Analyst

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Journal:	*Analyst*
Manuscript ID:	AN-ART-08-2014-001593.R2
Article Type:	Paper
Date Submitted by the Author:	22-Jun-2015

Complete List of Authors:	Gessei, Tomoko; Tokyo Metropolitan Industrial Technology Research Institute, ; Tokyo Medical and Dental University, Graduate School of Medical and Dental Sciences Arakawa, Takahiro; Tokyo Medical and Dental University, Institute of Biomaterials and Bioengineering Kudo, Hiroyuki; Tokyo Medical and Dental University, Institute of Biomaterials and Bioengineering Mitsubayashi, Kohji; Tokyo Medical and Dental University, Graduate School of Medical and Dental Sciences; Tokyo Medical and Dental University, Institute of Biomaterials and Bioengineering

Title:

Fiber-optic sorbitol biosensor based on NADH fluorescence detection toward rapid diagnosis of diabetic complications

AUTHORS:

Tomoko GESSEI[1,2], Takahiro ARAKAWA[3], Hiroyuki KUDO[3], Kohji MITSUBAYASHI[2,3]

AFFILIATIONS:

[1]Tokyo Metropolitan Industrial Technology Research Institute, 2-4-10 Aomi, Koto-ku, Tokyo, Japan

[2]Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

[3]Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo, Japan

***corresponding author**

Kohji MITSUBAYASHI is a professor at Department of Biomedical Devices and Instrumentation, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University

2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan

Phone: +81-3-5280-8091, Fax: +81-3-5280-8094, E-mail: m.bdi@tmd.ac.jp

ABSTRACT

More accumulation of sorbitol in tissues has been suspected as one of the causative factors of 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 enzyme reaction. An ultraviolet light emitting diode (UV-LED) was used as the excitation light source of NADH. The fluorescence of NADH was detected by a spectrometer or a photomultiplier tube (PMT). The UV-LED and the photodetector were coupled by 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 oxidized form of nicotinamide adenine dinucleotide (NAD^+). The changes of NADH fluorescence intensity were measured when a standard sorbitol solution had been added. 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 and 1.0 – 1000 μmol/l, respectively. The sorbitol biosensor system using the sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* obtains high selectivity and sensitivity compared with from sheep liver. The sorbitol biosensor allows for point-of-care testing applications or daily health care tests for diabetes patients.

Keywords

biosensor, fiber-optic, NADH, UV-LED, sorbitol, diabetic complications

1. INTRODUCTION

Diabetes population of the world 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 the diabetes treatment is to prevent these serious complications. The diabetes complications are separated 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 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]. Figure 1 shows the pathogenic mechanism of 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 increased by the polyol pathway flux is expected as one biomarker of diabetic complications.

Enzymatic or chromatographic (HPLC, GC-MS, etc.) methods are

typically used to analyze sorbitol levels in diagnosis of diabetes complications [6-8]. 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 evaluation of sorbitol levels in diabetes state [9, 10]. However, considering the sorbitol measurement in health care testing or point-of-care testing (POCT) test, simple, compact and relatively accurate sorbitol sensor is strongly required. Some approaches of the sorbitol sensing system have been proposed. Saccharides detection based on the affinity of phenylboronic acid compounds were reported [11-13]. Although synthetic chemosensors have good stability and reliability in a variety of conditions, these approaches have less selective than enzyme based biosensors. While, some electrochemical biosensors based on carbon nanotubes were reported. W Etienne et al. have reported the dehydrogenase-based biosensors using carbon nanotube and sol-gel thin film on glassy carbon electrode [14, 15]. J Tkac et al. developed the hyaluronic acid dispersed carbon nanotube electrode and applied the electrode to an amperometric NADH biosensor [16, 17]. However, these biosensors need improvement for application of the clinical examination in terms of sensitivity and selectivity.

In our previous work, nicotinamide adenine dinucleotide (NADH) -dependent fiber-optic biosensors with high-sensitive for various substances have been reported [18, 19]. The biosensing device is expected to be applied

for 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, the sorbitol biosensor based on an NADH fluorescence detection system for easy evaluation of diabetic complications was developed and demonstrated.

2. EXPERIMENTAL SECTION

2.1. Chemicals and apparatus

β-NADH (reduced form (di sodium salt)) and β-NAD^+ (oxidized form (free acid)) were obtained from Oriental Yeast Co., Ltd., Japan. Two types of sorbitol dehydrogenase (E.C. 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 enzyme, the standard solution was prepared in tris-HCl buffer (pH 9.0, 100 mmol/L). The pH of the buffer was adjusted to the desired value by adding of 6N HCl solution into 100 mmol/L tris(hydroxymethyl)aminomethane solution while being monitored by 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) was used for sorbitol dehydrogenase immobilization. PMEH is a copolymer of 2-Methacryloyloxyethyl phosphorylcholine (MPC) and 2-ethylhexyl methacrylate (EHMA) and was synthesized using 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 assemblies (BIF600-UV/VIS), an optical fiber probe (F1000-900) and 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.

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

Figure 2 shows the 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 an UV-LED ($\lambda_p \fallingdotseq 335$ nm) and the spectrometer which were filtered with a band-pass filter (band-pass wavelength: 330–350 nm) and long-pass filter (cut-off wavelength: 400 nm), respectively. In the case of using the photomultiplier tube, the band-pass filter (band-pass wavelength:

480-500nm) 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 sheep liver immobilized membrane was attached on the end of the optical fiber probe in order to apply to sorbitol measurement. The sorbitol dehydrogenase membrane was first prepared by coating sorbitol dehydrogenase solution dissolved in tris-HCl buffer (16 units/cm^2) and PMEH solution dissolved in ethanol (MPC:EHMA = 3:7, 5%) on the H-PTFE membrane filter (0.5 x 0.5 cm). It was cured overnight (approx. 12 hours) in the refrigerator (4^oC).

