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An enzyme-chromogenic surface plasmon resonance biosensor probe for hydrogen peroxide determination using a modified Trinder's reagent

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ABSTRACT

An absorption-based surface plasmon resonance (SPR_{Abs}) biosensor probe has been developed for simple and reproducible measurements of hydrogen peroxide using a modified Trinder's reagent (a chromogenic reagent). The reagent enabled the determination of the hydrogen peroxide concentration by the development of deep color dyes (λ_{max} = 630 nm) through the oxidative coupling reaction with *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethylaniline sodium salt monohydrate (MAOS; $C_{13}H_{20}NNaO_4S{\cdot}H_2O$) and 4-aminoantipyrine (4-AA) in the presence of hydrogen peroxide and horseradish peroxidase (HRP). In the present study, urea as an adduct of hydrogen peroxide for color development could be omitted from the measurement solution. The measurement solution containing 5 mM hydrogen peroxide was deeply colored at a high absorbance value calculated as 46.7 cm^{-1} and was directly applied to the SPR_{Abs} biosensing without dilution. The measurement was simply performed by dropping the measurement solution onto the surface of the SPR sensor probe, and the SPR_{Abs} biosensor response to hydrogen peroxide was obtained as a reflectivity change in the SPR spectrum. After investigation of the pH profiles in the SPR_{Abs} biosensor probe, a linear calibration curve was obtained between 1.0 and 50 mM hydrogen peroxide (r = 0.991, six points, average of relative standard deviation; 0.152%, n = 3) with a detection limit of 0.5 mM. To examine the applicability of this SPR_{Abs} biosensor probe, 20 mM glucose detection using glucose oxidase was also confirmed without influence of the refractive index in the measurement solution. Thus, the SPR_{Abs} biosensor probe employing the modified Trinder's reagent demonstrated applicability to other analyte biosensing tools.

1. Introduction

As biosensing tools, hydrogen peroxide measurement methods have been widely used for environmental, food, pharmaceutical, and clinical analyses and studied for applications to other analyte measurement methods combined with a large number of oxidase enzymes, which produce hydrogen peroxide (Nakamura and Karube, 2003).

Guilbault and Lubrano (1973) performed the direct electrochemical detection of hydrogen peroxide produced by a glucose oxidase (GOD) reaction using a metal electrode. In other electrochemical methods, Vijayakumar et al. (1996) employed an osmium complex for the determination of hydrogen peroxide using horseradish peroxidase (HRP). Tangkuaram et al. (2007) developed a hydrogen peroxide biosensor using screen-printed carbon electrode immobilized HRP and gold nanoparticles in the matrix of chitosan. However, in general, the electrochemical reaction has to address the influences of electroactive substances dissolved in sample.

In optical biosensing methods, several core techniques for hydrogen peroxide have been studied. In chemiluminescence methods, we previously developed a highly sensitive method with 1 nM hydrogen peroxide detection employing a flow injection analysis (FIA) system combining an *Arthromyces ramosus* peroxidase-luminol reaction system (Nakamura et al., 1999) and a compactly integrated flow cell with a chemiluminescence FIA system as a micro-total analysis system (μ-TAS) for hydrogen peroxide produced by a lactate oxidase reaction (Nakamura et al., 2001). Zhou et al. (2007) also developed a chemiluminescence biosensor based on the immobilization of HRP on a biocompatible chitosan membrane. As a fluorescence method, Xu and Zhang (2001) studied the determination of hydrogen peroxide using a thiamine-hydrogen peroxide-hemoglobin (thiamine-H_2O_2-Hb) system. Wu

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et al. (1995) studied a hydrogen peroxide measurement method utilizing heat-induced optical beam deflection, and Fernandes et al. (2005) studied an absorbance-based hydrogen peroxide measurement method. These studies enhanced the necessity to determine hydrogen peroxide, especially for developments in the biosensing fields, although the influence of scattering, caused by the sustained substances dissolved in the samples, has to be addressed. In addition, the procedure of sample dilution has to add in the case of that analyte concentration in the sample exceeds a detectable range.

