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Short communication

Simultaneous determination of charge number and diffusion coefficient of 2-chloro-1,4-dimethoxybenzene in acetonitrile using potential-step chronoamperometry

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Abstract

Potential-step chronoamperometry using a microdisk electrode was carried out for the simultaneous determination of the charge number and diffusion coefficients of natural electron-mediator dimethoxybenzene analogues during electro-oxidation. The process was verified as a one-electron oxidation during the radical cation formation.

Keywords: Potential-step chronoamperometry; Microdisk electrode; Lignin; Electron mediator; Diffusion coefficient

1. Introduction

Aromatic hydrocarbons, which have methoxy moieties, are secondary metabolites produced by white-rot fungus *Phanerochaete chrysosporium* during lignin biodegradation [1]. As pioneering work, the radical cations of dimethoxybenzenes were detected and confirmed by ESR measurement during the enzymatic reaction [2]. Later studies showed that the one-electron oxidized form, i.e., the corresponding cation radicals of methoxybenzyl alcohols and methoxybenzenes, is not only an intermediate but also works as natural electron mediator from the substrate, lignin polymer to the enzyme, lignin peroxidase (LiP) [3]. The three-dimensional X-ray diffraction structure of LiP substantiates the above mechanism, which indicates that direct interaction between the lignin and the active site of the heme prosthetic group of the enzyme is difficult since the heme in LiP is buried inside the protein [4,5]. However, there arises another question that the lifetime estimated from the first-order rate constant of the decay seems too short for the radical cation to be an efficient diffusible mediator [6]. In recent developments, it has been proposed that the formation of a complex of LiP and the cation radical extends the lifetime [7,8]. Thus, the investigation of the properties of the cation radical formed from the aromatic compounds with methoxy groups is an important subject, however, it is not easy to obtain parameters for quantitative kinetic analysis of the reaction of a typical compound such as veratryl alcohol (3,4-dimethoxybenzyl alcohol) since it undergoes a one-electron oxidation followed by a chemical reaction combined with an additional electron transfer reaction [9]. To our knowledge, even the charge number and the diffusion coefficient, which are the essential parameters for the quantitative kinetic analysis, have not been previously obtained. As mentioned above, though dimethoxybenzenes work as mediators, a typical compound, 1,4-dimethoxybenze, is not naturally produced by white-rot fungus [10]. On the other hand, chlorodimethoxybenzenes are secondary metabolites and known to be naturally produced mediators [11]. Baciocchi et al. [12] reported that the role of mediator structure during LiP catalytic process, and the efficiency of 2-chloro-1,4-dimethoxybenzene is second-best to veratryl alcohol in 16 mediators. In order to estimate the yields in LiP-catalysed oxidation of synthetic lignin, model compound, 2-chloro-1,4-dimethoxybenzene, was selected as a mediator since it enabled the distinction between the oxidative products from the substrate and the mediator [13]. Functions of 2-chloro-1,4-dimethoxybenzene and other low-molecular-weight compounds involved in lignin degradation have

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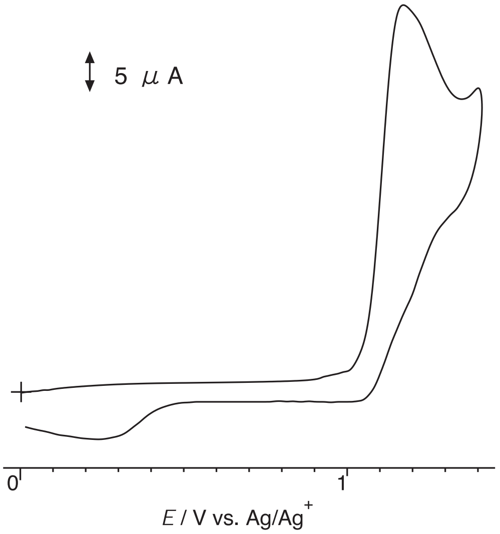


