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ORIGINAL PAPER

A novel, disposable, screen-printed amperometric biosensor for ketone 3-β-hydroxybutyrate fabricated using a 3-β-hydroxybutyrate dehydrogenase–mesoporous silica conjugate

Takeshi Shimomura · Touru Sumiya · Masatoshi Ono · Tetsuji Ito · Taka-aki Hanaoka

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Abstract A disposable amperometric biosensor for ketone 3-β-hydroxybutyrate (3HB) has been developed successfully. The sensor is based on a screen-printed carbon electrode containing Meldola's Blue (MB) and sensing components containing nicotinamide adenine dinucleotide (NAD^+) and 3-β-hydroxybutyrate dehydrogenase (3HBDH) immobilized in mesoporous silica (FSM8.0) using an aqueous photo-cross-linkable polymer matrix of polyvinyl alcohol (O-391), and it requires only a small sample volume of 10 μL for the measurement. The behavior of a resulting biosensor, i.e., 3HBDH–FSM8.0/NAD^+/MB-SPCE, was examined in terms of NAD^+ concentration for construction, pH, applied potential, operational range, selectivity, and storage stability. The sensor showed an optimum response at a pH of 7.6 and at an applied potential of −50 mV. The determination range and the response time for 3HB were from 30 μM to 8 mM and approximately 30 s, respectively. In addition, the sensor was quite stable and maintained >90 % of its initial response after being stored for over 6 months. This result implies that our method provides a novel biosensor for ketone 3-β-hydroxybutyrate which is easy-to-use, cost-effective, and has good reproducibility, which are vital for commercial purposes.

T. Shimomura (✉) · T. Sumiya · M. Ono
Funai Electric Advanced Applied Technology Research Institute Inc. (FEAT),
TCI 37A, 2-1-6, Sengen,
Tsukuba-shi, Ibaraki 305-0047, Japan
e-mail: shimomura.t@funai-atri.co.jp

T. Ito · T.-a. Hanaoka
National Institute of Advanced Industrial Science and Technology (AIST),
4-2-1 Nigatake, Miyagino-ku,
Sendai-shi, Miyagi 983-8551, Japan

Keywords Screen-printed carbon electrode · Meldola's Blue · Nicotinamide adenine dinucleotide · 3-β-Hydroxybutyrate dehydrogenase · Mesoporous silica · Aqueous photo-cross-linkable polymer

Introduction

Great attention has been drawn to the development of methods for blood 3-β-hydroxybutyrate determination because it is important for diagnosing and monitoring ketoacidosis for diabetic patient management [1, 2]. Ketone bodies refer to 3-β-hydroxybutyrate (3HB), acetoacetic acid, and acetone, and they are produced by incomplete fatty acid metabolism within the mitochondria of hepatocytes and used as an energy source when glucose cannot sufficiently provide the energy for the body's cells. Under pathological conditions involving disorders of glucose metabolism, alcoholism, and so on, excessive amounts of fatty acids are metabolized. Consequently, ketone bodies accumulate in the blood and ketoacidosis occurs. During periods of ketoacidosis, 3-β-hydroxybutyrate, which accounts for approximately 75 % of ketone bodies in the blood [3, 4], increases more markedly than acetoacetic acid and acetone and has been shown to be a better index of ketoacidosis [5–7]. Furthermore, a clinical significance, shown as ketone body ratio (acetoacetic acid/3-β-hydroxybutyrate), has been found between the concentration of 3-β-hydroxybutyrate and the pathophysiological state of the liver [8]. The concentration of 3-β-hydroxybutyrate reflects the balance between fat mobilization and the animal's capacity to utilize the ketone bodies produced. It has become an index for showing the health and nutritional status of the animal [9, 10]. Therefore, it is important to determine the concentration of 3-β-

hydroxybutyrate for clinical fields including the diagnosis and management of ketoacidosis for clinical diagnosis and management of disorders of ketoacid metabolism [11, 12].

Various methods have been developed for the determination of 3HB concentrations in the urine, serum, and blood samples. Conventionally, these methods use colorimetric or chromatographic measurements [13–16], but these methods are either time-consuming or require expensive apparatus and are not convenient for family use. Thus, the development of enzyme-based, amperometric biosensors is of considerable interest recently because their use involve several advantages, such as simple and direct measurements, rapid response, high selectivity, inexpensive, and convenience. At present, only a few biosensors for rapid and sensitive 3HB detection have been reported [17–21], and these biosensors are commonly based on the use of immobilized 3-β-hydroxybutyrate dehydrogenase (3HBDH), where 3HBDH catalyzes the conversion of 3-β-hydroxybutyrate and NAD^+ (nicotinamide adenine dinucleotide, oxidized form) to acetoacetate and NADH (nicotinamide adenine dinucleotide, reduced form), which can be oxidized at the electrode surface directly either through a mediator reaction or through another enzymatic reaction.

However, these biosensors have serious disadvantages in that their responses degrade over a period of time due to the loss of enzymatic activity of 3HBDH, which could be caused by inhibition of the mediator [17], and that they are affected by interference of electroactive substances due to the high applied potential required for detection [22, 23]. In particular, the improvement of stability is of great importance for the success of 3HB biosensors as practical analytical instruments. Therefore, there are considerable advantages to develop a sensitive, stable, and selective biosensor for the quantification of 3HB.

