Rapid Determination of Ascorbic acid, Dehydroascorbic acid and Total Vitamin C by Electrochemiluminescence with a Thin-layer Electrochemical Cell

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Abstract This paper reports on a rapid and sensitive method for the simultaneous determination ascorbic acid (H_2A), dehydroascorbic acid (DHA) and total vitamin C by electrochemiluminescnece (ECL) using a thin-layer electrochemical cell. Significant ECL signals can be generated by the anodic oxidation of $Ru(bpy)_3^{2+}$ in presence of H_2A or DHA in pH 8.8 phosphate buffer solution. Because of the extremely small dead volume of the thin-layer cell (ca. 1.5 μL), almost amount of H_2A is assumed to be completely oxidized to DHA with a short pre-electrolysis step. As a result, it is possible to determine the reductive vitamin C (H_2A) by square wave voltammetry before the pre-electrolysis step, while total vitamin C (sum of H_2A and DHA) is able to be determined at a subsequent ECL step. The method was employed for the determination of vitamin C in commercial beverages with the analytical results in good agreement with the certified values.

Keywords *Electrochemiluminescence; tris(2,2'-bipyridine)ruthenium; thin-layer electrochemical cell; ascorbic acid; dehydroascorbic acid; vitamin C*

1. Introduction

Vitamin C is an umbrella term for ascorbic acid (H_2A, a diproton weak acid) and dehydroascorbic acid (DHA). H_2A is the dominant reduced form of vitamin C while DHA is the oxidized form. They are found to be in equilibrium in most food products. It is well known that H_2A plays an important role in a number of biological functions, such as anti-oxidant activity, a role in the metabolism of several biologically important compounds, and a potential role in anti-cancer activity. DHA is also an important, interesting but somewhat enigmatic compound in biological systems [1]. Numerous analytical methods, including spectrophotometry [2,3], amperometry [4,5], voltammetry [6,7], iodometric titration [8], have been developed for the determination of H_2A in foods, pharmaceuticals and biological samples. High performance liquid chromatography (HPLC) is the most widely used technique for vitamin C determination. H_2A is known as an electroactive species which has been easily detected in amperometric and colometric systems. However, DHA is difficult to be detected by electrochemistry because of its low electroactivity. Moreover, it has only weak absorbance in UV region. Therefore, electrochemical detection of DHA is usually carried out by reducing of DHA to H_2A with dithiothreitol or enzymes [9-11]. The concentration of DHA can thereafter be calculated by subtraction of the H_2A concentration from the total vitamin (H_2A + DHA) concentration measured in a different chromatographic run. Similarly, in photometric detection procedure, DHA can be detected after precolumn derivation using regents such as *o*-phenylenediamine or 4,5-dimethyl-*o*-phenylenediamine, to form a qninoxaline derivative that absorbs light in the ultraviolet region [15,16]. But these procedures are complicated; the impurities have an influence on sensitivity and reproducibility of the measurement significantly.

Electrochemiluminescence (ECL) is the production of light from an electrolytic system. It has been accepted by the analytical chemist as a powerful tool for detection of many inorganic and organic compounds [17-19]. For example, ECL from $Ru(bpy)_3^{2+}$ (where bpy = 2,2'-bipyridine) has been used to measure the concentrations of coreactants such as oxalate and TPA to levels as low as 10^{-8} M [20]. The determination of H_2A in HPLC system with ECL detection was first introduced by Chen and Sato [21]. Thereafter, Zorzi *et al* reported that DHA was also detectable in $Ru(bpy)_3^{2+}$ based ECL system, and demonstrated that H_2A and DHA were able to be determined simultaneously in a single chromatoghraphic run [22].

In the present study, an alternative method was proposed for the determination of H_2A, DHA and total vitamin C by ECL using a thin-layer electrochemical cell, without the separation producers. Because of the extremely small dead volume of the cell, almost the amount of H_2A can be electrochemically oxidized to DHA in with a short pre-electrolysis step. It is possible to determinate the reductive vitamin C (H_2A) by square wave voltammetry before pre-electrolysis step, while the total amount of vitamin C (sum of H_2A and DHA) can be determined by the subsequent ECL step. The approach has several clearly advantages such as simple, rapid, cost-effective, and sensitive features. It was then employed for the determination of vitamin C in commercial beverages. Meanwhile, the features of ECL in the thin-layer cell were primarily discussed in this study.

2. Experimental

2.1. Chemicals

Tris(2,2'-bipyridyl)ruthenium(II) chloride [$Ru(bpy)_3Cl_2 \cdot 6H_2O$] was purchased from Aldrich, H_2A and DHA were purchased at the highest grade available from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used as received. The other reagents were of analytical grade purchased from Nacalai Tesque (Osaka, Japan). Working standard solutions were prepared by precise dilution of stock solutions with water. Phosphate buffer solution (PBS) was prepared by equimolar amount of disodium hydrogenphosphate (Na_2HPO_4) and potassium dihydrogenphosphate (KH_2PO_4). The appropriate pH of the buffer was adjusted with orthophosphoric acid or sodium hydroxide. All solutions were with distilled water purified by a WS200 distillation system (Yamato Scientific Co., Tokyo, Japan).

