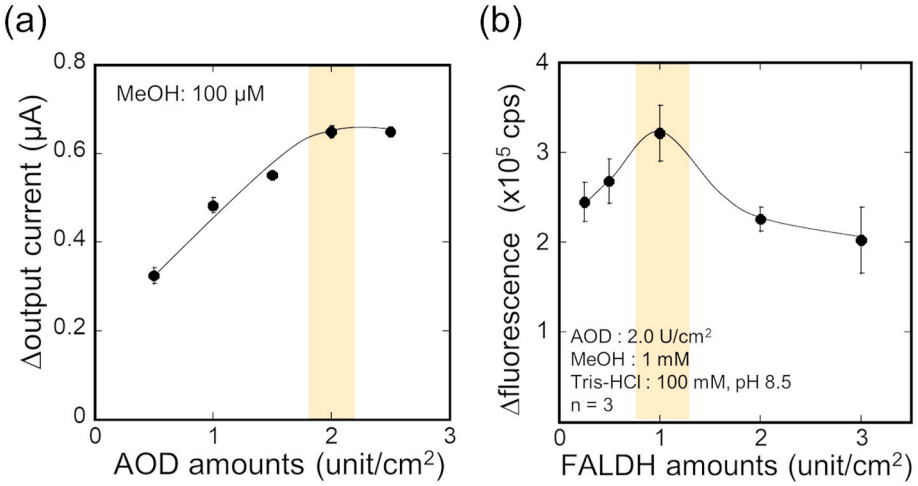

Fig. 4. Influences of the amounts of (a) AOD and (b) FALDH on the sensor outputs, respectively. The optimum AOD amount was studied with an AOD membrane, and that of FALDH was done with an AOD-FALDH membrane at the fixed AOD concentration.

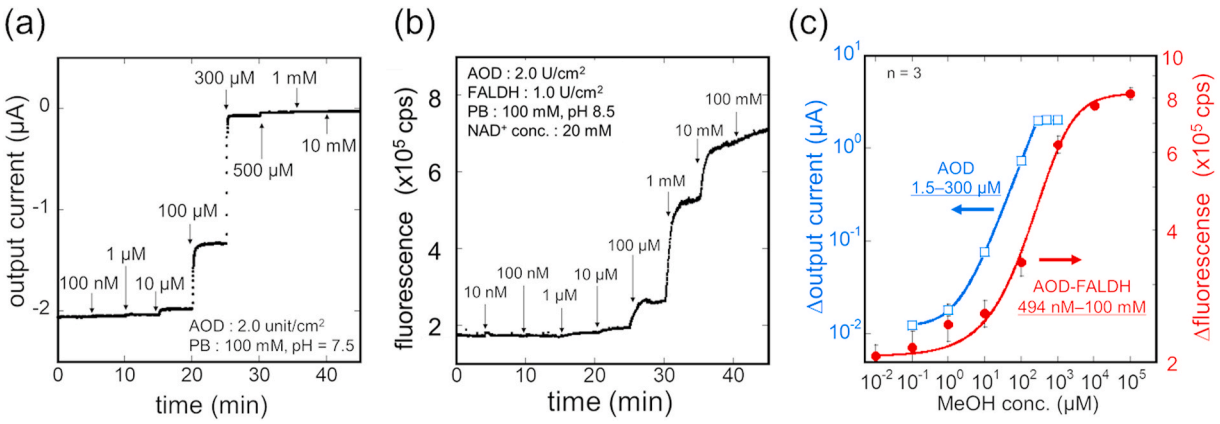

Fig. 5. Sensor responses to various concentrations of MeOH solution for (a) the AOD electrosensor and (b) AOD-FALDH fluorosensor, and (c) their calibration curves.

AOD-FALDH: $\Delta FI\ (cps) = A2 + (B2 - A2) / \{1 + (C2 / [MeOH\ (\mu M)])^{D2}\}^{E2}$, (2)

where A1 = 0.0121, B1 = 2.02, C1 = 272, D1 = 13.2, and E1 = 0.0788 are the coefficients for the AOD electrosensor with a coefficient of correlation (R) of 0.992; $A2 = 1.89 \times 10^4$, $B2 = 6.56 \times 10^5$, C2 = 858, D2 = 0.996, and E2 = 0.716 are the coefficients for the AOD-FALDH fluorosensor with R of 0.995; [MeOH] is the concentration of MeOH solution in μM. From the calibration curves, the dynamic ranges are determined to be 1.5–300 μM for the AOD electrosensor and 494 nM–100 mM for the AOD-FALDH fluorosensor, respectively. Here, the limit of quantification (LOQ) was determined as the concentration where ten times the standard deviation of the sensor baseline signal without the sample was equal to the sensor output. These results indicated that the cascade reaction with AOD and FALDH in conjunction with the fluorometry improved the LOQ by 3 times and made the dynamic range 335 times wider compared to a single reaction with AOD and oxygen electrode.