In this article, we describe the fabrication and characterization of a disposable screen-printed 3HB amperometric biosensor which significantly improved the performance of biosensors for 3HB determination in the past. Our biosensor is based on a screen-printed carbon electrode containing Meldola Blue (MB-SPCE) and coated with NAD^+ and an enzyme immobilized in mesoporous silica material (MPS) using an aqueous photo-cross-linkable polymer matrix of polyvinyl alcohol. The use of screen-printing technology is gaining great popularity in biosensor construction because it offers the possibility of mass-producing biosensors at low costs in a wide variety of geometries [24–27]. NAD^+ served as the cofactor of 3HBDH and was reduced to NADH. MB is used as the electron transfer mediator to effectively transfer electrons to the electrode [28–30], which promotes the oxidation of NADH to NAD^+ at a low operating voltage and minimizes the contribution of interfering substances to the biosensor response based on the detection of NADH because direct detection of NADH requires high overvoltage for NADH oxidation on carbon electrodes. MPS is used as a material for enzyme (3HBDH) immobilization in order to enhance the enzyme's stability. In fact, the MPS materials have inherent characteristics of high surface area and pore volume, tunable pore size accommodating dimensions of enzymes, and mechanical stability; encapsulating enzymes into MPS is considered to be one of the best immobilization methods to obtain enzyme stability with good catalytic activity [31, 32]. Our previous studies using enzyme-immobilized electrode in a stirred batch cell showed that high-performance biosensors could be constructed by the use of immobilized enzymes within MPS materials [33–36]. In this study, we adopt MPS powder (pore diameter, 8.0 nm, i.e., FSM8.0) as a host matrix for the encapsulation of 3HBDH from *Pseudomonas* sp. consisting of four subunits with a molecular weight of 130,000 (size, approx. 7.0×8.0×4.0 nm) [37]. The MPS materials encapsulating 3HBDH and NAD^+ are entrapped within a polymer matrix of modified polyvinyl alcohol cross-linked to each other by UV curing and formed on an underlying MB-SPCE electrode. On top of the screen-printed carbon electrode (SPCE), a reaction chamber, i.e., a screen-printed electrochemical cell with a circular container was attached which defines the electrode area and requires only a small sample volume for the measurement. The electrochemical performance of the resulting biosensor (3HBDH–FSM8.0/NAD^+/MB-SPCEs) has been investigated using cyclic voltammetry (CV) and amperometric detection. The response time, determination range, and operating range of the fabricated biosensor were investigated. In addition, the effects of NAD^+ concentration, pH value, applied potential, selectivity, and storage stability on the amperometric response of the biosensor were discussed.

Experimental

Chemicals and reagents

3HBDH (E.C. 1.1.1.30, from *Pseudomonas* sp., 100 U/mg) was purchased from Toyobo Co., Ltd. (Osaka, Japan). NAD^+, NADH, and 3-β-hydroxybutyrate were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Dococyltrimethylammonium chloride [$C_{22}H_{45}N(CH_3)_3Cl$] (C_{22}TMA) was kindly donated by the Lion Corporation (Tokyo, Japan). Sodium silicate (SiO_2/Na_2O=2.07) and tetraethyl orthosilicate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,3,5-Triisopropylbenzene (TIPB) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Several inks for the fabrication of SPCEs, namely, C2030519P4 (carbon/graphite ink), C2030519P5 (mediated carbon ink containing Meldola's Blue), C61003P7 (Ag/AgCl ink), and D2081009D6 (dielectric ink), were purchased from Gwent Electronic Materials Ltd. (Torfaen, UK). An aqueous photo-

cross-linkable polymer solution (O-391) was kindly donated by Chukyo Yushi Co., Ltd. (Nagoya, Japan). Glass epoxy sheets (thickness, 0.3 mm) were supplied by Sumitomo Bakelite Co., Ltd. (Tokyo, Japan). All other reagents were of analytical grade and were obtained from commercial sources.

Synthesis of mesoporous silica powder (FSM8.0)

MPS powder, FSM8.0, with a pore diameter of 8.0 nm, was prepared from sodium silicate using dococyltrimethylammonium chloride and TIPB, as we previously reported [33]. Of C_{22}TMA, 16 g was added to 200 mL water at 70 °C; to this mixture, 60 g of TIPB was added. The resultant mixture was vigorously stirred for 30 min at 70 °C. It was then added to 200 mL of water at 80 °C in the presence of 54.31 g of sodium silicate; the pH of this mixture was adjusted to 8.5 by slowly adding a 2-mol/L HCl aqueous solution. After the suspension was stirred for 3 h at 70 °C, the solid product was filtered out, washed three times with 300 mL distilled water at 70 °C, and dried. The sample was calcined at 150 °C for 2 h in air and the calcined product (300 g) dissolved in a mixture of ethanol (35.4 mL) and HCl (1.7 mL) solution. The suspension was then stirred for 5 h at 70 °C to remove the organic fraction. The solid product was filtered out, washed with ethanol three times, and dried; thus, we obtained FSM8.0.

Preparation of screen-printed carbon electrodes

An LS-56TVA semi-automatic screen-printing machine (Newlong Seimitsu Kogyo Co., Ltd., Japan), equipped with a 250-threads/in. polyester screen, a polyurethane squeegee, and stainless steel flood blade, was used to prepare the SPCEs. The SPCEs were printed in groups of 80 (reference, working, and auxiliary electrodes) onto glass epoxy sheets. They are fabricated in four printing steps: (1) the carbon ink was printed to form a conducting layer and also work as the counter electrode, (2) the Ag/AgCl ink was printed on the reference electrodes, (3) the carbon ink containing the MB layer was printed on the working electrode to form an activated surface, and (4) the insulating ink was printed to cover the non-working and non-conducting region of the SPCE in order to define the working area. After each deposition, the electrodes were dried at 60 °C for 30 min. The individual MB-SPCE was cut from the sheets into the size of 12×25 mm, and reaction chamber composed of white PET (ϕ=9.0 mm, 12×12×1.6 mm), which defines the working area and total sample volume of 100 μL, was bonded on the top of each MB-SPCE with an epoxy adhesive. By using this procedure, batch production of MB-SPCEs with low cost and stable quality was prepared. A picture of the manufactured electrode is presented in Fig. 1a. As can be seen, the working electrode was a 3.5-mm diameter disk; also the auxiliary electrode and the Ag/AgCl reference electrode were large and small portion of a ϕ=7.0×1.0-mm curved line, respectively.

