Highlight Review

Fabrication of Amperometric Biosensing Systems Focusing on Attachment of High Substrate Selectivity

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Abstract

The techniques for attaching high substrate selectivity to the amperometric biosensing systems are summarized with classification of membrane techniques, electron mediation techniques, electrodes, and electrochemical techniques.

♦ Introduction

The electrochemical biosensing systems have been extensively investigated to fabricate portable biosensors that enable facile examination of the bodily fluid for health check-up or diagnosis of inveterate diseases. There are two sensing modes; potentiometry and amperometry. In particular, the latter mode has become now more popular than the former because of its fast responses, high sensitivities, and high precision. The amperometry mode has been first developed to fabricate the glucose sensing system based on the glucose oxidase (GOx),[1] the principle of which is illustrated in Figure 1. Glucose cannot be directly oxidized on any conventional electrode, whereas GOx easily oxidizes it in the presence of O_2 as an oxidizing agent, generating gluconolactone and H_2O_2. Since H_2O_2 can be oxidized by a conventional electrode, determination of amount of H_2O_2 by electrochemical means allows indirect measurement of glucose concentration. Furthermore, some kinds of GOx possess relatively low selectivity for the oxidizing agent, artificial redox reagents in their oxidized form (M_{OX}) work well as electron acceptors for GOx in place of O_2. Then, glucose concentration can be determined by oxidation of the redox reagent in their reduced from (M_R). Based on the same principle, several kinds of electrochemical biosensing have been developed using redox reagents for the electron exchanges, which are called an electron mediator or an electron shuttle.[2] Among them, the amperometric glucose sensor is now commercially available as portable sensor systems using disposal sensor chips.

As well known, the enzyme is a natural catalyst having high substrate selectivity. However, the amperometric biosensing systems mentioned above have inherent drawback for the selectivity because some components in the bodily fluid, which are readily oxidized on the electrode, causes the measurement errors. In order to exclude such the interference in the measurements, several ways have been contrived. The developed techniques utilize elaborately chemical materials and electrochemical techniques in order to attach the substrate selectivity to the biosensing systems. In this review, those advance techniques are summarized since they should be useful not only for the biosensing systems but also other analytical systems and chemical devices.

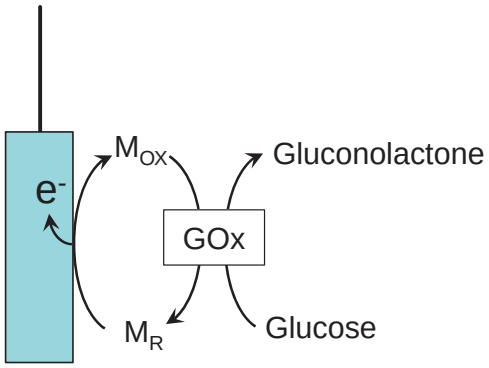

Figure 1. Schematic illustration showing the principle of amperometric biosensing system.

♦ Membrane Techniques

The substances, which give serious interference to the amperometric determination, are shown in Figure 2. In Figure 2, their structures in neutral aqueous solution are given. Ascorbic acid (Vitamin C), uric acid, and L-cysteine are always included in the bodily fluid. Since *p*-acetamidophenol possesses antipyretic and analgesic actions, it is widely used as a component of several medicines including cold medicine for popular use. Therefore, interference by this species arises for persons who conduct their bodily fluid check after taking such the drug.

Coating an electrode with a membrane having permselectivity of substances is a straightforward way for avoiding approach of the undesired species to the electrode surface. The membranes that have been used so far are summarized in Table 1.

The first attempt was made using dialysis membrane including cellulose and cellulose acetates. Their semipermeablity due to pore sizes is effective for excluding the interference substances as well as components of the fluid that adsorb on the electrode surface. However, the presence of the relatively thick membrane decreases glucose mass transport, leading to reduced sensitivity.