On the other hand, surface plasmon resonance (SPR) sensing methods have been developed for binding assays of immunoreactions by the detection of changes in a refractive index. In recent years, SPR sensing methods have been applied to simultaneous electrochemical SPR measurements (Iwasaki et al., 1998, 1999, 2001, 2002; Koide et al., 2000) or chromogenic measurements (Fujii et al., 2002; Mavri et al., 2007). In the former developments, two analytical tools were combined. In the latter developments, Fujii et al. (2002) achieved ammonium ion biosensing with an enzyme reaction with urease. They demonstrated the relationship between absorption and reflectivity in the SPR signal. Thus, the possibilities of absorption-based SPR sensing were shown. In addition, these SPR sensor developments reported above employed a prism as a sensing part.

In other studies on SPR sensor developments, probe-type chemical sensors without the use of a prism have been reported (Jorgenson and Yee, 1993; Cahill et al., 1997; Kurihara et al., 2004; Mavri et al., 2007). In fact, the development of probe-type sensors is of great importance, as they are essential for monitoring techniques, such as the environmental monitoring of pH, dissolved oxygen, conductivity, and biological oxygen demand (BOD) (Karube et al., 1977; Chee et al., 2000) and the clinical monitoring of glucose (Moscone et al., 1992). Studies on probe-type sensors have been conducted to the development of SPR biosensor probes. Akimoto et al. (2000, 2003, 2008) used an SPR sensor probe to detect immunoreactions, and its possible application to the measurement of small sample volumes or continuous monitoring was demonstrated.

In the present study, an absorption-based chromogenic SPR (SPR_{Abs}) biosensor probe for the simple and reproducible measurement of hydrogen peroxide was developed by applying the method, which employed the prism, reported by Fujii et al. (2002). In their study, these researchers demonstrated that the absorbance changes in the measurement solution responded proportionally to the changes in the reflectivity of the SPR signal. In this study, we experimented with a reagent to conduct a dye-forming reaction using peroxidase. The original reagents were proposed by Trinder (1969), and improved reagents, i.e., modified Trinder's reagents, were studied by Tamaoku et al. (1982a,b). Using the modified Trinder's reagent as a chromogenic reagent, the probe-type SPR_{Abs} sensor was applied to hydrogen peroxide measurements. Then, we examined the possibility to determine hydrogen peroxide directly without diluting the measurement solution; this cannot be directly measured in the spectrophotometric method due to extremely high absorbance. For experimental use, the sample dilution might not be much troublesome by using volumetric tools. However, for practical use, the determination method without need of the sample dilution has many advantageous in home, diagnosis, and field monitoring, and the determining procedure can absolutely be simplified. Finally, we applied the SPR_{Abs} biosensor probe for glucose detection using an oxidase enzyme GOD, and then the influence of the refractive index change on the SPR_{Abs} sensor response was discussed for further developments of the probe.

2. Experimental

2.1. Materials and reagents

N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethylaniline sodium salt monohydrate (MAOS; $C_{13}H_{20}NNaO_4S$-H_2O; CAS No. 82692-97-5) was purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). HRP (from horseradish root, 396 units mg^{-1}, Cat. No. 303-50991), 4-aminoantipyrine (4-AA), and a 30% hydrogen peroxide solution were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). The other chemicals used in this study were of reagent grade. Water was used after deionization.

The concentration of hydrogen peroxide as a standard was determined by the permanganate titration method (JIS K 8230, 1994). Each concentration of the hydrogen peroxide solution was prepared as a sample solution using a 10 mM sodium phosphate buffer (PB; pH 7.0) solution. To determine 50 mM hydrogen peroxide as an upper limit, 50 mM MAOS, 50 mM 4-AA, and 4.0 units μL^{-1} HRP were prepared using the PB solution. These solutions were freshly prepared on the day of the experiments.

