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A fiber-optic sorbitol biosensor based on NADH fluorescence detection toward rapid diagnosis of diabetic complications†

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Accumulation of sorbitol in the tissue is known to cause microvascular diabetic complications. In this paper, a fiber-optic biosensor for sorbitol which is used as a biomarker of diabetic complications was developed and tested. The biosensor used a sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* with high substrate specificity and detected the fluorescence of reduced nicotinamide adenine dinucleotide (NADH) by the enzymatic reaction. An ultraviolet light emitting diode (UV-LED) was used as the excitation light source of NADH. The fluorescence of NADH was detected using a spectrometer or a photomultiplier tube (PMT). The UV-LED and the photodetector were coupled using a Y-shaped optical fiber. In the experiment, an optical fiber probe with a sorbitol dehydrogenase immobilized membrane was placed in a cuvette filled with a phosphate buffer containing the oxidized form of nicotinamide adenine dinucleotide (NAD^+). The changes in NADH fluorescence intensity were measured after adding a standard sorbitol solution. According to the experimental assessment, the calibration range of the sorbitol biosensor systems using a spectrometer and a PMT was 5.0–1000 μmol L^{-1} and 1.0–1000 μmol L^{-1}, respectively. The sorbitol biosensor system using the sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* has high selectivity and sensitivity compared with that from sheep liver. The sorbitol biosensor allows for point-of-care testing applications or daily health care tests for diabetes patients.

1. Introduction

The population of the world with diabetes is steadily increasing, which is one of the major social issues. Diabetes is a chronic condition characterized by high concentration of glucose in the blood, and the chronic hyperglycemia causes serious complications. Thus, the main purpose of diabetes treatment is to prevent these serious complications. Diabetes complications are divided into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy).[1] The pathogenesis of these complications induced by hyperglycemia has been proposed to involve five major mechanisms: increased polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) informs, increased hexosamine pathway flux and overproduction of superoxide. In recent years, it has been revealed that the five hypotheses are closely implicated.[2–5] Fig. 1 shows the pathogenic mechanism of

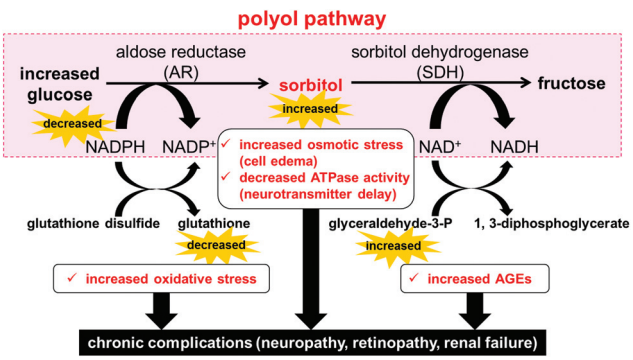

Fig. 1 Pathogenic mechanism of diabetic complications caused by polyol pathway flux. The polyol pathway is a two-step metabolic pathway in which glucose is reduced to sorbitol, which is then converted into fructose. In a hyperglycemia environment, increased sorbitol levels within cells induce an increase of osmotic stress and a decrease of ATPase activity.

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diabetes complications related to the polyol pathway flux. The polyol pathway flux is found to be associated with an increase of oxidative stress and AGEs. The increased polyol pathway flux is observed in an early stage of diabetes and it is expected that the increased polyol pathway flux is a starting point of metabolic abnormality. Therefore, sorbitol increase by the polyol pathway flux is expected to be one biomarker of diabetic complications.

Enzymatic or chromatographic (HPLC, GC-MS, *etc.*) methods are typically used to analyze sorbitol levels in the diagnosis of diabetes complications.[6–8] An enzymatic method using a fluorescence microplate reader is usually applied to clinical examination. In the enzymatic method, simplification of the sample processing methods was reported and the improved method was used for the evaluation of sorbitol levels in the diabetes state.[9,10] However, considering the use of sorbitol measurement in daily health care testing or point-of-care testing (POCT) tests, a simple, compact and relatively accurate sorbitol sensor is strongly required. Some approaches to the sorbitol sensing system have been proposed. Saccharide detection based on the affinity of phenylboronic acid compounds was reported.[11–13] Although synthetic chemosensors have good stability and reliability under a variety of conditions, these approaches have less selectivity than enzyme based biosensors. Besides, some electrochemical biosensors based on carbon nanotubes were reported. M. Etienne *et al.* have reported dehydrogenase-based biosensors using carbon nanotubes and sol–gel thin films on a glassy carbon electrode.[14,15] J. Tkac *et al.* developed the hyaluronic acid dispersed carbon nanotube electrode and applied this electrode to an amperometric NADH biosensor.[16,17] However, these biosensors need improvement for their application in clinical examination in terms of sensitivity and selectivity.

