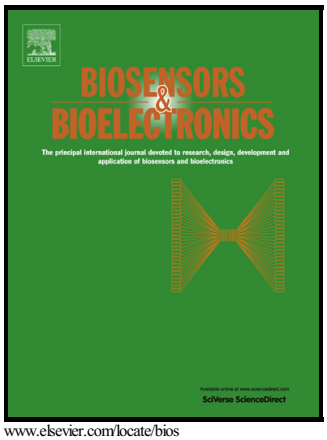

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Title:

An acetone bio-sniffer (gas phase biosensor) enabling assessment of lipid metabolism from exhaled breath

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Abstract

Several volatile organic compounds (VOCs) are released from human breath or skin. Like chemical substances in blood or urine, some of these vapors can provide valuable information regarding the state of the human body. A highly sensitive acetone biochemical gas sensor (bio-sniffer) based on the consumption of nicotinamide adenine dinucleotide (NADH), was developed and used to measure exhaled breath acetone concentration, and how this changed in response to lipid metabolism.

A fiber-optic biochemical gas sensing system was constructed with an ultraviolet-light emitting diode (UV-LED) excitation system (λ=340 nm), a photomultiplier tube (PMT) and an optical fiber probe. NADH-dependent secondary alcohol dehydrogenase (S-ADH) was immobilized on the optical fiber probe. Measurement of acetone concentration was carried out by immersing the sensor probe in phosphate buffer solution containing NADH. NADH is consumed by the enzymatic reaction of S-ADH, and the consumption is proportional to the concentration of acetone vapor. The change of fluorescent emitted from NADH

is analyzed by the PMT. Hence, fluorescence intensity decreased as the acetone concentration increased. The relationship between fluorescence intensity and acetone concentration was identified from 20 ppb to 5300 ppb. This interval included the concentration of acetone vapor in the breath of healthy people and those suffering from disorders of carbohydrate metabolism. Finally, the acetone bio-sniffer was used to measure breath acetone during an exercise stress test on an ergometer after a period of fasting. The concentration of acetone in breath was shown to significantly increase after exercise. This biosensor allows rapid, highly sensitive and selective measurement of lipid metabolism.

Introduction

Blood tests are common diagnostic tools enabling acquisition of valuable information regarding the metabolic function of the human body. However, they are invasive, potentially causing physical and psychological distress. In recent years, breath analysis as a tool for diagnosis and physiological assessment is evolving rapidly on a global scale (Hunter and Dweik 2008; Manolis 1983). Exhaled breath condensate (EBC) and volatile organic compounds (VOCs) are two major topics in breath analysis (Kuban and Foret 2013). EBC is the liquid phase of the exhaled breath. It predominantly consists of water vapor and aerosol particles from the lower respiratory tract (Di Natale et al. 2014). EBC has been used increasingly as a sample to detect viruses or bacteria (e.g., detection of influenza viruses in EBC samples using silicon nanowire sensor devices) (Shen et al. 2012). There are hundreds of VOCs in exhaled breath, some of which are also present in the bloodstream (Amann et al. 2014). Hence breath analysis offers a unique opportunity for non-invasive measurement (Bloemen et al. 2009; Riess et al. 2010; Yeh et al. 2008).

Acetone is a typical VOC in exhaled breath. It is affected by fasting, exercise and diabetes mellitus (Blaikie et al. 2014; Mork and Johanson 2006; Schubert et al. 2012; Wang and Wang 2013). Biochemical pathways of acetone production are well understood. Acetone is metabolized by 2 pathways: Decarboxylation of acetoacetate or dehydrogenation of 2-propanol. Acetoacetate is generated by dextrose metabolism and lipolysis, and is a major source of acetone in the human body (Musa-Veloso et al. 2002; Musa-Veloso et al. 2006). Acetone concentrations in exhaled breath have previously been shown to correlate strongly with acetone concentrations in blood, as well as with other ketones such as beta-hydroxybutyrate (Blaikie et al. 2014; Tanda et al. 2014). A correlation between acetone concentration in exhaled breath and blood glucose concentration has also been reported (Righettoni et al. 2013). The acetone concentration of exhaled breath in healthy people ranges from 200 to 900 ppb in previous research (Righettoni and Tricoli 2011; Toyooka et al. 2013). Increases in breath acetone concentration are usually a sign that cells lack insulin or are unable to effectively use available insulin; this occurs in diabetes. The breath

acetone concentrations of diabetic patients are higher than those of healthy people, and can exceed 900 ppb (Rydosz 2014; Sun et al. 2015). For this reason, acetone in exhaled breath is a potential biomarker for non-invasive diagnosis of diabetes.

There are several methods for determining acetone concentration. Low concentrations of acetone can be quantified by gas chromatography with mass spectrometry (GC/MS) (Deng et al. 2004; Fan et al. 2014; Phillips 1997; Qin et al. 1997). Though these methods are accurate, they have various disadvantages: They are expensive, time-consuming, operator-dependent and they are also unable to provide continuous measurements. Proton Transfer Reaction Mass Spectrometry (PTR-MS) is a continuous detection method for analysis of exhaled breath samples using gas phase hydronium ions as ion source reagents (Schwarz et al. 2009a; Schwarz et al. 2009b). However, species identification and quantification can be complicated by the presence of other compounds and fragments with the same mass to charge ratio. Nevertheless, a cell-based gas biosensor for detecting acetone vapor by direct exposure of gaseous substances

has been recently designed and validated (Bohrn et al. 2013).