2.2. SPR_{Abs} biosensor probe and apparatus

The design of the SPR_{Abs} sensor probe was identical to that already reported by Akimoto et al. (2000) (Fig. 1). Briefly, the SPR_{Abs} sensor probe was made with a cylindrical glass (BK7, $n = 1.516$) with a diameter of 3 mm and a length of 15 mm. The sensor probe has two surfaces; one is a sensor surface prepared by sputtering 54-nm-thick gold and 2-nm-thick chromium, and the other is a reflection surface prepared by sputtering 100-nm-thick chromium. The sputtering was performed using a CFS-4ES (Shibaura Engineering Works Co., Ltd., Tokyo, Japan). The surface plasmon is excited by a collimated polychromatic light with an incident angle of 68° to the sensor surface. The reflection surface is worked as a mirror to reflect the light with no influence on the surface plasmon. This incident angle was defined to appear as a local minimum point of reflectivity at around 645 nm.

Analysis of the SPR spectrum obtained by the SPR_{Abs} biosensor probe was performed with an original optical instrument composed of a halogen light (PHL-50, Mejiro Precision, Inc., Tokyo, Japan), a spectrometer (CT-10S, JASCO Co., Ltd., Tokyo, Japan), a beam splitter, and a polarizer. This instrument was based on a previously reported SPR probe system with slight modifications (Akimoto et al., 2008). Polychromatic light from the halogen light was collimated and then passed through the beam splitter to illuminate the sensor probe. This beam splitter can divide the incident light into two non-polarized reflected lights. Reflection light from the sensor surface of the probe was reflected by the beam splitter and then guided to the spectrometer. The polarizer was placed between the beam splitter and the spectrometer so that only the

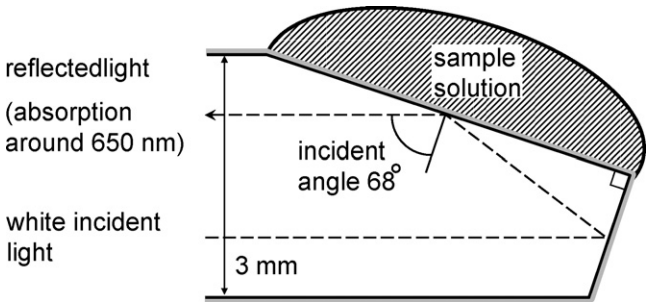

Fig. 1. Schematic representation of the SPR_{Abs} biosensing probe.

p-polarized light with respect to the sensor surface would be guided to the spectrometer.

2.3. Experimental procedures

For the measurement of hydrogen peroxide, three kinds of solutions (5 μL each, containing a 10 mM PB solution of pH 7.0), i.e., 50 mM MAOS, 50 mM 4-AA, and the sample solution containing hydrogen peroxide, were mixed on a plastic tray. Next, the HRP solution (5 μL) in PB was added to the measurement solution as an initiating reagent for color development. Then, the final concentrations of each reagent in the measurement solution (total, 20 μL) were 12.5 mM MAOS, 12.5 mM 4-AA, and 1.0 unit μL^{-1} HRP. Exactly, 1:00 min later, 10 μL of the measurement solution was put on the measurement surface of the SPR sensor probe, and 3:00 additional minutes were spent for the solvent exchange of the surface with the measurement solution and the completion of the enzymatic reaction (Tamaoku et al., 1982b). Four minutes from the start of the reaction, the SPR_{Abs} biosensor signal to hydrogen peroxide was obtained. The local minimum point of reflectivity appeared at around 645 nm and was defined as the SPR_{Abs} biosensor signal. All SPR_{Abs} measurements were repeated three times ($n = 3$) for each kind of measurement solution.