In our previous work, nicotinamide adenine dinucleotide (NADH)-dependent fiber-optic biosensors with high-sensitivity for various substances have been reported.[18,19] The biosensing device is expected to be applied to point-of-care testing applications and daily health care tests, because it is simple and compact by utilizing the LED as a light source. Therefore, in this study, a sorbitol biosensor based on an NADH fluorescence detection system for easy evaluation of diabetic complications has been developed and demonstrated.

2. Experimental section

2.1. Chemicals and apparatus

β-NADH (reduced form (disodium salt)) and β-NAD^+ (oxidized form (free acid)) were obtained from Oriental Yeast Co., Ltd, Japan. Two types of sorbitol dehydrogenases (EC 1.1.1.14, from microorganisms of the genus *Flavimonas* and from sheep liver) were provided by Wako Pure Chemical Industries Ltd, Japan and Roche Diagnostics K.K., Japan. Sorbitol, xylitol, glucose, fructose and mannitol were obtained from Wako Pure Chemical Industries Ltd, Japan. The standard solution was prepared in deionized water obtained from a Milli-Q purification system (Millipore Co., USA). In experiments with enzymes, the standard solution was prepared in Tris-HCl buffer (pH 9.0, 100 mmol L^{-1}). The pH of the buffer was adjusted to the desired value by adding 6 N HCl solution into 100 mmol L^{-1} tris(hydroxymethyl)aminomethane solution while being monitored using a pH meter (D-25, 801031, Horiba, Ltd, Japan). A hydrophilic PTFE (H-PTFE) membrane filter (porosity: 80%, thickness: 80 μm, pore size: 0.2 μm, JGWP14225, Millipore Co., USA) and MPC-*co*-EHMA (PMEH) were used for sorbitol dehydrogenase immobilization. PMEH is a copolymer of 2-methacryloyloxyethyl phosphorylcholine (MPC) and 2-ethylhexyl methacrylate (EHMA) and was synthesized using a free radical-polymerization method.[20]

The NADH excitation system was constructed with a UV-LED (UVTOP® BL335, Sensor Electronic Technology, Inc., USA) and a custom-fabricated UV-LED power supply circuit produced by KLV Co., Ltd (Japan). The fluorescence of NADH was detected using a spectrometer (USB2000+, Ocean Optics Inc., USA) or a photomultiplier tube (C9692, Hamamatsu Photonics K.K., Japan). Two optical fibers, a bifurcated optical fiber assembly (BIF600-UV/VIS), an optical fiber probe (F1000-900) and a filter holder (FHS-UV) were purchased from Ocean Optics Inc. (USA) and utilized for the fiber-optic measurement system. A long-pass filter and two band-pass filters were purchased from Asahi Spectra Co. Ltd, Japan.

2.2. Construction of the sorbitol measurement system

The sorbitol biosensor utilized the fluorescence of NADH (491 nm), which is produced by the following sorbitol dehydrogenase reaction as follows.

$$\text{D-sorbitol} + NAD^+ \rightarrow \text{D-fructose} + NADH + H^+$$

Fig. 2 shows a schematic illustration of the sorbitol measurement system. The sorbitol measurement system was constructed based on a fiber-optic NADH measurement system.[21] Two-way optical fiber was connected to the excitation system using a UV-LED (λ_p = 335 nm) and a spectrometer which were filtered with a band-pass filter (band-pass wave-