The perfluorinated ionomer, Nafion® membrane, was most widely attempted be used for enhancing selectivity of the glucose sensors. Since the principle interference substances are negatively charged in neutral condition, as shown in Figure 2, electrostatic repulsion between these substances and the Nafion® membrane possessing negatively charged sulfonate

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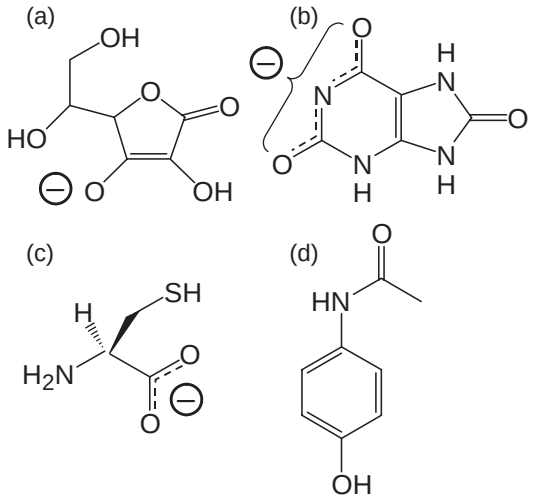

Figure 2. Chemical structures of ascorbic acid (a), uric acid (b), L-cysteine (c), *p*-acetamidophenol (d) in neutral condition.

Table 1. Membranes coated on the electrodes of the amperometric glucose sensors for excluding the interfering substances

Membrane	References
Millipore + Dialysis	3
Cellulose, Cellulose acetates	4, 5, 6
Polyurethane	7
Nafion®	8, 9, 10, 11, 12, 13
$[Os(bpy)_2Cl]$-polymer + HRP	14
Artificial bilayer	15

groups prohibits the undesired electrochemical oxidation on the electrode surface. It is easy to prepare the Nafion® membrane having desired thickness because the Nafion® membrane can be prepared by casting the commercially available Nafion® solution on the electrode. Therefore, loss of sensitivity of the sensor can be kept to minimum by tuning the membrane thickness. Furthermore, it is another merit that GOx can be immobilized in the Nafion® membrane. In this case, when O_2/H_2O_2 couple as an electron mediator, the amperometric glucose sensor is fabricated without addition of any reagent in analyte. However, since there are some interference substances having no charge even in neutral condition like *p*-acetamidophenol, as shown in Figure 2d, it is required to combine the Nafion® coating with another technique.

Heller et al. have developed a redox polymer membrane which works effectively for electron exchanges between the electrode and GOx. The poly(vinylpyridine) or poly(vinylimidazole), to which $[Os(bpy)_2Cl]^{+/2+}$ complex is attached (Os-polymer), can also immobilize covalently several kinds of enzymes including GOx. Then, the electrode coated with the Os-polymer immobizing GOx exhibits the amperometric glucose sensing properties without any reagent in the analyte. However, requirement of application potential higher than 0.3 V vs. SCE for oxidation of the $[Os(bpy)_2Cl]^+$ group is positive enough to oxidize the electrochemically active interference substances. In order to exclude errors due to the interference substances, it was attempted to chemically oxidize these species by coating a membrane including horse radish peroxidase (HRP) on the

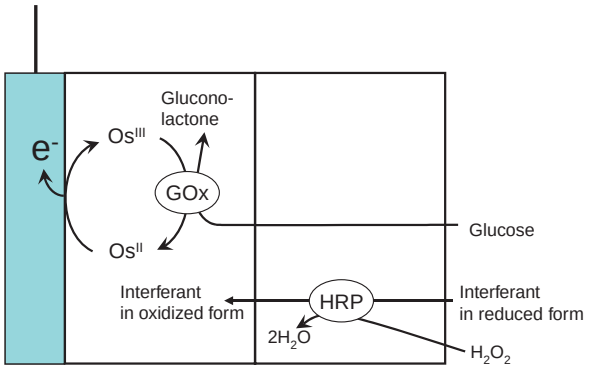

Figure 3. Selective glucose sensing with an electrode coated with two polymer layers containing GOx and HRP.

Os-polymer layer and dissolving H_2O_2 in the analyte, as shown in Figure 3. The oxidation of the interference substances including ascorbate, urate, and *p*-acetamidophenol by HRP and H_2O_2 in the outer layer suppressed errors caused by their oxidation.

A biomimetic membrane was attempted to be used for fabricating the amperometric uric acid sensor with high selectivity. The sensor was prepared using an artificial bilayer membrane composed of a self-assembled monolayer (SAM) of alkanethiol and phosphatidylcholine (PC) molecules, which was prepared by immersing an alkanethiol SAM-coated Au electrode in a solution containing PC vesicle.[16,17] It is known that self-organized fusion of the PC vesicle to the alkanethiol SAM takes place, forming the alkanethiol SAM/PC bilayer. Uricase (urate oxidase, UOx), which is a membrane protein, is readily immobilized in the alkanethiol SAM/PC bilayer, as schematically illustrated in Figure 4. Then, the electrode immobilizing UOx and 1-methoxy-5-methylphenazinium (MMP) that works as an electron mediator for UOx exhibits amperometric biosensing to uric acid. As shown in Figure 5, the electrode is not influenced by addition of ascorbic acid owing to the presence of the bilayer.

