New Colorimetric Detection of Glucose by Means of Electron-Accepting Indicators: Ligand Substitution of $[Fe(acac)_{3-n}(phen)_n]^{n+}$ Complexes Triggered by Electron Transfer from Glucose Oxidase

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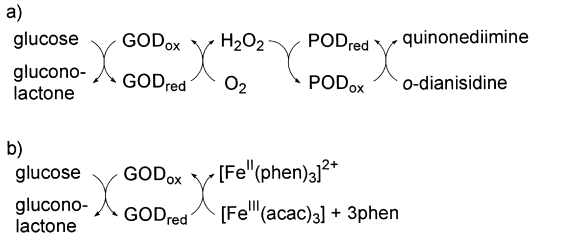
Abstract: A new colorimetric detection technique for glucose, based on electron transfer from glucose oxidase (GOD_{red}) to iron(III) acetylacetonate(acac)/phenanthroline(phen) mixed complexes, is developed. When GOD is added to an aqueous mixture that contains tris(acetylacetonato)iron(III) complex ($Fe^{III}(acac)_3$), 1,10-phenanthroline (phen), and glucose, the color immediately changes from pale yellow to red. The red color originates from formation of tris(1,10-phenanthroline)iron(II) complex ($[Fe^{II}(phen)_3]^{2+}$). Differential pulse voltammetry indicates that cationic, mixed-ligand complexes of $[Fe(acac)_{3-n}(phen)_n]^{n+}$ are formed upon mixing the labile $Fe^{III}(acac)_3$ complex and phenanthroline. The cationic mixed-ligand complexes electrostatically bind to GOD (pI 4.2), and are easily reduced by electron transfer from GOD_{red}. This electron transfer is not affected by the presence of oxygen. The reduced complex $[Fe^{II}(acac)_{3-n}(phen)_n]^{(n-1)+}$ then undergoes rapid ligand exchange to $Fe^{II}(phen)_3$. Formation of the colored Fe^{II} complex is repressed when the salt concentration in the mixture is increased, or when anionic bathophenanthroline disulfonate (BPS) is employed in place of phenanthroline. The use of labile metal complexes as electron acceptors would be widely applicable to the design of new biochromic detection systems.

Keywords: analytical methods · biosensors · electron transport · glucose oxidase · iron

Introduction

The development of sensing systems for biomolecules continues to be an important area of research.[1–6] The recognition of biomolecules has been achieved by the use of enzymes, as exemplified by glucose sensors.[7–10] The enzyme glucose oxidase (GOD) specifically converts glucose into gluconolactone, and this oxidation reaction is accompanied by reduction of the cofactor flavine adenine dinucleotide (FAD). $FADH_2$ in GOD_{red} is re-oxidized by oxygen and the hydrogen peroxide produced is electrically detectable. Amperometric glucose sensors have also been devised by immobilizing GOD in conducting polymer matrices such as polypyrroles.[8] Cyanide-bridged mixed-valence complexes of iron, cobalt, nickel, and ruthenium,[9] and ferrocene derivatives[10] have been also employed as electron mediators. In addition, colorimetric detection of glucose has frequently been used in laboratories. The popular scheme for the colorimetric detection of glucose is shown in Scheme 1a.[11] Hydrogen peroxide formed by the oxidation of glucose is decomposed by peroxidase (POD_{red}), and ensuing oxidation of *o*-dianisidine by POD_{ox} yields the colored product. This scheme consists of multiple chain reactions, and relies on POD



Scheme 1. Reaction schemes for the colorimetric detection of glucose. a) The conventional POD-based colorimetric detection system. b) The present POD-free system. Electron transfer from GOD_{red} to $[Fe(acac)_3]$ is promoted by the spontaneous formation of cationic mixed-ligand complexes $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$.