2.2. Apparatus

The electrochemical experiments were carried out by model 660 CHI electrochemical analyzer (CH Instruments, Austin, TX, USA). The fabricated thin-layer ECL cell is schematically represented in Fig. 1. The cell was sandwiched by 2 Teflon blocks, a Teflon spacer (25 μm in thickness) and a glass cover plate, giving a total cell volume of approximate 1.5 μL. A glassy carbon disk electrode (ϕ 1 mm) was served as working electrode which was housed into a Teflon block. The electrode was polished with aqueous slurries of alumina particles with size of 0.03 μm (BAS, Japan) prior to use. The sample solution was filled into the cell cavity through the stainless tube by a 2-mL syringe. A glass tube (id. 10 mm, length 40 mm) was served as solution overflow reservoir, where a reference silver/silver chloride (Ag/AgCl) electrode and a Pt counter electrode were placed. The light emitting from the electrode surface was measured with a H7468-1 photomultiplier tube (PMT) module (Hamamatsu Photonics, Shizuoka, Japan) via an optical fiber which was positioned opposite to the working electrode. The PMT module was controlled by a notebook computer and a laboratory-written software package via RS-232C interface. The program was developed by Microsoft Visual Basic 6.0. All ECL measurements were conducted in a light proof box.

2.3. Sample preparation

The commercial beverages were used as samples for vitamin C determination. The samples were first diluted 1:10 with 0.1 M PBS (pH 8.8), and then filtrated through a syringe filter (pore size: 0.2 μm, Fuji Photo Film, Japan). After that, the filtrate was filtered through a NEXUS solid-phase extraction (SPE) cartridge (Varian, USA) to eliminate some hydrophobic organic substances. The sample was mixed then with equivalent volume of 1.0 mM $Ru(bpy)_3^{2+}$ (v/v=1:1) for ECL measurement.

3. Results and discussion

3.1 ECL behavior of H_2A / $Ru(bpy)_3^{2+}$ system in a thin- layer cell

The feature of the thin-layer electrochemical cell was first evaluated by cyclic voltammetry. Figure 2 shows the cyclic voltammograms of 1.0 mM $[Fe(CN)_6]^{3-}$ in 0.1 M HCl at various scan rates. A symmetrical redox pair for $[Fe(CN)_6]^{3-}$ / $[Fe(CN)_6]^{4-}$ were observed around +0.21 V *vs*. Ag/AgCl, and the value of the peak potential separation (ΔE_p) was ca. 20 mV, which was much less than 59 mV as expected in a semi-infinite diffusion system [23]. Both the anodic and the cathodic peak currents were found to be proportional to the scan rate from 1 to 20 mV/s. The observations revealed the characteristic of a diffusion-limited mass transport in a thin layer cell, where the diffusion layer is larger than the cell thickness [24].

Figure 3(A) shows the voltammograms of 0.50 mM H_2A and 0.50 mM $Ru(bpy)_3^{2+}$ in 0.1 M PBS (pH 8.8) measured in a thin-layer (solid line) and a conventional electrochemical cell under diffusion-controlled condition (dashed line) with a same GC electrode. Figure 3(B) is the corresponding ECL intensity – potential profiles. In Fig.3(A), two oxidative waves around + 0.3 and +1.1 V *vs*. Ag/AgCl, are due to the oxidization of H_2A and $Ru(bpy)_3^{2+}$ at GC electrode. No reduction wave of H_2A was observed because the oxidized product was immediately hydrolyzed to DHA with low electoactivity. It can be seen that the thin-layer cell resulted in much better symmetrical voltammteric peaks and an improved baseline current. This was due to the fact that the current dropped not to a diffusion-limited finite value but effectively to zero after the depletion of the oxidative species at the electrode surface. In Fig. 3(B), the distinguished ECL signals were observed around at +1.15 V in both thin-layer and diffusion controlled conditions. The ECL mechanism of H_2A/$Ru(bpy)_3^{2+}$ can be interpreted by Scheme 1, in which the excited state $*Ru(bpy)_3^{2+}$ was produced by the co-reaction between the electrochemically intermediate product of H_2A and the electrogenerated $Ru(bpy)_3^{3+}$ following the electrode reaction.

