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Utilization of AC Impedance Measurements for Electrochemical Glucose Sensing Using Glucose Oxidase to Improve Detection Selectivity

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The electrochemical detection of glucose using glucose oxidase (GOx) has been conducted by means of AC impedance measurements. In the absence of glucose, the Nyquist plots were straight line due to a reversible redox reaction involving the electron mediator. On the other hand, the Nyquist plots became a circular arc in the presence of glucose, and its radius decreased with an increase in the glucose concentration. The shape of the Nyquist plots were explained with assistance of numerical calculations. Since the reaction impedance due to direct oxidation of interfering species was significantly higher than that of glucose oxidation catalyzed by glucose oxidase, unfavorable influence from interfering species could be eliminated without needing a complicated sensor device. When ferrocenecarboxylic acid was used as an electron mediator, the calibration curve obtained from the Nyquist plots was not affected by the presence of ascorbic acid. Based on those findings, a sensing system, which has no interference from both ascorbic acid and uric acid, was fabricated by using $[Os(bpy)_3]Cl_2$, which has a highly positive redox potential (0.65 V), as an electron mediator. A method to prepare calibration curves from AC impedance measurements with a fixed frequency was established so that this technique can be put to practical use.

Electrochemical biosensing has been widely investigated by combining an appropriate enzyme protein as a catalyst with a redox species that works as an electron mediator between the enzyme and an electrode.[1–13] The enzyme itself possesses high reaction selectivity, however, electrochemically active species in body fluids interfere with electrochemical detection. For example, ascorbic acid and uric acid are well-known to interfere with the electrochemical detection of glucose oxidation using glucose oxidase (GOx) because these species are directly oxidized regardless of the presence of GOx on the electrode surface.[14]

To avoid such interference, several methods have been developed. Membranes or microtubules having selective permeability are put in the sample inlet of a sensor tip to exclude such active species.[15–21] Another way is to fabricate a reaction system, which can detect the substrate by electrochemical reduction, though glucose is oxidized by the enzyme.[22,23] This can be made by introducing another enzyme, such as peroxidase. The oxidation of glucose at GOx is accompanied by reduction of oxygen to hydrogen peroxide. The peroxidase induces the reduction of generated hydrogen peroxide and oxidation of a redox species, the latter of which is electrochemically reduced at the electrode. In this case, the electrode potential can be set negative enough to avoid oxidation of the interfering species. However, these methods use modified electrodes as the sensor tips which are disposed after only one measurement. If the cost of the materials for the modification are expensive, then the price of one measurement will be very expensive. Thus, it is economically favorable to improve selectivity by designing a measurement circuit that uses a conventional and simple sensor tip.

Electrochemical measurements that use alternating current (AC) signals to an electrode are very useful for obtaining detailed information of the electrode reaction.[24–26] Using impedance measurements conducted by scanning the frequency, resistance and capacitance components can be determined from the electrochemical system, from which kinetic parameters can be evaluated.

Since electrochemical sensing of glucose catalyzed by GOx is a typical catalytic reaction involving the regeneration of the reduced form of an electrochemically active species via a chemical reaction (E_rC_i' type reaction), it can be easily characterized by using simple electrochemical measurements such as cyclic voltammetry and chronoamperometry. However, there are only a few reports concerning analysis of the reaction using the impedance technique that aimed to elucidate kinetic parameters of sensor systems using specific electrodes.[27–31] In our previous paper, we have reported that electrochemical determination of glucose using GOx and ferrocenecarboxylic acid as an electron mediator can be conducted by using AC impedance measurements.[32] A calibration curve has been drawn by using the reaction impedance obtained from the Nyquist plots and presence of ascorbic acid did not affect the calibration curve. The present paper expands on the previous study. The changes in shape of the Nyquist plots in the presence of glucose are elucidated through assistance of numerical calcula-

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tions on the AC impedance. Also, an electron mediator having more positive redox potential was chosen more kinds of interfering species to avoid. With the information obtained from the Nyquist plots, determination of glucose concentration is also conducted by using AC measurement with a single frequency, which makes this technique more useful in practical applications. This technique will be also widely applicable to various biosensing devices using enzymatic reactions because a complicated design for each reaction system is not needed.

Experimental

Glucose oxidase from *Aspergillus niger* (GOx, EC 1.1.3.4, 190 units mg^{-1}, type X-S) was purchased from Sigma and used as received. Ferrocenecarboxylic acid (Fc-COOH), potassium hexachloroosmate(IV) ($K_2[OsCl_6]$) and 2,2′-bipyridine (bpy) from Aldrich were purchased as reagent grade and used without further purification. Water that was used for preparation of all aqueous solutions was purified by a Milli-Q system gradient A10 (Millipore). Tris(2,2′-bipyridyl)osmium(II) chloride ($[Os(bpy)_3]Cl_2$) was synthesized by mixing $K_2[OsCl_6]$ and bpy in ethylene glycol according to the literature.[33,34] All other chemicals were purchased as analytical grade from Wako Pure Chemicals.