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. Measurement of NADH concentration was carried out by immersing the optical fiber probe in 300 µl of stirred DI water and dropping NADH solution. Fluorescence of NADH was measured intensity changes of 491 nm by the spectrometer. The reaction of sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* was confirmed by dropping sorbitol solution in Tris-HCl buffer mixed with sorbitol dehydrogenase and β-NAD^+ and measuring fluorescence intensity change of NADH using a spectrometer. The measurement of 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 sorbitol dehydrogenase immobilized membrane was monitored by a spectrometer or a photomultiplier tube. Optimization of β-NAD^+ concentrations was determined by the fluorescence output value obtained by dropping β-NAD^+ solution in sorbitol solution of 100 μmol/L. The sensor output comparison by the difference in 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 photomultiplier were recorded by 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. The sorbitol solution of a constant concentration was added to the blood plasma including heparin, the mixture solution was used for the evaluation of sorbitol measurement in the biological sample. Biological sample was 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) was poured into a microtube and mixed. Then, the mixture solution was centrifuged for 10 min at 3000 rpm. 60 μl of potassium hydroxide solution (1.0 mol/L) was added to

100 µl of the supernatant solution to adjust the pH 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 previous study [21]. The UV-LED system is rather small comparing with conventional UV lighting systems, which does not require any additional cooling mechanisms. Total power consumption was reduced down to 150 mW, which is a mere one percent in comparison with a mercury lamp. For those 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, it was confirmed on fiber-optic NADH measurement system using an UV-LED and a spectrometer. A peak wavelength of NADH was 491 nm and the full width half maximum of the emission bandwidth was approximately 12 nm.

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

$$\text{intensity (counts)} = 3.98 \times [\text{NADH } (\mu\text{mol/L})]^{0.98}$$

where a correlation coefficient of 0.991. The calibration range was 1.0 – 200 μmol/L. Under high NADH concentrations conditions than 200 μmol/L NADH, the fluorescence intensity was saturated. The result shows that the

constructed NADH fluorescence measurement system is significant role for NADH determination.

3.2. Optimization of the sorbitol biosensor

The results of confirming the enzymatic reaction using sorbitol dehydrogenase solution is shown in Figure 4. The experiments were performed by using the constructed NADH fluorescence measurement system and phosphate buffer dissolved sorbitol dehydrogenase. The fluorescence intensity of NADH (491nm) was increased immediately after dropping of sorbitol solution. 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 enzyme reaction. The similar results as in Figure 4 have been obtained even when using sorbitol dehydrogenase from sheep liver. In experiments with sorbitol dehydrogenase solution, the calibration range to sorbitol was 1.0-100 μmol/L. The correlation coefficient was 0.994.

The effect of the concentration of β-NAD^+ on the response intensity of the sorbitol biosensor was investigated. Figure 5 shows the fluorescence intensity to the variation in the concentration of β-NAD^+. The fluorescence intensity indicates the sensor output of after 10 minutes in addition of the sorbitol solution. For low concentration of β-NAD^+, the enzymatic reaction

rate and NADH production decreased. On the other hand, under the condition of high concentration of β-NAD^+, the reaction rate was relatively fast, but the stable fluorescence intensity was decreased with increasing to β-NAD^+ concentration. β-NAD^+ of high concentrations is known to be an inhibitor of NAD^+/NADH dependent dehydrogenase reaction [22]. Therefore, we used β-NAD^+ concentrations of 10.0 mmol/L in our experiments described below.

Figure 6 shows the responsiveness to sorbitol of 10 μmol/L using a spectrometer and a photomultiplier tube as the photo detector. Fluorescence intensity of the system using the photomultiplier was about 16000 counts, whereas that of the system using the spectrometer was about 10 counts. The use of the photomultiplier, the output signal-to-noise ratio improved about 2.6 times. We verified that the fluorescence detection for sorbitol in the photomultiplier tube was 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 low concentration: 101 ± 24 μmol/day (the mean urinary sorbitol excretion) and 75.6 ± 11.5 nmol/g Hb (the mean sorbitol level in whole blood) [10]. Thus, a photomultiplier tube was required to measure at low concentration of sorbitol.

3.3. Analytical characteristics of the sorbitol biosensor

Figure 7 shows the responses of the sorbitol biosensor with the

photomultiplier based on the results of the preliminary experiments. The inset of Figure 7 shows the sensor output at low sorbitol concentrations. The fluorescence intensity of the sorbitol biosensor increased in relation to increasing sorbitol concentration. In the sorbitol concentration of 1.0 μmol/L, the sensor output was stabilized at about 5.0 minutes. As the sorbitol concentration increased, the time required to stabilize of the sensor output was longer. In the sorbitol concentration of 1.0 mmol/L, the sensor output was stabilized at about 10 minutes. However, even as at the sorbitol concentration of 1.0 mmol/L, the 95% response time of the sorbitol biosensor was about 4.5 minutes. Therefore, it can be said 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 PMEH polymer used for the enzyme immobilization was negatively affected. When the calibration curve of the sorbitol biosensor based on steady value of the output was calculated, the correlation coefficient was 0.989 and the calibration range to sorbitol was 1.0-1000 μmol/L.

Then, we analyzed the rate of the intensity increase in order to reduce the detection time. The changes in fluorescence intensity per minute are shown in Figure 8. The inset of Figure 8 shows the responses of 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 spectroscope or a photomultiplier, after dropping sorbitol solution, the fluorescence intensity was significantly increased and gradually attenuated thereafter. The slope of the fluorescence intensity of the sorbitol biosensor using a spectrometer and a photomultiplier 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.

Figure 9 shows the calibration curves of two type sorbitol biosensors obtained from peak value of slope of the fluorescence intensity. The fluorescence intensity of the sorbitol biosensor using a photomultiplier was described by the equation:

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

where a correlation coefficient of 0.999. The calibration range was 1.0 – 1000 μmol/L. In comparison with the sorbitol biosensor using a spectrometer, the sensitivity was improved by about 5 times. The sorbitol biosensor using a spectroscope was insufficient sensitivity to measure sorbitol in the blood, but it had a sufficient sensitivity for 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 testing in homes and hospitals. The calibration range of the sorbitol biosensor using a photomultiplier was included the standard value in blood of diabetes complications. It is considered that the sorbitol biosensor using a photomultiplier is possible to apply to the diagnosis of more accurate diabetes complications.

Figure 10 shows a comparison of the output to sugars (100 μmol/L) due to a difference in the origin of sorbitol dehydrogenase. The sorbitol biosensor using 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*. Sorbitol dehydrogenase from microorganisms of the genus *Flavimonas* has advantages of simple, accurate and high-sensitive sorbitol measurement.