Scheme 1

$$H_2A \rightarrow HA^- + H^+ \quad (pK_{a1} = 4.17,\ pK_{a2} = 11.57) \qquad (1)$$

$$HA^- \rightarrow HA\cdot + e^- \text{ (rate-determining step)} \qquad (2)$$

$$HA \rightarrow A\cdot^- + H^+ \quad (pK_{a3} = -0.45, \text{ fast}) \qquad (3)$$

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \qquad (4)$$

$$A\cdot^- + Ru(bpy)_3^{3+} \rightarrow *Ru(bpy)_3^{2+} + \text{product} \qquad (5)$$

$$*Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{2+} + h\upsilon\,(620\text{ nm}) \qquad (6)$$

As was discussed in our previous study [25], at weak basic condition (pH 8~9), H_2A was dissociated to form ascorbate monoanion (HA^-), which was oxidized to the ascorbate radical ($HA\cdot$) at the rate-determining step (Eq (2)). Since $HA\cdot$ is a very strong acid (pK_a = –0.45), it subsequently underwent a rapid dissociate to form of a relatively stable radical anion ($A\cdot^-$) [26]. $*Ru(bpy)_3^{2+}$ was suggested to be produced by the energetic electron transfer between electrochemically generated $Ru(bpy)_3^{3+}$ and the radical anion $A\cdot^-$. The

deprotonation step of H_2A was considered as an important factor in the ECL reaction. The effect of the buffer pH on the ECL intensity of H_2A was investigated over pH range of 6 ~ 11. When pH was 8.8, the ECL intensity reached the maximum value. When pH was greater than 10, the background signal was increased dramatically. This can be attributed to the competitive reaction between $Ru(bpy)_3^{3+}$ and OH^- [27].

Comparing with the diffusion-controlled condition, the ECL intensity in a thin-layer cell decayed more rapidly when the potential was scanned more positive than +1.2 V vs. Ag/AgCl in thin layer cell. According to Scheme 1, $Ru(bpy)_3^{2+}$ should not be consumed during ECL reaction because it was regenerated at the electrode surface even though in a cell with extremely small volume [28]. Since the coreactant HA^- was not able to be diffused from the bulk solution in a thin layer cell, its concentration was depleted entirely in the cell cavity with increasing of potential which consequently resulted in a rapid decrease of ECL intensity.

Figure 4 shows the dependence of ECL intensities on H_2A concentration under thin-layer and the diffusion-controlled conditions, respectively. Under the diffusion controlled condition, the light intensity was proportional to H_2A concentration in a low concentration region (0 - 1.0 mM), but was significantly quenched when the concentration was higher than 2 mM. It was noted in our previous study that the quenching effect became more evidence under the forced mass transport condition. We suggested that $*Ru(bpy)_3^{2+}$ was quenched by HA^- diffusing from the bulk solution due to the electron transfer (ET) route.

Scheme 2

$$*Ru(bpy)_3^{2+} + HA^- \rightarrow [Ru(bpy)_3^{+}\cdots HA\cdot] \quad (7)$$

$$\rightarrow Ru(bpy)_3^{+} + A^- + H^+ \quad (7a)$$

$$\rightarrow Ru(bpy)_3^{2+} + HA^- \quad (7b)$$

The intermediate state, $[Ru(bpy)_3^{+}\cdots HA\cdot]$ was assumed to be formed in diffusional control process. Once the intermediate state was formed, both the decomposition reaction (7a) and the back electron transfer reaction (7b) could occur. Because the collision frequency between $*Ru(bpy)_3^{2+}$ and HA^- can be enhanced in the presence of a large amount of HA^- or under the forced mass transport conditions, significant quench effect was observed [25]. In contract, however, the ECL quenching was not observed in a thin-layer cell even though the H_2A concentration was higher than 5 mM. This can be explained by the limited-diffusion feature of thin-layer cell because there was no diffusional HA^- from the bulk solution.

3.2 ECL behavior of DHA / $Ru(bpy)_3^{2+}$ system in a thin-layer cell

In order to determination of total vitamin C, the quantitative determination of DHA was examined in a thin-layer cell. Previous studies have shown that DHA could be served as a coreactant in $Ru(bpy)_3^{2+}$ based ECL system but the light emitting intensity was weaker in comparison with H_2A [22, 29]. DHA was oxidized slowly at the GC electrode, and resulted in a low oxidation current around + 0.8 V vs. Ag/AgCl in pH 8.8 PBS [29]. The generation of $*Ru(bpy)_3^{2+}$

in DHA / $Ru(bpy)_3^{2+}$ system was supposed to be the homogeneous chemical co-reaction between the electrogenerated $Ru(bpy)_3^{3+}$ and an oxidative intermediate product of DHA (R), as is shown in Scheme 3.

Scheme 3

$$DHA \rightarrow R + ne^- \quad \text{(slow)} \quad (8)$$

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (4)$$
$$Ru(bpy)_3^{3+} + R \rightarrow {}^*Ru(bpy)_3^{2+} + \text{products} \quad (9)$$
$${}^*Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{2+} + h\upsilon \quad (5)$$

In Fig. 5, it shows the corresponding ECL – potential profiles of DHA with various concentrations and a calibration curve. ECL intensity was found to be proportional to the DHA concentration in a range from 0 to 1 mM, with a correlation coefficient of 0.989 and a detection limit of ca. 25 μM (S/N ≥3). Since H_2A could be readily converted into DHA by electrochemical oxidization in a thin layer cell, it is possible to determination of total vitamin C (H_2A + DHA) based on DHA quantitation.