Fabrication of the 3-β-hydroxybutyrate biosensor (3HBDH–FSM8.0/NAD^+/MB-SPCEs)

A 3HBDH-immobilized FSM8.0, i.e., 3HBDH–FSM8.0 conjugate, was prepared as follows [31]: An FSM8.0 powder (100 mg) was added to 4.0 mL of 10 mg/mL 3HBDH solution (MES buffer, pH 5.5). The suspension was then shaken for 24 h at 4 °C to establish an adsorption equilibrium. The 3HBDH–FSM8.0 conjugate was collected by centrifugation and then washed with distilled water. The amount of 3HBDH adsorbed into the pores of the FSM8.0 was estimated to be 197.8 mg/g of the 3HBDH–FSM8.0, as determined by spectrophotometric analysis [33].

The sensing layer of the 3HB biosensor was obtained according to the following procedure: (1) The 3HBDH–FSM8.0 conjugate was shaken for 12 h at 4 °C in 4 mL of phosphate buffer (pH 7.6) containing various amounts of NAD^+. Of this solution, 1,000 μL (i.e., 29.9 mg of 3HB–FSM8.0) and 500 μL of the O-391 solution were mixed together and stirred for 5 min. Then, the resulting mixture (10-μL aliquots) was spin-coated on the MB-SPCEs at a spin rate of 700 rpm for 1 min and allowed to dry for 1 h in the dark at 4 °C. (2) After that, UV light was illuminated to cure O-391 for 1 min. The obtained 3HBDH–FSM8.0/NAD^+/MB-SPCEs were left to dry overnight and kept at 4 °C until used for the measurements. Figure 1b shows the fabrication process of our biosensor. The final amount of immobilized 3HBDH was estimated to be approx. 32.9 μg (3.29 U) per electrode.

Apparatus and electrochemical measurements

CV and constant potential amperometric measurements were performed with a PalmSens handheld potentiostat (Palm Instruments BV, the Netherlands) at room temperature (25 °C). All electrochemical experiments were carried out using a reaction chamber attached on the SPCEs containing 90 μL of 0.1 M phosphate buffer (pH 7.6). CV experiments were performed with and without 3HB solution, in which the potential range used was −0.4/+0.4 V at a scan rate of 10 mV/s. The amperometric experiments were carried out by applying a constant potential to the working electrode of the biosensor. After the transient current had settled (t=200 s), 10-μL aliquots of known concentrations of 3HB standard solutions were injected into the reaction chamber by micropipette and the sensor response was measured until the resulting current reached a steady-state value.

