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Bio-sniffer (gas-phase biosensor) with secondary alcohol dehydrogenase (S-ADH) for determination of isopropanol in exhaled air as a potential volatile biomarker

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ABSTRACT

Exhaled breath analysis has attracted lots of researchers attention in the past decades due to its advantages such as its non-invasive property and the possibility of continuous monitoring. In addition, several volatile organic compounds in breath have been identified as biomarkers for some diseases. Particularly, studies have pointed out that concentration of isopropanol (IPA) in exhaled air might relate with certain illnesses such as liver disease, chronic obstructive pulmonary (COPD), and lung cancer. In this study, a highly sensitive and selective biochemical gas sensor (bio-sniffer) for the breath IPA concentration determination was constructed and optimized. This bio-sniffer measures the concentration of IPA according to the fluorescence intensity of oxidized nicotinamide adenine dinucleotide (NADH), which was produced by an enzymatic reaction of secondary alcohol dehydrogenase (S-ADH). The NADH detection system employed an UV–LED as the excitation light, and a highly sensitive photomultiplier tube (PMT) as a fluorescence intensity detector. A gas-sensing region was developed using an optical fiber probe equipped with a flow-cell and enzyme immobilized membrane, and connected to the NADH measurement system. The calibration range of the IPA bio-sniffer was confirmed from 1 ppb to 9060 ppb that was comparable to other IPA analysis methods. The results of the analysis of breath IPA concentration in healthy subjects using the bio-sniffer showed a mean concentration of 16.0 ppb, which was similar to other studies. These results have demonstrated that this highly sensitive and selective bio-sniffer could be used to measure the IPA in exhaled air, and it is expected to apply for breath IPA research and investigation of biomarkers for clinical diagnosis.

1. Introduction

Over the last few decades, researchers have shown increasing interest in analyzing human breath because the exhaled air contains a variety of bio-information, such as traces of eating, signals of the status of the body's metabolism, biological clocks and signs of some diseases (Cao and Duan, 2006; Risby and Solga, 2006; Sinues et al., 2013). Additionally, compared to blood examination, non-invasive property of the breath measuring not only can reduce the uncomfortable feeling of a patient but also provides a continuous pattern for monitoring the changing of the human body. Researchers have identified more than two hundred volatile organic compounds (VOCs) in the composition of breath (Pauling et al., 1971), and some of them have the potential to be biomarkers for indicating the presence of certain diseases (Cheepsattayakorn and Cheepsattayakorn, 2013; Konvalina and Haick, 2014). For example, nitric oxide is one of the well-known indicators for asthma (Kharitonov et al., 1994), and a higher concentration of breath acetone, which is related to lipid metabolism, was observed in patient with diabetes mellitus (Reyes-Reyes et al., 2015; Tassopou et al., 1969). Discovering biomarkers for cancer screening in an early stage is always an important issue in the health care industry. Recently, several researchers have found that the isopropanol (IPA) concentrations in the breath may relate to some illnesses. For instance, Hanouneh et al. discovered that the breath IPA

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concentration of patients with liver disease was higher than in healthy people (Hanouneh et al., 2014), Phillips et al. used breath IPA levels to predict breast cancer (Phillips et al., 2006), and Cikach et al. observed an increasing trend of IPA concentration in the breath of patients with pulmonary arterial hypertension.(Cikach et al., 2014). Several other studies have also demonstrated that lung cancer patients might exhale a greater concentration of IPA (Buszewski et al., 2012a; Rudnicka et al., 2011, 2014; Ulanowska et al., 2011; Wehinger et al., 2007). Not only the breath IPA, some studies also pointed out that the blood IPA concentration would increase in ketoacidosis patients (Bailey, 1990; Palmiere et al., 2012). However, most studies mentioned above analyzed dozens of substances in the breath at the same time. Not much research exists that concentrates specifically on isopropanol in the breath and its implications for clinical diagnosis. A comprehensive investigation of the relationship between a status of human health and the exhaled IPA is necessary and extremely valuable.

Currently, direct breath IPA analysis methods include gas chromatography-mass spectrometry (GC-MS) (Rudnicka et al., 2014), GC-MS with time-of-flight mass spectrometry (GC-TOF-MS or GC-MS x TPF-MS) (Rudnicka et al., 2011), proton transfer reaction-MS (PTR–MS) (Wehinger et al., 2007), solid-phase microextraction with mass spectrometry (SPME-MS) (Kischkel et al., 2010), and selected ion flow tube-MS (SIFT-MS) (Cikach et al., 2014; Hanouneh et al., 2014) are used. These techniques, usually with high sensitivity, can detect targets at a very low concentration, but there still exists some deficiencies, including difficulties in continuous breath monitoring and inconvenience of a large amount of detection due to the complicated operations and the high cost of instruments. In addition, most of these analyzers identify the target molecules by mass-to-charge ratio (m/z); it will cause confusion when the substances display an identical m/z fragment. Other reported IPA detectors like ZnO–CdO composites (Cai et al., 2014) or a transmission sensor (Elosua et al., 2013) may be used more easily than GC related methods. But their sensitivity, usually at the ppm (parts per million) levels, was insufficient for breath measurement because the breath IPA is mostly identified at ppb (parts per billion) concentrations.