Due to the shortcomings of the above mentioned methods, a rapid, convenient, non-invasive and highly selective method for gaseous acetone detection is strongly required. This might be achieved using an enzyme-based biosensor. Several enzymes such as acetone carboxylase catalyze the oxidation of acetone. One such enzyme, nicotinamide adenine dinucleotide (NADH)-dependent dehydrogenase, is suitable for use in biosensors. Secondary alcohol dehydrogenase (S-ADH) catalyzes the reduction of acetone in the presence of NADH, which acts as an electron donor. 2-propanol and oxidized nicotinamide adenine dinucleotide (NAD^+) are produced through the following enzymatic reaction:

$$\text{acetone} + \text{NADH} \overset{\text{S-ADH}}{\rightleftharpoons} \text{2-propanol} + NAD^+ \qquad (1)$$

Research regarding NADH-dependent amperometric biosensors has already been undertaken (Sluis and Ensign 1997; Sutak et al. 2012; Yamazaki et

al. 2001). Although this type of sensor is superior in terms of sampling volume, structure and measurement cost, there are several disadvantages to be considered. Most of the electro-active biomolecules such as catecholamines, ascorbic acid and uric acid are oxidized at an electrical potential similar to that of NADH oxidation. In addition, direct oxidation of NADH at bare electrodes results in surface contamination of the electrodes (fouling) due to the adsorption of oxidized products of NADH, resulting in poor sensitivity (Musameh et al. 2002; Nowall and Kuhr 1995). Optical methods are also commonly used for NADH detection. It is known that NADH can be excited by 340 nm ultraviolet (UV) radiation and it has a fluorescent emission peak at 490 nm, while NAD^+ has no fluorescent emission or optical properties.

We have previously reported a fiber-optic NADH sensor (Kudo et al. 2010), which uses a UV-light emitting diode (UV-LED) excitation system and a primary alcohol dehydrogenase (ADH) immobilized membrane for measuring the concentration of ethanol in gaseous samples. Fluorescence intensity increases as NADH is produced though an enzymatic reaction.

Based on this ethanol biosensor, a rapid, compact and highly selective biosensor for acetone vapor was developed using NADH-dependent S-ADH. The biosensor consists of a high-intensity UV-LED excitation system, a photomultiplier tube (PMT) and an optical fiber probe with a S-ADH immobilized membrane. The UV-LED excitation system has a simple and compact structure, due to its low power consumption and low heat generation. LEDs also provide advantages in safety and controllability compared with UV lamps.

Hence, a novel bio-sniffer for continuous monitoring of gaseous acetone by the reverse catalytic reaction of S-ADH was developed. When S-ADH catalyzes the reduction of acetone in the presence of NADH, which acts as an electron donor, 2-propanol and oxidized NAD^+ are produced. This study aims to describe the biosensor design, characteristics and validation through analysis of breath acetone concentration in test subjects at rest and during exercise according to lipid metabolism.

2. Experimental Methods

2.1 Reagents and chemicals

NADH-dependent secondary alcohol dehydrogenase (EC. 1.1.1.x, 1 unit mg^{-1}, E001) originating from yeast, was purchased from Daicel Chiral Technologies, Japan. NADH (the reduced form) was from Oriental Yeast, Japan. Hydrophilic PTFE (H-PTFE, porosity: 80%, pore size: 0.2 μm, JGWP14225), an immobilization enzyme carrier, was from Millipore, USA. 2-methacryloyloxyethyl phosphorylcholine copolymerized with 2-ethylhexyl methacrylate (PMEH) was synthesized according to methods used in previous research (Kudo et al. 2008). pH 7.0, 0.1 M phosphate buffer solution (PB) was prepared by gradually adding 0.1 M dipotassium hydrogen phosphate (K_2HPO_4, 98.0%, 164-04315, Wako Pure Chemical Industries, Japan) to 0.1 M potassium dihydrogen phosphate (KH_2PO_4, 98%, 160-04275, Wako Pure Chemical Industries, Japan) to obtain the specified pH value. All solutions were prepared using deionized distilled water obtained from a Milli-Q purification system (Millipore, USA). A 50 μM

NADH solution was prepared using the PB (pH7.0, 0.1 M), to act as circulating buffer. A standard acetone vapor was prepared using a standard gas generator (PD-1B-2, GASTEC corporation, Japan).

2.2 Construction of the fiber optic S-ADH bio-sniffer

The acetone bio-sniffer consisted of an excitation source, a photodetector and an optode that produces NAD^+ from acetone. The excitation system utilized a UV-LED (UVTOP® BL335, Sensor Electronic Technology Inc., USA) with a UV-LED power supply circuit (GS200, Yokogawa Electric Corporation, Japan). A PMT (C9692, Hamamatsu corporation, Japan) was used as the photodetector. These components were connected to separated ends of a bifurcated optical fiber assembly (core size 600 ± 10 μm, BIF600-UV/VIS, Ocean Optics Inc. USA). An optical fiber probe (F1000-900) was connected to the common end of the optical fiber assembly. In order to reduce the background signal, two band-pass filters (λ=340 ± 10 nm and λ=490 ± 10 nm, BPF, Asahi Spectra, Japan) were used in excitation and detection, respectively.

The S-ADH sensitive optode was fabricated by attaching a flow-cell with an S-ADH immobilized membrane to the end of the optical fiber probe. The flow-cell had a cylindrical structure consisting of a silicone tube (Φ2 mm) and a PMMA cell (Φ4 mm), forming a socket for the optical fiber probe. Micro flow-channels supplying NADH-containing PB, were formed on the boundary between the silicone and PMMA, depicted in Figure 1. The S-ADH membrane was attached onto the end of the flow-cell as a sensing diaphragm. S-ADH (0.5 unit cm^{-2}) and PMEH solution (10%, 25 μl cm^{-2}) was mixed and spread carefully onto the H-PTFE membrane filter. The membrane was cured in a refrigerator for 3 hours at 4 ^{o}C. The S-ADH immobilized membrane was then cut into 2 × 2 cm and fixed onto the flow-cell with a silicone O-ring.