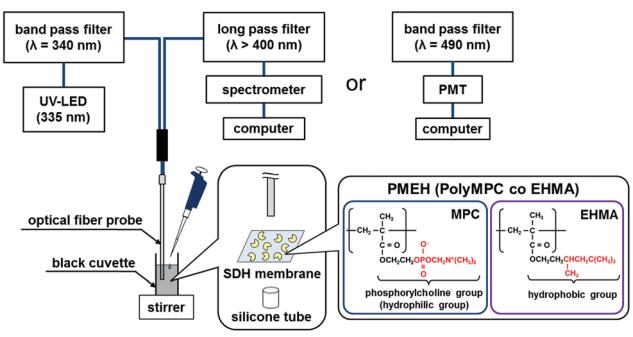

Fig. 2 Schematic illustration of the sorbitol measurement system. Two-way optical fiber was connected to the excitation system using a UV-LED and a spectrometer which were filtered with a band-pass filter and a long-pass filter, respectively. The SDH was immobilized by the PMEH polymer.

length: 330–350 nm) and a long-pass filter (cut-off wavelength: 400 nm), respectively. In the case of using a photomultiplier tube, the band-pass filter (band-pass wavelength: 480–500 nm) was used. The assembled terminal of the two-way optical fiber assembly was connected to the optical fiber probe. Each sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* and from a sheep liver immobilized membrane was attached to the end of the optical fiber probe in order to apply it to sorbitol measurements. The sorbitol dehydrogenase membrane was first prepared by coating a sorbitol dehydrogenase solution dissolved in tris-HCl buffer (16 units per cm^2) and PMEH solution dissolved in ethanol (MPC : EHMA = 3 : 7, 5%) on the H-PTFE membrane filter (0.5 × 0.5 cm). It was cured overnight (approx. 12 hours) in the refrigerator (4 °C).

2.3. Assessment methods of the sorbitol biosensor

In the preliminary experiments, the characteristics of the constructed fiber-optic NADH measurement system were evaluated using deionized water which contains various concentrations of NADH. Measurements of the NADH concentration were carried out by immersing the optical fiber probe in 300 μl of stirred DI water and adding NADH solution dropwise. The changes of NADH fluorescence intensity were measured at 491 nm by using a spectrometer. The reaction of the sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* was confirmed by adding sorbitol solution dropwise into Tris-HCl buffer mixed with sorbitol dehydrogenase and β-NAD^+ and measuring the fluorescence intensity changes of NADH using a spectrometer. The measurement of the sorbitol concentration was carried out by immersing the sensor probes in Tris-HCl buffer which contained β-NAD^+. The fluorescence change of NADH induced by enzymatic reaction of the sorbitol dehydrogenase immobilized membrane was monitored using a spectrometer or a photomultiplier tube. Optimization of β-NAD^+ concentrations was determined from the fluorescence output value obtained by adding β-NAD^+ solution dropwise into a sorbitol solution of 100 μmol L^{-1}. The comparison of the sensor output by the difference in the origin of sorbitol dehydrogenase was examined using xylitol, glucose, fructose and mannitol. All experiments were performed in a dark box. The sensor outputs obtained from the spectrometer or the photomultiplier tube were recorded using a laptop PC.

2.4. Sorbitol measurement in biological samples

As an example of biological samples, rat whole blood including heparin was obtained from Nippon Bio-Supp. Center, Japan. The blood plasma was obtained by centrifugation of the whole blood. A sorbitol solution of a constant concentration was added to the blood plasma including heparin, and the mixture solution was used for the evaluation of sorbitol measurements in the biological sample. Biological samples were pretreated based on the F-kit (J. K. International) preparation method. 100 μl of the blood plasma and 100 μl of perchloric acid solution (1.0 mol L^{-1}) were poured into a microtube and mixed. Then, the mixture solution was centrifuged for 10 min at 3000 rpm. Then 60 μl of potassium hydroxide solution (1.0 mol L^{-1}) was added to 100 μl of the supernatant solution to adjust the pH to 7–9. The mixture solution was centrifuged for 10 min at 5000 rpm and the supernatant solution (200 μl) was used as a pre-treated biological sample.