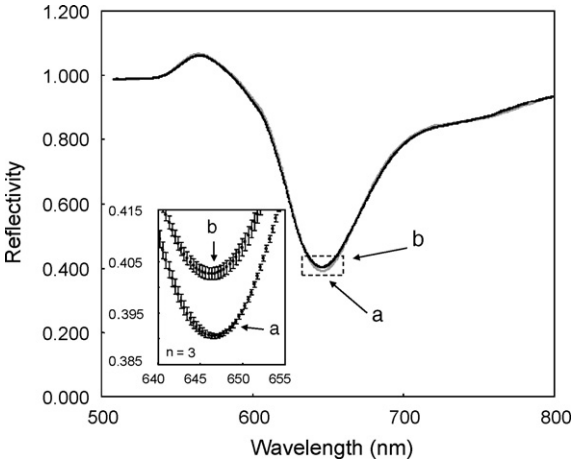

Fig. 3. SPR_{Abs} biosensor responses to 20 mM hydrogen peroxide (a, 0 mM; b, 20 mM H_2O_2). In 20 μL of the measurement solution, 12.5 mM MAOS, 12.5 mM 4-AA, and 1.0 unit μL^{-1} HRP were combined with 5 μL of a sample solution containing 0 or 20 mM hydrogen peroxide. Then, a 10 mM PB solution of pH 7.0 was added to all solutions as a solvent. The error bars are the standard deviations of three measurements.

3. Results and discussion

3.1. Response to hydrogen peroxide

In the present study, a probe-type SPR sensor was applied to an absorption-based biosensing for hydrogen peroxide. For the determination of hydrogen peroxide, water-soluble and stable dye gave high absorbance at visible wavelength ($\lambda_{max} = 630$ nm, deep blue) in a measurement solution and was produced through the oxidative coupling reaction with MAOS and 4-AA in the presence of hydrogen peroxide and HRP (Tamaoku et al., 1982b). The reaction scheme is shown in Fig. 2.

The SPR sensor probe was designed to appear at a local minimum point of reflectivity at around 645 nm with around 65 nm of half-width. The value of the half-width was then defined as the half-value of the wavelength width at the half-value of the reflectivity between 1.0 and the local minimum point. The dye after color development has a wavelength spectrum in the light absorbance between 500 and 700 nm (Tamaoku et al., 1982b). The SPR spectrum of the dye was observed from the absorbance spectrum. Changes in reflectivity of the SPR spectrum could then be induced by changes in the absorbance of the color-developed dye.

In Fig. 3, the SPR_{Abs} biosensor responses to 0 mM hydrogen peroxide, obtained from a transparent measurement solution (absorbance: 0 cm^{-1}), are shown. The local minimum points of reflectivity appeared at around the expected point of 646.72 nm. The SPR_{Abs} biosensor responses to 20 mM hydrogen peroxide were also obtained. The measurement solution was then deeply colored with blue, and SPR_{Abs} biosensing was performed without dilution of the measurement solution. In the experiments, differences in the SPR_{Abs} biosensor responses in reflectivity between 0 and 20 mM hydrogen peroxide were clearly observed, and the value in reflectivity was 0.39052 ± 0.00046 (relative standard deviation, R.S.D. = 0.117%, $n = 3$) at 0 mM and 0.40266 ± 0.00119 (R.S.D. = 0.295%) at 20 mM hydrogen peroxide. The increase in the reflectivity by the increase in absorbance in the measurement solution supported the results of the study by Fujii et al. (2002). In addition, no notable wavelength shift caused by the changes in the refractive index was observed in the SPR_{Abs} biosensor responses between 0 and 20 mM hydrogen peroxide (less than 0.5 nm). Thus, the measurability of hydrogen peroxide was achieved under the simplest constituent of the modified Trinder's reagent in this examination.