2.3 Characterization of the fiber optic S-ADH bio-sniffer

Figure 1 shows the experimental setup for monitoring of standard acetone vapor. In this system, the acetone vapor was supplied from the standard gas generator. The flow-rate was controlled using a mass flow controller with a

needle-bulb regulator (Type: RK1200, Koflok, Tokyo, Japan). NADH-containing PB was circulated into the flow-cell by an intelligent pump (INTELLIGENT PUMP, FLOM Co. Ltd., Japan). S-ADH loses its specific activity if chemical conditions are not adequate, such as in dry conditions, and at an inappropriate temperature or pH. The flow of PB kept the enzyme membrane wet, supplied NADH, and also efficiently removed the reaction products (including NAD^+) and excessive substrates. Hence, reaction conditions were optimized by the circulating PB.

Characterization of the acetone bio-sniffer was carried out by supplying the standard gas to the sensing region via a Teflon tube. High purity air flowed into the Teflon tube first, and subsequently the acetone vapor was applied to the bio-sniffer. The total flow rate was kept at 200 ml min^{-1}. High purity air was also used as the carrier gas to adjust the acetone levels. Changes in NADH fluorescence intensity were measured using a PMT. The experiment was repeated for five cycles.

2.4 The effect of pH and NADH concentration, and bio-sniffer selectivity

The bio-sniffer's response to PB of different pH was evaluated first. 50 μM NADH was added to 0.1 M PB solutions of different pH (pH 5.5, 6.0, 6.5, 6.7, 7.0, 7.2 and 7.5) to prepare circulating PB solution. 1 ppm acetone vapor was applied to the bio-sniffer and the prepared solutions of different pH values were circulated into the flow-cell.

Subsequently, the effect of NADH concentration was evaluated under optimized pH conditions. Various NADH concentrations (20, 50 and 100 μM) were prepared in 0.1 M pH 7.0 PB, . 1 ppm acetone vapor was applied to the bio-sniffer and the prepared solutions of different NADH concentration were circulated into the flow-cell.

The bio-sniffer's selectivity was then evaluated with eleven single gases and two mixed gases. The following single gases were used at a concentration of 1 ppm: acetone, 2-butanone, 2-pentanone, acetaldehyde, formaldehyde, methanol, 2-propanol, 1-propanol, ethanol, 1-butanol, methyl mercaptan. The mixed gases included 1 ppm acetone and either 0.5 ppb 2-pentanone or 2-butanone. These gases are typically found in human breath. Each gas was

supplied from the standard gas generator.

2.5 Measurement of breath acetone during an exercise stress test

Authorization for this part of the study was granted by the Human Investigations Committee of the Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University (authorization code: 2014-01), conforming to standards stipulated in the Declaration of Helsinki. Informed consent was obtained from all subjects.

Subjects attended the laboratory in the morning after an overnight fast of at least 6 hours. Expired air samples were collected at rest at 0 minutes and later at 30 minutes. The subjects then cycled for 30 minutes on a bicycle ergometer, with a constant load of 50 watts. Heart rate was continuously recorded during exercise and expired air samples were collected at 10 minute intervals. Air samples were also collected at 15 or 30 minute intervals after 150 minutes of exercising. Each subject was asked to inspire normally, hold their breath for 5 seconds, and then exhale slowly. Exhalation from the first 3 seconds was

discarded and the remainder of expired air was collected using a sampling bag with a mouthpiece (Bikov et al. 2013) The bio-sniffer was subsequently exposed to the samples of expired air using an air pump at a flow rate of 200 ml min^{-1} (S1).

3. Results and discussion

3.1 Characteristics of the acetone bio-sniffer

The fluorescent response to acetone vapor was investigated using the standard gas measurement system. Figure 2 shows real-time changes in fluorescence intensity (Δintensity) at various concentrations of gaseous acetone. When the sensing region was exposed to acetone vapor, fluorescence intensity decreased and reached steady state values immediately. Fluorescence intensity returned to the initial state upon cessation of acetone vapor flow. These responses indicate that NADH decreases at the enzyme membrane as it is consumed through the catalytic reaction involving S-ADH and acetone vapor, and immediately removed from the sensing region by the buffer flow.

The bio-sniffer measures the gas level as a balance between consumption and supply rates of NADH. The optimal flow rate of PB was found to be 1.5 ml min^{-1}. When the PB flow rate is below this value, supplied NADH is insufficient resulting in a smaller ∆intensity at the steady state and a longer recovery time. Conversely, when the PB flow rate is high, supplied acetone vapor is insufficient to fully react with NADH, resulting in a smaller ∆intensity at the steady state and a shorter recovery time. The fast response to acetone vapor confers an advantage in on-site measurement compared to conventional chromatographic methods such as GC and other biochemical alternatives, such as colorimetric methods (Worrall et al. 2013). The time to reach 95% of the steady state was 35–70 seconds; this is on par with solid state gas sensors used for monitoring purposes (Righettoni et al. 2012). Hence, this demonstrates that the bio-sniffer enables real-time monitoring of acetone vapor concentration.

The calibration curve for acetone vapor is shown in Figure 3. The analytical curve follows the sigmoidal shape described by the following equation:

$$\Delta\text{intensity (counts)} = 165197 / (1 + (466 / \text{acetone vapor [ppb]})^{0.97})^{0.86} \quad (2)$$

This equation has a correlation coefficient of 0.999. The dynamic range was from 20 to 5300 ppb, which includes breath acetone concentrations of healthy (200–900 ppb) and diabetic (> 900 ppb) individuals. Fluorescent output of the bio-sniffer also demonstrated high reproducibility. For acetone vapor at 500 ppb, a coefficient of variation of 2.6% (n = 5) was obtained. These values provide evidence supporting the use of the bio-sniffer to measure acetone in exhaled human breath.