3. Results and discussion

3.1. Characteristics of the fiber-optic NADH measurement system

The UV-LED based excitation system and the NADH detection system have been optimized in a previous study.[21] The UV-LED system is rather small compared with conventional UV lighting systems, which do not require any additional cooling mechanisms. The total power consumption was reduced down to 150 mW, which is a mere one percent in comparison with a mercury lamp. For these advantages, the UV-LED based excitation system is also considered to be suitable for practical applications such as point-of-care testing applications and daily health care tests. In this paper, this was confirmed on the fiber-optic NADH measurement system using a UV-LED and a spectrometer. The peak wavelength of NADH was 491 nm and the full width at half maximum of the emission bandwidth was approximately 12 nm.

Fig. 3 shows responses to various concentrations of NADH. The fluorescence output increased with increasing the NADH concentration by adding NADH solution dropwise every 3 minutes. The inset of Fig. 3 shows the calibration curve of the fiber-optic NADH measurement system. The fluorescence intensity was described using the equation:

$$\text{Intensity (counts)} = 3.98 \times [\text{NADH (μmol L}^{-1})]^{0.98}$$

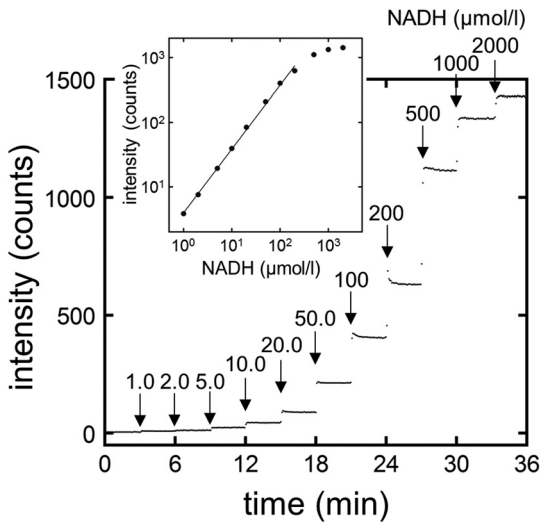

Fig. 3 Typical response to various concentrations of NADH. The fluorescence output increased with increasing the NADH concentration by adding NADH solution dropwise every 3 minutes. The inset shows the calibration curve of the fiber-optic NADH measurement system. The calibration range of 1.0–200 μmol L^{-1} was obtained.

where the correlation coefficient is 0.991. The calibration range was 1.0–200 μmol L^{-1}. Under higher NADH concentration conditions than 200 μmol L^{-1} NADH, the fluorescence intensity was saturated. The result shows that the constructed NADH fluorescence measurement system has a significant role in NADH determination.

3.2. Optimization of the sorbitol biosensor

The results confirming the enzymatic reaction using sorbitol dehydrogenase solution are shown in Fig. 4. The experiments were performed using the constructed NADH fluorescence measurement system and phosphate buffer dissolved sorbitol dehydrogenase. The fluorescence intensity of NADH (491 nm) was increased immediately after adding sorbitol solution dropwise. The intensity was stabilized at about 3 minutes. It takes a little time to stabilize the fluorescence intensity output as compared with the experimental results using NADH solution. The time lag is considered to be the time in which NADH is generated by the enzymatic reaction. Similar results to those in Fig. 4 have been obtained even when using the sorbitol dehydrogenase from sheep liver. In experiments with sorbitol dehydrogenase solution, the calibration range of sorbitol was 1.0–100 μmol L^{-1}. The correlation coefficient was 0.994.

The effect of the concentration of β-NAD^+ on the response intensity of the sorbitol biosensor was investigated. Fig. 5 shows the fluorescence intensity *versus* the variation in the concentration of β-NAD^+. The fluorescence intensity indicates the sensor output after 10 minutes of addition of the sorbitol solution. For low concentrations of β-NAD^+, the enzymatic reaction rate and the NADH production decreased. On the other hand, under the conditions of high concentration of β-NAD^+, the reaction rate was relatively fast, but the stable fluorescence intensity decreased with increasing β-NAD^+ concentration. β-NAD^+ of high concentrations is known to be an inhibitor of NAD^+/NADH dependent dehydrogenase reaction.[22] Therefore, we used a β-NAD^+ concentration of 10.0 mmol L^{-1} in our experiments described below.

