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# Electrochemical analysis of autodisplayed adrenodoxin (Adx) on the outer membrane of *E. coli*

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ABSTRACT

In this work, adrenodoxin (Adx) was expressed on the outer membrane of *E. coli* by autodisplay and then the iron–sulfur cluster was incorporated into apo-Adx by an anaerobic reconstitution process. For the determination of the redox potentials of the iron–sulfur clusters of the autodisplayed Adx, *E. coli* cells with autodisplayed Adx were immobilized on a gold electrode modified with a self-assembled monolayer of mercaptoundecanoic acid (MUA). From the repeated cyclic voltammetry (CV) analysis, the *E. coli* (10 mM HEPES buffer, pH 7.0) with autodisplayed Adx showed significant changes in shape with an oxidation peak at +0.4 V (vs. Ag/AgCl) and a reduction peak at −0.3 V (vs. Ag/AgCl) after the reconstitution process for the incorporation of the iron–sulfur cluster. From the repeated CV analysis in the reduction and oxidation potential ranges, the iron–sulfur clusters of the autodisplayed Adx were observed to undergo reversible redox reactions via direct electron transfer to the MUA-modified gold electrode.



## 1. Introduction

Adrenodoxin (Adx) is a small, soluble, acidic mitochondrial protein that contains one iron–sulfur cluster as a prosthetic group and usually acts as an electron transfer protein [1]. Adx had essential roles in the biosynthesis of mammalian steroids in the adrenal glands, including glucocorticoids, mineralocorticoids, and androgens [2]. As a component of the class I cytochrome P450 system in the adrenal mitochondria, Adx is known to transfer electrons from a NADPH-dependent adrenodoxin reductase (AdR) to CYP11A1 and the enzymes of the CYP11B family [3].

Recently, bovine adrenodoxin (Adx) has been expressed on the outer membrane of *E. coli* by autodisplay technology, with the iron–sulfur cluster then incorporated into apo-Adx by an anaerobic reconstitution process [4]. By adding purified adrenodoxin reductase and P450 CYP11A1, a whole-cell biocatalyst system was established, which effectively synthesizes pregnenolone from cholesterol. The addition of artificial membrane constituents or detergents, which have previously been indispensable for obtaining functional steroidal P450 enzymes, was not necessary. The activity of the whole cell (0.21 nmol $h^{-1}$ $nmol^{-1}$ CYP11A1) was in the same range as those obtained earlier in reconstitution assays. The whole-cell system has been reported to be an easy-to-handle and stable tool for the expression of membrane-associated P450 enzymes without the need for microsome preparation or the reconstitution of artificial membrane vesicles [5,6].

In this work, *E. coli* cells with autodisplayed Adx were immobilized onto gold electrodes modified with a self-assembled monolayer of mercaptoundecanoic acid (MUA) for the determination of the redox potentials of the iron–sulfur cluster of the autodisplayed Adx [3,7,8]. The oxidation and reduction peaks were estimated from the repeated CV analysis and the iron–sulfur cluster of the autodisplayed Adx were observed to undergo reversible redox reactions via direct electron transfer to the MUA-modified gold electrode.

## 2. Materials and methods

### 2.1. Materials

LB broth was purchased from Duchefa (Haarlem, Netherlands), Aprotinin was purchased from Roche Korea (Seoul, Korea), and carbenicillin, lysozyme, DNase, and all of the other analytical grade chemicals were purchased from Sigma-Aldrich Korea (Seoul, Korea).

### 2.2. Autodisplay of Adx

*E. coli* UT5600 (F-ara14 leuB6 azi-6 lacY1 proC14 tsx-67 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi1, DompT-fepC266) was used for the expression of the autotransporter fusion proteins. The plasmid pJM7, which encodes the AIDA-I autotransporter, and the plasmid pATAdx 04H366, which encodes bovine adrenodoxin, have been previously described [4–6]. Bacteria were routinely grown at 37 °C in Luria-Bertani (LB) broth containing 100 mg of carbenicillin per liter,

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and ethylenediaminetetraacetate (EDTA) was added to a final concentration of 10 μM and β-mercaptoethanol was added to a final concentration of 10 mM [9–14].

### 2.3. Reconstitution of the iron–sulfur cluster [4,5]

The chemical reconstitution of the iron–sulfur cluster and the refolding of Adx on the surface of the *E. coli* cells was performed under strictly anaerobic conditions in 50 mM Tris–Cl buffer (pH 7.4) at ambient temperature, as previously described [4,5]. For chemical reconstitution, the bacterial suspension with a calculated final $OD_{578}$ of 50 (4 ml) was concentrated in 6 M guanidine hydrochloride, supplemented with 1 mM β-mercaptoethanol and 0.2 mM ferrous ammonium sulfate, and was slowly titrated with a solution containing 100 mM $Li_2S$ and 10 mM dithiothreitol. Cells were incubated for 15 min, washed twice in 100 mM potassium phosphate buffer (pH 7.4), and then used for the whole-cell activity assays.