3.2 The effect of pH and NADH concentration, and bio-sniffer selectivity

To optimize measurement conditions for the acetone bio-sniffer, signal dependence on the pH value of PB and the NADH concentration were investigated. For pH value, Δintensity at different PB pH values were measured for a given acetone vapor concentration. PB pH values were varied from 5.5 to 8.0 whilst keeping concentrations of PB and NADH at 0.1 M and 50 μM

respectively. As shown in S2, Δintensity peaks at pH 7.0 for three different acetone concentrations (250, 500 and 900 ppb). This result can be explained by the following two reasons: Firstly, the enzymatic activity of S-ADH is dependent on PB pH, as pH can alter the three-dimensional conformation of an enzyme and its active site, which influences its stability and efficacy. Hence, pH optimization confers greater stability for the sensor response. Secondly, NADH is an unstable reagent in mildly acidic conditions; hence pH 7.0 is more suitable for NADH stability.

Subsequently, with a PB pH of 7.0, the effect of NADH concentration on the dynamic range for the acetone bio-sniffer was examined. As indicated in Figure 4, for 50 μM NADH the dynamic range for acetone vapor was from 20 to 5300 ppb. This implies that a low concentration of NADH results in a low background, which is important for measuring acetone at a low concentration. With 20 μM NADH the dynamic range was lower from 7 to 900 ppb. However, when the concentration of NADH is below 20 μM there is insufficient NADH for the enzymatic reaction, and sensitivity becomes poorer. On the other hand,

when the concentration of NADH is increased to 100 μM the dynamic range broadens to 50 to 10000 ppb, with a substantial increase at the upper limit. For NADH concentrations exceeding 100 μM, the upper limit is saturated and the lower limit detrimentally increases. By fine-tuning the NADH concentration, the acetone bio-sniffer can be adjusted to measure acetone vapor at concentrations from 7 to 10000 ppb.

The selectivity of the acetone bio-sniffer was also determined. Since NADH-dependent S-ADH is reactive to ketones, 2-butanone, 2-pentanone and other aldehydes, thirteen different gaseous species including eleven single gases and two mixed gases were tested to compare responses. Figure 5 shows relative changes in fluorescence intensity for various gaseous species in comparison to acetone vapor. Fluorescence intensities for 2-butanone and 2-pentanone exceeded that of acetone, reaching 139% and 117% respectively, while little fluorescence was detected for other single gases. This demonstrates that the bio-sniffer exhibits high selectivity due to substrate specificity of the enzyme.

Although high responses to 2-butanone and 2-pentanone were observed, the output interference is negligible as only minute quantities of these gases (0.38 ppb) are present in the exhaled breath of healthy people (Van den Velde et al. 2008). Evidence also indicates that 2-butanone and 2-pentanone are volatile biomarkers of lung cancer found in the exhaled breath of lung cancer patients (Buszewski et al. 2012). Given the high responses to both gases, the acetone bio-sniffer might have the potential to be used as a diagnostic tool for lung cancer screening.

3.3 Breath acetone analysis during the exercise stress test

Unlike chromatographic techniques, the acetone bio-sniffer enables real-time measurement of acetone vapor. We harnessed this function to assess lipid metabolism based on breath acetone analysis. Figure 6 depicts the time courses of acetone concentration in exhaled breath before, during and after the exercise stress test. Subjects were fasted for at least 6 hours beforehand. Acetone concentrations increased during exercise, and peaked at 15–30

minutes after exercise. At the peaks, acetone concentrations reached 110–140% of the initial values. Thereafter, they gradually decreased to steady states.

Elevated acetone concentration in exhaled breath correlates with increased lipolysis due to the exercise. During exercise after a period of fasting, fatty acids are released from adipose tissue and enter the bloodstream as free fatty acids (FFAs). Increased FFAs are broken down by beta-oxidation to produce acetyl CoA, from which ketone bodies (acetoacetate, beta-hydroxybutyric acid and acetone) are synthesized. Volatile acetone can then enter alveolar airspaces during gaseous exchange, and is consequently exhaled. After exercise, ketone body concentration in blood continues to increase. Thus, breath acetone concentration peaked after the exercise, and only decreased thereafter. This proof of concept demonstrates that the acetone bio-sniffer can be used to assess lipid metabolism during exercise.

4. Conclusion

In this study, we developed a highly sensitive and selective biosensor for

the measurement of acetone in exhaled breath based on the reverse (reduction) catalytic reaction of S-ADH. The acetone bio-sniffer employed a UV-LED as a light source to facilitate miniaturization and ease of use. Real-time monitoring of acetone vapor was successfully performed using a cylindrical flow-cell with an enzyme immobilized membrane. The fluorescence signal exhibited a clear relationship with acetone concentration from 20 to 5300 ppb; this encompasses acetone concentrations in the exhaled breath of both healthy and diabetic individuals. The response time to reach 95% of the steady state was 35–70 seconds. Furthermore, the dynamic range of the bio-sniffer could range from 7–10000 ppb by fine-tuning the NADH concentration. Since the bio-sniffer also detects 2-butanone and 2-pentanone, it could potentially be used as a diagnostic tool for lung cancer screening. When assessing lipid metabolism based on exhaled breath, increased acetone concentration due to exercise was observed. We anticipate future use of the acetone bio-sniffer as a non-invasive analytical tool for assessment of lipid metabolism in exercise, fasting and diabetes mellitus.

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Figure captions

Figure 1

Experimental setup for characterization of the fiber-optic acetone bio-sniffer. The bio-sniffer measures acetone vapor as fluorescence of NADH, which is consumed at the enzyme membrane. PB that contained NADH was circulated at a flow-rate of 1.5 ml min $^{-1}$. The capacity of the reservoir is 1000 ml. The inset shows the cross-sectional structure of the flow-cell with the S-ADH immobilized membrane.

Figure 2

Changes in various concentrations of standard acetone vapor over time. The steady state value is determined by the balance consumption rates of NADH. The response time to reach 95% of the steady state was 35–70 seconds.