3.3 Determination of total vitamin C by thin-layer ECL with pre-electrolysis step

Figure 6 shows the effect of pre-electrolysis time on the cyclic voltammograms of 0.5 mM H_2A in 0.1 M PBS (pH=8.8) containing 0.5 mM $Ru(bpy)_3^{2+}$, as well as the corresponding ECL profiles in a thin-layer cell. The pre-electrolysis was occurred at +0.6 V where H_2A could be oxidized to DHA. It was seen that the voltammetric peak current of H_2A (at +0.2 V) decreased rapidly with increasing of the pre-electrolysis time, and almost disappeared after 3 s electrolysis time, indicating that H_2A was efficiently oxidized to DHA within a short electrolysis time. Meanwhile, the ECL intensity was also decreased with increasing of the pre-electrolysis time, and the signal was no longer alternated when the pre-electrolysis time was over 3 s. As has been discussed above, the weaker ECL intensity of DHA can be due to the relatively lower kinetics of the electron transfer for the electrochemical oxidation of DHA at GC electrode. For a $Ru(bpy)_3^{2+}$ / DHA system, however, both voltammetric and ECL responses were not changed after the pre-electrolysis step, as is shown in Fig. 6(b, d). To verify if the total vitamin C can be determined by ECL as a form of DHA, thin-layer ECL with pre-electrolysis step was employed for the determination of the samples mixed with a certain amount of H_2A and DHA. In this experiment, the concentration of the total vitamin C ([H_2A] + [DHA]) was kept constant (= 0.5 mM), while the H_2A fractions {f_{H2A} = [H_2A]/([H_2A]+[DHA])} were varied. Square wave voltammetry (SWV) was conducted before the thin-layer ECL measurements. The pre-electrolysis time was 3 s. In Fig. 7, the ECL intensities along with the SWV signals for the oxidation of H_2A were plotted against f_{H2A}. It is interesting to note that the SWV peak current at +0.2 V increased linearly with f_{H2A}, while the ECL intensity was not dependent on f_{H2A}. We thus suggested that the concentration of H_2A could be selectively determined by SWV in a H_2A / DHA mixture solution, while the total concentration of vitamin C could be determined by a subsequent ECL measurement, because (H_2A in) vitamin C was efficiently converted to DHA in a pre-electrolysis step.

3.4 Determination of H_2A, DHA and total vitamin C in commercial beverages

The thin-layer ECL combined with SWV was applied to determination of total vitamin C, H_2A, DHA in the commercially available beverages. The samples were lemon water (House Wellness, Japan), acerola drink (Nichirei co., Japan) and Orange juice (Dole co., Japan) respectively. The analytical results (averaged form 4 measurements) are given in Table 1. The relative standard deviations (RSDs) of the determination were below 5.0%. Since H_2A is easily oxidized to DHA when exposed to air, DHA is present in natural fruits, but at low level. Good agreements were achieved in comparison with the recommended values provided by the manufactories. It thus proved that the method in drinks assay was practical usage. Since the total time required for the analysis of one sample (including the sample preparation) was less than 20 minutes, the improvement of the analytical reliability is expected in vitamin C assay.

4. Conclusions

A thin-layer cell was developed and characterized in the application for ECL measurement. The present study has shown that significant ECL signals could be generated by the anodic oxidation of $Ru(bpy)_3^{2+}$ in presence both H_2A and DHA in pH 8.8 phosphate buffer solution. Because of the extremely small dead volume of the thin-layer cell, almost amount of H_2A was assumed to be completely oxidized to DHA with a short pre-electrolysis step. As a result, it was possible to determine the reductive vitamin C (H_2A) by square wave voltammetry before the pre-electrolysis step, while total vitamin C (sum of H_2A and DHA) was able to be determined in a subsequent ECL step. The method was employed for the determination of vitamin C in commercial beverages with the analytical results in good agreement with the certified values.

Acknowledgements

This work was partially supported by a Grant-in-Aid (No. 18655030) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

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Figure legends

Fig. 1 Schematic diagrams of the thin-layer ECL cell. (A) the over view; (B) the cross-sectional view. (a) Ag/AgCl reference electrode, (b) Pt counter electrode, (c) glassy carbon working electrode (1 mm diameter), (d) inlet for sample solution, (e) syringe for sample injection, (f) waste receiver (a glass tube), (g) electrolyte solution, (h) Teflon blocks, (i) Teflon spacer (25 μm), (j) glass slide, (k) screw for clamp, and (l) optical fiber (1 mm core).

Fig. 2 Cyclic voltammograms of 1.0 mM $Fe(CN)_6^{3-}$ in 0.1 M HCl. (A) Under diffusion-limited condition (thin-layer cell); (B) under semi-infinite diffusion conditions (observed in a conventional electrochemical cell). Inset shows the plots of peak currents vs. scan rate.

Fig. 3 Cyclic voltammograms and corresponding ECL responses of 0.50 mM H_2A in PBS containing 0.50 mM $Ru(bpy)_3^{2+}$. The solid lines are obtained under diffusion-limited condition (thin-layer cell) while the dashed lines are measured under semi-infinite diffusion condition. Potential scan rate was 50 mV/s and voltage of PMT was biased at +700 V.