♦ *Electron Mediation Techniques*

The O_2/H_2O_2 couple was used as the electron mediator in the initial stage of fabrication of the biosensing systems, especially the amperometric glucose sensors. Oxidation of H_2O_2 requires relatively positive potential, causing oxidation of other electrochemically active compounds. The improvement of the electron mediation system for solving interference problem is something like perceptional change. The first attempt was made by introducing another enzyme, HRP, in order to fabricate the biosensing system that detected substrate with electrochemical reduction although the main enzymatic reaction was oxidation, as shown in Figure 6. The HRP catalyzes reduction of H_2O_2 and an appropriate electron donor. Then, the detection of glucose without causing any other electrochemical reaction has been achieved using GOx and HPR with $[Fe(CN)_6]^{3-/4-}$ as an electron mediator.[18,19]

If there is a redox species, which works as an electron mediator for an enzyme and has redox potential negative enough to avoid oxidation of other compounds, choice of it is another way to fabricate the amperometric biosensing system having high selectivity. Such an attempt was made to fabricate the cholesterol sensor based on cholesterol oxidase (ChOx).[20] The survey of the redox species have revealed that phenazine and phe-

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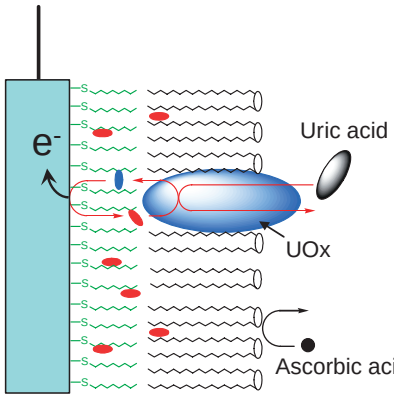

Figure 4. Schematic illustration of the PC/UOx/MMP/OT/Au electrode exhibiting selective amperometric detection of uric acid.

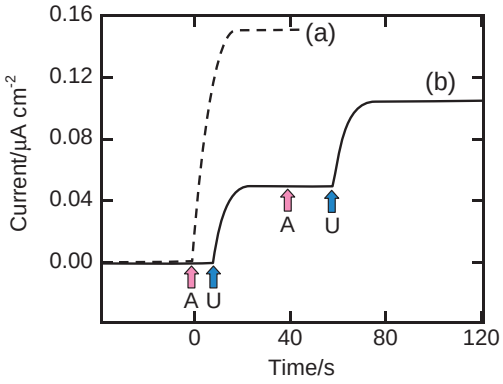

Figure 5. Time course of current responses of bare Au (a) and PC/UOx/MMP/OT/Au (b) electrodes obtained for stepwise addition of 0.1 mmol dm^{-3} ascorbic acid (indicated by A with arrows) and 0.1 mmol dm^{-3} uric acid (indicated by U with arrows). The both electrodes were polarized at 0 V vs. SCE in N_2-saturated 0.1 mol dm^{-3} borate buffer (pH 8.5).[15]

nothiazine derivatives having redox potentials ranging between −0.22 and −0.17 V vs. SCE worked as electron acceptors for ChOx in solution containing 2-propanol as a solubilizing agent for cholesterol. The amperometric sensing of cholesterol using such the electron mediator enableds detection of cholesterol with exclusion of influence by 12 compounds including ascorbic acid and *p*-acetamidophenol. In addition, the amperometric biosensing system enabling quantitative measurements of total cholesterol (free cholesterol and cholesterol ester) has been accomplished by introducing cholesterol esterase.[21]

As mentioned above, coating of the Nafion® membrane on the electrode is effective to exclude the negatively charged substances but neutral *p*-acetamidophenol can permeate the membrane. This problem was solved by using *p*-acetamidophenol itself as an electron mediator.[22] Since *p*-acetamidophenol exhibits redox reaction at around +0.4 V vs. Ag/AgCl, it works well as an electron mediator for GOx. The enzymatic reaction using *N*-acetyl-*p*-benzoquinone monoimine, which is oxidation form of *p*-acetamidophenol, gave the results that the reaction rate reached V_{max} at the concentration higher than 5 mmol dm^{-3}. Therefore, when the glucose sensor was fabricated using the