3.4. Evaluation of the sorbitol biosensor using the biological samples

The bar graph comparing the output value of the sorbitol in blood plasma and the sorbitol standard solution was shown in supplemental figure 1. When the output of the sorbitol biosensor for sorbitol standard solution was 100%, the sensor output in pre-treated blood plasma was 86.7%. The sensor output value of sorbitol in whole blood of biological samples for solvent was slightly reduced. The sensor surface and enzymes were strongly influenced by adsorption of some protein in whole blood. Meanwhile, we confirmed that the developed sorbitol biosensor might be detected sorbitol in biological samples of whole blood and plasma. In the future, we will proceed with examines for accurate sorbitol detection in biological samples toward diagnosis of diabetes complications.

4. Conclusions

The sorbitol biosensor based on an NADH fluorescence detection system for 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 to NADH of the constructed system was 1.0 – 200 μmol/L. Next, the detection of NADH generated by sorbitol dehydrogenase reaction was confirmed in state of sorbitol dehydrogenase solution. As a result, it was found that the sorbitol calibration range was 1.0 μmol/L by detection of NADH generated by the enzyme reaction. Further, β-NAD^+ concentration in the enzyme reaction was optimized. The optimal β-NAD^+ concentrations were found to be 10.0 mmol/L. Based on the preliminary experiments, the sorbitol biosensor was constructed by attaching the sorbitol dehydrogenase immobilized membrane to the optical fiber probe of NADH fluorescence detection system. The calibration range of the sorbitol biosensor was 1.0 – 1000 μmol/L. The detection time of the sorbitol biosensor was about 1.5 minutes by analyzing the rate of the fluorescence intensity increase. In addition, 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 diagnosis of diabetes complications.

ACKNOWLEDGMENTS

I am particularly grateful for the assistance given by Tomoka NAGASHIMA and Ken IWAMIYA. I am also deeply grateful for the support given by Professor Kiyoko SHIBA. This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 25790070, Grants-in-Aid for Scientific Research System, by Japan Science and Technology Agency (JST) and by MEXT (Ministry of Education, Culture, Sports, Science and Technology) Special Funds for Education and Research “Advanced Research Program in Sensing Biology”.

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LEGENDS FOR ILLUSTRATES

Figure 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 to fructose. In hyperglycemia environment, increased sorbitol levels within cells induce the increase of the osmotic stress and the decrease of the ATPase activity.

Figure 2.

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

Figure 3.

Typical response to various concentrations of NADH. The fluorescence output increased with increasing NADH concentration by dropping NADH solution 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 was obtained.

Figure 4.

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

Figure 5.

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

Figure 6.

Responsiveness to sorbitol of 100 μmol/L in the case of using a spectrometer and a photomultiplier tube as the photo detector. The use of the photomultiplier, the output signal-to-noise ratio improved about 2.6 times.

Figure 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 to sorbitol was 1.0-1000 μmol/L.

Figure 8.

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

Figure 9.

Calibration curves of two type sorbitol biosensors. The calibration range of the sorbitol biosensor using a photomultiplier was 1.0 – 1000 μmol/L, which was included the standard value in blood of diabetes complications.

Figure 10.

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

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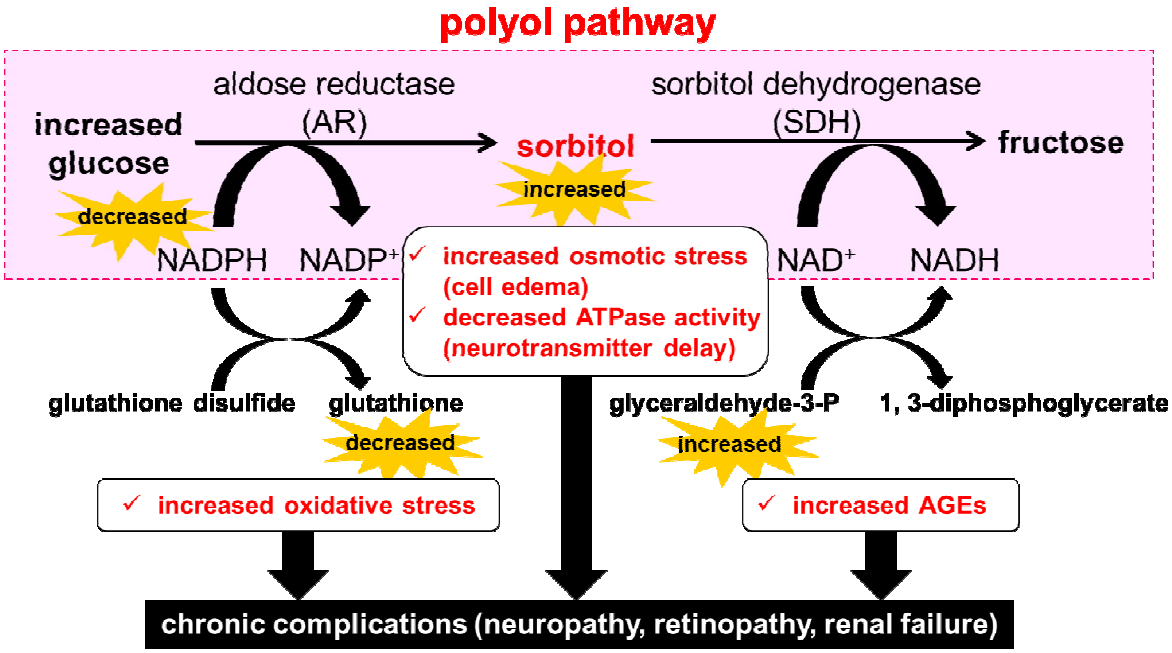

Figure 1.