### 2.4. Electrochemical analysis

The gold electrode was prepared by sequential sputtering of Ti layer with a thickness of 2 nm and Au layer with a thickness of 100 nm on a glass substrate with an area of $1.5 \times 0.5$ $cm^2$. The clean gold electrodes were rinsed with absolute ethanol several times and then incubated in 5 mM MUA in ethanol overnight at 4 °C. The MUA-modified electrode was then rinsed with ethanol and 10 mM HEPES, and then dried in $N_2$ gas. After the modification of the electrode, *E. coli* was immobilized on the modified surface at a concentration of $1 \times 10^9$ cells/ml ($OD_{578}$ = 1.0) for 4 h and then washed three times with 10 mM HEPES buffer. Finally, the electrode was transferred onto the electrochemical cell for measurement [3,7,8]. The background buffer solution (10 mM HEPES) in the electrochemical cell was carefully bubbled with argon to remove the oxygen before measurement. All electrochemical measurements were performed using a potentiostat from iVIUM Technologies (Compactstat, Netherlands). An external reference electrode of Ag/AgCl and a counter electrode made of Pt (Metrohm, Switzerland) were used for analysis of the redox couple. The potentials mentioned here are all in comparison with this reference electrode [15–18]. For the cyclic voltammetry (CV), the scan rate was controlled to be 50 mV/s and the scan range was set to be between +0.6 V and −0.6 V against Ag/AgCl reference electrode as described in each graph.

## 3. Results and discussion

### 3.1. Autodisplay of Adx and reconstitution of the Fe–S cluster

In this work, adrenodoxin (Adx) was expressed on the outer membrane of *E. coli* by the transformation of an autodisplay vector with the Adx gene (pAT-ADX 04H366) into *E. coli* (UT5600). Adx is known to be a small acidic protein of 14 kDa with 14 aspartates and 10 g lutamates and only 6 lysines and 5 arginines out of its total 128 amino acid residues [1]. As shown in Fig. 1(A), Adx is a passenger protein that is autotransported with the β-barrel protein, and is connected to the β-barrel protein through a linker peptide with 70 glycine residues