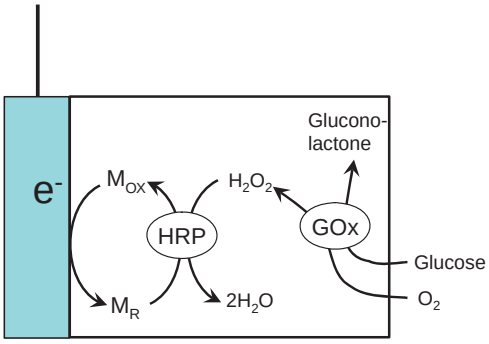

Figure 6. The amperometric glucose sensor system using GOx and HRP for conducting the substrate detection by electrochemical reduction.

Nafion®-coated electrode and sufficient amount of *p*-acetamidophenol as an electron mediator, measurement errors caused by interfusion of further *p*-acetamidophenol into the analyte was completely suppressed.

♦ Electrodes

If an electrode induces the desired electrochemical reaction alone, it should be useful to make the electrochemical biosensing systems having high selectivity. Investigation focusing on such the purpose was initiated with the systems using O_2/H_2O_2 couple as the electron mediator. It has been found that deposition of noble metals such as Rh, Ru, Pd, Ir, and their alloys on a carbon electrode put high catalytic activity for oxidation of H_2O_2 to the electrode.[23–26] Furthermore, use of the deposition bath in the presence of an enzyme allowed to co-deposition of the enzyme with metals.[25–29] This way is expedient for fabricating a microbiosensor used for in vivo analysis. Because the metal-deposited electrode possesses highest activity for H_2O_2 oxidation than electrochemically active species inherent in the biological media, the resultant biosensing systems exhibit relatively high selectivity. To exclude completely some errors due to oxidation of the interference substances, the electrode was coated with the permselective polymer membrane. In this case, since electrochemical deposition of the polymer was possible, the conducting polymers, such as polypyrrole, polyaniline, polythiophene, and poly(*p*-phenylenediamine), which are prepared by electrochemical oxidation of the corresponding monomers, have been attempted. Among them, a carbon fiber electrode, on which GOx and Ru were codeposited (GOx-Ru|CF) exhibited specific selectivity.[30] The amount of GOx included in the deposited Ru increased with an increase in GOx concentration in the deposition bath. Figure 7 shows plots of current responses of the GOx-Ru|CF electrode as a function of GOx concentration in the deposition bath. The Ru-deposited CF electrode without GOx gave distinct oxidation currents for ascorbic acid and uric acid. Their current responses decreased as the GOx concentration of the deposition bath increased, while the current responses to glucose increased. Although the mechanism enhancing selectivity has not yet been elucidated, the optimized biosensor exhibited high curent responses for glucose with suppression of errors due to five interference compounds less than 1% of the glucose responses.

The use of electron mediator is one way for electrochemically inducing enzymatic reaction, as shown in Figure 1. At the

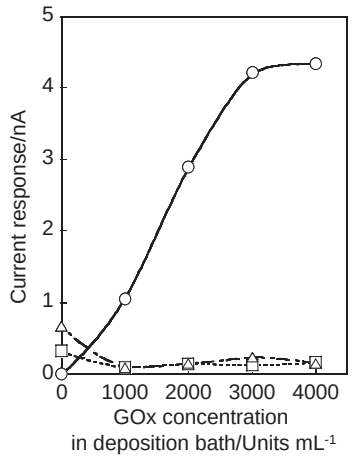

Figure 7. Current responses for oxidation of (○) 15 mmol dm^{-3} glucose, (□) 0.5 mmol dm^{-3} ascorbic acid, and (△) 0.5 mmol dm^{-3} uric acid.[30]

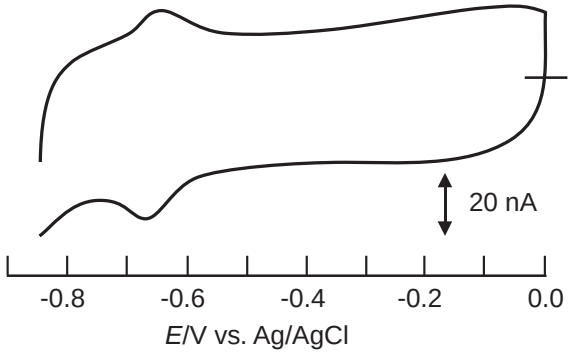

Figure 8. Cyclic voltammogram of GOx/CNT-modified Pt electrode taken in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) at a scan rate of 10 mV s^{-1}.[32]