Fig.4 Dependence of ECL intensity on concentration of H_2A, measured under thin-layer condition (●) and semi-infinite diffusion condition (▲), respectively. The other conditions were the same as in Fig.3.

Fig. 5 ECL potential profiles of DHA with different concentrations in a thin-layer cell. Inset shows the calibrations curve. The otherconditions were the same as in Fig. 3.

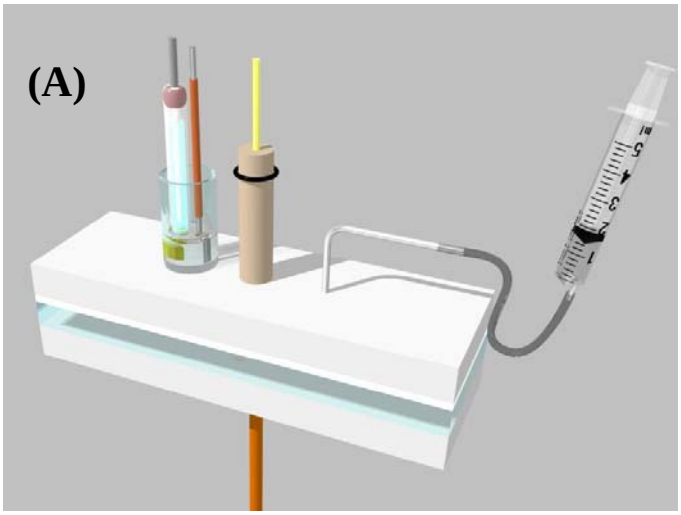
Fig. 6 Effect of the pre-electrolysis time on (A) cyclic voltammograms and (B) ECL responses observed for (a) $Ru(bpy)_3^{2+}$ / H_2A and (b) $Ru(bpy)_3^{2+}$ / DHA systems in a thin-layer cell. The pre-electrolysis step was carried out at +0.6 V *vs.* Ag/AgCl. Other conditions were the same as in Fig. 3.

Fig. 7 Plots of ECL intensity and the SWV peak current as a function of H_2A fraction (f_{H2A}). The concentration of total vitamin C was kept 0.5 mM, and the pre-electrolysis step was carried out at +0.6 V for 3 s. Parameters for SWV measurement: initial potential 0.0 V, end potential 1.0 V, pulse amplitude 25.0 mV, step potential 4 mV, and frequency 15 Hz. The SWV oxidation peak currents were measured at +0.2 V *vs.* Ag/AgCl.

Table 1. Determination of vitamin C contents ($\mu g\ mL^{-1}$) in commercial beverages.

Samples	Recommended value [a] ($\mu g\ mL^{-1}$)	Total vitamin C (H_2A + DHA) [b] ($\mu g\ mL^{-1}$)	H_2A [c] ($\mu g\ mL^{-1}$)	DHA [d] ($\mu g\ mL^{-1}$)
Lemon water	2000	1800	1490	310
Acerola drink	1200	1180	1090	110
Orange Juice	440	440	410	30

a) certified values from the manufactures.
b) measured by thin-layer ECL; results are the average value of four measurements.
c) measured by SWV; results are the average value of four measurements.
d) concentration difference between the total vitamin and H_2A.