Electrochemical measurements were carried out using a normal three-electrode system. A glassy carbon (GC) disk having 1.5 mm radius (7.07×10^{-2} cm^2) was used as a working electrode. Its surface was polished with alumina powder (0.3 μm) dispersed in water prior to use. A Pt foil and an Ag|AgCl electrode in saturated KCl aqueous solution were used as counter and reference electrodes, respectively. All electrode potentials in this study are given relative to the Ag|AgCl reference electrode. The base electrolyte solution contained 0.1 M (1 M = 1 mol dm^{-3}) phosphate buffer (pH = 7.0) and 0.1 M KCl as a supporting electrolyte to reduce solution resistance. GOx and the electron mediator of prescribed amounts were added, and N_2 gas was bubbled for at least 15 min to remove oxygen. A 1 M glucose solution made with the base electrolyte solution was used as a stock solution and injected in the electrolyte solution so as to give the desired concentration. Cyclic voltammetry, chronoamperometry, and impedance measurements were conducted at 30 °C using a Voltalab PZG-402 electrochemical analyzer. AC impedance measurements were performed in the frequency range from 15.8 mHz to 100 kHz, and the amplitude of AC potential was 10 $mV_{p\text{-}p}$ (peak to peak).

Numerical calculations involving electrochemical and enzymatic reactions with diffusion of the electron mediators were conducted by calculating the mass balance in one-dimensional discrete cells according to the finite difference method reported by Yokoyama et al.[35,36] All calculations were conducted by using homemade software running on an IBM compatible PC programmed with Microsoft Visual Basic.NET.

Results and Discussion

Electrochemical Detection of Glucose Using GOx and Fc-COOH. Electrochemically active species, such as hexacyanoferrate ($[Fe(CN)_6]^{3-/4-}$)[37–39] or ferrocene derivatives,[40–42] are good electron mediators in the electrochemical detection of some enzymatic reactions. Figure 1 (solid lines) shows cyclic voltammograms of the electrolyte solution containing 750 unit mL^{-1} GOx, 0.2 mM Fc-COOH, and glucose at different concentrations. Currents due to electrochemical oxidation of Fc-COOH increased with an increase in glucose concentration, indicating clearly that oxidation of GOx by the oxidized form of Fc-COOH took place. At electrode potentials positive of 0.4 V, oxidation of glucose by GOx determined the reaction rate, giving the limiting currents. As shown in Fig. 1, the shape of the voltammogram became sigmoid and hysteresis between anodic and cathodic potential scans became small as glucose concentration increased.

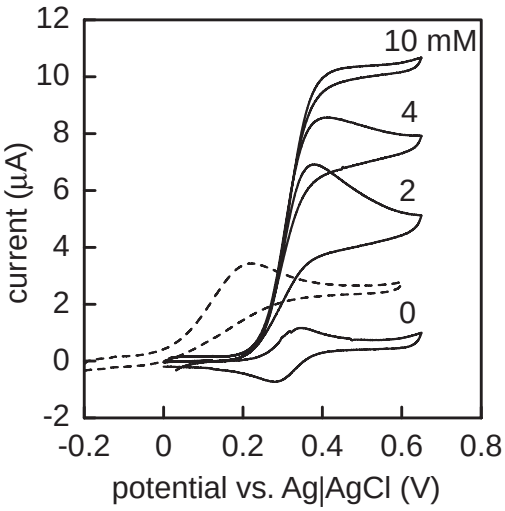

Fig. 1. Cyclic voltammograms obtained for a glassy carbon electrode in the electrolyte solution (pH = 7) containing 750 unit mL^{-1} GOx, 0.2 mM Fc-COOH, and glucose at different concentrations (solid lines), and in the electrolyte solution (pH = 7) containing 0.5 mM ascorbic acid (broken line). All voltammograms were taken at scan rate of 10 mV s^{-1}.

The influence of an interfering species on the glucose oxidation was investigated. Figure 1 (broken line) shows a voltammogram obtained for 0.5 mM ascorbic acid. Currents due to direct oxidation of ascorbic acid, which gave a current peak at 0.2 V, exhibited that it was easily oxidized in the potential region where the determination of glucose concentration was conducted. Therefore, ascorbic acid must interfere with the amperometric determination of glucose.

AC Impedance Measurements. AC impedance measurements were applied to the electrochemical oxidation of glucose in the presence of GOx and Fc-COOH. The open circle with solid lines in Fig. 2 show the Nyquist plots obtained for different concentrations of glucose. The DC bias potential (E_{dc}) was 0.36 V, which was slightly more positive than the redox potential of Fc-COOH (0.32 V). In the absence of glucose, the plots showed a straight line, indicating that the Warburg impedance (Z_w) due to the reversible redox reaction of Fc-COOH was dominant in the impedance measurements. When glucose was added to the electrolyte solution, the shapes of the Nyquist plots looked like a circular arc, and its radius became small with an increase in glucose concentration.

Numerical calculations were attempted in order to understand the changes in shape of the Nyquist plots caused by addition of glucose. We found in the present study that the Nyquist plots could be calculated by digital simulation based on the method of finite difference.[43,44]

Yokoyama et al. have reported that digital simulation of cyclic voltammograms obtained for an electrochemically mediat-