Fig. 1. Cyclic voltammogram for 5 mM 2-chloro-1,4-dimethoxybenzene in 0.6 M $NaClO_4/CH_3CN$ solution at a GC electrode. The sweep rate was 0.1 V s^{-1}.

been discussed in a review article [14]. From the viewpoint of electroanalytical chemistry, dimethoxybenzenes have an advantage because their electrode processes are somewhat simpler compared to that of veratryl alcohol [15]. In this study, we employ the model compound 2-chloro-1,4-dimethoxybenzene and we demonstrate the charge number of the electrode reaction and the diffusion coefficient which are simultaneously determined in the electro-oxidation by performing the potential-step chronoamperometry using a microdisk electrode [16].

2. Experimental

2-Chloro-1,4-dimethoxybenzen (CDMB, Tokyo Kasei), 1,4-dimethoxybenzene (DMB, Kanto Chemicals) and ferrocene (Fc, Tokyo Kasei) were used as received. As a model of a hydrophobic environment, the solvent acetonitrile (Infinity pure grade, Wako) was used. Sodium perchlorate (Wako) as a supporting electrolyte was used since it was reported that the use of typical alkyl ammonium salts like tetrabutylammonium tetrafluoroborate in acetonitrile leads to a deposition on the electrode surface of a p-doped polyphenylene film [17].

Cyclic voltammetry and potential-step chronoamperometry were carried out using the conventional three-electrode cell configuration. The electrode potential was controlled by a potentiostat (Hokuto Denko, HA-501G) and a function generator (Kenwood, FG-281), and the voltammograms and the chronoamperograms were displayed and stored using a digital storage oscilloscope (Tektronics, TDS-1012). A glassy carbon (GC) disk electrode (diameter 1 mm, BAS) and a carbon fiber (CF) microdisk electrode (diameter 33μm, BAS) were used as the working electrode. A Pt spiral electrode and Ag/Ag^+ (0.01 M (mol dm^{-3}) $AgNO_3$ in acetonitrile) electrode were the auxiliary and reference electrodes, respectively. The surfaces of the working GC and CF electrodes were polished on lapping films (9, 6, 3, 2 and 1 μm, 3M Imperial) and then sonicated in water for 3 min. All experiments were performed at room temperature (22 ± 1 °C).

3. Results and discussion

The redox potentials of the substrates were first confirmed by cyclic voltammetry. Fig. 1 shows the cyclic voltammogram of CDMB at the GC working electrode on a conventional time scale. An essentially similar redox property was observed for the oxidation of DMB in acetonitrile except for the oxidation potential ($E_{pa} = 0.99$ V). Though DMB is reported to have a reversible redox property [15], the reverse cathodic current could not observed, suggesting that the corresponding radical cation decompo-

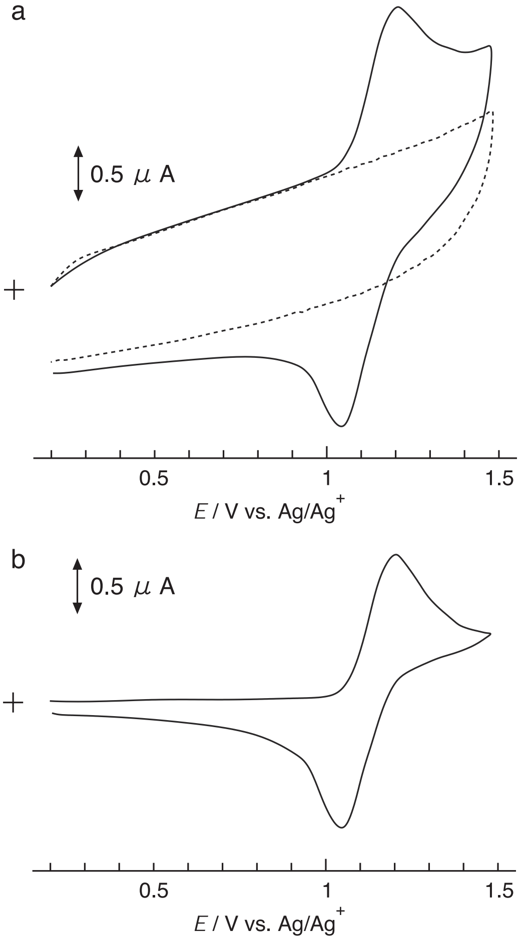
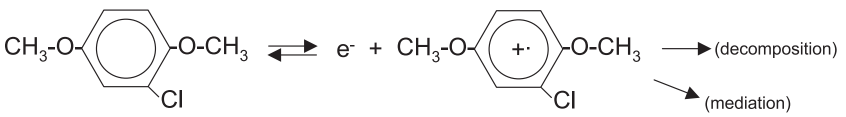