Next, the absorbance of the same measurement solution (20 mM H_2O_2) of the Trinder's reagent used in the previous experiment was measured using a spectrophotometer (Model DU-800, Beckman Coulter, Inc., Fullerton, CA, USA). The measurement solution containing 20 mM H_2O_2 was then diluted 100-fold for the absorbance measurements. The maximum absorption of the solution was measured and found at 630 nm. The peak wavelength corresponded well with the results obtained by Tamaoku et al. (1982b). The absorbance of the Trinder's reagent was then calculated as 46.7 cm^{-1}, and the molar extinction coefficient (ε) of the chromogenic complex consisting of MAOS and 4-AA was calculated as 9.82×10^3 cm^{-1} M^{-1}. The result of the absorbance measurement did not correspond with the results obtained by Tamaoku et al. (1982b). In their results, the molar

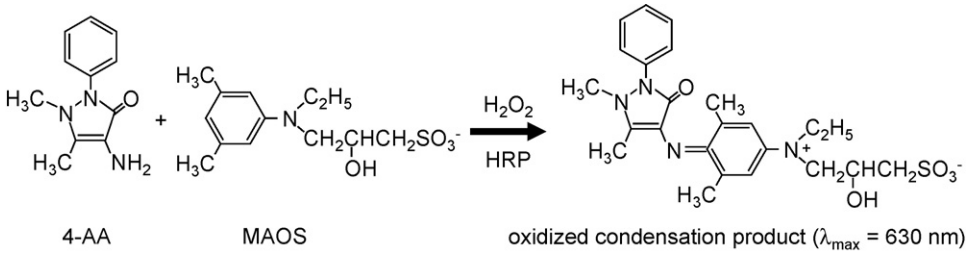

Fig. 2. Measurement principle of the modified Trinder's reagent for hydrogen peroxide.

extinction coefficient (ε) of the Trinder's reagent was obtained at $2.25 \times 10^4\ cm^{-1}\ M^{-1}$. On the other hand, the value of the molar extinction coefficient obtained in the present study was calculated from the slope of the linear regression curve ($r = 0.9998$, $n = 3$, 0 μM H_2O_2; $0\ cm^{-1}$, 12.5 μM H_2O_2; $0.1287 \pm 0.0087\ cm^{-1}$, 25 μM H_2O_2; $0.2556 \pm 0.0046\ cm^{-1}$, 50 μM H_2O_2; $0.4895 \pm 0.0408\ cm^{-1}$, and 75 μM H_2O_2; $0.7332 \pm 0.0191\ cm^{-1}$). The differences of two values in the molar extinction coefficient might have been caused by the lack of the urea adduct of hydrogen peroxide ($(NH_2)_2CO{\cdot}H_2O_2$). However, a sufficient SPR_{Abs} biosensor response to 20 mM hydrogen peroxide could be successfully obtained in the absence of urea. In these examinations, the SPR_{Abs} biosensor probe method showed applicability to the direct biosensing of an analyte dissolved in a deeply colored sample.

3.2. Effects of pH

In the experiments by Tamaoku et al. (1982b), the color dye consisted of MAOS, and 4-AA could be stably developed under the condition between pH 5.5 and 9.5. To apply the hydrogen peroxide biosensor probe to other analyte measurements combined with oxidase enzymes, the effects of pH on the SPR_{Abs} biosensor responses to hydrogen peroxide were investigated. In the experiments, 0 and 20 mM hydrogen peroxide solutions were used as sample solutions, and the differences of the SPR_{Abs} biosensor responses to the sample solution containing 0 or 20 mM hydrogen peroxide were also examined for the characterization.