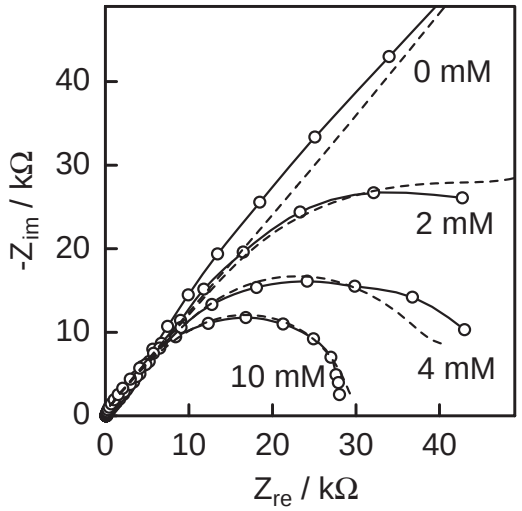

Fig. 2. Nyquist plots obtained for a glassy carbon electrode in the electrolyte solution (pH = 7) containing 750 unit mL^{-1} GOx, 0.2 mM Fc-COOH, and glucose of given concentrations (solid lines and open circles). E_{dc}, AC amplitude and frequency range of applied signals were set to 0.36 V vs Ag|AgCl, 10 $mV_{p\text{-}p}$ and 100 kHz–15.8 mHz, respectively. Broken lines show the results of numerical simulation.

ed enzyme reaction is possible based on a diffusion equation, the Butler–Volmer equation, and kinetics of enzymatic reaction (Eqs. 1–3).[36]

$$\frac{\partial C_R}{\partial t} = D_{med}\frac{\partial^2 C_O}{\partial x^2} + \frac{2k_{cat}C_E}{\dfrac{K_{M,med}}{C_O} + \dfrac{K_{M,sub}}{C_{sub}} + 1} \quad (1)$$

$$\frac{\partial C_{sub}}{\partial t} = D_{sub}\frac{\partial^2 C_{sub}}{\partial x^2} - \frac{k_{cat}C_E}{\dfrac{K_{M,med}}{C_O} + \dfrac{K_{M,sub}}{C_{sub}} + 1} \quad (2)$$

$$D_{med}\frac{\partial^2 C_R}{\partial x^2} = k^0\left[C_R \exp\left\{\frac{\alpha nF}{RT}(E - E^0)\right\} - C_O \exp\left\{-\frac{(1-\alpha)nF}{RT}(E - E^0)\right\}\right] \quad (3)$$

where C_O, C_R, and C_{sub} are the concentrations of oxidized mediator, reduced mediator, and substrate of enzymatic reaction, respectively; D_{med} and D_{sub} are the diffusion coefficients of mediator and substrate, respectively; $k_{cat}C_E$ is the rate constant of the enzymatic reaction; $K_{M,med}$ and $K_{M,sub}$ are the Michaelis constants of GOx for mediator and substrate, respectively. Numerical calculations are conducted by changing the electrode potential linearly with time.

Digital simulation of the Nyquist plots can be conducted by application of potential changes associated with sinusoidal wave expressed by Eq. 4 in place of the potential changes associated with triangular wave.

$$E = E_{dc} + \frac{E_{amp}}{2}\sin(2\pi ft) \quad (4)$$

where E_{amp} is the amplitude of AC potential (10 $mV_{p\text{-}p}$) and f is the frequency. Numerical calculations gave current responses in the sinusoidal wave (I_{ac}), and absolute impedance values ($|Z|$) were calculated from the amplitude of electrode potential (E_{amp}) and current (I_{amp}), as expressed by Eq. 5.

$$|Z| = \frac{E_{amp}}{I_{amp}} \quad (5)$$

The phase difference (θ) is determined from time difference of the peaks between E_{ac} and I_{ac}. Then, real and imaginary parts of the impedance calculated by Eqs. 6 and 7 are plotted on the complex plane by changing f from 63.3 mHz to 100 Hz.

$$Z_{re} = |Z|\cos\theta \quad (6)$$

$$Z_{im} = |Z|\sin\theta \quad (7)$$

The diffusion coefficient (D_{med}) and kinetic constant of the electrode reaction (k^0) of Fc-COOH have been reported by several groups.[43–49] However, those values were variant due to differences in the composition of solution and/or electrode materials. Therefore, D_{med} used in the present study was estimated from the peak currents of cyclic voltammograms taken at different scan rates (5 to 50 mV s^{-1}), where the electrode reaction could be regarded as reversible. Plots of the peak currents as a function of square root of scan rate (v) allowed estimation of D_{med} (6.2×10^{-6} cm^2 s^{-1}).[50]

k^0 (6.3×10^{-2} cm s^{-1}) was estimated from charge-transfer resistance (R_{CT}) determined by AC impedance measurements taken at the standard electrode potential (E^0) of Fc-COOH (0.32 V).[51] The diffusion coefficient of glucose ($D_{glu} = 6.8 \times 10^{-6}$ cm^2 s^{-1}) has been reported by several groups.[52–54] Since some enzymatic constants ($K_{M,med}$, $K_{M,sub}$, and $k_{cat}C_E$) are unknown, a nonlinear method of least squares was used to estimate these parameters. In this study, a brute force least square method was employed to fit the parameters. After repetitious calculations, the sum of squares of the absolute differences (SSAD) between experimentally obtained values and calculated ones afforded the minimum value with a set of parameters ($K_{M,med} = 200\,\mu M$, $K_{M,sub} = 8$ mM, and $k_{cat}C_E = 1.0 \times 10^{-3}$ M s^{-1}). The results obtained for different glucose concentrations are shown by broken lines in Fig. 2. As shown, the simulated lines fit well the shape changes from straight line to circular arcs with a decrease in the radius with an increase in glucose concentration. Deviations of $K_{M,sub}$, $K_{M,med}$, and k_{cat} causing a 10% change in SSAD were 8.0 ± 0.3 mM ($\pm 3.8\%$), $200 \pm 30\,\mu M$ ($\pm 15\%$), and $(1.00 \pm 0.03) \times 10^{-3}$ M s^{-1} ($\pm 3.0\%$), respectively. These results indicated that $K_{M,med}$ has the smallest effect on the shape of the Nyquist plots. In other words, it is not guaranteed that the $K_{M,med}$ value (200 μM) was accurately estimated in this simulation.