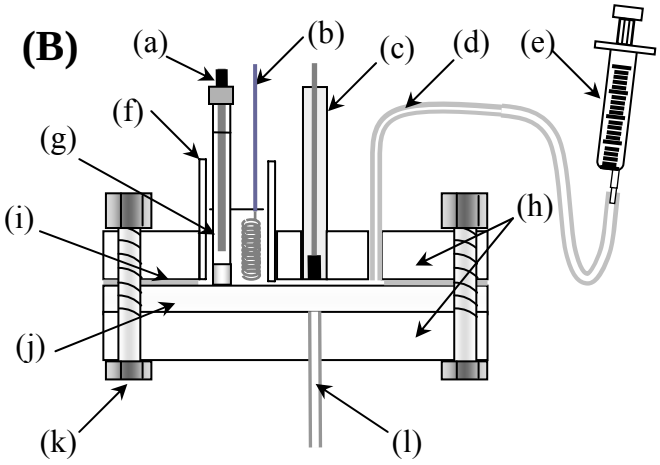
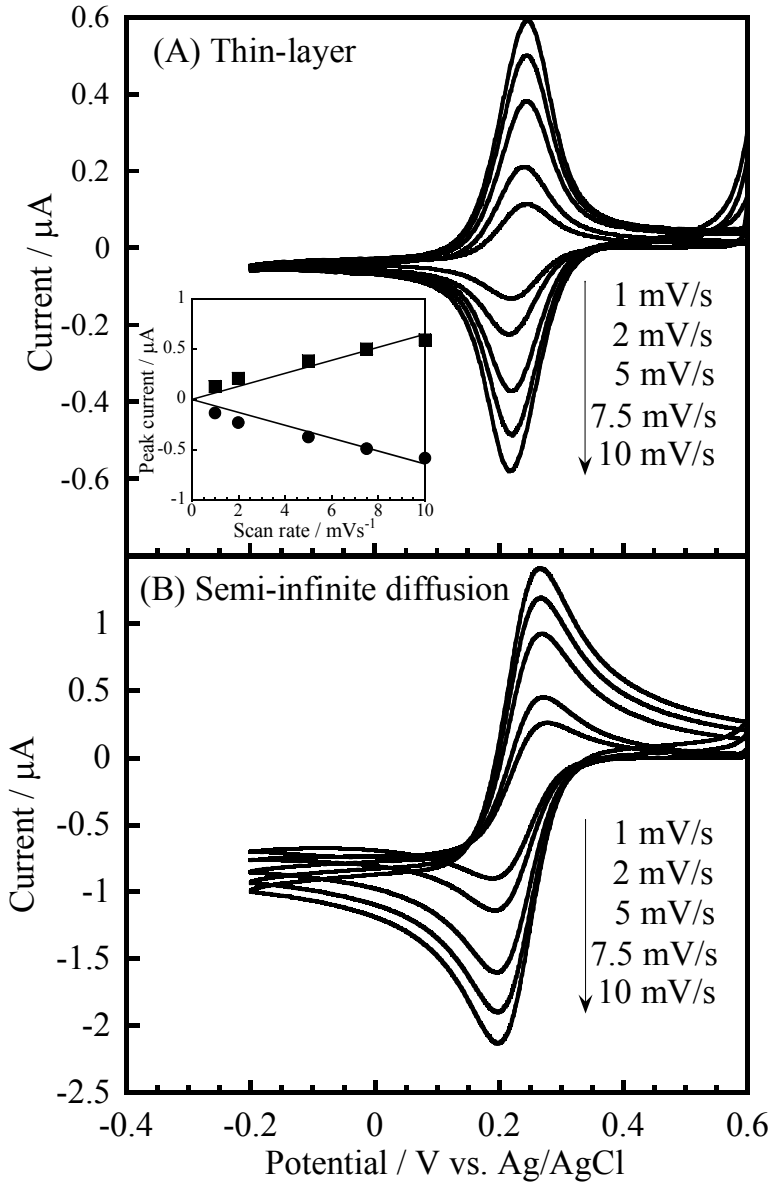


Fig. 1 F. Takahashi



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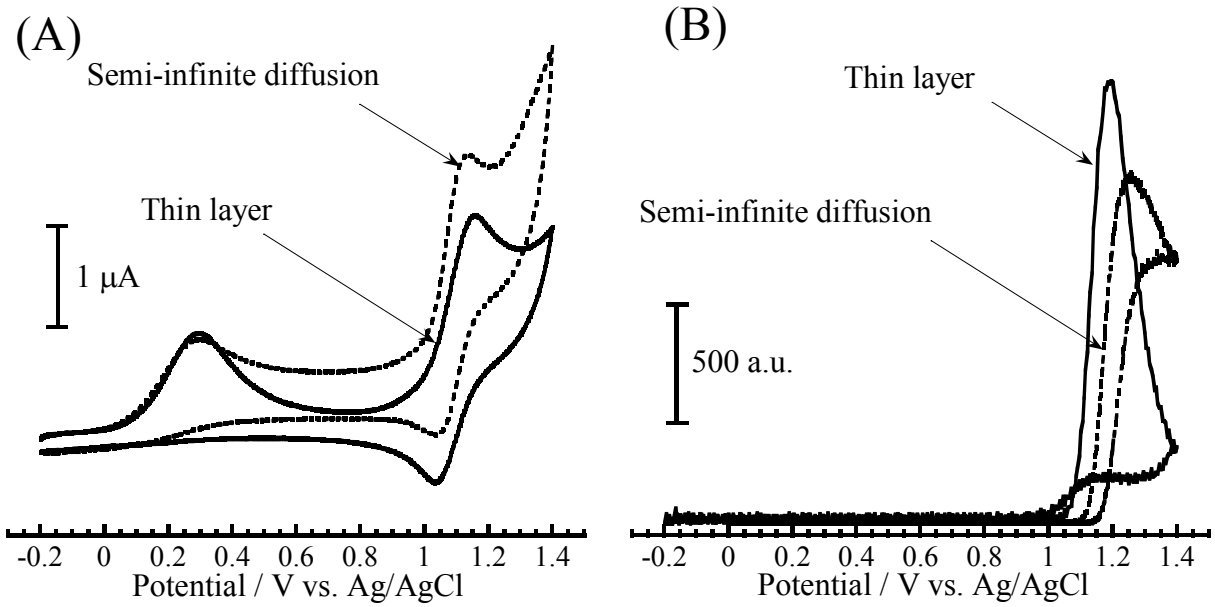