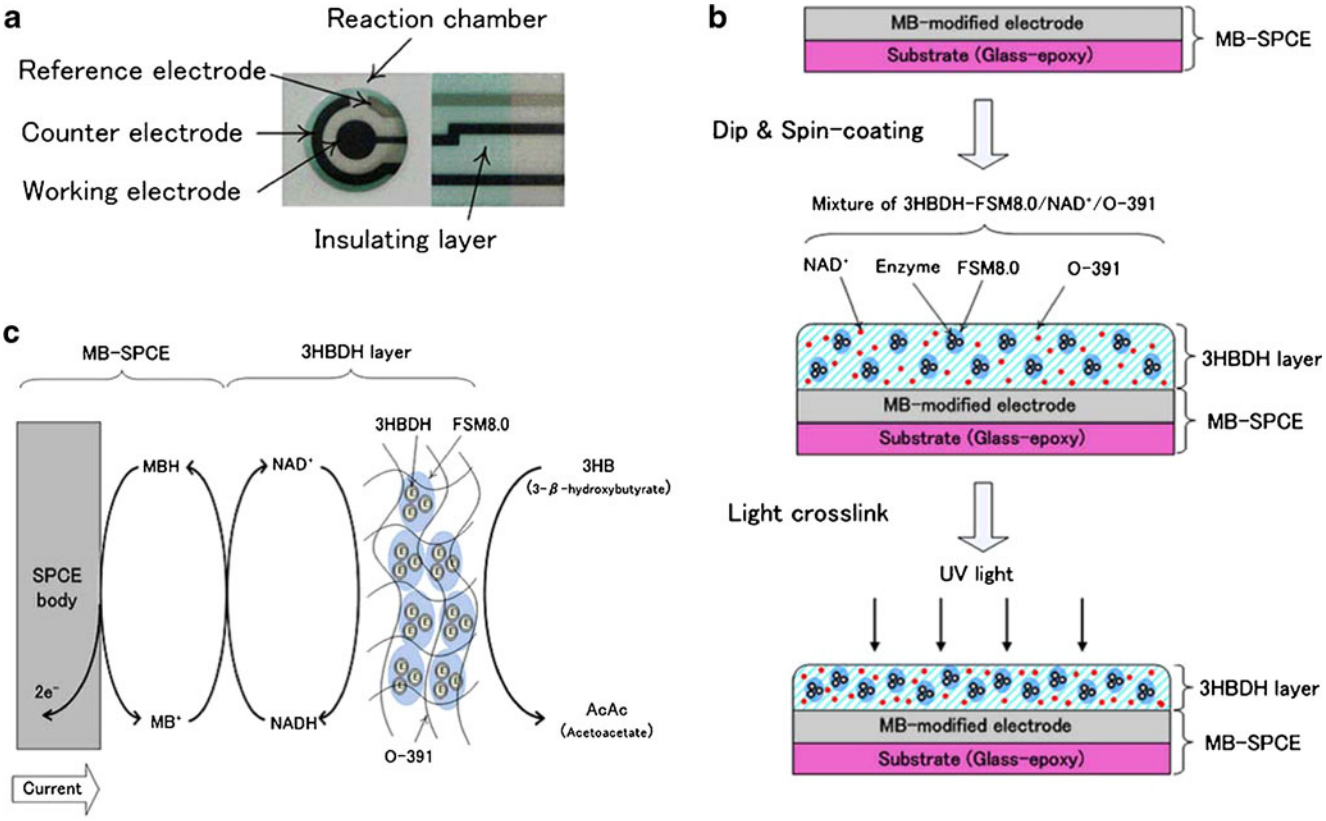

Fig. 1 **a** Structure of the manufactured MB-SPCE electrode on a glass epoxy substrate. **b** Fabrication process of the 3HBDH–FSM8.0/NAD^+/MB-SPCE biosensor. **c** Schematic representation of the operation mechanism of our biosensor

Results and discussion

Reaction principle

The sequence of reactions involved in the amperometric detection of 3HB here can be described according to the following reactions [17–21, 28–30]:

$$3-\beta-\text{Hydrobutyrate} + NAD^+ \xrightarrow{3HBDH} Acetoacetate + NADH + H^+ \quad (1)$$

$$NADH + MB^+ \longrightarrow NAD^+ + MBH \quad (2)$$

$$MBH \xrightarrow{electrode} MB^+ + H^+ + 2e^- \quad (3)$$

where immobilized 3HBDH catalyzes the oxidation of 3HB to acetoacetate and NAD^+ is reduced to NADH. The produced NADH donates electrons to MB^+ and is oxidized to NAD^+, which is involved in reaction 1 again, and MB^+ receives electrons and is changed to a reduced form, MBH. MBH is finally oxidized to MB^+ by a working electrode with a proper potential. This reaction produces a flow of electrons to the working electrode and, thus, a measurable current, the magnitude of which is directly related to the 3HB concentration. The operation mechanism of our biosensor is illustrated in Fig. 1c.

Electrochemical characterization of the 3HBDH–FSM8.0/NAD^+/MB-SPCEs

The electrochemical behavior of the MB-SPCEs and plain SPCEs was first evaluated using the NADH solution by performing CV experiments in order to establish the catalytic ability toward the oxidation of NADH by Meldola's Blue. Figure 2a (1)–(4) shows the CVs of the plain SPCEs and MB-SPCEs in the absence and the presence of 5.0 mM NADH, respectively. The scan was initiated at −0.4 V to a switching potential of +0.4 V at a scan rate of 10 mV/s. These figures show that an important increase in anodic current was observed in the presence of NADH for MB-SPCEs. This electrocatalytic current in the presence of NADH is due to electron shuttling by MB from NADH to the electrode surface, according to the reactions in Eqs. 2 and 3, and show the catalytically ability of the MB-SPCE electrode for NADH oxidation.

Next, CVs were obtained with the 3HBDH–FSM8.0/NAD^+/MB-SPCE biosensor in the absence (Fig. 2b (1)) and the presence (Fig. 2b (2)) of 5.0 mM 3HB solution (pH 7.6), respectively. This behavior clearly demonstrates

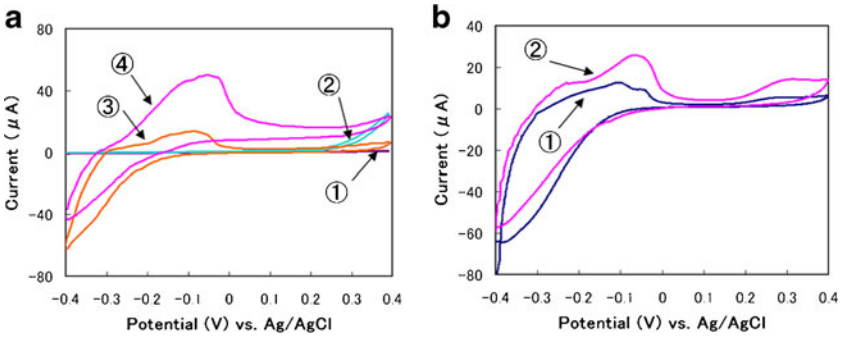

Fig. 2 **a** Cyclic voltammograms obtained with plain SPCE in phosphate buffer solution (pH 7.6) (*1*), with 5.0 mM NADH added (*2*), MB-SPCE in phosphate buffer solution (pH 7.6) (*3*), and with 5.0 mM NADH added (*4*). Potential range, −0.4/+0.4 V; scan rate, 10 mV/s. **b** Cyclic voltammograms obtained with the 3-HBDH–FSM8.0/NAD^+/MB-SPCE biosensor in the absence (*1*) and in the presence (*2*) of 3HB (5.0 mM) in phosphate buffer solution (pH 7.6). Potential range, −0.4/+0.4 V; scan rate, 10 mV/s

that enzymatically generated NADH from the reaction of NAD^+ and 3HB catalyzed by 3HBDH, as schematized in Fig. 1c, can diffuse to the underlying MB-SPCE and undergo electrocatalytic oxidation. Such behavior is a prerequisite for the successful operation of an amperometric 3HB biosensor, which is discussed in subsequent sections of this paper.