Concentration profiles of the reduce form of mediator in the vicinity of the electrode surface, which are obtained during numerical calculation, are helpful in understanding the shape changes in the Nyquist plots. The results obtained for frequencies of 0.1, 1, and 10 Hz are shown in Fig. 3. To make small concentration changes due to AC signal conspicuous, the concentration gradient of the mediator caused by application of E_{dc} was omitted by subtracting the average value during one cycle of the AC signal. The distributions of the mediator concentration at the electrode surface are depicted at intervals of 45° phase angle of AC potential. In the absence of glucose, as shown by broken lines in the figures, the finite diffusion rate of the mediator delays propagation of the concentration change against the AC potential, which causes a phase difference of

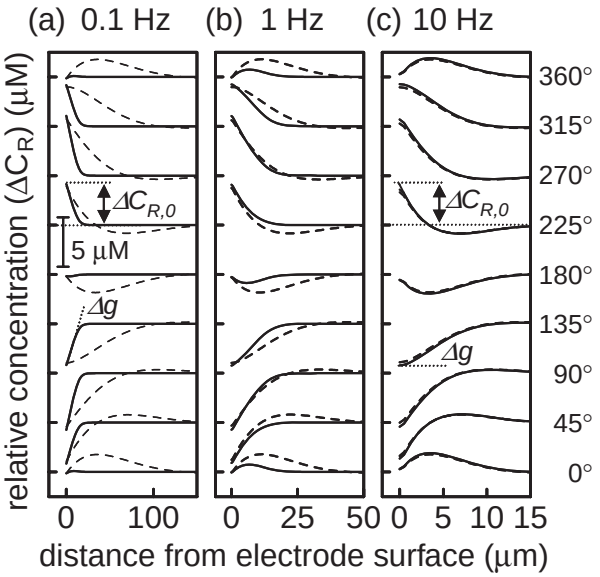

Fig. 3. Relative concentration distribution of reduced form of electron mediator around the electrode surface by the numerical calculation (finite difference method) of electrochemical and enzymatic reaction at (a) 0.1 Hz, (b) 1 Hz, and (c) 10 Hz, respectively. The concentration distribution in the absence and the presence of glucose (10 mM) are shown by broken and solid lines, respectively.

45° between the change in surface concentration ($\Delta C_{R,0}$) and that in concentration gradient (Δg). The surface concentration is determined by the Nernst equation, and the concentration gradient determines current values. Thus, the AC currents are 45° out-of-phase from AC potentials.

On the other hand, the electron mediator, which is oxidized at the electrode surface, is reduced by the enzymatic reaction around the electrode surface in the presence of glucose. If the rate of enzymatic reaction is sufficiently fast, the concentration profile surface instantly reaches a steady state around the electrode surface, and the concentration change does not propagate to the bulk solution as shown by solid lines in Fig. 3a. Then, the change in concentration gradient at the surface is almost in phase with that of the surface concentration at lower frequency. However, when the frequency increases, the rate of enzymatic reaction gradually becomes insufficient to achieve a steady state concentration profile, giving the same situation as the case in the absence of glucose as shown in Figs. 3b and 3c.

AC Impedance Measurements in the Presence of Ascorbic Acid. Figure 4 shows the Nyquist plots taken at $E_{dc} = 0.36$ V for a solution containing GOx, Fc-COOH, and glucose at different concentrations in the absence (a) and presence (b) of 0.5 mM ascorbic acid as an interfering species. Quite similar profiles were obtained, whether or not ascorbic acid was present. In our previous report, it has been shown that the calibration curve obtained by plotting diameters of the Nyquist plots as a function of glucose oxidation is not affected by addition of ascorbic acid.[32]

This feature can be quantitatively understood by comparing the Nyquist plots shown in Fig. 5a. The Nyquist plots for electrochemical oxidation of 15 mM glucose catalyzed by GOx