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employed in excess to keep the reaction of GOD rate-determining. The development of artificial electron acceptors may replace POD and would lead to more simple colorimetric techniques. In this study, we devised a novel POD-free method for the colorimetric detection of glucose, by introducing an inorganic electron acceptor that changes color upon receiving electrons from GOD_{red}.

Our reaction scheme is shown in Scheme 1b. We have sought electron acceptors that display color changes upon receiving an electron from GOD_{red}. In this scheme, it is desirable that the electron-transfer process is not affected by the presence of oxygen. Association of GOD and the acceptor molecule should be favorable, since it would facilitate the electron transfer between them. As GOD is anionic at neutral pH (pI 4.2), the acceptor should preferably possess cationic charges. A neutral complex, acetylacetonato iron(III) ($[Fe^{III}(acac)_3]$), was chosen as a starting material, since it converts to cationic mixed-ligand complexes in the presence of 1, 10-phenanthroline (phen). The enzymatic reduction of the mixed-ligand complex is expected to trigger the formation of $[Fe^{II}(phen)_3]$, which can easily be detected by its intense metal-to-ligand charge transfer (MLCT) absorption.

Results and Discussion

Spectroscopic studies: When GOD (0.16 mg mL^{-1}) was added to a solution of $[Fe^{III}(acac)_3]$ (0.5 mM), 1, 10-phenanthroline (phen, 4.0 mM), and glucose (200 mM) in phosphate buffer (10 mM, pH 7.0), an immediate color change from light yellow to red was observed. Figure 1A displays the UV-visible absorption spectrum of the above mixture. An absorption peak is apparent at 510 nm with a shoulder at 480 nm, and its intensity increases with time. Figure 1B (curve a) shows the time course of absorbance increase at 510 nm after the addition of GOD. When the mixture is devoid of either GOD (curve b), glucose (curve c) or 1, 10-phenanthroline (curve d), the absorption intensity at 510 nm does not increase. Thus, the presence of all components is required for the color change.

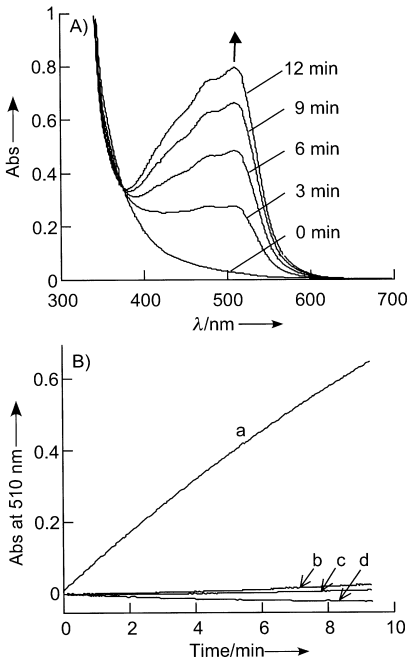

Figure 1. A) Spectral changes after the addition of GOD to phosphate buffer (10 mM) containing $[Fe^{III}(acac)_3]$ (0.5 mM), phenanthroline (4.0 mM), and glucose (200 mM); pH 7.0; [GOD] = 0.16 mg mL^{-1}. B) Time courses of absorption intensity changes at 510 nm. a) Absorbance increase shown for the sample examined in Figure 1A, b) without GOD, c) without glucose, and d) without phenanthroline.

The dependence of absorption intensity at 510 nm on the concentration of phenanthroline was investigated (Figure 2A, concentration of $[Fe^{III}(acac)_3]$, 0.5 mM). These spectra were recorded when the steady state was attained, that is, after