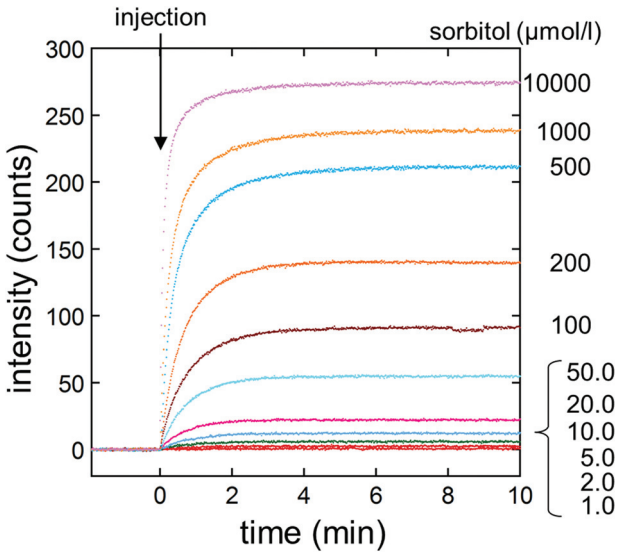

Fig. 4 Typical response to various concentrations of sorbitol in a sorbitol dehydrogenase solution. The fluorescence intensity of NADH (491 nm) was increased immediately after adding sorbitol solution dropwise. The intensity was stabilized at approximately 3 minutes.

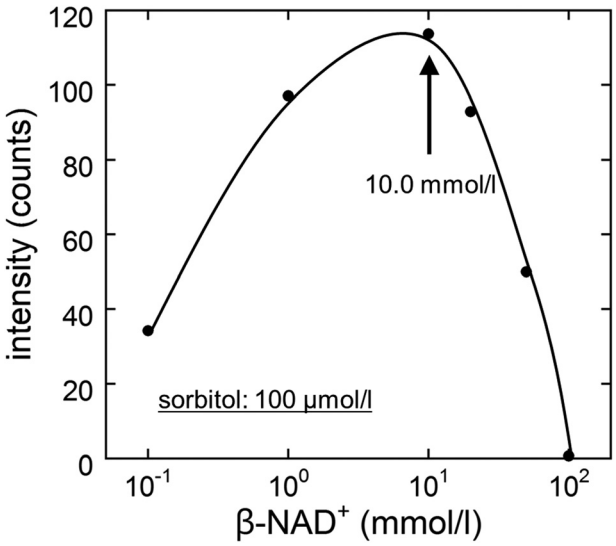

Fig. 5 Fluorescence intensity *versus* the variation in the concentration of β-NAD^+. For low concentrations of β-NAD^+, the enzymatic reaction rate and NADH production decreased. On the other hand, under the conditions of high concentration of β-NAD^+, the reaction rate was relatively fast, but the stable fluorescence intensity was decreased with increasing the β-NAD^+ concentration.

Fig. 6 shows the responsiveness to sorbitol of 10 μmol L^{-1} using a spectrometer and a photomultiplier tube as the photodetector. The fluorescence intensity of the system using the photomultiplier tube was about 16 000 counts, whereas that of the system using the spectrometer was about 10 counts. With the use of the photomultiplier tube, the output signal-to-noise ratio improved by about 2.6 times. We verified that the fluorescence detection for sorbitol in the photomultiplier tube was of very high intensity compared to the spectrometer. In the photosensor field, photomultiplier tubes are known to have particularly high sensitivity. The sorbitol in biological fluids is known to be of low concentration: 101 ± 24 μmol per day (the mean urinary sorbitol excretion) and 75.6 ± 11.5 nmol g^{-1} Hb (the mean sorbitol level in whole blood).[10] Thus, a photomultiplier tube was required for measuring at low concentration of sorbitol.