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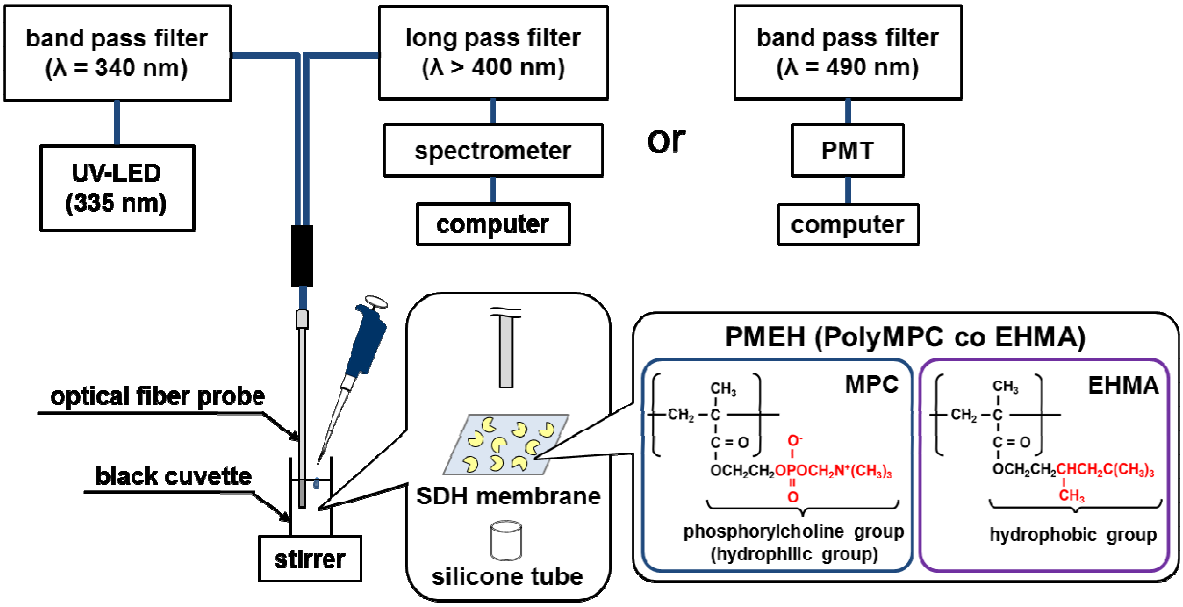

Figure 2.

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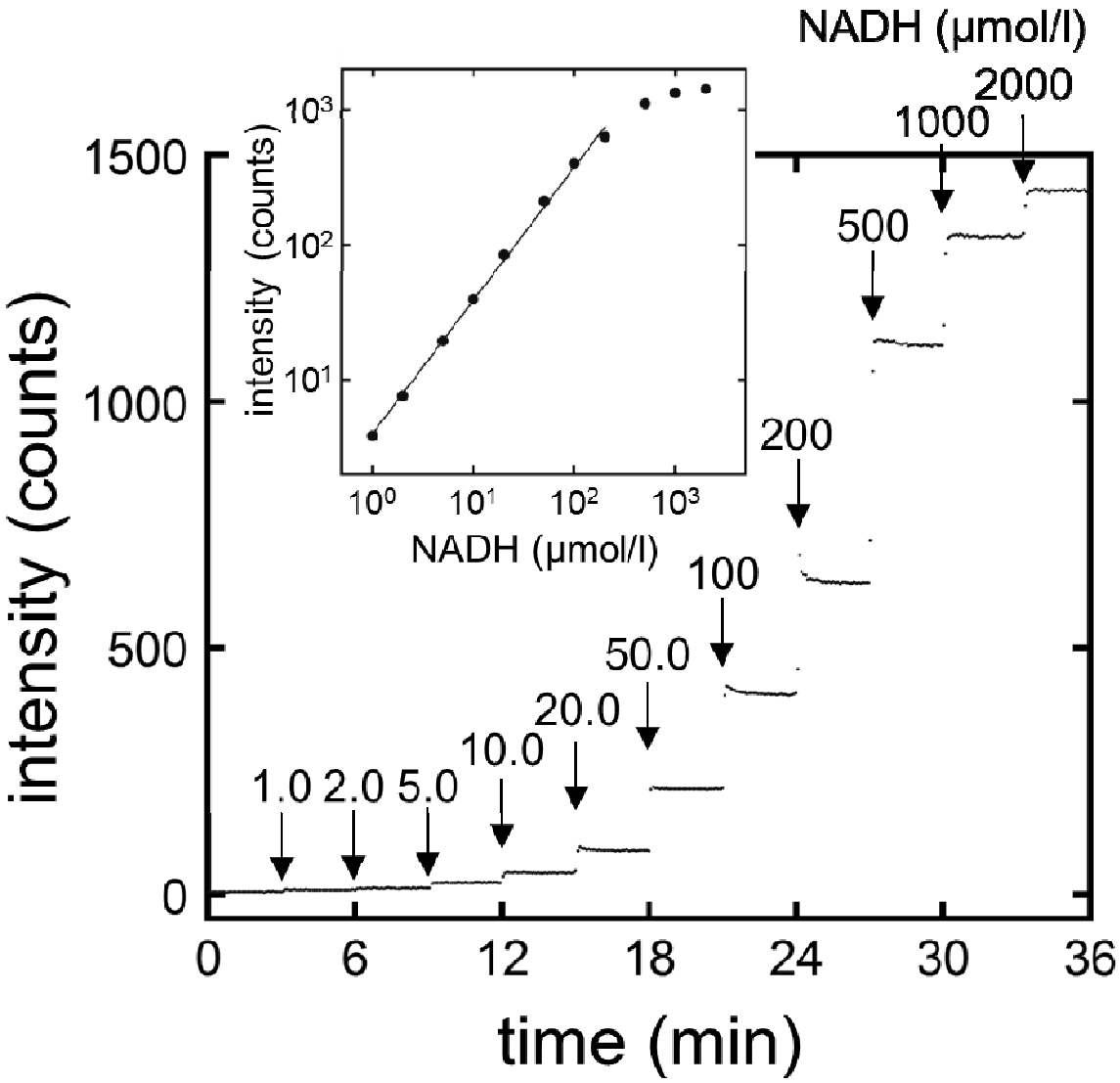

Figure 3.