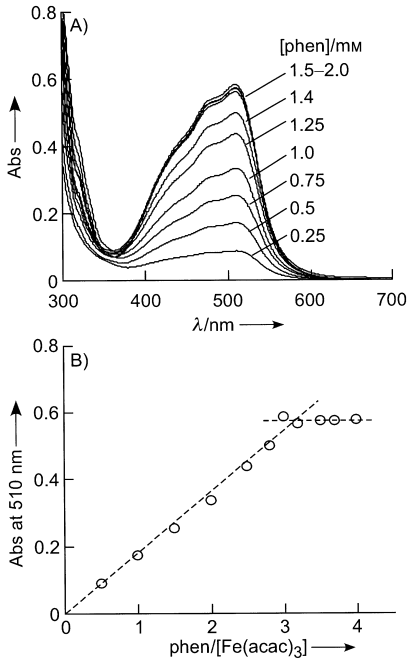

Figure 2. A) Spectral changes observed upon the addition of GOD (0.32 mg mL^{-1}) to phosphate buffer (10 mM). $[Fe(acac)_3]$ (0.5 mM), phenanthroline (0.25–2.0 mM), glucose (200 mM). Each spectrum was obtained after reaching the equilibrium (ca. 120 minutes after mixing). Cell length, 1 mm. B) Dependence of absorption intensity on the molar ratio of phen$[Fe(acac)_3]$. Absorbance was measured at 510 nm.

120 minutes of mixing. The absorption intensity at 510 nm increased linearly with the increase in the concentration of phenanthroline from 0.25 to 1.5 mM, and was saturated above 1.5 mM. Figure 2B displays absorbance at 510 nm as a function of the molar ratio of phen to $[Fe^{III}(acac)_3]$. The absorption intensity at 510 nm levels off when the molar ratio of phen to $[Fe^{III}(acac)_3] = 3$, indicating that the stoichiometry of the complex formed is Fe^{II}/phen = 1:3. To confirm the formation of $[Fe^{II}(phen)_3]^{2+}$ complex, we separately synthesized the complex by mixing $FeCl_2$ and three equivalents of phenanthroline. The synthesized $[Fe^{II}(phen)_3]Cl_2$ showed a MLCT band at 510 nm (molecular extinction coefficient $\varepsilon = 1.11 \times 10^4\ M^{-1}\ cm^{-1}$),[12], which is identical with that observed in Figure 2A ($[Fe^{III}(acac)_3]$ (0.5 mM), phen (1.5 mM)). These results clearly indicate that the $[Fe^{III}(acac)_3]$ complex is converted to $[Fe^{II}(phen)_3]^{2+}$ by the enzymatic reaction of GOD, and this conversion is responsible for the observed color change. The magnetic moment of $[Fe^{III}(acac)_3]$ complex

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is known to be approximately 5.9 μ_B at 80–300 K,[13, 14] indicating that the Fe^{III} ion is in the high-spin state $[d^5:(t_{2g})^3(e_g)^2]$. Generally, high-spin complexes are labile to ligand substitution reactions. On the other hand, the strong crystal field of phenanthroline ligand renders the $[Fe^{II}(phen)_3]^{2+}$ complex in the low-spin state, which is inert to ligand substitution. Thus the present colorimetric detection of glucose is based on the ingenious combination of ligand exchange and redox behavior of iron complexes.

Association of iron complexes with GOD: To investigate whether the ligand exchange of $[Fe^{III}(acac)_3]$ complex precedes the electron transfer, or is triggered by the electron transfer from GOD_{red}, the phenanthroline complex $[Fe^{III}(phen)_3]Cl_3$ was synthesized and was used as an electron acceptor. Figure 3A shows the time courses of the absorption