3.3. Analytical characteristics of the sorbitol biosensor

Fig. 7 shows the responses of the sorbitol biosensor with the photomultiplier tube based on the results of the preliminary experiments. The inset of Fig. 7 shows the sensor output at low sorbitol concentrations. The fluorescence intensity of the sorbitol biosensor increased in relation to increasing sorbitol concentration. At the sorbitol concentration of 1.0 μmol L^{-1},

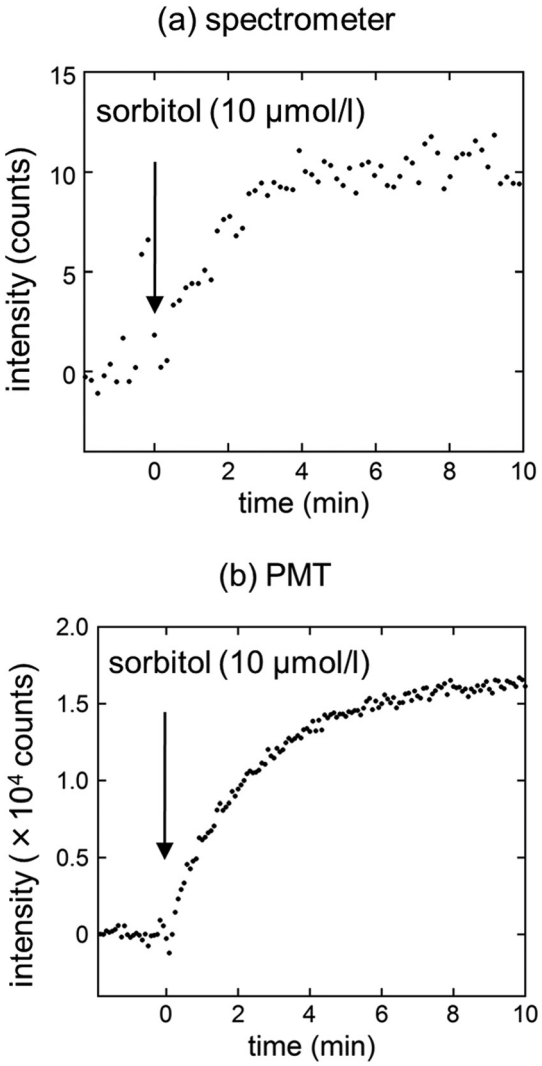

Fig. 6 Responsiveness to sorbitol of 100 μmol L^{-1} in the case of using a spectrometer and a photomultiplier tube as the photodetector. With the use of the photomultiplier tube, the output signal-to-noise ratio improved by about 2.6 times.

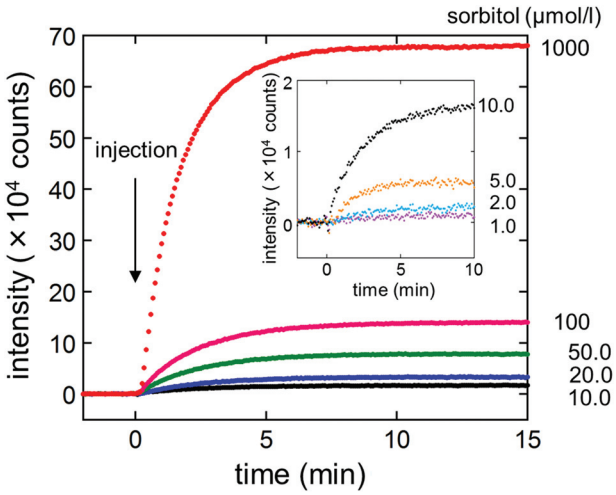

Fig. 7 Typical response of the sorbitol biosensor. The inset shows the sensor output at low sorbitol concentrations. The 95% response time of the sorbitol biosensor was about 4.5 minutes. The correlation coefficient was 0.989 and the calibration range of sorbitol was 1.0–1000 μmol L^{-1}.