Fig. 4 shows the results. The SPR_{Abs} biosensor responses to 0 mM hydrogen peroxide were increased by increasing the pH; in contrast, the SPR_{Abs} biosensor responses to 20 mM hydrogen peroxide were decreased by increasing the pH. In these results, the differences in the reflectivity obtained from 0 to 20 mM hydrogen peroxide decreased by increasing the pH. The local minimum points of the wavelength in the SPR_{Abs} biosensor response to 0 mM hydrogen peroxide (pH 5.0, 6.0, 7.0, 8.0, and 9.0) were then observed at 646.36, 646.36, 646.72, 646.72, and 646.72 nm, respectively. In addition, the local minimum points of the wavelength in the SPR_{Abs} biosensor response to 20 mM hydrogen peroxide (pH 5.0, 6.0, 7.0, 8.0, and 9.0) were observed at 646.00, 646.36, 646.36, 646.72, and 646.72 nm, respectively. In these results, no remarkable changes in the local minimum points of wavelength were observed. Therefore, under the experimental conditions of the present study, it was concluded that a clear influence of the pH on the SPR_{Abs} biosensor response was found as the reflectivity changed. Furthermore, such characteristics of the influence of the pH on the reflectivity changes might have occurred by an unknown factor in the SPR phenomenon or as a result of the absence of an urea adduct in the measurement solution. In addition, color fading of the developed chromogenic complex was observed in the experimental results by Tamaoku et al. (1982b), although they did not investigate the influence of the pH on color fading in detail. The HRP activity changes at different pHs in the measurement solution might also be considered. In fact, the HRP activity was high at around pH 7.0 and low at alkaline pH. In these results, characteristic SPR_{Abs} biosensor responses at different pHs were observed. Therefore, our SPR_{Abs} biosensor probe can be applied to hydrogen peroxide measurements in clinical analyses because the biological fluids had a mostly stable pH in comparison with other applications, such as those involving environmental (Nakamura et al., 1999, 2007d,e,f,g; Nakamura and Suzuki, 2007a,b) and food (Volotovskya and Kim, 1998; Huang and Wu, 2006) analyses.

3.3. Calibration curve for hydrogen peroxide

To characterize and utilize our SPR_{Abs} biosensor method for other analyte applications, we investigated the property of the responses to hydrogen peroxide. Fig. 5 shows two calibration curves for hydrogen peroxide. In Fig. 5(a), a linear calibration curve was successfully obtained between 1.0 and 50 mM hydrogen peroxide ($r = 0.991$, six points, average of R.S.D.; 0.152%, $n = 3$) with a detection limit of 0.5 mM hydrogen peroxide. Then, the data of Fig. 5(a) shown as the local minimum point of reflectivity to each concentration of hydrogen peroxide were extracted from the SPR_{Abs} biosensor spectrums (see Fig. S1 in the supplementary data). In addition, the local minimum point of the wavelength was observed from 646.36 to 647.44 nm, and no relationship between the local minimum point of wavelength and the hydrogen peroxide concentration was observed ($r = 0.6619$). Therefore, as in the case of the results obtained by Fujii et al. (2002), the absorbance in the measurement solution could only be detected as the reflectivity in the SPR_{Abs} biosensor spectrums. From the characteristics in the

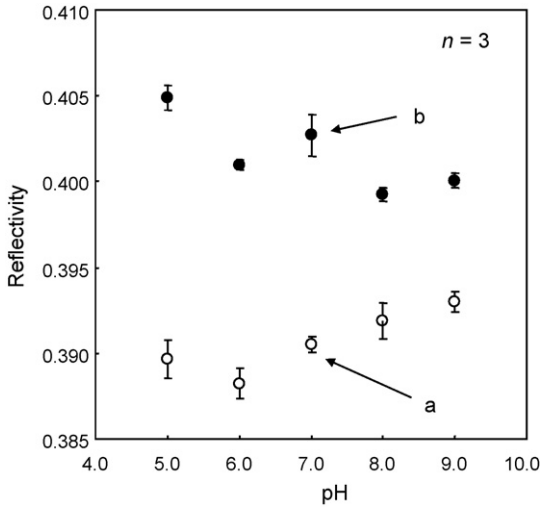

Fig. 4. Effects of the pH on SPR_{Abs} biosensor responses (a, 0 mM; b, 20 mM H_2O_2). A 10 mM PB buffer of each pH was added to four kinds of solution (MAOS, 4-AA, HRP, and sample) as the solvent. Other measurement conditions were the same as those in the experiment in Fig. 3.