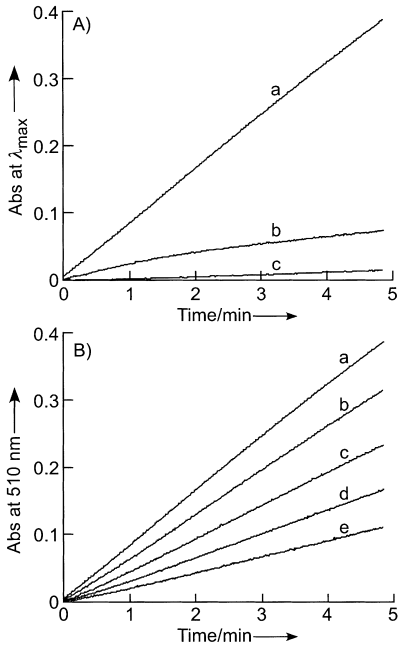

Figure 3. A) Time courses of the absorption maximum intensity upon the addition of GOD (0.16 mg mL^{-1}) to aqueous glucose (glucose; 200 mM in 10 mM phosphate buffer, pH 7.0). a) $[Fe^{III}(acac)_3]$ (0.5 mM) + phenanthroline (4.0 mM), b) $[Fe^{III}(phen)_3]Cl_3$ (0.5 mM), c) $[Fe^{III}(acac)_3]$ (0.5 mM) + BPS (4.0 mM). B) Dependence of absorption increase on the added salt concentration. [GOD] = 0.16 mg mL^{-1}, [glucose] = 200 mM, phosphate buffer (10 mM, pH 7.0). Concentrations of NaCl; a) 0, b) 10, c) 25, d) 50, and e) 100 mM.

band at λ_{max} based on the enzymatic reaction of GOD. When $[Fe^{III}(phen)_3]Cl_3$ was used in place of $[Fe^{III}(acac)_3]$ (curve b), formation of $[Fe^{II}(phen)_3]$ was repressed compared to the standard enzymatic reaction system (curve a, phen was added to the mixture containing GOD, glucose and $[Fe^{III}(acac)_3]$). Therefore, $[Fe^{III}(acac)_3]$ is not totally converted to $[Fe^{III}(phen)_3]^{3+}$ prior to the electron transfer,[15] but instead undergoes partial ligand substitution and changes to cationic, mixed-ligand Fe^{III} complexes, that is, $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$ ($n = 1$ or 2), as discussed later.

When anionic bathophenanthroline disulfonate (BPS) was used instead of 1,10-phenanthroline, the absorption increase due to the formation of $[Fe^{II}(BPS)_3]^{4-}$ complex was almost negligible (curve c), in spite of the larger molecular coefficient (MLCT, λ_{max}, 535 nm, $\varepsilon = 22{,}400\ M^{-1}\ cm^{-1}$).[12] The suppressed electron transfer would probably be ascribed to the electrostatic repulsion between the negatively charged GOD and the BPS-substituted iron complexes.

Complexation of the cationic mixed-ligand complexes $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$ and GOD_{red} is further supported by the effect of ionic strength. Figure 3B shows the time courses of the absorption increase at 510 nm at different NaCl concentrations (0–100 mM). Apparently, the formation of $[Fe^{II}(phen)_3]$ complex is suppressed at higher NaCl concentrations. Generally, electrostatic interactions are shielded under high salt concentrations, and, therefore, the electrostatic interaction between GOD and the iron(III) complexes plays an important role in the efficiency of electron transfer. These observations support the formation of cationic mixed-ligand complexes $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$, which are electrostatically bound to GOD, as schematically shown in Figure 4. The reduction of $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$ by GOD_{red} provides $[Fe^{II}(acac)_{3-n}(phen)_n]^{(n-1)+}$, which subsequently undergoes ligand-substitution to $[Fe^{II}(phen)_3]^{2+}$.

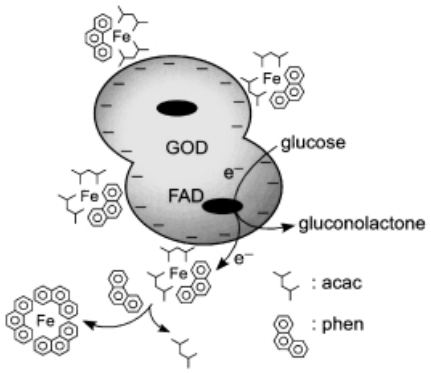

Figure 4. Schematic illustration of the glucose colorimetry system triggered by the electron transfer from GOD_{red} to Fe^{III} complexes. The cationic mixed-ligand Fe^{III} complexes bind to GOD and receive an electron from GOD_{red}. The formed Fe^{II} complexes undergo ligand substitution with phen and a $[Fe^{II}(phen)_3]^{2+}$ complex is formed.