Optimization of the sensor response

In order to optimize the sensor response, the influence of three variables, i.e., cofactor concentration, applied potential value, and solution pH, related to the performance of the 3HBDH–FSM8.0/NAD^+/MB-SPCE biosensor is investigated under suitable conditions in this subsection. Although there are many experimental variables affecting biosensor response, we think that the simplified approach using these variables below distill the essential of the optimization procedure for our disposable 3HB biosensor for commercial purposes [17, 20].

Effect of NAD^+ concentration on the response current

Experiments were carried out to establish the optimal cofactor concentrations for the 3HBDH–FSM8.0/NAD^+/MB-SPCE biosensor. Figure 3 shows the dependence of the current responses on the cofactor concentrations, where the NAD^+ concentration in the fabrication process was varied from 0.5 to 20 mM and the experiments were carried out by adding 10-μL aliquots of aqueous 3HB solutions to the reaction chamber containing 90 μL of phosphate buffer (pH 7.6). The optimal NAD^+ concentrations were found to be 5 mM for this sensor. At a lower concentration, it would be the rate limiting factor in reaction 1, so that the response current would be small and the response of the biosensor would decrease. At higher concentrations of NAD^+, the current responses decreased because NAD^+ is a known inhibitor of the electrochemical oxidation process of NADH [38]. Therefore, we used NAD^+ concentrations of 5 mM for the fabrication process of our biosensor.

Effect of applied potential to the sensor

The effect of the applied potential on the 3HBDH–FSM8.0/NAD^+/MB-SPCE biosensor response was studied in order to identify the most suitable potential for amperometric detection. For this purpose, 1.0 mM 3HB solution (10-μL aliquots) was added to the reaction chamber filled with 90 μL of phosphate buffer at pH 7.6 and the current was monitored. The values of the current response were plotted against the applied potential, as shown in Fig. 4a, and it can be seen that th maximum current response was observed at an applied potential of −50 mV. Thus, we adopted −50 mV vs. Ag/AgCl as an operating potential for further amperometric study.

Effect of solution pH to the sensor

The effect of the pH on the 3HBDH–FSM8.0/NAD^+/MB-SPCE biosensor response to 3HB was studied in the pH range between 5.8 and 9.0. The results are shown in Fig. 4b,

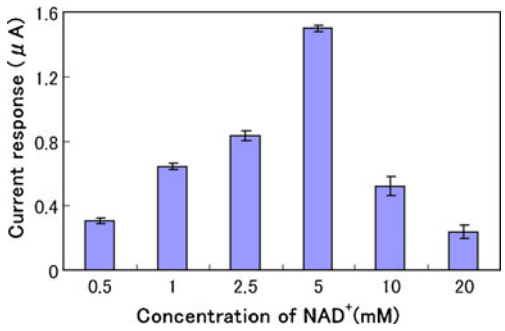

Fig. 3 Effect of NAD^+ concentration on the response of the 3HBDH–FSM8.0/NAD^+/MB-SPCE biosensor in phosphate buffer (pH 7.6) containing 1.0 mM of the 3HB solution. Applied potential is −50 mV vs. Ag/Ag/Cl. *Error bars* show deviations within six measurements

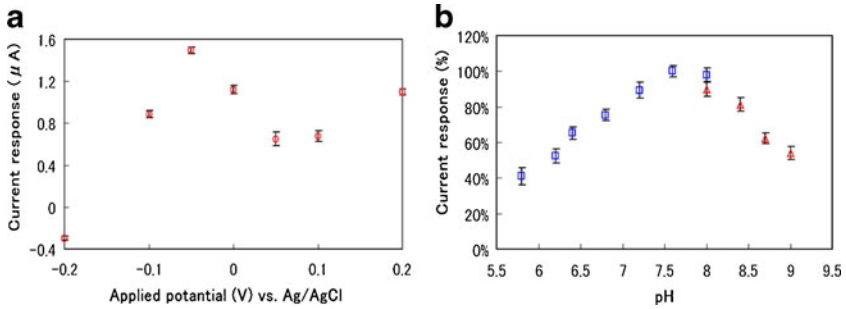

Fig. 4 **a** Response of the 3HBDH–FSM8.0/NAD$^+$/MB-SPCE biosensor in phosphate buffer (pH 7.6) at several applied potentials containing 1.0 mM of the 3HB solution. *Error bars* show deviations within six measurements. **b** Response of the 3HBDH–FSM8.0/NAD$^+$/MB-SPCE biosensor in different buffer solutions at several pH values (pH 5.8–8.0, phosphate buffer (*square*); pH 8.0–9.0, borate buffer (*triangle*)) containing 1.0 mM of the 3HB solution. Applied potential is −50 mV vs. Ag/Ag/Cl. *Error bars* show deviations within six measurements

in which the output current of the sensor is expressed as the percentage of the maximum response attained at an appropriate optimal pH. We can see that the maximum response was obtained in the pH range of 7.6–8.0. Hence, a pH of 7.6 was selected in our experiments so as to ensure high sensitivity for sensor response.