Figure 3

Calibration curve for the acetone bio-sniffer using acetone vapor. The fluorescence intensity relates to the concentration of acetone vapor from 20 to

5300 ppb.

Figure 4

Effect of the concentration of NADH on the range of the acetone bio-sniffer. For 50 µM NADH the dynamic range for acetone vapor was 20 to 5300 ppb. For 20 µM NADH the dynamic range was 7 to 900 ppb. Increasing the concentration of NADH to 100 uM improves the upper limit of the dynamic range and expands it to 50 to 10000 ppb.

Figure 5

Gas selectivity of the S-ADH bio-sniffer. The bio-sniffer response to 2-butanone and 2-pentanone was 139% and 117% respectively. A smaller response was detected for other chemical substances.

Figure 6

Time course of acetone concentration in exhaled breath before, during and after

exercise. The subjects were fasted for at least 6 hours before exercising. Breath acetone concentrations increased during exercise, and peaked 15–30 minutes after exercise. At the peaks, acetone concentrations reached 110–140% of the initial values.

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Figures:

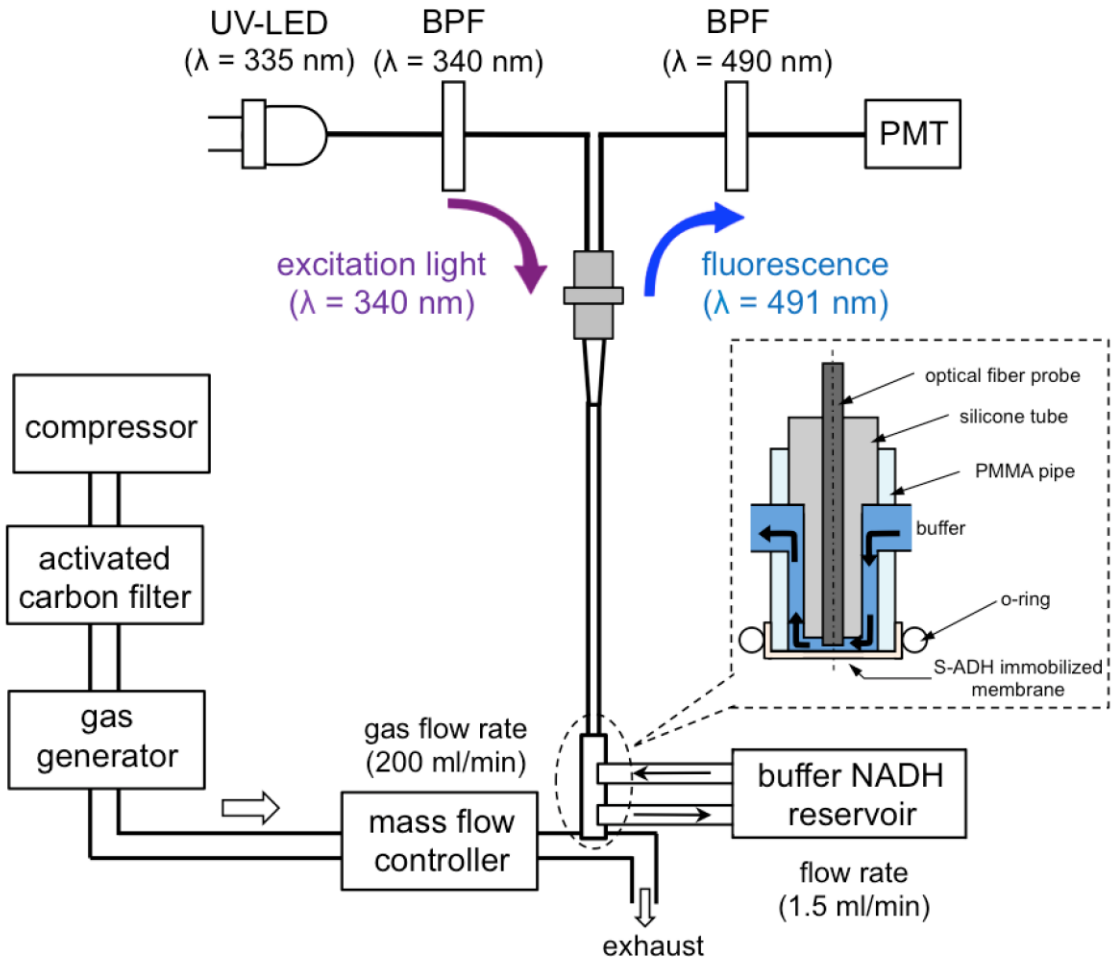

Figure 1