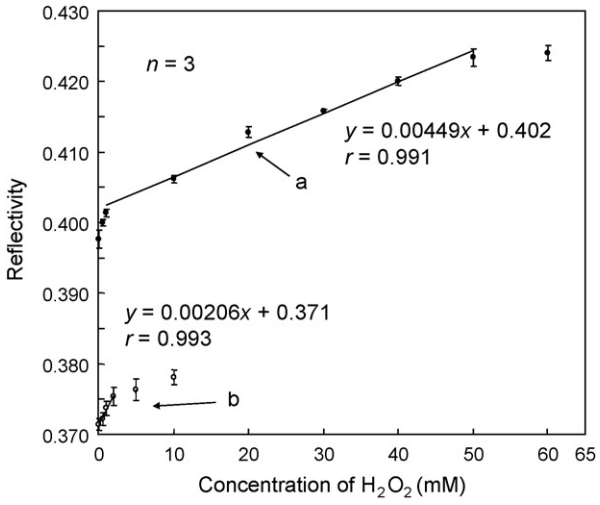

Fig. 5. Calibration curve for hydrogen peroxide (a, 12.5 mM MAOS, 12.5 mM 4-AA, and 1.0 unit μL^{-1} HRP; b, 1.25 mM MAOS, 1.25 mM 4-AA, and 1.0 unit μL^{-1} HRP were contained in each measurement solution). Then, a 10 mM PB solution of pH 7.0 was added to all solutions as a solvent. The error bars are the standard deviations of three measurements.

calibration range, we expected the applicability of the method to the determination of blood sugar or urine sugar.

In addition, to investigate the characteristics of the SPR_{Abs} biosensor probe, another calibration curve was also examined under different conditions by a 10-times dilution of MAOS and 4-AA in the measurement solution. However, the practical characteristics of the calibration range and the detection limit (obtained around 1 mM H_2O_2) of the hydrogen peroxide concentration could not be obtained (Fig. 5(b)). The local minimum point of the wavelength then appeared in a narrow range from 643.48 to 643.84 nm, and no relationship between the local minimum point of wavelength and the hydrogen peroxide concentration was observed ($r = 0.5904$). In these experiments, it was confirmed that the SPR_{Abs} responses to hydrogen peroxide could be obtained without influence of the refraction index in the samples, although a remarkable shift in the wavelength of about 4 nm was observed between two calibrations that were probably due to differences in the refraction index in MAOS and 4-AA.

Thus, the calibration curve for hydrogen peroxide was successfully obtained by the present SPR_{Abs} biosensor probe. Considering the calibration range for hydrogen peroxide, we expected the applicability of the SPR_{Abs} biosensor probe to clinical use because several constituent levels in human fluids are applicable to this sensor's properties for the measurements. In a subsequent study, the application of our sensor probe was examined.

3.4. Application to glucose sensing

Utilizing the characteristics obtained in the calibration range of hydrogen peroxide, we focused on blood glucose as the other analyte. In general, the blood glucose level is between 4.2 and 7.8 mM in healthy persons; however, the glucose level is elevated around 20 mM in individuals with hyperglycemia. Then, the SPR_{Abs} probe was applied to glucose detection using GOD (from *Aspergillus niger*; 217 units mg^{-1}, Amano Enzyme, Inc., Nagoya, Japan), which was added to the HRP solution to have the same catalytic activity (4.0 units μL^{-1}) as HRP. The results are shown in Fig. 6. As in the case of the SPR_{Abs} biosensor responses to the same concentration of hydrogen peroxide, obvious SPR_{Abs} biosensor responses to 20 mM glucose in comparison with the responses to 0 mM glucose were observed. The value of reflectivity was 0.39689 ± 0.00060 (R.S.D. = 0.151%, $n = 3$) at 0 mM and 0.40744 ± 0.00077 (R.S.D. = 0.189%) at 20 mM glucose.