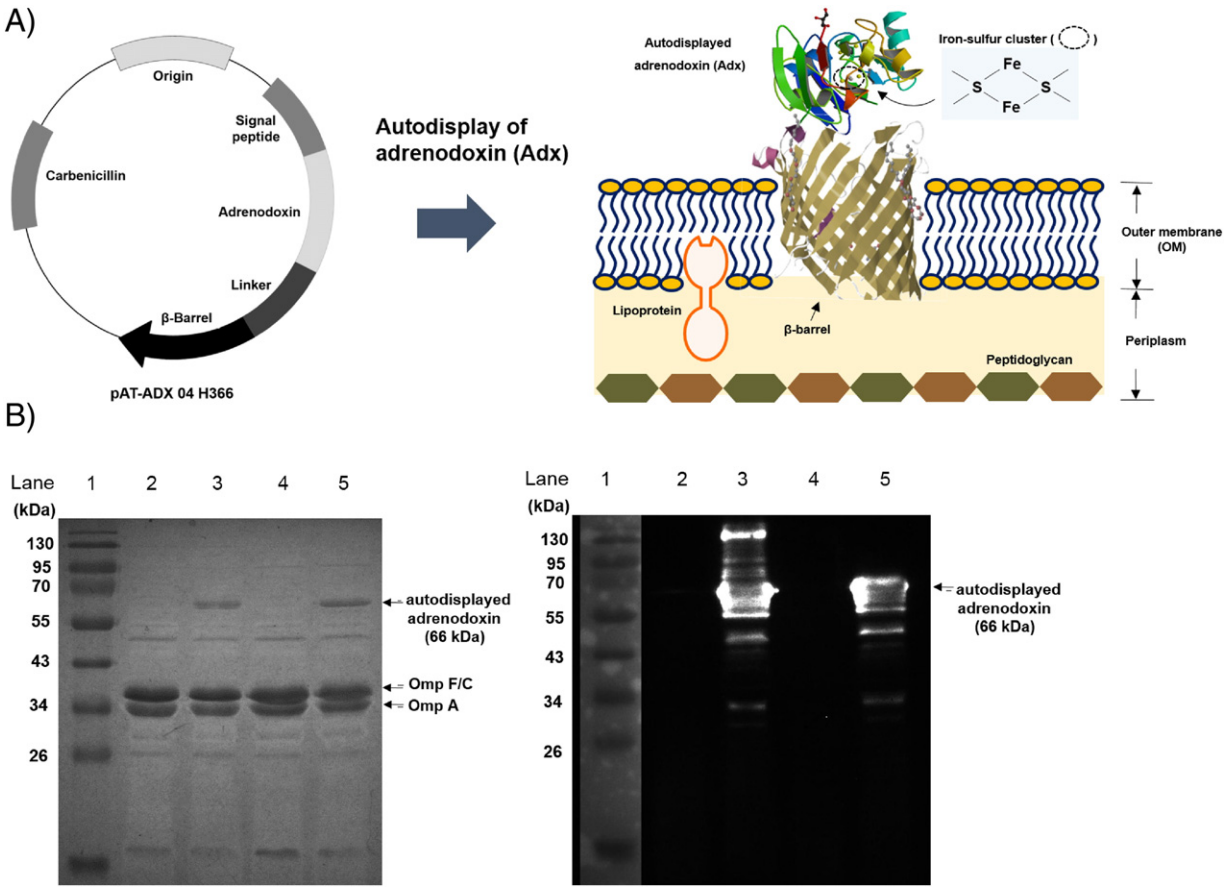


**Fig. 1.** Autodisplay of adrenodoxin (Adx). (A) Transformation of autodisplay vector of Adx composed of a signal peptide, Adx, linker, and β-barrel. (B) SDS-PAGE (left) and Western blotting (right) after the autodisplay of Adx. Lane 1: protein ladder, Lane 2: proteins from intact *E. coli* (UT5600) without dithiotreitol (DTT) treatment, Lane 3: proteins from *E. coli* with autodisplayed Adx without DTT treatment, Lane 4: proteins from intact *E. coli* (UT5600) with DTT treatment. The Western blotting was carried out using anti-Adx antibodies (polyclonal) and anti-rabbit IgG antibodies labeled with horseradish peroxidase (HRP). The chemi-luminescent protein bands were created by treatment with luminol.

[9–14]. As shown in Fig. 1(B), the protein band of Adx was confirmed from Western blotting with anti-Adx antibodies and SDS-PAGE of the outer membrane proteins of *E. coli* showed that the fusion protein of Adx was expressed with an expected molecular weight of 66 kDa. In the case of Omp A, the surface density on the outer membrane of *E. coli* is known to be $1.5 \times 10^5$ molecules/$\mu m^2$. Through densitometry of the protein bands, the number of Adx proteins on the outer membrane of *E. coli* was estimated to be 4.8 molecules/*E. coli* cell [19,20].

The ability of Adx to accept and donate electrons depends on the redox potentials of the iron–sulfur clusters. Due to the autotransport mechanism, Adx can only be expressed on the outer membrane of *E. coli* in an unfolded state, as apo-Adx without a prosthetic group (e.g. Adx devoid of the 2[Fe–S] cluster) is biologically inactive [1,2]. As previously described, the iron–sulfur cluster was effectively incorporated on the apo-Adx on the *E. coli* surface [3–5]. The iron–sulfur clusters formed and were immediately incorporated into the apo-Adx displayed on the bacterial surface when the color of the solution changed to black, as shown in Fig. 2(A). Cells that survived this procedure could be handled under aerobic conditions without a loss of activity [4,5]. The incorporation of the iron–sulfur cluster after the reconstitution process was tested using energy-dispersive X-ray spectroscopy (EDX). As shown in Fig. 2(B), *E. coli* cells were immobilized on the glass substrate and then a SEM image was taken before and after the reconstitution process. At the same time, the EDX spectrum was obtained, which clearly showed the iron species peaks (FeKα E = 6.4 eV) of the *E. coli* cells with the autodisplayed Adx after the reconstitution process. These results showed that Adx was autodisplayed on the outer membrane of *E. coli* and the iron–sulfur cluster was incorporated by the reconstitution process.