A biochemical gas-sensor exploiting advantages of specific enzymatic reactions could be a good candidate for the determination of VOCs in exhaled breath. Nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase enzyme is one of the appropriate choices for developing a biosensor. This kind of enzymes can normally divide hydrogen atoms from a target substance, while transferring electrons to a coenzyme which is usually NAD (Ying, 2008). Reduced NAD (NADH) has a unique fluorescence property releasing visible fluorescence when it is exposed to 340 nm wavelength ultraviolet (UV) light (Kudo et al., 2009). However, the oxidized form of NAD (NAD^+) does not have this similar feature. Therefore, utilizing the NAD-dependent enzymatic reaction and the NADH optical property could allow for the construction of a sensor capable of measuring the concentration of the enzyme reactants. Previously, our groups developed a bio-sniffer for measuring of formaldehyde (FA) and acetone concentration in a gas phase (Kudo et al., 2012; Ye et al., 2015). The FA bio-sniffer combined a UV light-emitting diode (UV-LED) and a photomultiplier tube (PMT) with a two-way branch optical fiber for NADH fluorescence measurements, and employed the formaldehyde dehydrogenase to distinguish and catalyze FA. One of the difficulties of constructing an enzyme-based gas sensor is that this kind of NADH catalytic reaction prefers to react in a liquid medium, which can cause a dilemma for the enzyme to contact with the coenzyme and gas substance at the same time. Our FA biosensor overcomes this problem by using an optical fiber equipped with a specialized flow cell and a formaldehyde dehydrogenase immobilized membrane as a diaphragm. Circulated NAD^+ contained buffer through the flow channel permitted the enzyme to interact with NAD^+ and FA gas simultaneously. Furthermore, designing of the flow cell also allowed the bio-sniffer to continuously monitor instead of single point detect because the circulated buffer could remove the produced NADH and immediately supply fresh NAD^+ to the sensing region.

NAD-dependent secondary alcohol dehydrogenase (S-ADH) is an enzyme that can catalyze secondary alcohols to ketones, and the main substrate is IPA. The enzymatic reaction of the S-ADH is presented at the following equation:

$$\text{Isopropanol} + NAD^+ \underset{\text{pH 6}}{\overset{\text{S-ADH}}{\underset{\text{pH 8}}{\rightleftharpoons}}} \text{acetone} + \text{NADH} \quad (1)$$

In a weak alkaline environment, S-ADH prefers to catalyze IPA to acetone and simultaneously produces proportional NADH. Thus, according to the fluorescence intensity of NADH, the concentration of the IPA can be calculated. Recently, we have developed an optical biosensor utilizing the enzymatic reaction of S-ADH as described in the Eq. (1) for determination of the IPA concentration in the liquid phase (Chien et al., 2016). The composition of this biosensor is similar to the bio-sniffer including the portion of UV-LED, PMT and optical fibers. But this IPA biosensor can only perform in liquid phase detection because it lacks of the flow cell equipment. The results of this liquid phase IPA biosensor demonstrate that measuring the concentration of the IPA by increasing NADH fluorescence intensity is feasible. Therefore, by combining the technique of bio-sniffer and this IPA biosensor, it is possible to construct a new bio-sniffer to detect the IPA in the gas phase.

The aims of this study include: (1) construction of an bio-sniffer for IPA vapor measurements using an S-ADH immobilized membrane, a high power UV-LED, a high sensitivity PMT, and an optical fiber with flow-cell, (2) to investigate and optimize the factors that may interfere with the performance of the bio-sniffer, and (3) to apply the bio-sniffer in analyzing the IPA concentration in the exhaled breath supplied by healthy subjects.

2. Materials and methods

2.1. Standards and reagents

Dipotassium hydrogen phosphate (K_2HPO_4, 98%), potassium dihydrogen phosphate (KH_2PO_4, 98%), 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris, 99.9%, Biochemistry grade), Hydrochloric Acid (HCl, 1 mol L^{-1}), sodium hydrogen carbonate ($NaHCO_4$, 99.5%, JIS special grade), sodium carbonate (Na_2CO_4, 99.8%, JIS special grade) and standard isopropanol (99.7%, JIS special grade) solution were purchased from Wako Pure Chemical Industries, Japan. β-NAD^+ were bought from Oriental Yeast, Co., Ltd., Japan. NADH-dependent secondary alcohol dehydrogenase (S-ADH, EC. 1.1.1.x, 1 unit mg^{-1}, E001) from yeast was obtained from Daicel Chiral Technologies, Co., Japan. All agents are analytical grade and no further purification is needed before used. Deionized (DI) distilled water collected from Milli-Q purification system (Millipore, Co., USA) was used for all buffer solution. Hydrophilic polytetrafluoroethene filter membrane (H-PTFE, pore size: 0.2 μm, porosity: 80%, JGWP14225) employed for S-ADH immobilization was obtained from Millipore, USA. The synthesis method of the polymer using 2-methacryloyloxyethyl phosphorylcholine polymerized with 2-ethylhexyl methacrylate (PMEH) was reported in our previous study (Kudo et al., 2009).

2.2. Construction of the IPA bio-sniffer

The IPA bio-sniffer comprised a photon detection unit, an excitation light source, a bifurcated optical fiber, an optical fiber probe equipped with cylindrical flow cell and an enzyme immobilized membrane. The NADH excitation equipment contains a high power UV-LED (UVTOP® BL335, Sensor Electronic Technology, Inc., USA), which could emit UV-light with the central wavelength at 340 nm, and a controllable power supplier (GS200, Yokogawa Electric Corporation,