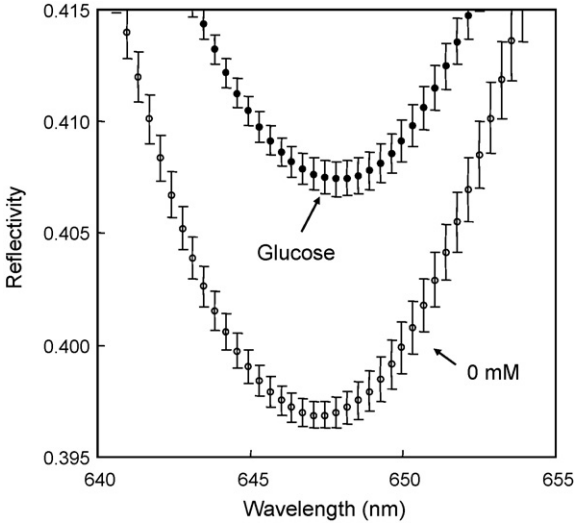

Fig. 6. Detection of the SPR_{Abs} biosensor responses to 20 mM glucose. In 20 μL of the measurement solution, 12.5 mM MAOS, 12.5 mM 4-AA, 1.0 unit μL^{-1} HRP, and 1.0 unit μL^{-1} GOD were combined with 5 μL of a sample solution containing 0 or 20 mM glucose. Then, a 10 mM PB solution of pH 7.0 was added to all solutions as a solvent. HRP and GOD were dissolved in a solution with the same enzymatic activity of 20 units in 5 μL volume. The error bars are the standard deviations of three measurements.

A slight wavelength shift of +0.36 nm in the SPR_{Abs} biosensor responses between 20 mM hydrogen peroxide (data not shown) and 20 mM glucose was then observed, and the wavelength shift might have occurred as a result of a small influence of glucose in the refractive index. A wavelength shift caused by the addition of GOD to the measurement solution could also be expected, as well as the difference in the refractive indexes. The influence of the dissolution of GOD in the measurement solution might also be observed in the wavelength shift of 0.72 nm in the SPR_{Abs} biosensor responses to 0 mM glucose in comparison with the results obtained in Fig. 3 (0 mM H_2O_2). In addition, the wavelength shift by the existence of GOD was relatively small compared with the wavelength shift by that of MAOS and 4-AA in the measurement solution.

From these results, the possibility to measure samples directly without dilution and to determine the analyte concentration without the influences of the refractive index in the measurement sample was demonstrated. Thus, these results demonstrate that our SPR_{Abs} biosensor method has potential as an analyte biosensing tool, especially for clinical use, because many constituent levels in human fluids were in the range of the hydrogen peroxide calibration obtained in this study. Indeed, the properties of the present method might be applicable to the continuous monitoring of analytes (Petrou et al., 2003) or enzyme activity (Posthuma-Trumpie et al., 2007) if the present SPR_{Abs} biosensor probe is improved. In addition, the specific properties leading to direct analysis can definitely simplify the sensing procedure and are applicable for home and clinical use as well as field monitoring.

4. Conclusion

In the present study, an enzyme-chromogenic SPR biosensor probe for hydrogen peroxide was successfully developed using the modified Trinder's reagent without influences of the refractive index change in the measurement solution, the probe was calibrated between 1.0 and 50 mM hydrogen peroxide without a sample dilution, and the assignment to the sensitivity was found. According to the characteristics of the calibration range, 20 mM glucose detection was performed as a possible concentration for clinical use. As a result, the applicability of the SPR_{Abs} biosensor probe to other analytes was shown. In addition, a deeply colored sample with high absorbance could be measured; thus, simplification was realized by the direct measurement of such a sample. The measurability of the high absorbance in the present method is superior to that of the spectrophotometric method. Furthermore, the measurability without the influence of the refractive index change, which was caused by the macromolecules in the sample in this method, might be applicable to implantable blood constituent monitoring systems (Newman and Turner, 2005). As a subsequent study, utilizing our knowledge in glucose biosensor developments (Kurusu et al., 2005; Nakamura et al., 2005, 2006, 2007a,b,c; Kaimori et al., 2006; Kitamura et al., 2006), the improvement of an SPR_{Abs} biosensor probe for continual cancer detection during laparotomy is now in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.04.022.

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