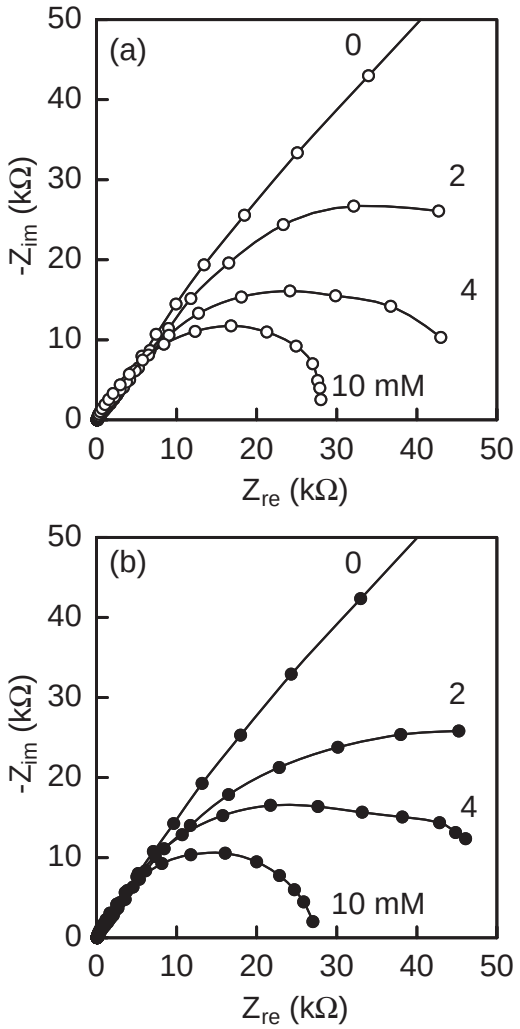

Fig. 4. Nyquist plots obtained for a glassy carbon electrode in an electrolyte solution (pH = 7) containing 750 unit mL^{-1} GOx, 0.2 mM Fc-COOH, and glucose at different concentrations in the (a) absence or (b) presence (0.5 mM) of ascorbic acid as an interfering species. E_{dc}, AC amplitude and frequency range of applied signal were set to 0.36 V vs Ag|AgCl, 10 mV_{p-p} and 100 kHz–15.8 mHz, respectively.

have a circular arc shape. In contrast, both real and imaginary parts of the impedance due to direct oxidation of 0.5 mM ascorbic acid increased monotonically with a decrease in frequency. Figure 5b shows the Bode representation of logarithm of absolute impedance values due to glucose oxidation ($|Z_{glu}|$) and direct oxidation of ascorbic acid ($|Z_{asc}|$) as a function of logarithm of frequency. As shown, $|Z_{asc}|$ drastically increased with decreasing frequency, while $|Z_{glu}|$ did not change largely even in the lower frequency region. These phenomena reflect well the shapes of the cyclic voltammograms shown in Fig. 1. For the case of glucose oxidation, the slope of current changes (dI/dE) was relatively steep at E_{dc} in the AC impedance measurements. In this case, it is expected that large current responses are obtained for the AC potential change around E_{dc}. Therefore, the reaction impedance ($Z = E_{amp}/I_{amp}$) should be low. On the other hand, the reaction impedance

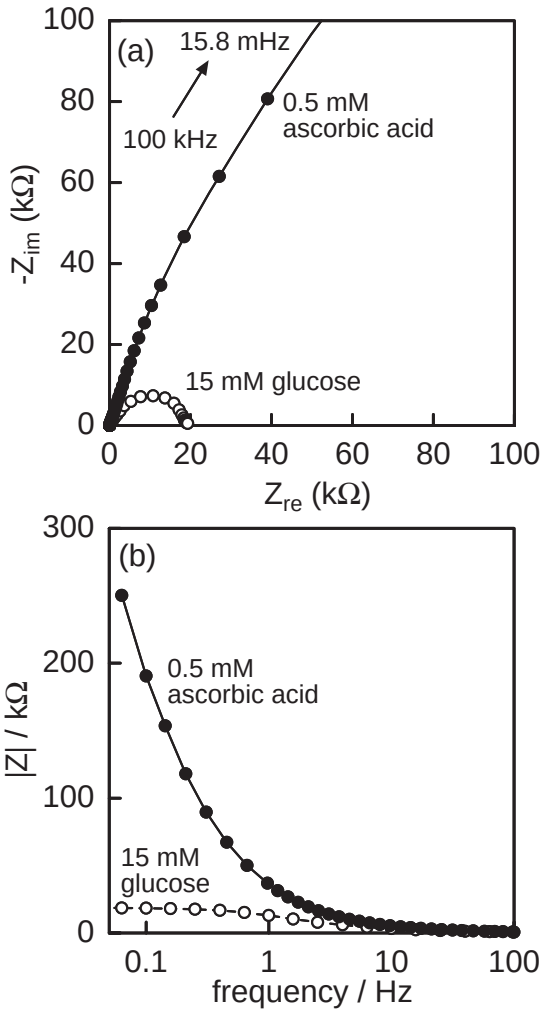

Fig. 5. (a) Nyquist plots obtained in the phosphate buffer solution (pH = 7) containing 750 unit mL^{-1} GOx, 0.2 mM Fc-COOH, and 15 mM glucose (white dots) and in the phosphate buffer solution containing 0.5 mM ascorbic acid (black dots). E_{dc}, AC amplitude and frequency range of applied signal were set to 0.36 V vs Ag|AgCl, 10 $mV_{p\text{-}p}$ and 100 kHz–15.8 mHz, respectively. (b) Absolute values of impedances due to electrochemical oxidation of glucose (white dots) and ascorbic acid (black dots) plotted against the frequency of AC signal.

for ascorbic acid oxidation became much higher than that of glucose because of small current changes. Both reactions are electrochemically independent, meaning that the total reaction impedance ($|Z_{total}|$) can be calculated as a parallel circuit of Z_{glu} and Z_{asc}. At a frequency of 63.3 mHz, $|Z_{glu}|$ and $|Z_{asc}|$ were 18.6 and 250 kΩ, respectively. Thus, total reaction impedance ($|Z_{total}|$) was 17.8 kΩ, which was 96% of $|Z_{glu}|$. Therefore, the contribution of Z_{asc} to Z_{total} should be quite small, i.e., the Nyquist plots mostly provided information about the glucose oxidation.