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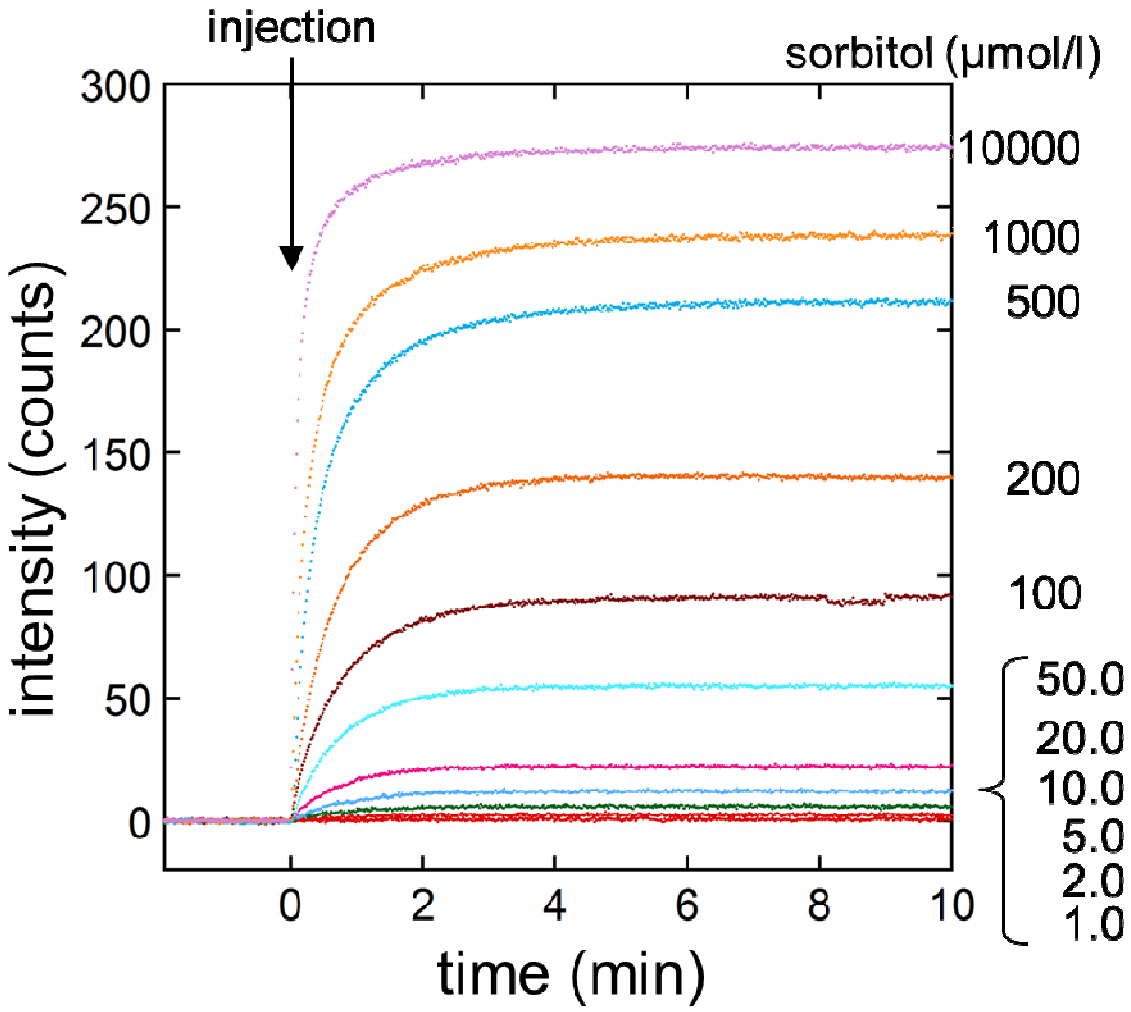

Figure 4.

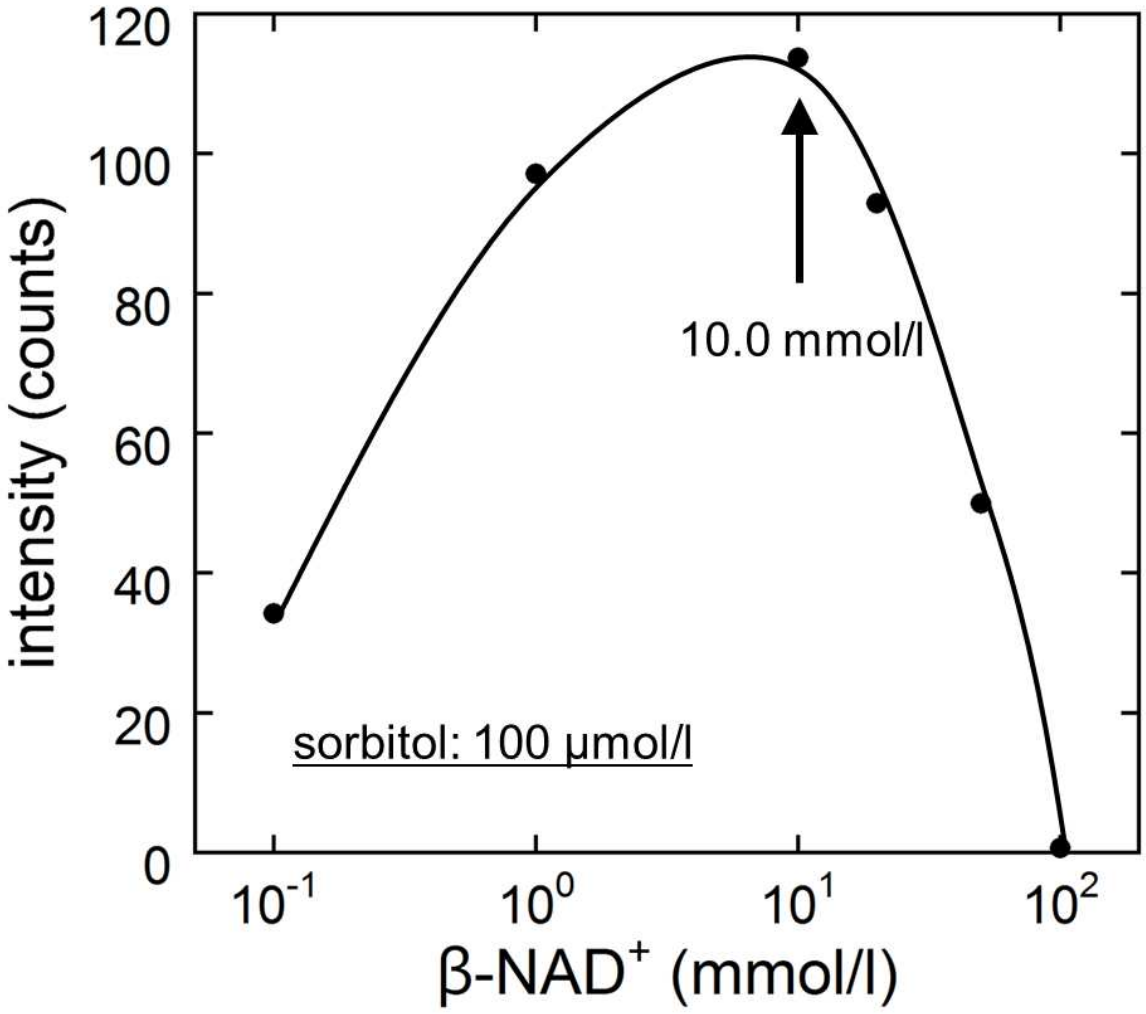
120
100
80
60
40
20
0
intensity (counts)
10.0 mmol/l
sorbitol: 100 µmol/l
10-1
100
101
102
β-NAD+ (mmol/l)

Figure 5.