Amperometric response and calibration curve of the sensor

The operational range and detection limit of the sensor were studied. Figure 5a shows the calibration curve and typical amperometric responses (inset) of the sensor to injections of different concentrations of the 3HB solution (10-μL aliquots), ranging from 1.0 μM to 20 mM, into the reaction chamber at an applied potential of −50 mV vs. Ag/AgCl in phosphate buffer at pH 7.6. The 3HBDH–FSM8.0/NAD$^+$/MB-SPCE biosensor indicated a good linear relation (y= 1.436x+0.068, where y represents the current in microampere and x is the concentration of the 3HB solution in millimolars) in the range from 30.0 μM to 8 mM, with a correlation coefficient of 0.997; the current became saturated above 10 mM. The lower detection limit was estimated to be 29.2 μM at a signal-to-noise ratio of 3, and the response time to reach a steady output current after applying the 3HB solution was approximately 30 s. The reproducibility of the biosensor was calculated in terms of the relative standard deviation (RSD) of the slopes and the RSD of the intercept values for the calibration curves, achieving values of 0.72 and 5.84 % (n=6), respectively. Thus, the 3HBDH–FSM8.0/NAD$^+$/MB-SPCE biosensor displayed a wide operational range, good detection limit, rapid response, and good reproducibility that can be successfully applied to the detection of 3HB. In fact, this linear range is wide enough for clinical purpose in the future because the concentration of 3HB in the blood is normally 50 μM, reaching a maximum of about 500 μM; in the case of untreated diabetes mellitus or excessive consumption of ethanol, the value may become higher. It is higher than 1 mM when hyperketonemia occurs and it is higher than 3 mM when ketoacidosis occurs [6]. The apparent Michaelis–Menten constant of the 3HBDH–FSM8.0/NAD$^+$/MB-SPCE sensor evaluated from an electrochemical version of the Lineweaver–Burk equation was calculated to be 2.8 mM, which is comparable to or lower than the corresponding values observed for some papers (2.3 mM:

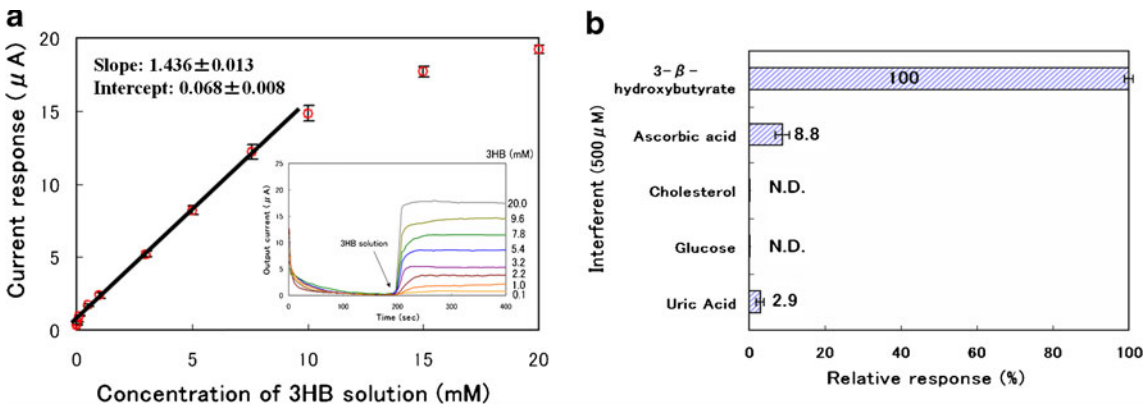

Fig. 5 **a** Typical responses (*inset*) and calibration curve of the 3HBDH–FSM8.0/NAD$^+$/MB-SPCE biosensor to injections of aqueous 3HB solutions with application of a potential of −50 mV vs. Ag/AgCl in phosphate buffer (pH 7.6). *Error bars* show deviations within six measurements. **b** Relative responses of possible interferents to the 3HBDH–FSM8.0/NAD$^+$/CoPC-SPCE sensor. *Error bars* show deviations within four measurements

Table 1 Comparisons of different methods to prepare 3HB biosensors based on screen-printed electrode and 3HBDH

Enzyme	Mediator, cofactor	Material of working electrode	Immobilization method	Potential	Linear range	Detection limit	Response time	Stability	Interference protection	Reference
3HBDH	4-Methyl-*o*-quinone, NAD^+	Carbon ink	Chemically covalent binding	0.35 V vs. Ag/Cl	0–5 mM	n.a.	n.a.	n.a.	Low potential	[18]
3HBDH	NAD^+	Carbon ink containing iridium	Physical adsorption	0.2 V vs. Ag/Cl	0–8 mM	n.a.	n.a.	n.a.	Cross talk; n.a.	[19]
3HBDH	Potassium ferricyanide, NAD^+	Carbon ink + CMC	Physical adsorption	0.3 V vs. Ag/Cl	14.4 μM–5.3 mM	14.4 μM	50 s	1 month (stored dry at RT)	Dilution of sample, low potential	[20]
3HBDH	NAD^+	Platinized carbon ink	Physical adsorption	0.15 V vs. Ag/Cl	0–5.0 mM	n.a.	30 s	>3 months (storage at 4 °C)	Low potential	[21]
3HBDH	NAD^+	Carbon ink containing Meldola's Blue	Entrapment in mesoporous silica	−0.05 V vs. Ag/Ag/Cl	30 μM–8 mM	29.2 μM	30 s	>6 months (stored dry at 4 °C)	Low potential	This paper

[19]; 3 mM: [18]: 5.4 mM: [21]), which indicates a high affinity between the 3HB and 3HBDH immobilized within the 3HBDH–FSM8.0/NAD^+/MB-SPCE sensor, mainly due to its biocompatibility and large surface area.