Electrochemical studies: Under aerobic conditions, it is possible that molecular oxygen competes with the electron transfer from GOD_{red} to the $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$ complex formed in situ. The effect of oxygen concentration on the formation of $[Fe^{II}(phen)_3]^{2+}$ complex was determined for solutions that had been bubbled with a) oxygen, b) air, and c) nitrogen (Figure 5). The initial coloring rates increase in proportion to the increase in GOD concentration, and the rates obtained for samples a–c were almost identical. Therefore, the electron transfer from GOD_{red} to the mixed-ligand complex $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$ is not interfered with by oxygen.

The formation of the mixed-ligand complex $[Fe^{III}(acac)_{3-n}(phen)_n]^{n+}$ and its reduction potential were evaluated by means of differential pulse voltammetry (DPV). Figure 6 displays the voltammograms obtained for

0947-6539/02/0824-5582 $ 20.00+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 24

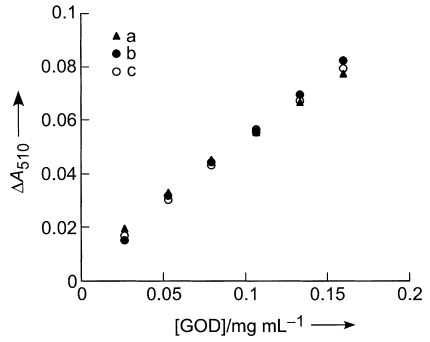

Figure 5. Initial absorption changes at 510 nm (ΔA_{510}) at varied GOD concentrations. GOD was added to a) O_2-bubbled, b) air-bubbled, and c) N_2-bubbled glucose solutions. Phosphate buffer (10 mM, pH 7.0), glucose (200 mM), [$Fe^{III}(acac)_3$] (0.5 mM), phenanthroline (4.0 mM).

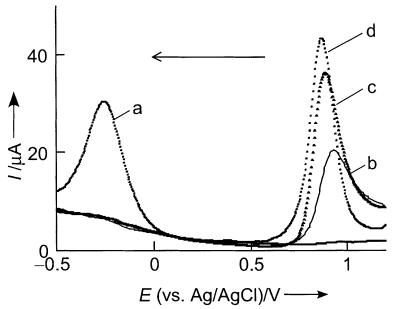

Figure 6. Differential pulse voltammograms in 10 mM phosphate buffer (pH 7.0, 0.5 M KCl). a) [$Fe^{III}(acac)_3$] (0.5 mM), b) [$Fe^{III}(acac)_3$] (0.5 mM) + phenanthroline (4.0 mM), c) [$Fe^{III}(acac)_3$](0.5 mM) + phenanthroline (4.0 mM) + glucose (50 mM) + GOD (0.16 mg mL^{-1}), d) separately prepared [$Fe^{II}(phen)_3$]Cl_2 (0.5 mM). The arrow indicates the direction of potential step.

a) [$Fe^{III}(acac)_3$], b) an aqueous mixture of [$Fe^{III}(acac)_3$] complex and phenanthroline, and c) [$Fe^{II}(phen)_3$]$^{2+}$ produced by the enzymatic reaction of GOD in an [$Fe^{III}(acac)_3$]/phen mixed system. A voltammogram of a separately prepared [$Fe^{II}(phen)_3$]Cl_2 complex is shown in Figure 6d for comparison. Complex [$Fe^{III}(acac)_3$] showed a single reduction wave centered at $E = -0.26$ V (vs Ag/AgCl, Figure 6a). On the other hand, the mixture of [$Fe^{III}(acac)_3$] and phenanthroline (without GOD and glucose) gave a remarkably positive-shifted wave at $E = +0.93$ V (Figure 6b). This peak is different from the reduction potential of [$Fe^{II}(phen)_3$]Cl_2 (reference sample, $E = +0.87$ V Figure 6d), and is consistent with the formation of the mixed-ligand complexes [$Fe^{III}(acac)_{3-n}(phen)_n$]$^{n+}$ ($n = 1$ or 2). The observed reduction potential of the mixed-ligand complexes is more positive than the standard redox potential of oxygen (0.48 V vs. Ag/AgCl), and therefore it is reasonable to suggest that they act as superior electron acceptors compared to oxygen.