Fig. 2. (a) Cyclic voltammogram for 5 mM 2-chloro-1,4-dimethoxybenzene (solid line) in 0.6 M $NaClO_4/CH_3CN$ solution at a CF electrode. Dashed line is the background. The sweep rate was 500 V s^{-1}. (b) Cyclic voltammogram obtained by background subtraction from (a).



Scheme 1.

ses in the given time scale. In Fig. 2, cyclic voltammograms are shown using a CF working electrode under a linear diffusion controlled condition. In these cases, chemically reversible voltammograms were obtained and the ratio of the cathodic and anodic peak currents (i_{pc}/i_{pa}) of the voltammogram approaches almost unity. Therefore, the effect of the chemical reaction following the electron transfer can be considered negligible. The estimation of the n value will confirm the elucidation of the above electrode reaction (vide infra) (Scheme 1).

During oxidation of dimethoxybenzenes, since the forward reaction for a potential step to the limiting current region would be unperturbed by the following reaction, even if it existed, we can determine the step potential from the inspection of Fig. 2 [18]. The potential was stepped from an initial value at which no faradaic current flows to a value at which the electrode reaction of the reacting species occurs at a mass transfer-controlled rate. The electrode potential was stepped from 0 to +1.3 V vs. Ag/Ag$^+$. At +1.3 V, CDMB is oxidized at a mass transfer-controlled rate since it is quite positive compared to the anodic peak potential. The chronoamperograms were corrected for the residual current by subtracting the corresponding chronoamperogram recorded with the supporting electrolyte solution. The typical chronoamperogram of the oxidation of CDMB is shown in Fig. 3. The intercept and the slope of Fig. 3 allow one to determine the charge number n involved in the electrode reaction and the diffusion coefficients D [16].

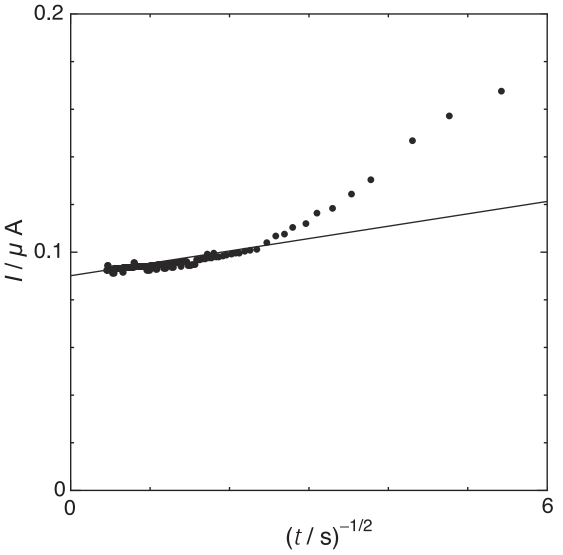


Fig. 3. Chronoamperogram for the oxidation of 5 mM 2-chloro-1,4-dimethoxybenzene in 0.6 M $NaClO_4/CH_3CN$ solution at a CF electrode. The electrode potential was stepped from 0 to +1.3 V vs. Ag/Ag$^+$.