Selectivity against interferences

The interference effects due to the most common electrochemical interfering species (500 μM; 3HB, ascorbic acid, cholesterol, glucose, and uric acid) were evaluated by adding each of them individually into the reaction chamber. The relative response gives a measure of the sensor's selectivity. The results are summarized in Fig. 5b and show that the relative responses obtained for most of these interferents were weak under the testing conditions. In particular, the responses to ascorbic acid and uric acid are only 8.8 and 2.9 % when the 3HB response was taken as 100 %. Furthermore, the interferences of ascorbic acid and uric acid to the 3HB response were examined in the presence of their physiological normal level. In concrete, different concentrations of the 3HB solution, ranging from 1.0 μM to 20 mM, were added into the solutions containing ascorbic acid and uric acid at their typical concentration levels in blood (50 and 500 μM, respectively) [6, 20]. The results showed that the background current (the current obtained in 0.1 M phosphate buffer (pH 7.6) in the absence of 3HB) increased slightly, but the presence of ascorbic acid and uric acid did not produce any obvious interference in the biosensor response and did not affect the sensitivity of our biosensor. These results show that the 3HBDH–FSM8.0/NAD^+/MB-SPCE sensor has good selectivity due to the combination of low working potential and the sensing layer with high catalytic ability for 3HB by densely immobilized 3HBDH within the FSM8.0.

Storage stability of the sensor

The storage stability of the 3HBDH–FSM8.0/NAD^+/MB-SPCE sensor was investigated by evaluating its response to 1.0 mM 3HB solution. The sensor was stored desiccated at 4 °C when not in use. As a result, the sensor was quite stable and maintained >90 % of its initial current response over a 6-month period. This is more stable than the 3HB biosensor based on a screen-printed electrode previously reported [20, 21]. Such excellent stability is crucial for any commercial biosensor device. The highly stable nature of this sensor is considered to result from the intimate integration of the enzyme with FSM8.0 and shows that FSM8.0 is very efficient for retaining the activity of 3HBDH.

A comparative study of different 3HB biosensors based on disposable/reusable screen-printed electrode and 3HBDH is presented in Table 1, showing that our proposed biosensor offers comprehensive electrochemical properties

compared with other biosensors reported previously in the literature. It shows that the proposed biosensor has good storage stability longer than that of other biosensors. Its linearity was comparable to [19] or better than [18, 19, 22], and the limit of detection was comparable to [20].

Conclusion

A disposable amperometric biosensor for ketone 3-β-hydroxybutyrate with sufficient performance (3HBDH–FSM8.0/NAD^+/MB-SPCE) has been developed successfully. Our biosensor is based on a screen-printed carbon electrode containing Meldola Blue and coated with NAD^+ and 3-β-hydroxybutyrate dehydrogenase immobilized in MPS using an aqueous photo-cross-linkable polymer matrix of polyvinyl alcohol (O-391). The response of the biosensor with 3HBDH–FSM8.0/NAD^+/MB-SPCE was linear with substrate concentrations from 30 μM to 8 mM and a response time of 30 s. The sensor showed an optimum response at a pH of 7.6 and at an applied potential of −50 mV. In addition, the sensor exhibited high selectivity and good stability, and it maintained >90 % of its initial current response after being stored for over 6 months in dry condition. This result implies that our method provides a novel disposable biosensor for ketone 3-β-hydroxybutyrate which is easy-to-use, cost-effective, and with good reproducibility, which are vital for the commercialization of the biosensor.