AC Impedance Measurements in the Presence of Uric Acid. Uric acid is another interfering species coexisting in normal test samples, such as human blood. The normal concentration of uric acid in the blood of a healthy human is in the range from 0.1 to 0.4 mM, and symptoms of gout appear if the blood concentration becomes larger than 0.4 mM.[18] Figure 6a (solid line) shows a cyclic voltammogram obtained in the phosphate buffer solution containing 0.5 mM uric acid. A large current due to direct oxidation of uric acid was observed, and the peak potential was almost the same as that of oxidation of Fc-COOH, shown by broken line in Fig. 6a. Figures 6b and 6c show the Nyquist plots and the Bode plots taken at $E_{dc} = 0.36$ V for 15 mM glucose (white dots) and 0.5 mM uric acid (black dots), respectively. Since the reaction impedances for both cases were significantly low, selective detection of glucose could not be achieved.

Glucose Oxidation Using GOx and $[Os(bpy)_3]Cl_2$. As recognized from measurements using Fc-COOH as an electron mediator, it is essential for selective detection to choose an E_{dc} where the oxidation current due to an interfering species reaches its limiting value and does not change upon the AC potential change, whereas changes in the currents due to glucose oxidation are sufficiently large. In AC impedance measurements, the effective range of E_{dc} is determined by the redox potential of the electron mediator. When Fc-COOH was used as a mediator, unfavorable interference due to uric acid could not be avoided, although that due to ascorbic acid was removed, as already described. However, interference from uric acid can be avoided if an electrochemically active species having a more positive redox potential is used. Basically, the electron mediator should enhance the rate of the oxidation reaction catalyzed by the enzyme and, thus, enhance the sensitivity. In the case of DC amperometric sensors, however, the potential must be more negative to avoid direct oxidation of the interfering species. To confirm the merit of the AC impedance measurement, $[Os(bpy)_3]Cl_2$, which has a redox potential of 0.65 V, was used as the electron mediator. It has already been reported that $[Os(bpy)_3]Cl_2$ works well as an electron mediator for glucose oxidation with GOx.[34,55,56]

Figure 7 (solid line) shows cyclic voltammograms obtained for the electrolyte solution containing $Os(bpy)_3Cl_2$ and GOx in the absence and presence of glucose. The oxidation currents increased upon the addition of 5 mM of glucose. When 0.5 mM uric acid was present in the electrolyte solution, currents due to its direct oxidation were superimposed on those of GOx-catalyzed reaction as shown in Fig. 7 (broken line). In addition, from a careful comparison between Fig. 6a and Fig. 7, it was noticed that the anodic wave due to uric acid oxidation in the presence of GOx and glucose slightly increased. This suggested that the uric acid worked as an electron mediator for GOx. From these results, it is obvious that selective detection of glucose cannot be conducted by conventional amperometric measurements.

Figure 8a shows the Nyquist plots obtained for glucose oxidation using $[Os(bpy)_3]Cl_2$ and GOx. E_{dc} was set to 0.65 V, at which the reaction impedance due to glucose oxidation was minimum. As in the case of glucose oxidation using Fc-COOH, the shape of the Nyquist plots became a circular arc when glucose was added to the electrolyte solution. It is noted that absolute value of the impedance ($|Z|$) was significantly lower than that obtained for the case using Fc-COOH. For example, $|Z|$ measured after addition of 10 mM glucose at $f =$ 63.3 mHz was estimated to be 13.2 kΩ for $[Os(bpy)_3]Cl_2$ system, whereas that value for the Fc-COOH system was 27.9 kΩ.