Chronoamperometric data were analyzed by the following equation [19,20]

$$I = nFADc\left\{\frac{4}{\pi r} + \frac{1.4367}{\pi\sqrt{Dt}} + \frac{0.02813r^2}{\pi(Dt)^{3/2}} - \frac{0.00081r^4}{\pi(Dt)^{5/2}}\right\}$$

$$\text{with } Dt/r^2 \geq 0.423 \qquad (1)$$

where F is the Faraday constant, A is the electrode area, c is the concentration of the electroactive species and r is the diameter of the working electrode. If we assumed that, for example, the diameter of the working electrode was 12.5 μm and the diffusion coefficient of the substrate was 5×10^{-5} cm^2 s^{-1}, then the third and fourth terms in Eq. (1) would occupy 4% of the whole during the electrolysis time of 50 ms and 2% during 100 ms and 0.9% during 200 ms, so the both terms can be negligible for the longer electrolysis in the analysis. The n and D values can be simultaneously determined when the electrode area and the concentration of the substrate are previously known. The electrode area in the study was checked by the potential-step chronoamperometric measurement for the 5 mM Fc/Fc$^+$ couple in 0.6 M $NaClO_4/CH_3CN$ using $n = 1$, and determined to be 32.8 ± 0.7 μm. For the curve in Fig. 3, a change in the slope was made over the electrolysis time domain between 0.2 s (2.24 s$^{-1/2}$) and 4.8 s (0.46 s$^{-1/2}$). This time domain is enough long to neglect the higher terms in Eq. (1). The slope and intercept in Fig. 3 were analyzed by using Eq. (1). Table 1 indicates the result of the n and D values. The n values can be regarded as almost unity for both cases and the electrode process of interest was verified to be radical cation formation. Our D value of DMB in acetonitrile is close to that in CH_2Cl_2/CF_3COOH reported by Fabre and co-workers [17]. In the case of veratryl alcohol (VA), the lifetime of the radical cations is too short-lived (half-life, 0.5 ms) to diffuse away from the active site of the enzyme [7,21]. Therefore, it was suggested that an enzyme-bound radical formation and it was demonstrated that the presence of an LiP compound II–VA$^+$ complex [3,7]. On the other hand, DMBs are oxidized to the corresponding cation radicals, implying a possible function as a diffusible medi-

Table 1

	n	D/10^{-5} cm^2 s^{-1}	$E^{o\prime}$ /V
DMB	0.94 ± 0.09	1.88 ± 0.07	0.99 ± 0.01
CDMB	0.96 ± 0.12	2.75 ± 0.14	1.12 ± 0.01

ator in LiP oxidations because of their relative stability as cation radicals confirmed by ESR spectroscopy [2,21]. The mediation efficiency is reported to be a function of the redox potential for the mediator and CDMB is more effective than DMB [12]. It is interesting that the order of the efficiency is consistent with that of *D* value in this study. This presumably means the difference of the mediation mechanism of VA and DMBs during LiP oxidation processes. In conclusion, for the oxidation of dimethoxybenzenes, the one-electron oxidation process was verified and the appropriate diffusion coefficients were obtained.

References

[1] J.R. Obst, T.K. Kirk, Isolation of lignin, in: W.A. Wood, S.T. Kellogg (Eds.), Biomass Part B, Lignin, Pectin, and Chitin, Methods in Enzymology, vol. 161, Academic Press, San Diego, 1988, pp. 3–6.

[2] P.J. Kersten, M. Tien, B. Kalyanaraman, T.K. Kirk, The ligninase of *Phanerochaetechrysosporium* generates cation radicals from methoxybenzenes, J. Biol. Chem. 260 (1985) 2609–2612.

[3] A. Khindaria, C. Nie, S.D. Aust, Determination and characterization of the lignin peroxidase compound II–veratryl alcohol cation radical complex, Biochemistry 36 (1997) 14181–14185.