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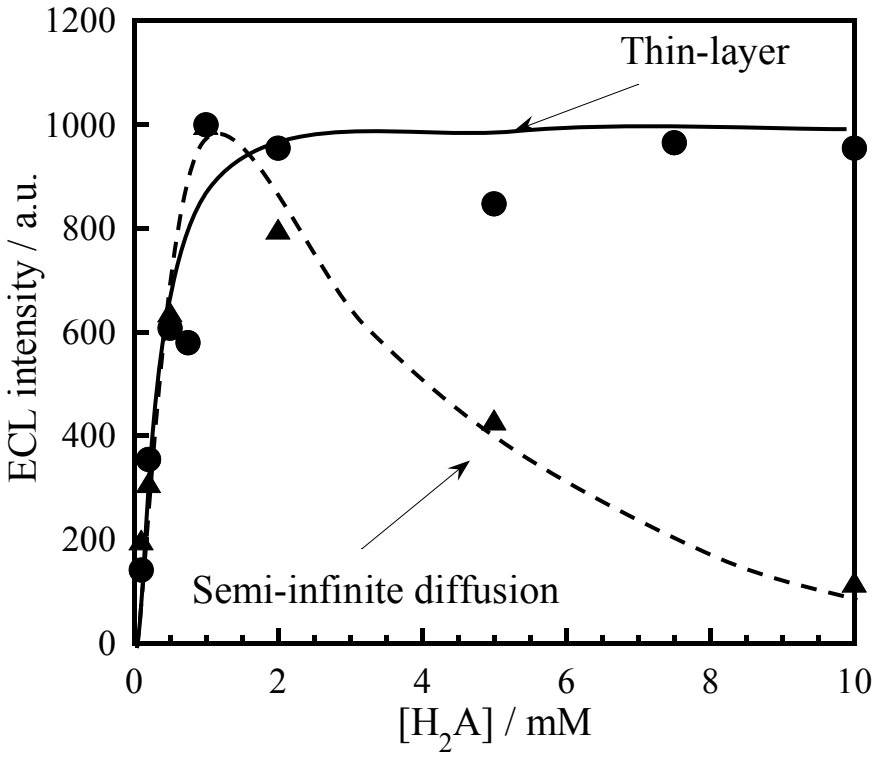

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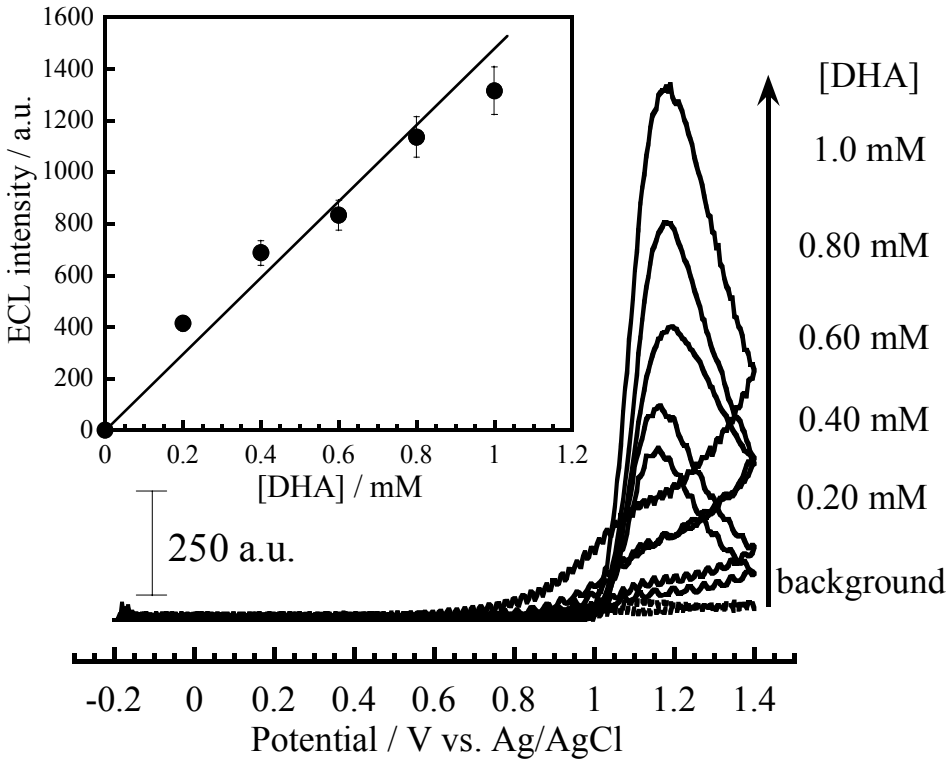