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References

1. Goldstein DE, Little RR, Lorenz RA, Malone JI, Nathan D, Peterson CM, Sacks DB (2004) Tests of glycemia in diabetes. Diabetes Care 27(7):1761–1773
2. American Diabetes Association (2002) Tests of glycemia in diabetics (position statement). Diabetes Care 25:S97–S99
3. Koch DD, Feldbruegge DH (1987) Optimized kinetic method for automated determination of β-hydroxybutyrate. Clin Chem 33: 1761–1766
4. Ma ZL, Zhang Y, Li WH, Wang YZ (2002) Significance of determining blood beta-hydroxybutyrate for diagnosis and therapy of diabetic ketoacidosis. Chin J Diabetes 10:235
5. Harano Y, Kosugi K, Hyosu T, Uno S, Ichikawa Y, Shigeta Y (1983) Sensitive and simplified method for the differential determination of serum levels of ketone bodies. Clin Chim Acta 134:327–336
6. Laffel L (1999) Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes Metab Res Rev 15(6):412–426
7. Csako G (1990) Causes, consequences, and recognition of false-positive reactions for ketones. Clin Chem 36(7):1388–1389
8. Uno S, Ito S, Kurono M, Yamaoka Y, Kamiyama Y, Ozawa K (1987) A simple and sensitive assay for blood ketone bodies using highly purified 3-hydroxybutyrate dehydrogenase. Clin Chim Acta 168:253–255
9. Palleschi G, Rathore HS, Mascini M (1988) Amperometric probe for 3-hydroxybutyrate with immobilized 3-hydroxybutyrate dehydrogenase. Anal Chim Acta 209:223–230
10. Pires CK, Martelli PB, Reis BF, Lima JL, Saraiva ML (2003) An automatic flow procedure for the determination of 3-hydroxybutyrate in animal serum and plasma. J Agric Food Chem 51:2457–2460
11. Lean IJ, Bruss ML, Baldwin RL, Troutt HF (1991) Bovine ketosis: a review. I. Epidemiology and pathogenesis. Vet Bull 61:1209–1218
12. Alberti KGMM, Cutbert C (1982) In: Porter R, Lawrenson G (eds) Metabolic acidosis (Ciba Foundation Symposium 87). Pitman Books, London
13. Varley H, Gowenlock AH, Bell M (1980) Practical clinical biochemistry, vol. 1, 5th edn. William Heinemann Med. Books, London
14. Harrison J, Hodson AW, Skillen AW, Stappenbeck R, Agius L, Alberti KGMM (1988) Blood glucose, lactate, pyruvate, glycerol, 3-hydroxybutyrate and acetoacetate measurements in man using a centrifugal analyser with a fluorimetric attachment. J Clin Chem Clin Biochem 26:141–146
15. Kiba N, Inoue Y, Tachibana M, Tani K, Koizumi H (2003) Simultaneous determination of d-glucose and 3-hydroxybutyrate by chemiluminescence detection with immobilized enzymes in a flow injection system. Anal Sci 19(8):1203–1206
16. Kimura M, Kobayashi K, Matsuoka A, Hayashi K, Kimura Y (1985) Headspace gas-chromatographic determination of 3-hydroxybutyrate in plasma after enzymic reactions, and the relationship among the three ketone bodies. Clin Chem 31(4):596–598
17. Kwan RCH, Hon PYT, Mak WC, Law LY, Hu J, Renneberg R (2006) Biosensor for rapid determination of 3-hydroxybutyrate using bienzyme system. Biosens Bioelectron 21(7):1101–1106
18. Batchelor MJ, Green MJ, Sketch CL (1989) Amperometric assay for the ketone body 3-hydroxybutyrate. Anal Chim Acta 221:289–294
19. Fang L, Wang SH, Liu CC (2008) An electrochemical biosensor of the ketone 3-β-hydroxybutyrate for potential diabetic patient management. Sen Actuators B Chem 129:818–825
20. Li G, Ma NZ, Wang Y (2005) A new handheld biosensor for monitoring blood ketones. Sens Actuators B Chem 109(2):285–290
21. McNeil CJ, Spoors JA, Cooper JM, George K, Alberti MM, Mullen WH (1990) Amperometric biosensor for rapid measurement of 3-hydroxybutyrate in undiluted whole blood and plasma. Anal Chim Acta 237:99–105
22. Moiroux J, Elving PJ (1978) Effects of adsorption, electrode material and operational variables on the oxidation of dihydronicotinamide adenine dinucleotide at carbon electrode. Anal Chem 50:1056–1062
23. Jaegfeldt H (1980) Adsorption and electrochemical oxidation behavior of NADH at a clean platinum electrode. J Electroanal Chem 110:295–302
24. Khan GF, Wernet W (1997) Design of enzyme electrodes for extended use and storage life. Anal Chem 69:2682–2687
25. Albareda-Sirvent M, Merkoçi A, Alegret S (2000) Configurations used in the design of screen-printed enzymatic biosensors. Sens Actuators B 69:153–163
26. Hart JP, Wring SA (1997) Recent developments in the design and application of screen-printed electrochemical sensors for biomedical, environmental and industrial analyses. TRAC 16:89–103
27. Renedo OD, Alonso-Lomillo MA, Arcos Martínez MJ (2007) Recent developments in the field of screen-printed electrodes and their related applications. Talanta 73:202–219

28. Sprules SD, Hart JP, Wring SA, Pittson R (1994) Development of a disposable amperometric sensor for reduced nicotinamide adenine dinucleotide based on a chemically modified screen-printed carbon electrode. Analyst 119:253–257
29. Avramescu A, Noguer T, Avramescu M, Marty JL (2001) Chronoamperometric determination of D-lactate using screen-printed enzyme electrodes. Anal Chim Acta 433:81–88
30. Bala C, Rotariu L, Vasilescu A, Magearu V (2004) Development of a new ethanol biosensor with electropolymerised Meldola Blue as mediator. An Univ Bucureşti Chimie, Anul XIII (Serie Nouă) I–II:19–25
31. Fan J, Yu C, Gao F, Lei J, Tian B, Wang L, Luo Q, Tu B, Zhou W, Zhao D (2003) Cubic mesoporous silica with large controllable entrance sizes and advanced adsorption properties. Angew Chem Int Ed 42:3146–3150
32. Blin JL, Gerardin C, Carteret C, Rondehuser L, Selve C, Stebe MJ (2005) Direct one-step immobilization of glucose oxidase in well-ordered mesostructured silica using a nonionic fluorinated surfactant. Chem Mater 17:1479–1486
33. Shimomura T, Itoh T, Sumiya T, Mizukami F, Ono M (2008) Electrochemical biosensor for the detection of formaldehyde based on enzyme immobilization in mesoporous silica materials. Sen Actuators B Chem 135:268–275
34. Shimomura T, Itoh T, Sumiya T, Mizukami F, Ono M (2009) Amperometric determination of choline with enzyme immobilized in a hybrid mesoporous membrane. Talanta 78:217–220
35. Shimomura T, Itoh T, Sumiya T, Mizukami F, Ono M (2009) Amperometric biosensor based on enzymes immobilized in hybrid mesoporous membranes for the determination of acetylcholine. Enzyme Microb Technol 45:443–448
36. Shimomura T, Itoh T, Sumiya T, Hanaoka T, Mizukami F, Ono M (2011) Amperometric detection of phenolic compounds with enzyme immobilized in mesoporous silica prepared by electrophoretic deposition. Sen Actuators B Chem 153:361–368
37. Paithankar KS, Feller C, Kuettner EB, Keim A, Grunow M, Strater N (2007) Cosubstrate-induced dynamics of D-3-hydroxybutyrate dehydrogenase from *Pseudomonas putida*. FEBS J 274(21):5767–5779
38. Gorton L, Persson B, Hale HL, Boguslavsky LI, Karan HI, Lee HS, Skotheim TA, Lan HL, Okamoto Y (1992) In: Edelman PG, Wang J (eds) Biosensors and chemical sensors (ACS Symposium Series. Maple Press, York, PA