[4] T.L. Poulos, S.L. Edwards, H. Wariishi, M.H. Gold, Crystallographic refinement of lignin peroxidase at 2 Å, J. Biol. Chem. 268 (1993) 4429–4440.

[5] T. Choinowski, W. Blodig, K.H. Winterhalter, K. Piontek, The crystal structure of lignin peroxidase at 1.7 Å resolution reveals a hydroxy group on the C^{β} of tryptophan 171: a novel radical site formed during the redox cycle, J. Mol. Biol. 286 (1999) 809–827.

[6] L.P. Candeias, P.J. Harvey, Lifetime and reactivity of the veratryl alcohol radical cation, J. Biol. Chem. 270 (1995) 16745–16748.

[7] A. Khindaria, I. Yamazaki, S.D. Aust, Veratryl alcohol oxidation by lignin peroxidase, Biochemistry 34 (1995) 16860–16869.

[8] D. Sheng, M.H. Gold, Oxidative polymerization of ribonuclease A by lignin peroxidase from *Phanerochaete chrysosporium*, Eur. J. Biochem. 259 (1999) 626–634.

[9] D.C. Goodwin, S.D. Aust, T.A. Grover, Evidence for veratryl alcohol as a redox mediator in lignin peroxidase-catalyzed oxidation, Biochemistry 34 (1995) 5060–5065.

[10] P.J.M. Teunissen, J.A. Field, 2-Chloro-1,4-dimethoxybenzene as a novel catalytic cofactor for oxidation of anisyl alcohol by lignin peroxidase, Appl. Environ. Microbiol. 64 (1998) 830–835.

[11] P.J.M. Teunissen, J.A. Field, 2-Chloro-1,4-dimethoxybenze as a mediator of lignin peroxidase catalyzed oxidations, FEBS Lett. 439 (1998) 219–223.

[12] E. Baciocchi, M.F. Cerini, O. Lanzalunga, S. Mancinelli, Lignin peroxidase catalyzed oxidation of 4-methoxymandelic acid. The role of mediator structure, Tetrahedron 58 (2002) 8087–8093.

[13] F. D'Acunzo, O. Lanzalunga, First synthesis of a polysaccharide-supported lignin model compound and study of its oxidation promoted by lignin peroxidase, Biochem. Biophys. Res. Commun. 313 (2004) 17–21.

[14] R. ten Have, P.J.M. Teunissen, Oxidative mechanisms involved in lignin degradation by white-rot fungi, Chem. Rev. 101 (2001) 3397–3413.

[15] D.T. Sawyer, A. Sobkowiak, J.L. Roberts Jr., Electrochemistry for Chemists, 2nd ed., Wiley, New York, 1995, pp. 459–462.

[16] M. Kakihana, H. Ikeuchi, G.P. Sato, K. Tokuda, Diffusion current at microdisk electrode-application to accurate measurement of diffusion coefficients, J. Electroanal. Chem. 117 (1981) 201–211.

[17] B. Fabre, K. Michelet, N. Simonet, J. Simonet, Electrochemical oxidation of 1,4-dialkoxybenzenes in organic media. Reversible one-electron process in a methylene chloride/trifluoroacetic acid mixture and electropolymerization reaction in dry acetonitrile, J. Electroanal. Chem. 425 (1997) 67–75.

[18] A.J. Bard, L.R. Faulkner, Electrochemical Methods Fundamentals and Applications, 2nd ed., Wiley, New York, 2001, p. 498.

[19] K. Aoki, J. Osteryoung, Diffusion-controlled current at the stationary finite disk electrode, J. Electroanal. Chem. 122 (1981) 19–35.

[20] K. Aoki, J. Osteryoung, Formulation of diffusion-controlled current at very small stationary disk electrodes, J. Electroanal. Chem. 160 (1984) 335–339.

[21] D.K. Joshi, M.H. Gold, Oxidation of dimethoxylated aromatic compounds by lignin peroxidase from *Phanerochaete chrysosporium*, Eur. J. Biochem. 237 (1996) 45–57.