The selectivity of both sensors was studied by comparing the relative sensor output from various VOC solutions to MeOH, including ethanol, 1-propanol, 1-butanol, 2-propanol, acetone, acetaldehyde, and formaldehyde (Fig. 6). The concentrations of the samples were 100 μM for the AOD electrosensor and 1 mM for the AOD-FALDH fluorosensor, respectively. The result shows that the relative output for primary alcohols other than MeOH, which showed the large output in the AOD electrosensor, was suppressed with the AOD-FALDH fluorosensor. On the other hand, in the AOD-FALDH fluorosensor, the relative output for aldehydes increased, which was almost absent in the AOD electrosensor. This is due to the presence of FALDH in the membranes. However, acetaldehyde (saliva: 0.48 μM, urine: 1.8 μM) and formaldehyde (saliva: 0.14 μM, urine: 2.6 μM) in body fluids are relatively small compared to MeOH (saliva: 4.0 μM, urine: 59 μM) [19-23]. Therefore, the influence on the sensor output is limited. In conclusion, the cascade reaction with AOD and FALDH allows highly selective measurement of MeOH.

4. Conclusion

In this study, two liquid phase methanol biosensors, the AOD electrosensor and AOD-FALDH fluorosensor, were developed and compared for non-invasive assessment of intestinal bacteria activity. Incorporating two-enzyme cascade reaction with AOD and FALDH into fluorometry, 3 times improvement in the sensitivity and 335 times broadening of the dynamic range (from 1.5–300 μM to 494 nM–100 mM) from the AOD electrosensor were achieved. Besides, the selectivity to methanol among other aliphatic alcohol with similar physical and chemical properties was improved owing to the cascade reaction.

On the other hand, presence of FALDH induced reactions to aldehydes which did not occur when using only AOD. This reactivity to aldehydes could be suppressed by selecting FALDH from another origin, which we will investigate in future study. Also, considering the concentrations of aldehydes in body fluids, the influence on the sensor output is supposed to be limited. These results supported the conclusion that the AOD-FALDH fluorosensor will be a useful tool for evaluation of colonic bacteria activity from a body fluid.

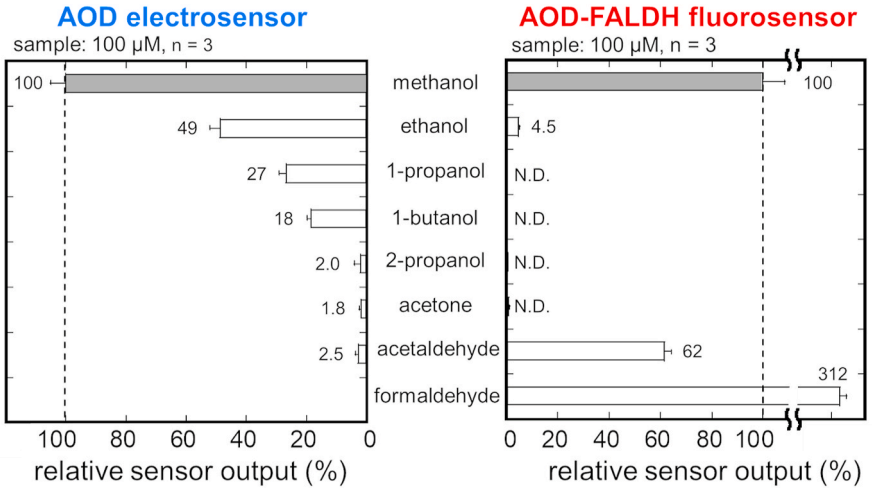

Fig. 6. Relative sensor outputs to MeOH for (a) the AOD electrosensor and (b) the AOD-FALDH fluorosensor.

Author contributions

Koji Toma: Writing – review & editing, Conceptualization. Kanako Iwasaki: Data curation, Writing – original draft. Takahiro Arakawa: Visualization, Methodology, Conceptualization. Yasuhiko Iwasaki: Methodology. Kohji Mitsubayashi: Conceptualization, Writing – review & editing, Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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