same time the direct electron transfer between redox enzymes and electrodes has been extensively studied. Among the several attempts, use of carbon nanotube (CNT) seems to be a successful way. It was found that GOx molecules were easily adsorbed on CNT by mixing CNT powder with GOx-contained buffer solution.[31] Putting the resulting mixture on a Pt or a glassy carbon electrode and drying it gave a GOx/CNT-modified electrode. Figure 8 shows cyclic voltammogram of a GOx/CNT-modified Pt electrode, giving distinct redox couple due to FAD at around −0.65 V vs. Ag/AgCl.[32] Similar behavior was confirmed by other research groups.[33,34] When O_2-dissolved electrolyte solution was used, reduction currents were observed, exhibiting occurrence of electrochemical O_2 reduction formulated by

$$\mathrm{GOx(FADH_2)} + \mathrm{O_2} \rightarrow \mathrm{GOx(FAD)} + \mathrm{H_2O_2} \quad (1)$$

$$\mathrm{GOx(FAD)} + 2\mathrm{H^+} + 2\mathrm{e^-} \rightarrow \mathrm{GOx(FADH_2)} \quad (2)$$

Polarization of the electrode at constant potential gave constant reduction current, which was decreased by addition of glucose into the solution because of the glucose oxidation (GOx(FAD) + glucose → GOx(FADH$_2$) + gluconolactone). Plots of magnitude of current decrease as a function of glucose gave a calibration line for quantitative analysis of glucose concentration. In this case, since the detection was conducted

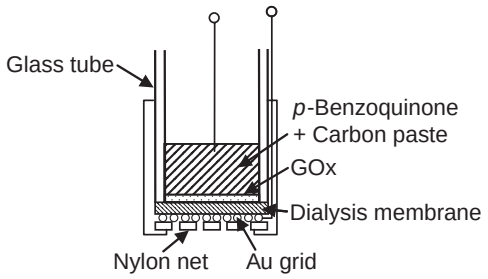

Figure 9. A minigrid-attached film-coated GOx-immobilized *p*-benzoquinone-mixed carbon paste electrode.[36]

by electrochemical reduction, influence by the interference substances was avoidable in the similar manner as that for the reductive detection of glucose using GOx and HP, as shown in Figure 6.[35]

♦ Electrochemical Techniques

Aggressive exclusion of the interference substances by electrochemical means has been proposed by fabricating the specifically designed sensor chip, as shown in Figure 9.[36] The sensing electrode was composed of carbon paste containing *p*-benzoquinone and GOx layer, on which a dialysis membrane was covered. Then, a gold grid electrode was put on the dialysis membrane and was fixed by a nylon sheet. Glucose sensing was conducted by polarizing the sensing electrode at +0.2 V vs. SCE. When potential of +0.5 V vs. SCE was applied to the gold grid electrode, influence by ascorbic acid was excluded by its oxidation before reaching the sensing electrode. This way was confirmed to be effective for the ascorbic acid concentration up to 180 mg dm^{-3}.

Attempt was made to apply the electrochemical AC impedance technique to the biosensing system for detecting the desired species with high selectivity.[37,38] The experiments were conducted using the electrolyte solution containing enzyme, electron mediator, and substrate. It was the key point to choose an electron mediator having redox potential that was more positive than oxidation potential of the interference substances, as shown in Figure 10. The AC impedance measurements were made while applying the DC bias potential (E_{DC}) to the electrode. The E_{DC} was set to around redox potential of the electron

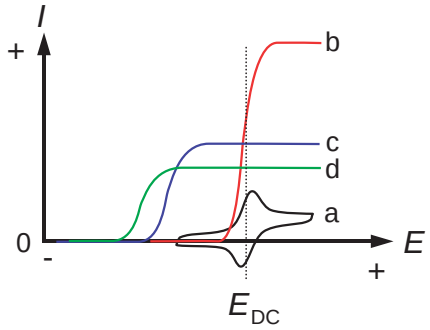

Figure 10. Schematic illustration displaying relationship of *I*–*E* profiles between redox reaction of the electron mediator (a), oxidation of the target substrate (b), and oxidation of interference substances (c), (d). E_{DC}: DC bias potential applied to the electrode in AC impedance measurements.