Interestingly, the [$Fe^{II}(phen)_3$]$^{2+}$ complex produced by the enzymatic reaction of GOD shows a negative shift in the reduction potential (from +0.93 V to +0.89 V) with enhanced intensity (Figure 6b → c). The observed reduction potential in Figure 6c is comparable to the separately prepared reference sample (Figure 6d), but its current intensity was slightly smaller. In general, current intensities depend on the diffusion coefficient of the redox species, and are affected by their concentration and the number of electrons that participate in reactions.[16] As these voltammograms were obtained under the same experimental conditions, the observed difference in the current intensity is ascribed to the difference in diffusion coefficients. It is probable that the [$Fe^{II}(phen)_3$]$^{2+}$ complex produced enzymatically is still bound to GOD and that it possesses a smaller diffusion coefficient than the monomeric [$Fe^{II}(phen)_3$]$^{2+}$ complex. A similar observation has been reported for the [$Fe^{II}(phen)_3$]$^{2+}$ complex bound to DNA.[17]

Effect of ligand and iron complexes to GOD: The inhibitory effect of phenanthroline, [$Fe^{III}(acac)_3$], and [$Fe^{II}(phen)_3$]$^{2+}$ complexes on the enzymatic activity of GOD depicted in Scheme 1a was investigated.[11] GOD (2.6×10^{-5} mg mL^{-1}) was added to an oxygen-bubbled phosphate buffer solution (10 mM, pH 7.0) that contained glucose (20–200 mM), POD (0.16 mg mL^{-1}), and *o*-dianisidine (0.15 mM). The absorption increase at 436 nm due to the oxidation of *o*-dianisidine was monitored and kinetic parameters (K_m, V_{max}) were obtained by a Lineweaver–Burk plot (Table 1).

Table 1. Kinetic parameters determined by the oxidation of *o*-dianisidine by GOD (Scheme 1a).

System	K_m [mM]	$V_{max} \times 10^{10}$ [mol s^{-1}]
Scheme 1a	98	7.3
Scheme 1a + phen[a]	98	7.3
Scheme 1a + $Fe^{II}(phen)_3$[b]	98	6.6

[a] Phenanthroline was added at a concentration of 1 mM. [b] [$Fe^{II}(phen)_3$]$^{2+}$ was added at a concentration of 0.05 mM.

As can be seen from Table 1, the obtained K_m and V_{max} values are not affected by the presence of phenanthroline (1 mM). When [$Fe^{II}(phen)_3$]$^{2+}$ is added at a concentration of 0.05 mM, the V_{max} value decreases slightly from 7.3×10^{-10} to 6.6×10^{-10} mol s^{-1}. This may be attributed to binding of [$Fe^{II}(phen)_3$]$^{2+}$ to GOD interfering with binding of the acceptor complex [$Fe^{III}(acac)_{3-n}(phen)_n$]$^{n+}$ to GOD. This may account for the lower detection limit in the present technique (ca. 5×10^{-4} M) compared to that in Scheme 1a (ca. 2×10^{-6} M). Though the sensitivity is not superior to the conventional method at this stage, the simpler reaction scheme and the lack of need for POD may find valuable applications. Design of a better electron-transfer system is now under investigation in these laboratories.