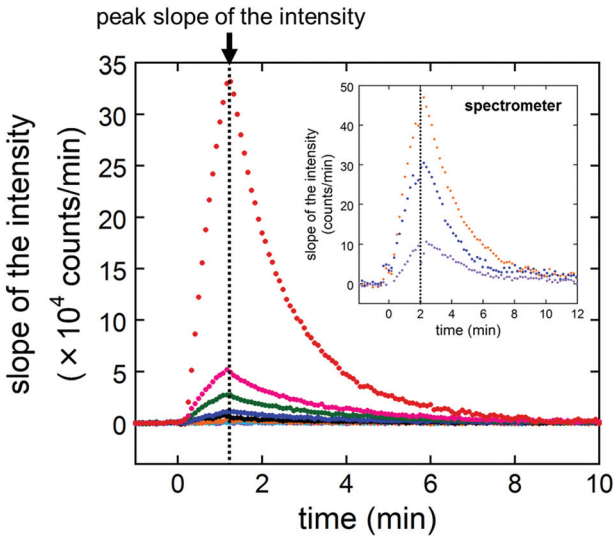

Fig. 8 Differential analysis of the sorbitol biosensor. The inset shows the responses of the spectrometer type sorbitol biosensor. The slope of the fluorescence intensity of the sorbitol biosensor using a spectrometer and a photomultiplier tube was maximized in about 2.0 minutes and 1.5 minutes, respectively.

the sensor output was stabilized at about 5.0 minutes. As the sorbitol concentration increased, the time required to stabilize the sensor output was longer. At the sorbitol concentration of 1.0 mmol L^{-1}, the sensor output was stabilized at about 10 minutes. However, even at the sorbitol concentration of 1.0 mmol L^{-1}, the 95% response time of the sorbitol biosensor was about 4.5 minutes. Therefore, it can be said that the response time of the constructed sorbitol biosensor system was less than 5.0 minutes. In comparison with the experiments using the enzyme solution, the responsiveness of the sorbitol biosensor was poor. It was considered that the PMEH polymer used for the enzyme immobilization was negatively affected. When the calibration curve of the sorbitol biosensor based on the steady value of the output was calculated, the correlation coefficient was 0.989 and the calibration range of sorbitol was 1.0–1000 μmol L^{-1}.

Then, we analyzed the rate of intensity increase in order to reduce the detection time. The changes in fluorescence intensity per minute are shown in Fig. 8. The inset of Fig. 8 shows the responses of the spectrometer type sorbitol biosensor analyzed similarly. The horizontal axis represents time and the vertical axis represents the slope of the fluorescence intensity. Even if it were using a spectrometer or a photomultiplier tube, after adding sorbitol solution dropwise, the fluorescence inten-

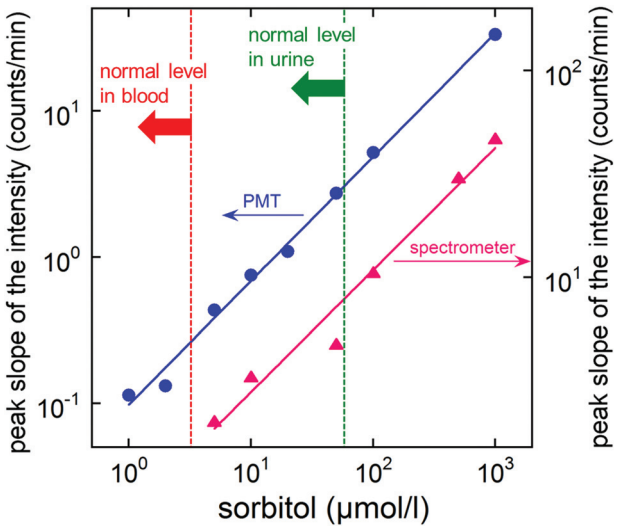

Fig. 9 Calibration curves of the two types of sorbitol biosensors. The calibration range of the sorbitol biosensor using a photomultiplier tube was 1.0–1000 μmol L^{-1}, which was included as the standard value in blood of diabetes complications.

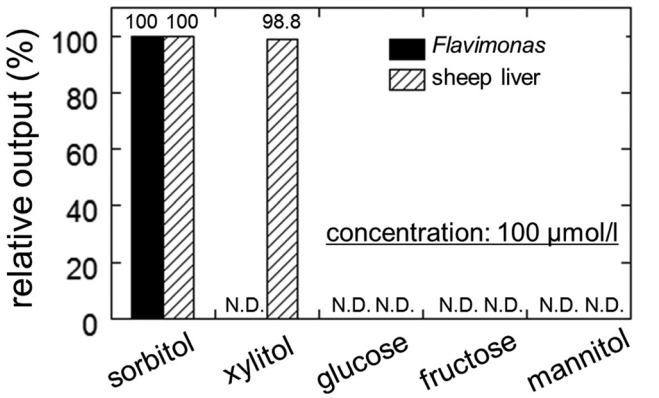

Fig. 10 Comparison of the output to sugars in the sorbitol biosensors using the sorbitol dehydrogenase from microorganisms or that from sheep liver. The sorbitol biosensor using the sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* showed high selectivity compared to that of sheep liver.

sity was significantly increased and was gradually attenuated thereafter. The slope of the fluorescence intensity of the sorbitol biosensor using a spectrometer and a photomultiplier tube was maximized in about 2.0 minutes and 1.5 minutes, respectively. Thus, the calculations shorten the detection time of the biosensor by about 8 minutes.