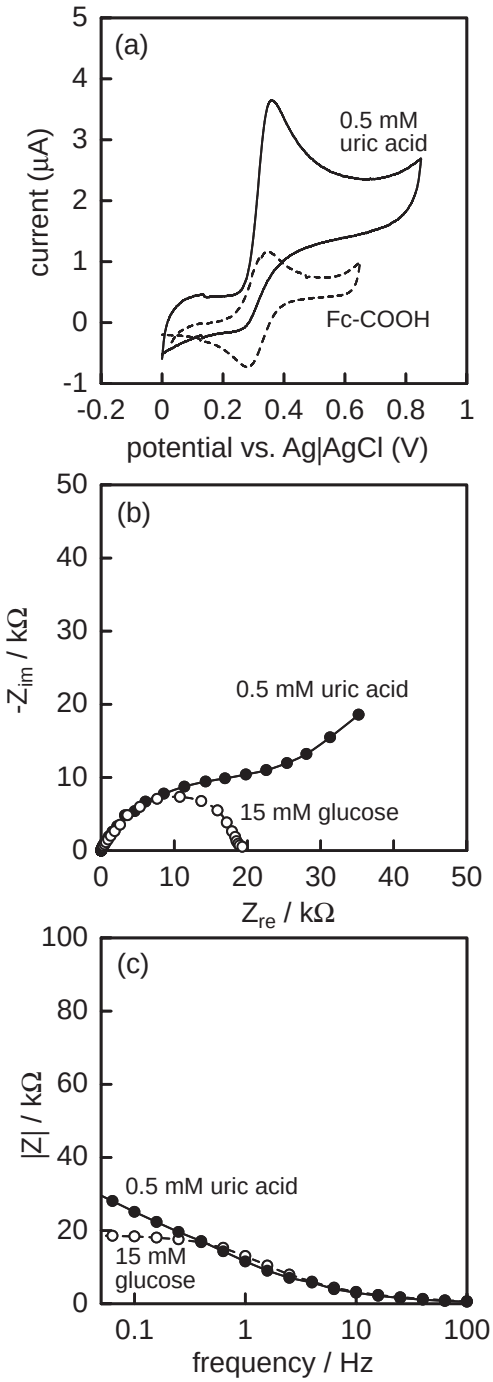

Fig. 6. (a) Cyclic voltammogram for direct oxidation of 0.5 mM uric acid on the glassy carbon electrode (solid line). Cyclic voltammogram for redox reaction of 0.2 mM Fc-COOH is also shown as broken line for comparison. (b) Nyquist plots obtained in a phosphate buffer solution (pH = 7) containing 750 unit mL^{-1} GOx, 0.2 mM Fc-COOH, and 15 mM glucose (white dots) and in a phosphate buffer solution containing 0.5 mM uric acid (black dots). E_{dc}, AC amplitude and frequency range of applied signal were set to 0.36 V vs Ag|AgCl, 10 $mV_{p\text{-}p}$ and 100 kHz–15.8 mHz, respectively. (c) Absolute values of impedances due to electrochemical oxidation of glucose (white dots) and uric acid (black dots) plotted against the frequency of AC signal.

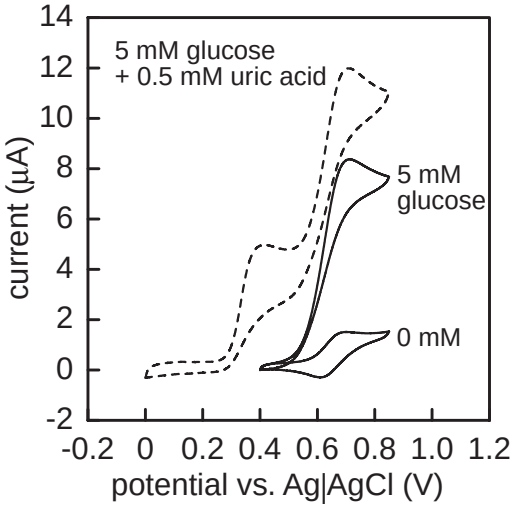

Fig. 7. Cyclic voltammograms taken in the phosphate buffer solution containing 0.2 mM $[Os(bpy)_3]Cl_2$ and 750 unit mL^{-1} GOx. Solid line shows voltammograms obtained for 0 and 5 mM glucose. Broken line shows the voltammogram obtained for 5 mM glucose after addition of 0.5 mM uric acid as an interfering species.

As mentioned above, the more positive redox potential of $[Os(byp)_3]^{2+/3+}$ caused an enhancement in oxidation currents. This implies that the reaction impedance decreases by positive shift in the redox potential of the electron mediator. Figure 8b shows the Nyquist plots for glucose oxidation with GOx and $[Os(bpy)_3]Cl_2$ in the presence of 0.5 mM uric acid. As expected, all of the profiles for different glucose concentrations were completely consistent with those obtained for the solution in the absence of uric acid. Furthermore, the profile of the Nyquist plots did not change as shown in Fig. 8c, even when ascorbic acid was added to the electrolyte solution as another interfering species.

AC Impedance Measurements at a Single Frequency for Detecting Glucose Concentration. AC impedance measurements revealed that this technique for electrochemical biosensing is very effective for suppressing the interfering species. However, requirements of changes in the AC frequency and measurements of both the absolute value and phase of impedance make this method impractical for fabricating sensors from an economical viewpoint. If the glucose concentration can be determined by measuring the absolute value of impedance with a fixed frequency, a biosensor having higher selectivity could be fabricated without much additional cost. We demonstrated its possibility, as shown in Fig. 9a. The measurements were conducted with $E_{dc} = 0.65$ V vs Ag|AgCl and $f = 63.3$ mHz. Plots of the reciprocals of the absolute impedance ($1/|Z|$) as a function of glucose concentration gave a straight line. The presence of the interfering species, i.e., both ascorbic acid and uric acid, did not have a significant effect, except for slight differences in the low concentration range. However, the calibration curve obtained from amperometric sensing was strongly affected, as shown in Fig. 9b, because of overlapping currents due to the unfavorable direct oxidation. In the impedance measurements at a single frequency, only two or three cycles of the AC signal was required for precise