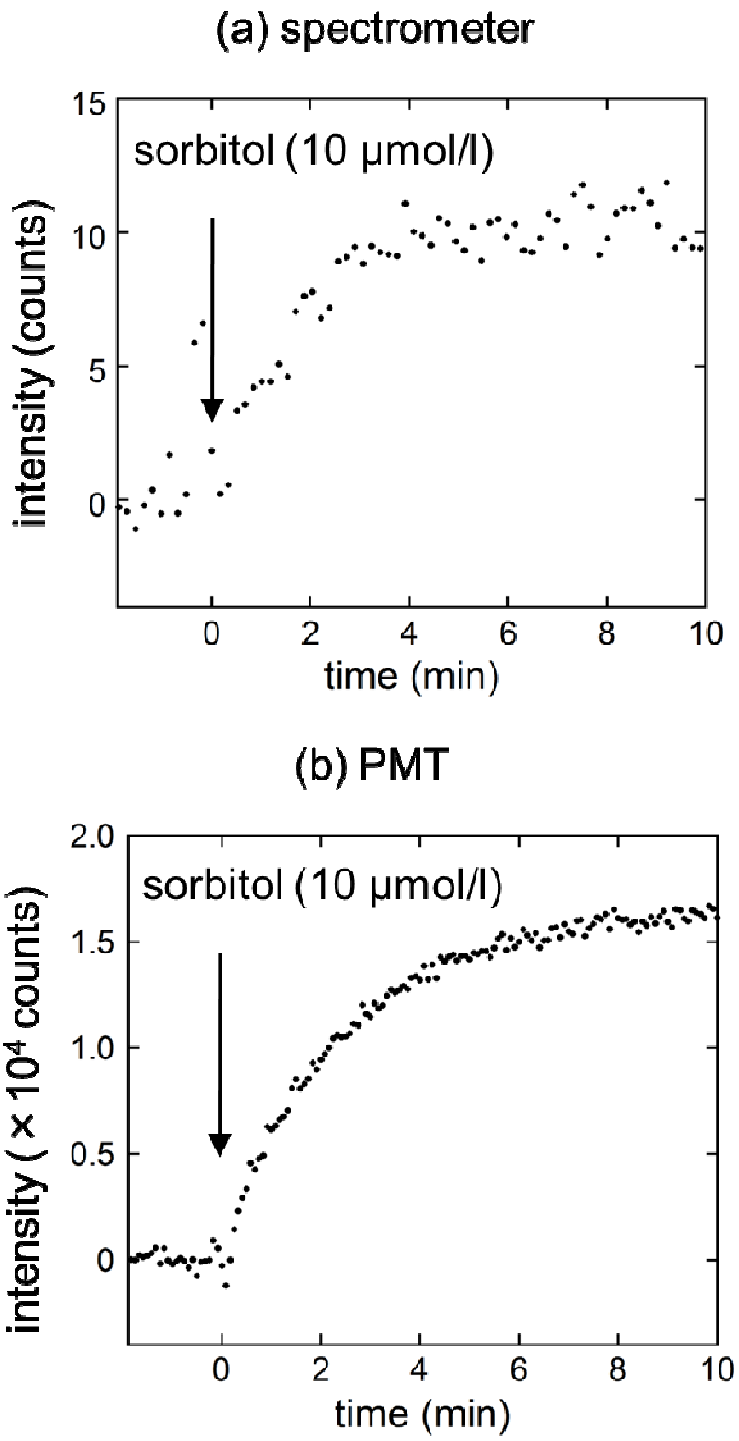
(a) spectrometer
sorbitol (10 μmol/l)
intensity (counts)
time (min)
(b) PMT
sorbitol (10 μmol/l)
intensity (× 10^4 counts)
time (min)

Figure 6.