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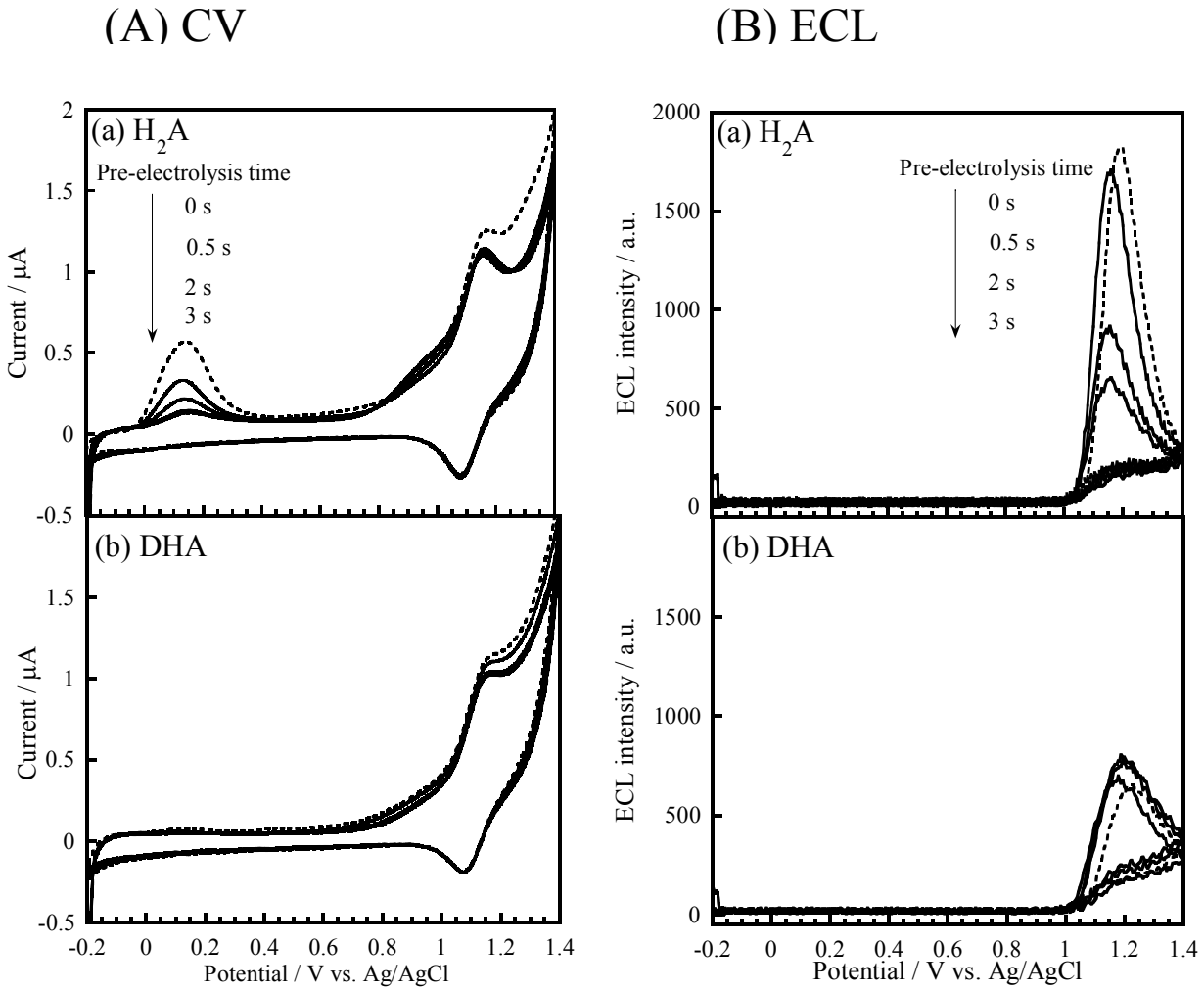

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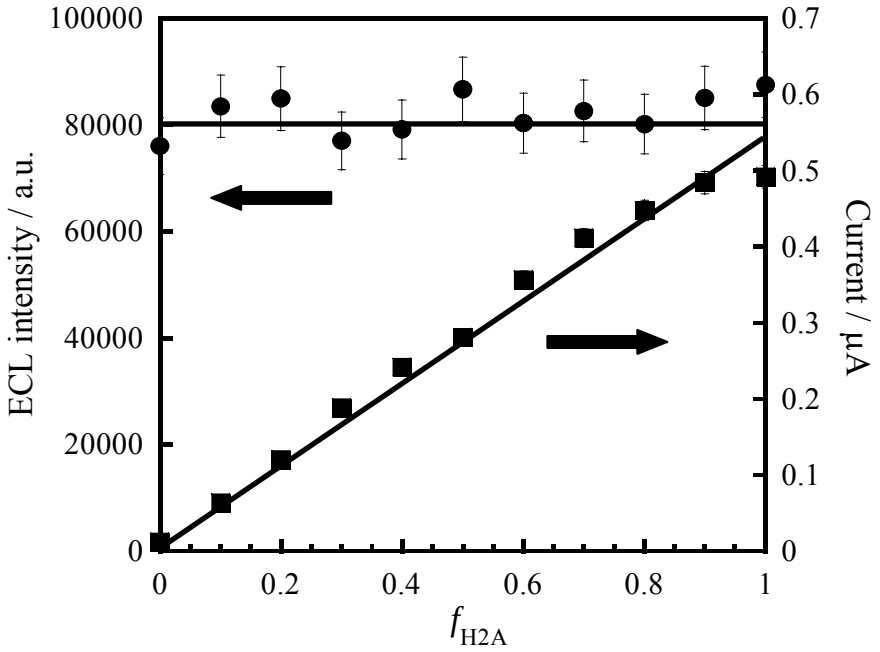

Fig. 7 F. Takahashi