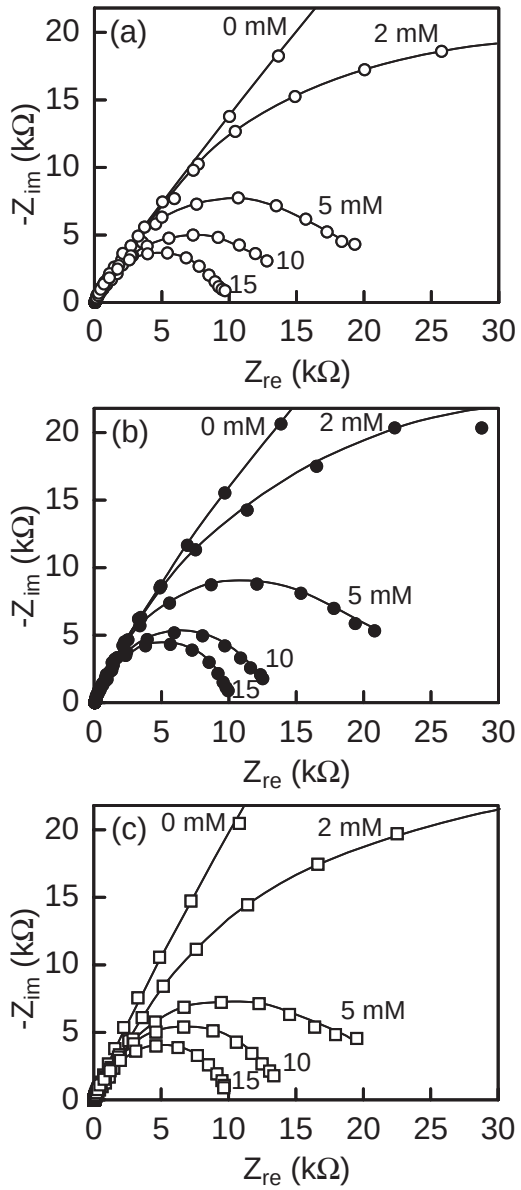

Fig. 8. Nyquist plots for different concentration of glucose obtained in the electrolyte solution containing no interfering species (a), uric acid (b), and uric acid and ascorbic acid (c). Concentration of both interfering species are 0.5 mM. These Nyquist plots were obtained for the phosphate buffer solution containing 0.2 mM $[Os(bpy)_3]Cl_2$ and 750 unit mL^{-1} GOx. E_{dc}, AC amplitude and frequency range of applied signal were set to 0.65 V vs Ag|AgCl, 10 $mV_{p\text{-}p}$ and 100 kHz–15.8 mHz, respectively.

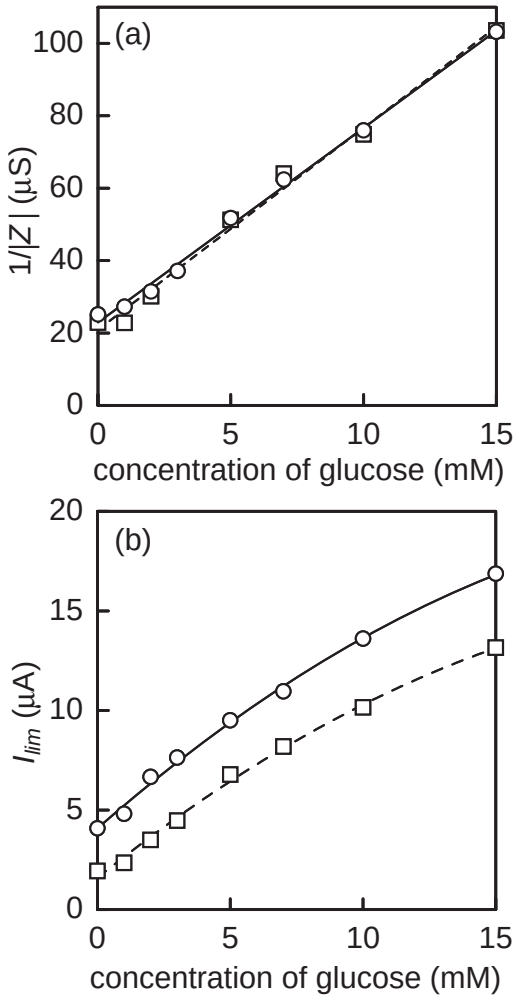

Fig. 9. Calibration curves obtained from absolute impedance measured at a fixed frequency (63.3 mHz) (a), and those obtained by conventional DC amperometric sensing (b). Broken and solid lines show the calibration curves in the absence and presence of interfering species (0.5 mM uric acid and 0.5 mM ascorbic acid). AC impedance and DC amperometric measurements were conducted in 0.1 M phosphate buffer solution containing 0.2 mM $[Os(bpy)_3]Cl_2$, 750 unit mL^{-1} GOx, and 0.1 M KCl, and the AC and DC electrode potentials were 0.65 and 0.73 V vs Ag|AgCl, respectively.

measurements. Thus, if the measurements are conducted at 63.3 mHz, sensing time should take only several tens of seconds, which are comparable to waiting time required for conventional amperometric sensing.

Conclusion

The selective determination of glucose concentration using GOx was conducted in the presence of interfering species, such as ascorbic acid and uric acid, by using AC impedance measurements. In the presence of glucose, the Nyquist plots became circular arcs because the concentration gradient caused by the AC current gradually became in phase with the surface concentration of reduced mediator determined by AC potential. When DC bias potential applied during impedance measurements was sufficiently more positive than oxidation potential of an interfering species, direct oxidation of the interfering species reached a diffusion limit. Therefore, since the reaction impedance for the direct oxidation of interfering species was much higher than that of the glucose oxidation, the toal impedances were unchanged in the presence of the interfering species. When $[Os(bpy)_3]Cl_2$, which has a highly positive redox potential (0.65 V), was used as an electron mediator, the calibration curves of glucose concentration were not affected by the presence of both ascorbic acid and uric acid, whereas the electrochemical determinations using the amperometric mea-

surements were.

Furthermore, determination of the glucose concentration could also be conducted by using AC measurements at a single frequency, which enables shortening measurement time and minimization of the sensing devices for practical use. This method should be also widely applicable to various biosensing devices using enzymatic reactions because it does not need a complicated design for each reaction system itself.

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