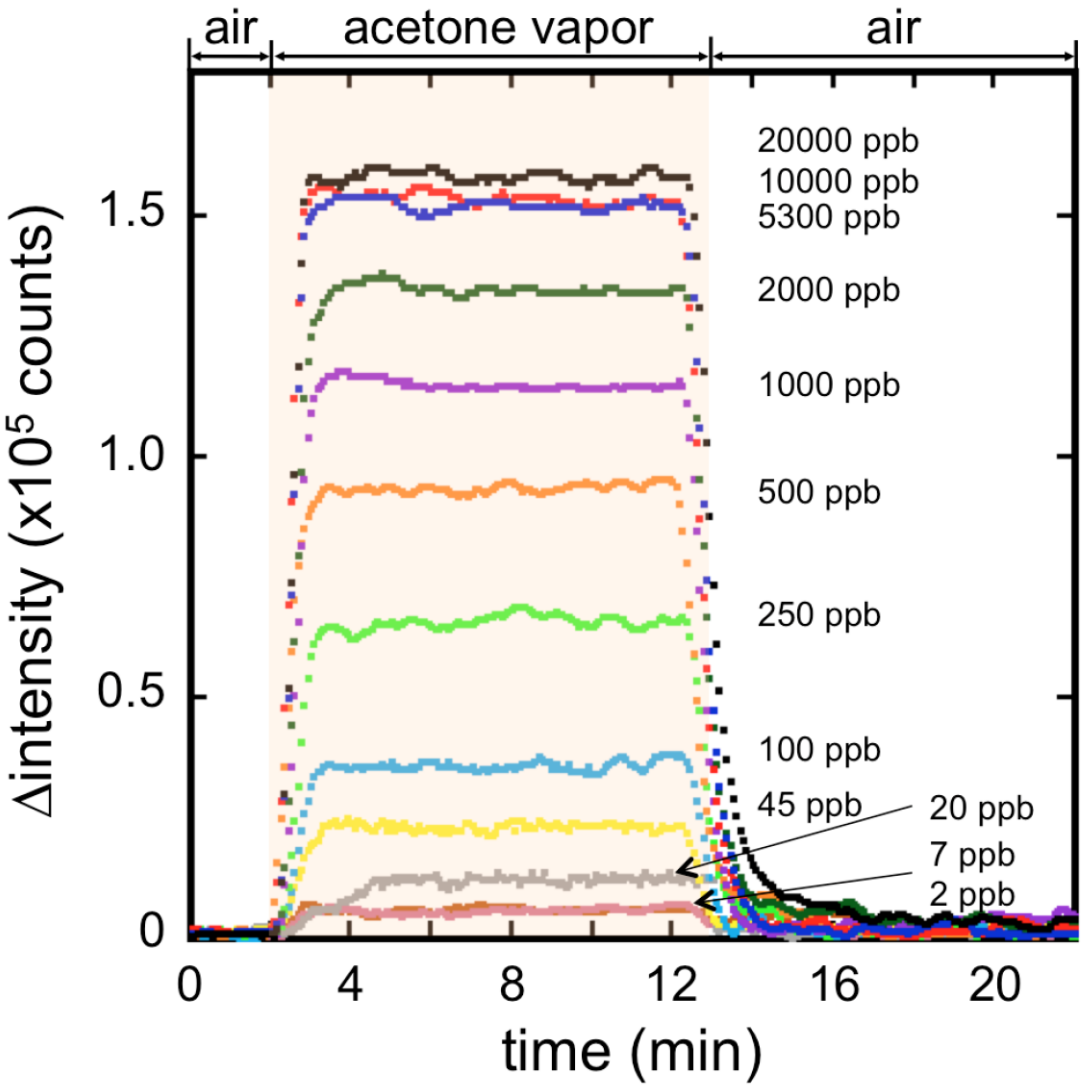

Figure 2

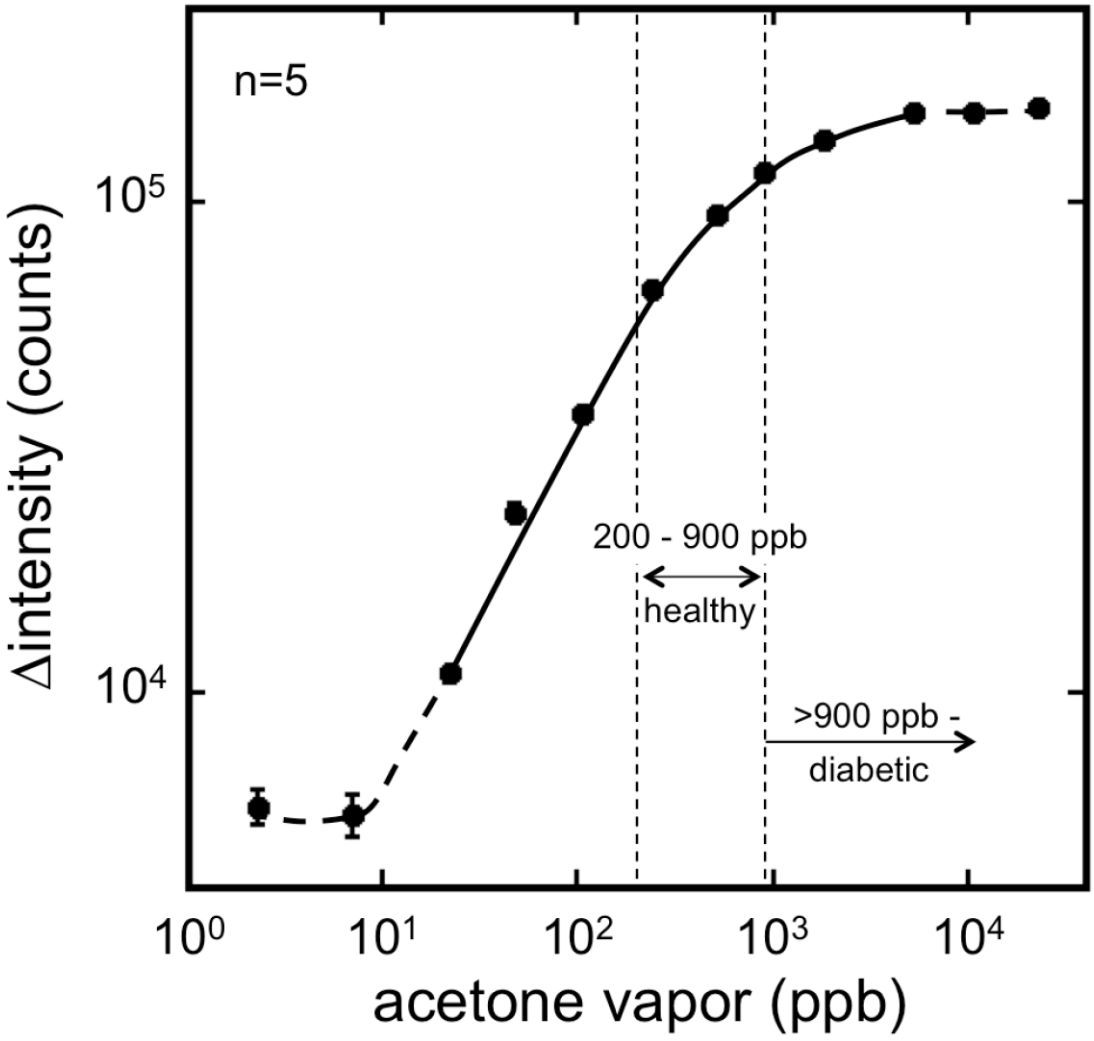

Figure 3

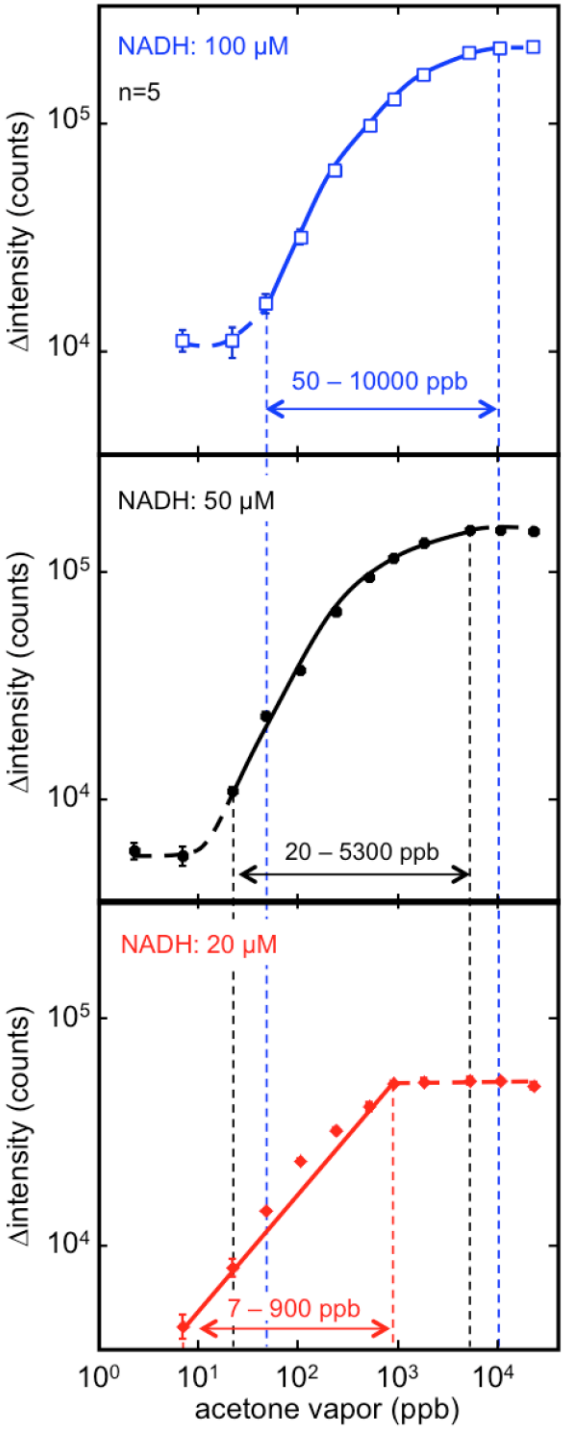
NADH: 100 µM
n=5
50 – 10000 ppb
NADH: 50 µM
20 – 5300 ppb
NADH: 20 µM
7 – 900 ppb
Δintensity (counts)
10^5
10^4
10^0
10^1
10^2
10^3
10^4
acetone vapor (ppb)

Figure 4

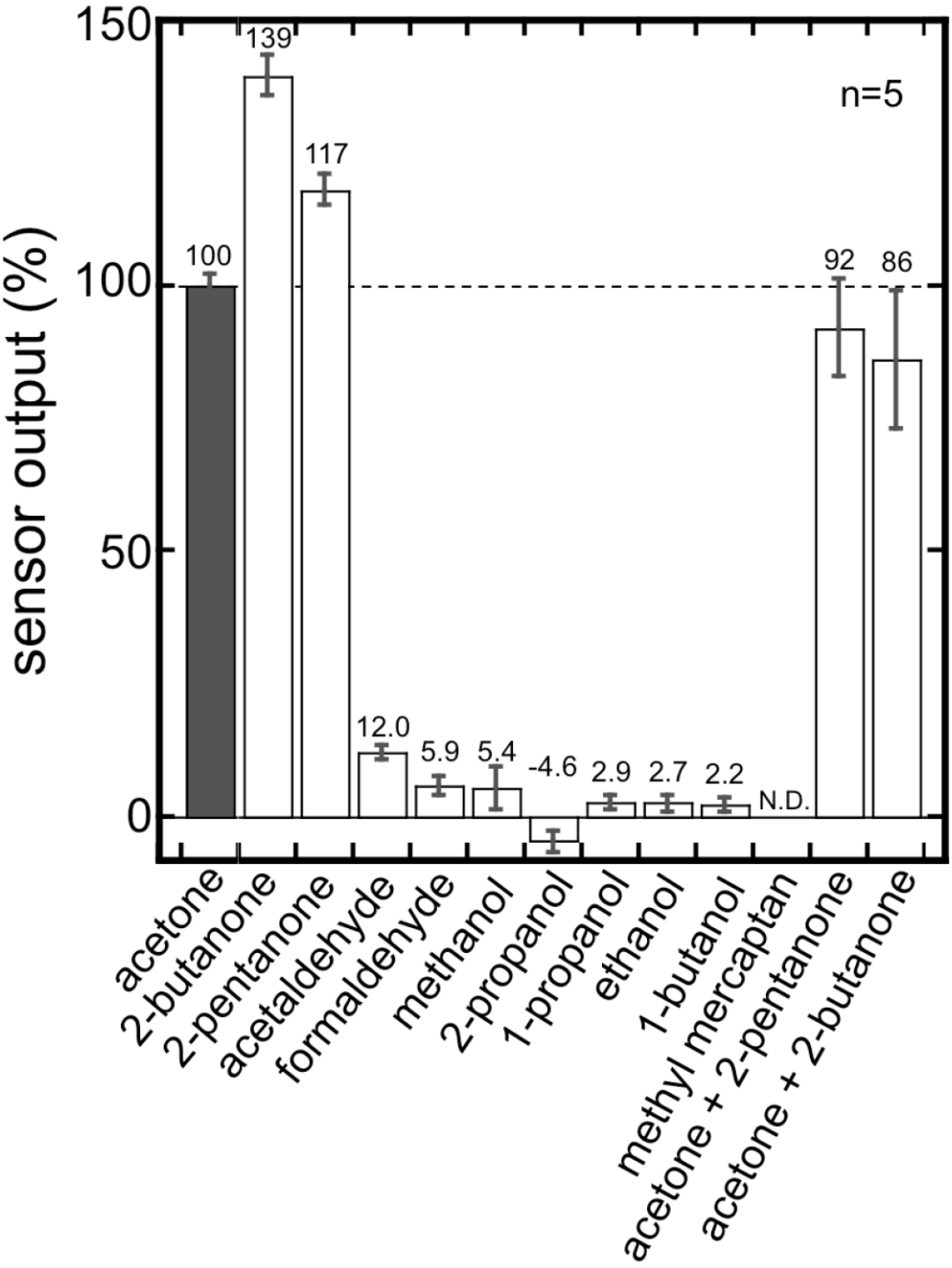
150
100
50
0
sensor output (%)
n=5
100
139
117
12.0
5.9
5.4
-4.6
2.9
2.7
2.2
N.D.
92
86
acetone
2-butanone
2-pentanone
acetaldehyde
formaldehyde
methanol
2-propanol
1-propanol
ethanol
1-butanol
methyl mercaptan
acetone + 2-pentanone
acetone + 2-butanone