### 3.2. Electrochemical analysis of Adx

Usually, proteins with iron–sulfur clusters are known to directly transfer unmediated electrons to redox partner proteins [1,2]. The electrode surface used for the study of electron transfer from the iron–sulfur cluster was modified with consideration of the properties of the redox partner proteins. As shown in Fig. 3(A), the self-assembled monolayer with MUA on a gold surface was reported to effectively promote direct

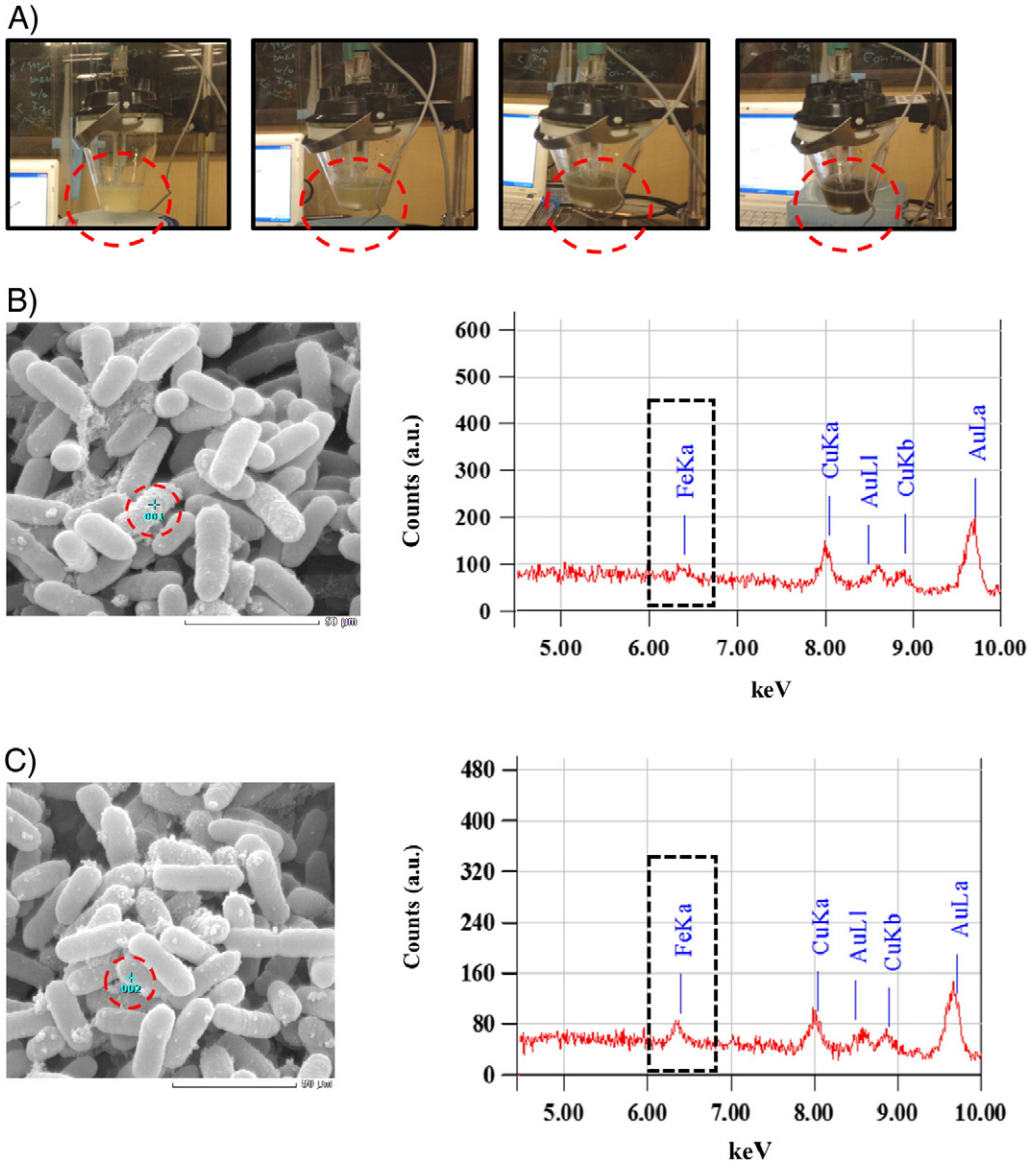


**Fig. 2.** Reconstitution of the iron–sulfur cluster. (A) Pictures of the reconstitution reaction. The reaction was carried out under anaerobic conditions by adding $LiS_2$ dropwise to Adx-expressing cells in a ferrous ammonium sulfate buffer at room temperature. (B) Energy-dispersive X-ray spectroscopy (EDX) of intact *E. coli* (UT5600) and (C) *E. coli* with autodisplayed Adx after the reconstitution process. The EDX spectra showed iron species peaks (FeKα E = 6.4 eV) in the *E. coli* cells with autodisplayed Adx after the reconstitution process.