Conclusion

Electron transfer from proteins to metal complexes is one of the fertile areas of supramolecular biochemistry, and the concept of electron-accepting indicators may provide useful tools for elucidating biological electron-transfer processes. The use of in situ formed, mixed-ligand complexes [$Fe^{III}(acac)_{3-n}(phen)_n$]$^{n+}$ provides a novel technique for colorimetric detection of glucose. The simpler reaction scheme and lack of need for peroxidases and *o*-dianisidine indicators add useful features to the present system. In addition, this

technique is not influenced by the presence of oxygen. The present method would be widely applicable to redox enzymes whose colorimetric technique has not been established. Such applications would be attractive targets for supramolecular and analytical chemists to contemplate.

Experimental Section

Materials: Tris(acetylacetonato)iron(III), 1,10-phenanthroline monohydrate (phen), disodium bathophenanthroline disulfonate (BPS), glucose oxidase (GOD, EC 1.1.3.4 from Aspergillus niger), peroxidase (POD, EC 1.11.1.7 from horseradish), and *o*-dianisidine dihydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). β-D-Glucose was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). They were used as received without further purification. Water was purified with a Direct-Q system (Millipore).

Tris(phenanthroline)iron complexes, namely, $[Fe^{II}(phen)_3]Cl_2$ and $[Fe^{III}(phen)_3]Cl_3$, were synthesized by mixing three equivalents of phenanthroline with $FeCl_2 \cdot 4H_2O$ or $FeCl_3 \cdot 6H_2O$, respectively, in pure water. These iron complexes were diluted with phosphate buffer (10 mM, pH 7.0, concentration, $[Fe^{II}(phen)_3]Cl_2 = [Fe^{III}(phen)_3]Cl_3 = 0.5$ mM) for the spectral measurements.

Measurements: UV-visible absorption spectra were recorded on a JASCO V-570 spectrophotometer equipped with a peltier type thermostatic cell holder ETC-505 (temperature, $25 \pm 0.1\,^{\circ}C$). Quartz cells of 1 cm path length were used. Tris(acetylacetonato)iron(III) (1.25 mM, 1.25 mL), phenanthroline (0.62 – 10 mM, 1.25 mL), glucose (3.1×10^{-3} – 1.24 M, 0.5 mL), and the enzyme GOD (0.78 – 5.0 mg mL^{-1}, 0.1 mL) in phosphate buffer solution (10 mM, pH 7.0) were freshly prepared and mixed in the quartz cells. The mixed solutions were kept stirring during the measurements. When the effect of oxygen concentration on the colorimetric detection was to be determined, the stock solutions of $[Fe^{III}(acac)_3]$, phenanthroline, and glucose were bubbled with oxygen, nitrogen, or air for 20 minutes before the mixing step.

The enzymatic activity of GOD was investigated by using the multiple chain reactions shown in Scheme 1a.[11] The oxidation reaction of glucose was achieved by adding GOD (8.0×10^{-4} mg mL^{-1}, 0.1 mL) to the oxygen-bubbled phosphate buffer solution (10 mM, pH 7.0, 3 mL) which contained glucose (20 – 200 mM), POD (0.16 mg mL^{-1}), and *o*-dianisidine (0.15 mM). The absorption increase at 436 nm due to the oxidation of *o*-dianisidine was monitored for 5 minutes, and the kinetic parameters (K_m, V_{max}) were obtained from the Lineweaver – Burk plot.

Differential pulse voltammetry (DPV) was performed with CV-50W (BAS). A standard three-electrode configuration was used with ITO as the working electrode, Ag/AgCl (3 M NaCl) as the reference electrode, and a platinum wire as the counter electrode in a glass vessel at room temperature. The electrolyte solution containing 0.5 M KCl was deoxygenated by purging nitrogen gas for 20 minutes. DPV parameters: scan rate, 20 mV s^{-1}; pulse amplitude, 50 mV; pulse width, 50 ms; pulse period, 200 ms; sampling width, 17 ms.

Acknowledgements

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