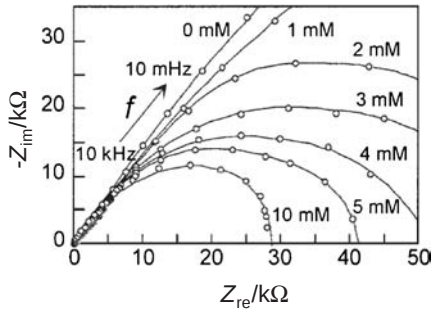

Figure 11. Nyquist plots obtained for a glassy carbon electrode polarized at 0.36 V vs. Ag/AgCl in the electrolyte solution (pH 7) containing 30 mM GOx, 0.2 mmol dm^{-3} ferrocenecarboxylic acid and glucose of the given concentrations.[37]

mediator where oxidation current of the target substrate did not reach its limiting value.

Figure 11 shows the Nyquist plots taken by a glassy carbon electrode polarized at 0.36 V vs. Ag/AgCl in the electrolyte solution containing 30 mmol dm^{-3} GOx, 0.2 mmol dm^{-3} ferrocenecarboxylic acid and glucose in its concentration from 0 to 10 mmol dm^{-3}.[37] A straight line is seen for the electrolyte solution in the absence of glucose, whereas the line tends to draw a semicircle by addition of glucose. Its diameter decreases with an increase in glucose concentration. In this case, the impedance value obtained by extrapolating the semicircle to $f \rightarrow 0$ (Z_{re}^{ex}) is the charge-transfer resistance. Comparison of the Nyquist plots and polarization curves of the same electrolyte solutions have revealed that the Z_{re}^{ex} value corresponded to dE/dI of the polarization curve at 0.36 V vs. Ag/AgCl in each solution. Validity of this relationship was proved by digital simulation studies.[38]

Furthermore, it has been found that the Nyquist profiles were not influence at all by addition of the interference substances. Since their oxidation currents reached the diffusion limiting currents, their dE/dI values (Z_{int}) were infinite at E_{DC}. Therefore, the total impedance value (Z) reflected only the impedance for glucose oxidation (Z_{glu}) because of the equation of $1/Z = 1/Z_{glu} + 1/Z_{int}$. From these viewpoints, the redox species having more positive redox potential would be better as an electron mediator. Then, $[Os(bpy)_3]Cl_2$ was chosen for quantitative analysis of glucose. As shown in Figure 12 a calibration line was prepared by plots of $1/Z$ at 63.3 mHz as a function of glucose concentration. The plots was not influenced at all by addition of both ascorbic acid and uric acid.[39]

♦ Conclusion

Although biological reactions possess perfect substrate selectivity, their artificial utilization sometime loses the inherent properties. In case of using the enzymes as transducers, requirement of electrodes causes unfortunate results. As summarized in this review, several attempts have been made to improve the selectivity with assistance of several techniques. Some techniques are as close as restructuring the vital functions, and others are human's technologies that are far from biological ways. Anyhow, the techniques providing high selectivity to the biosensing systems include useful nanotechnologies that could be useful for other analytical ways, bioreactors, and electronic devises. In other words, nanotechnologies that will be developed from

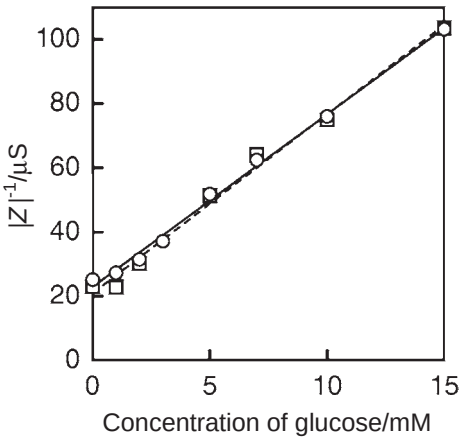

Figure 12. Calibration curves obtained from absolute impedance measured at 63.3 mHz in the absence (square) and presence (circle) of interfering species (0.5 mmol dm^{-3} ascorbic acid and 0.5 mmol dm^{-3} uric acid). Measurements were conducted by polarizing the electrode at 0.65 V vs. Ag/AgCl in 0.1 mol dm^{-3} phosphate buffer solution containing 0.2 mmol dm^{-3} $[Os(bpy)_3]Cl_2$, 750 unit mL^{-1} GOx and 0.1 mol dm^{-3} KCl.[38]

now on may open the new techniques for utilizing biomaterials without loss of their inherent properties. Such the comprehensive developments must be required for artificial utilization of the biomaterials.

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