Fig. 9 shows the calibration curves of two types of sorbitol biosensors obtained from peak values of the slope of the fluorescence intensity. The fluorescence intensity of the sorbitol biosensor using a photomultiplier tube was described using the equation:

$$\text{Peak slope of the intensity (counts)} = 0.098 \times [\text{sorbitol } (\mu\text{mol L}^{-1})]^{0.84}$$

where the correlation coefficient is 0.999. The calibration range was 1.0–1000 μmol L^{-1}. In comparison with the sorbitol biosensor using a spectrometer, the sensitivity was improved by about 5 times. The sorbitol biosensor using a spectrometer had insufficient sensitivity to measure sorbitol in the blood, but it had sufficient sensitivity for the detection of sorbitol in urine. On the other hand, it is considered that the sorbitol biosensor using a spectrometer is simple and compact and is suitable for POCT in homes and hospitals. The calibration range of the sorbitol biosensor using a photomultiplier tube was included as the standard value in blood of diabetes complications. It is considered that the sorbitol biosensor using a photomultiplier is capable of being applied to diagnose more accurately diabetes complications.

Fig. 10 shows a comparison of the output to sugars (100 μmol L^{-1}) due to the difference in the origin of sorbitol dehydrogenase. The sorbitol biosensor using the sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* showed high selectivity compared to that of sheep liver. The selectivity of the sorbitol biosensor was based on substrate specificity of the sorbitol dehydrogenase from microorganisms of the genus *Flavimonas*. The sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* has the advantage of a simple, accurate and highly-sensitive sorbitol measurement.

3.4. Evaluation of the sorbitol biosensor using the biological samples

The bar graph comparing the output values of the sorbitol in blood plasma and the sorbitol standard solution is shown in ESI Fig. 1.† When the output of the sorbitol biosensor for sorbitol standard solution was 100%, the sensor output in pretreated blood plasma was 86.7%. The sensor output value of sorbitol in whole blood of biological samples for the solvent was slightly reduced. The sensor surface and enzymes were strongly influenced by adsorption of some proteins in whole blood. Meanwhile, we confirmed that the developed sorbitol biosensor might detect sorbitol in biological samples of whole blood and plasma. In the future, we will proceed with studies for accurate sorbitol detection in biological samples toward the diagnosis of diabetes complications.

4. Conclusions

A sorbitol biosensor based on an NADH fluorescence detection system for the easy evaluation of diabetic complications was developed and tested. First, the NADH fluorescence detection system was constructed and the quantitative performance of the system was confirmed. The calibration range of NADH of the constructed system was 1.0–200 μmol L^{-1}. Next, the detection of NADH generated by the sorbitol dehydrogenase reaction

was confirmed in the state of sorbitol dehydrogenase solution. As a result, it was found that the sorbitol calibration range was 1.0–1000 μmol L^{-1} by detection of the NADH generated by the enzymatic reaction. Further, the β-NAD^+ concentration in the enzymatic reaction was optimized. The optimal β-NAD^+ concentrations were found to be 10.0 mmol L^{-1}. Based on the preliminary experiments, the sorbitol biosensor was constructed by attaching the sorbitol dehydrogenase immobilized membrane to the optical fiber probe of the NADH fluorescence detection system. The calibration range of the sorbitol biosensor was 1.0–1000 μmol L^{-1}. The detection time of the sorbitol biosensor was about 1.5 minutes by analyzing the rate of fluorescence intensity increase. In addition, a good selectivity of the sorbitol biosensor for saccharides was confirmed. In the future, the developed sorbitol biosensor is anticipated to be used to detect sorbitol in biological fluids for the diagnosis of diabetes complications.

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