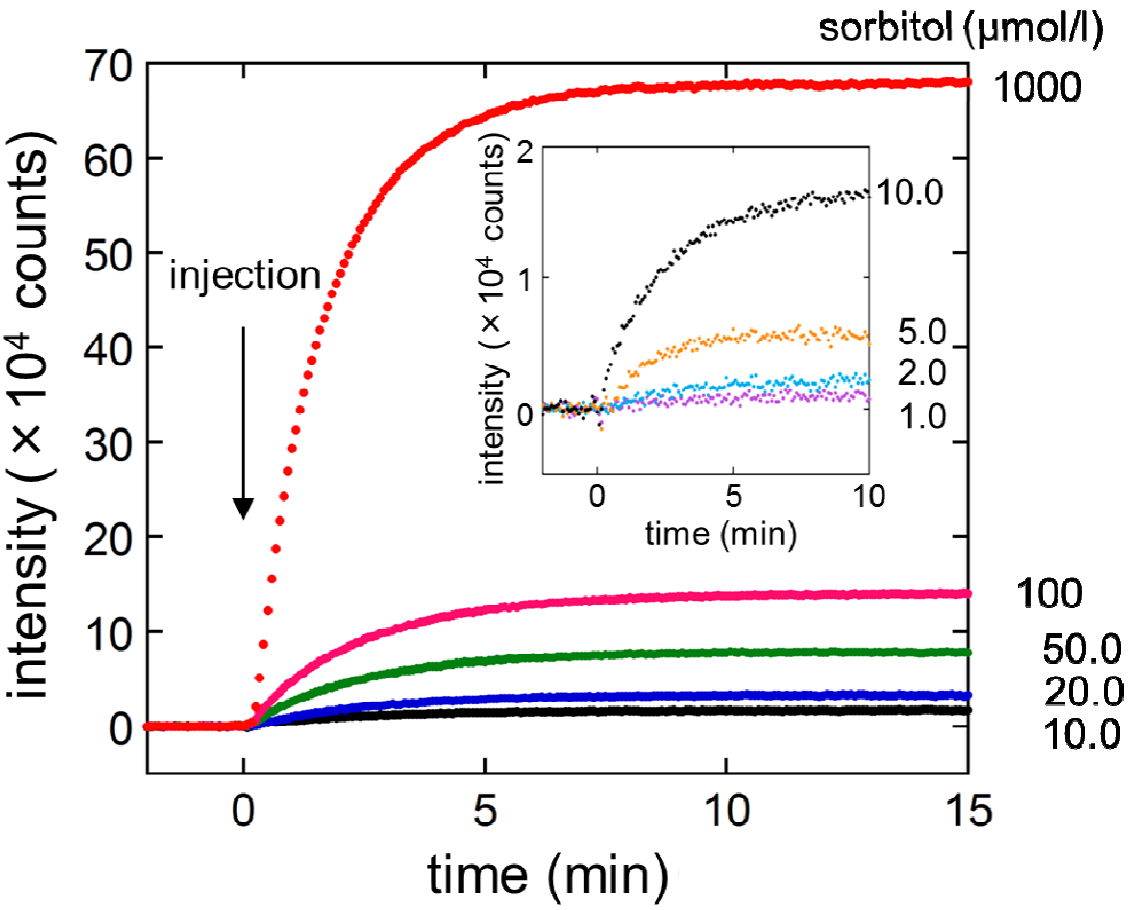
sorbitol (μmol/l)
1000
100
50.0
20.0
10.0
5.0
2.0
1.0
injection
intensity (× 10⁴ counts)
time (min)
0
10
20
30
40
50
60
70
0
5
10
15
intensity (× 10⁴ counts)
time (min)
0
1
2
0
5
10

Figure 7.

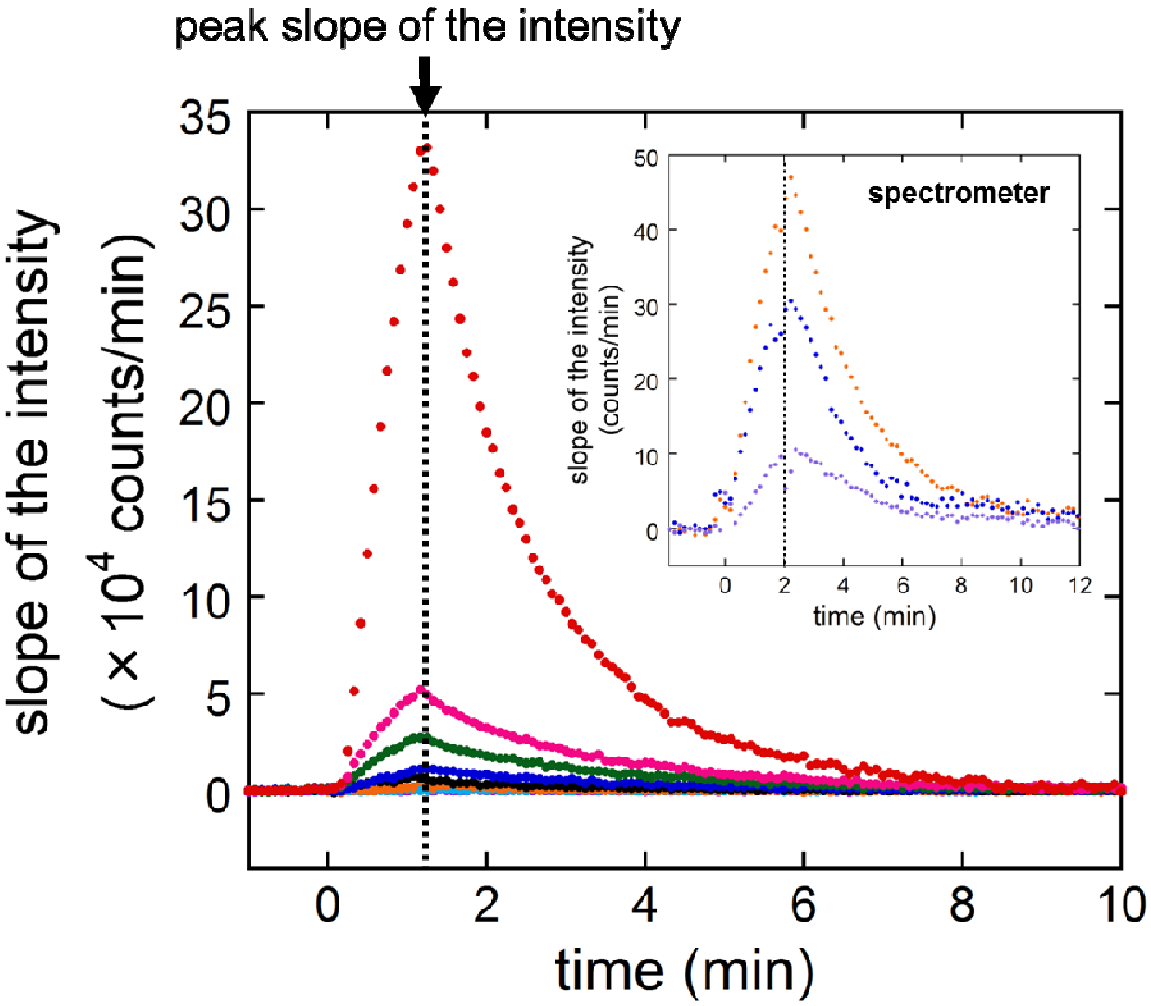
peak slope of the intensity
slope of the intensity
(x 10^4 counts/min)
time (min)
spectrometer
slope of the intensity
(counts/min)
time (min)

Figure 8.

Fiber-optic sorbitol biosensor based on NADH fluorescence detection toward rapid diagnosis of diabetic complications
Tomoko GESSEI, et al.