Figure 5

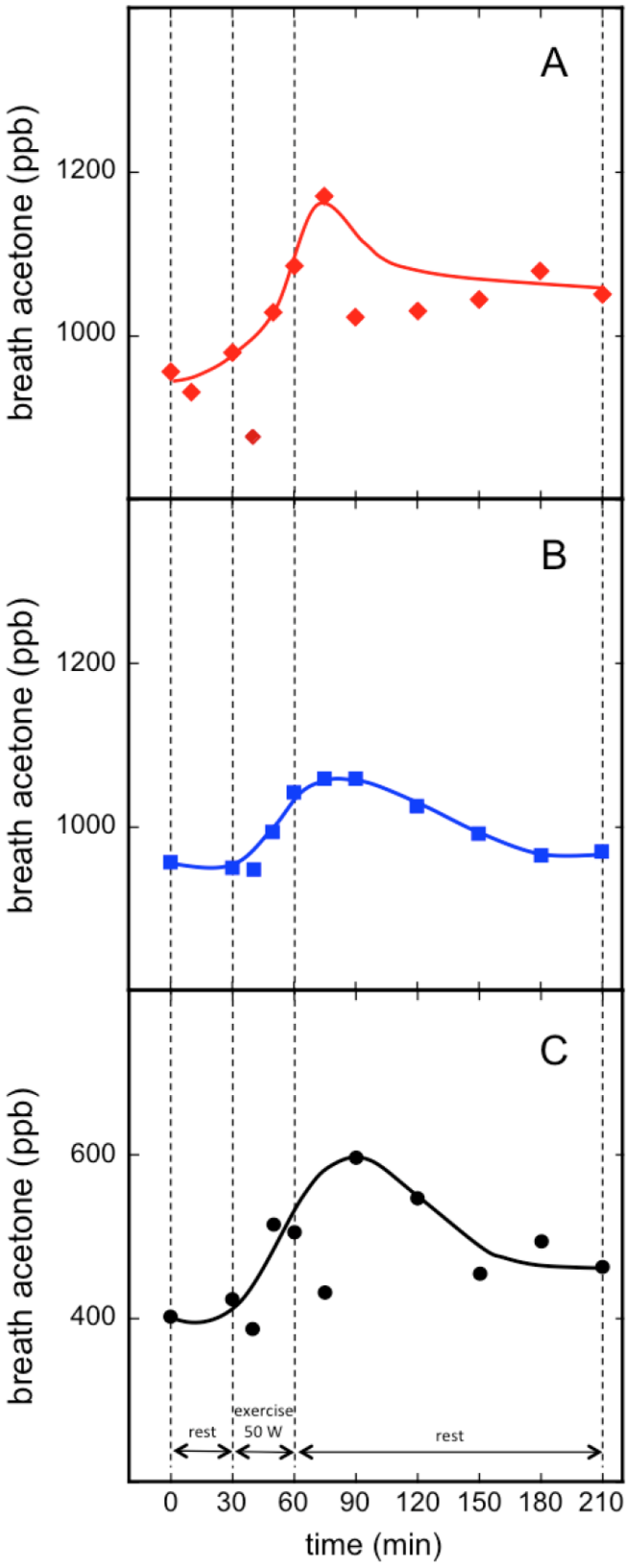
A
B
C
breath acetone (ppb)
1200
1000
600
400
rest
exercise
50 W
rest
0
30
60
90
120
150
180
210
time (min)

Figure 6

Highlights

- We develop acetone biosensor for monitoring of acetone vapor from human breath.
- The Acetone concentration is measured by fluorometry of reduced NADH consumption in reversible reaction by S-ADH
- Developed acetone bio-sniffer can be applied for assessment of lipid metabolism.