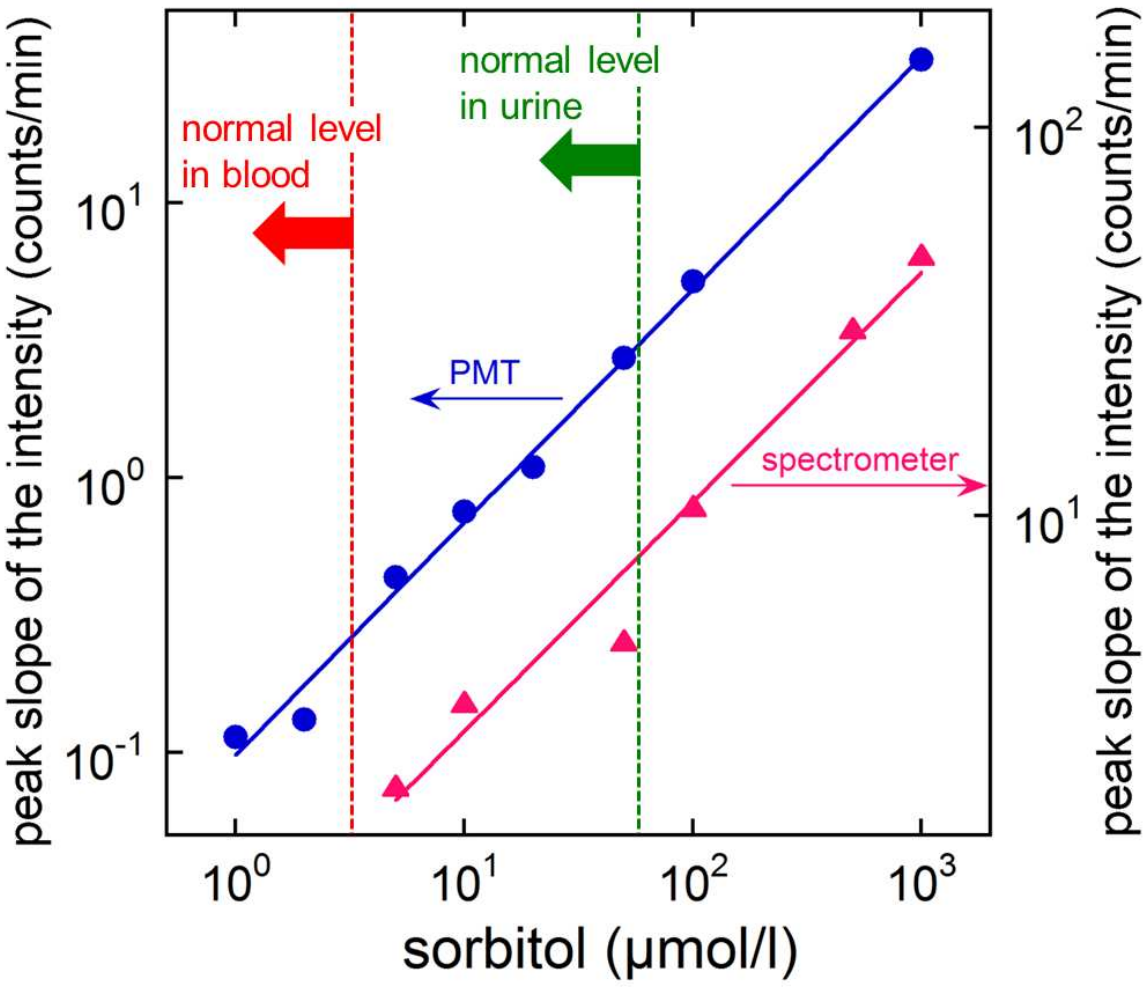

Figure 9.

Fiber-optic sorbitol biosensor based on NADH fluorescence detection toward rapid diagnosis of diabetic complications
Tomoko GESSEI, et al.

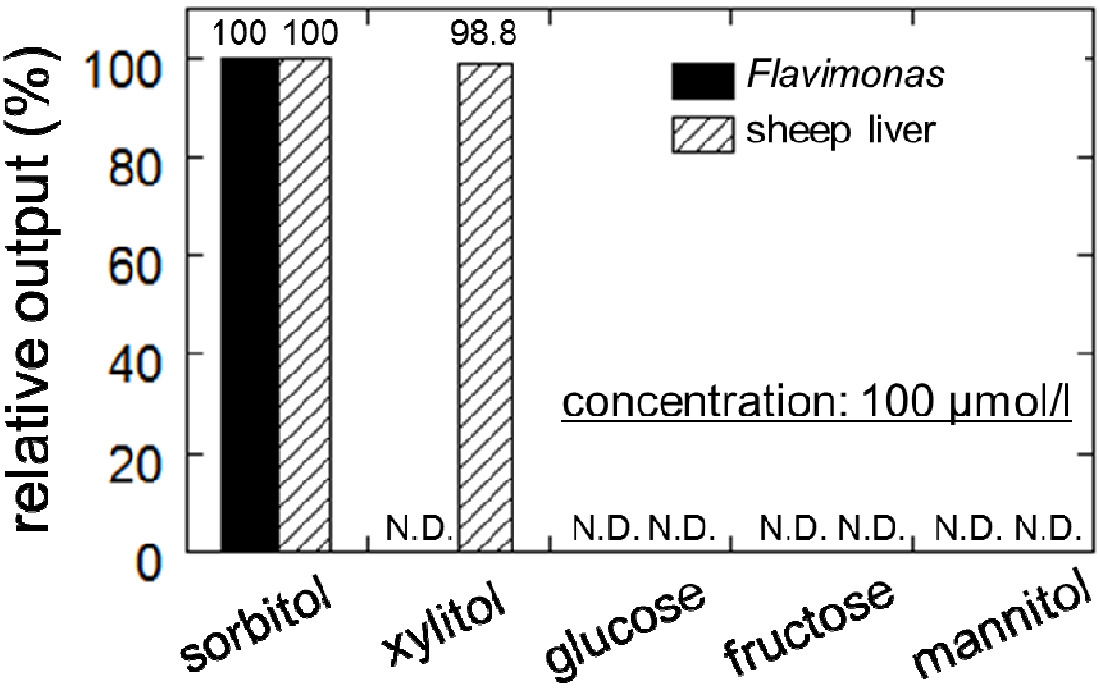

Figure 10.