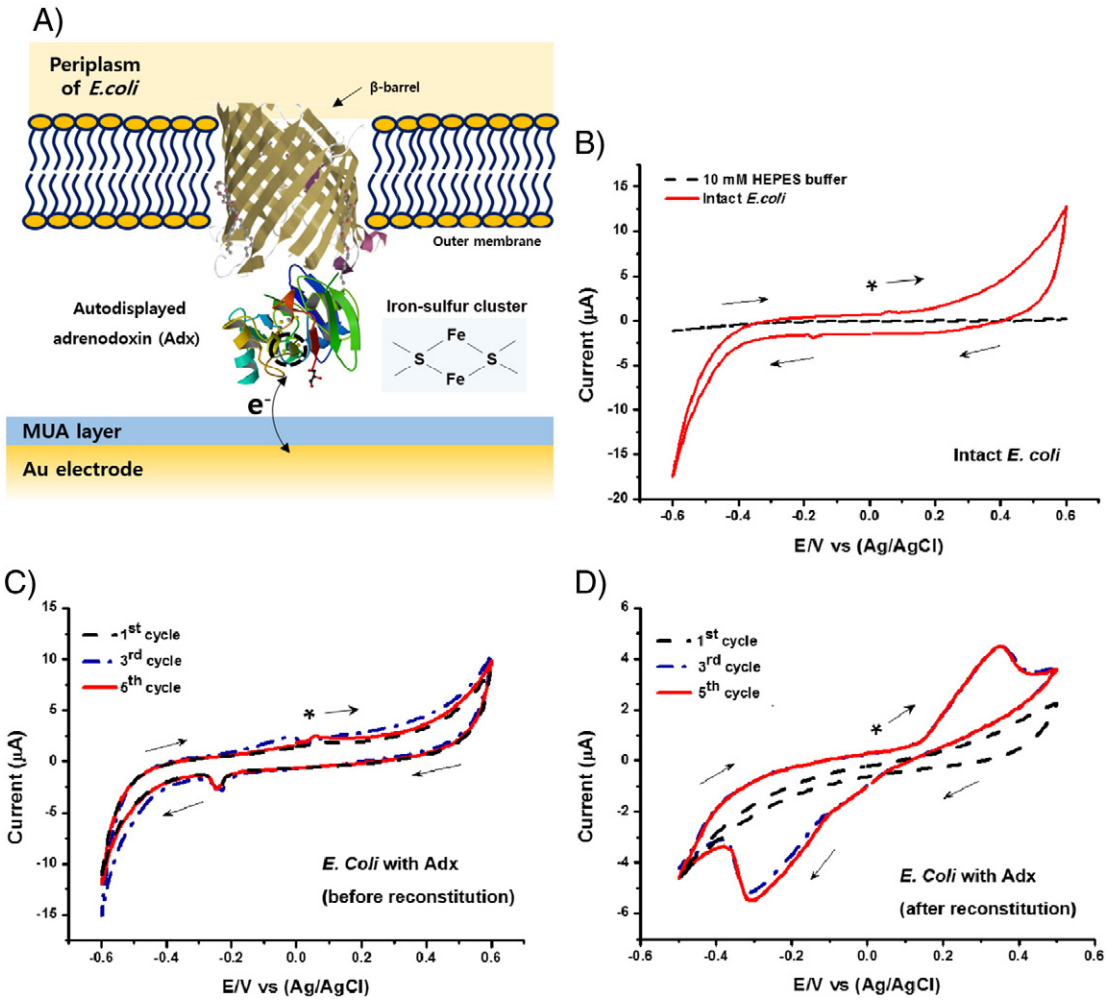


**Fig. 3.** Cyclic voltammetry (CV) analysis of the electron transfer of the autodisplayed Adx. (A) Schematic view of the electron transfer reaction between the autodisplayed Adx and the self-assembled monolayer of MUA. (B) CV analysis of the intact *E. coli* (UT5600). CV analysis of *E. coli* with the autodisplayed Adx (C) before and (D) after the reconstitution process. The scan rate was controlled to be 50 mV/s.

electron transfer from negatively charged proteins such as Adx and cytochrome $b_5$ [21–24]. For wild-type Adx, the redox potential was reported to have a midpoint potential of −0.27 V (vs. NHE) [3,4]. In the case of mutant Adx, the redox potentials were known to differ, especially when the amino acid residues related to the iron–sulfur cluster were modified. In this work, the gold electrode was also modified with a self-assembled monolayer of MUA and the *E. coli* cells with autodisplayed Adx were immobilized after the reconstitution process for the determination of the redox potentials of the iron–sulfur cluster of the autodisplayed Adx.

The intact *E. coli* (UT5600) without autodisplayed Adx underwent the reconstitution process for the incorporation of the iron–sulfur cluster, and was then immobilized on the MUA-modified gold electrode. For the analysis of the redox potentials of the autodisplayed Adx, CV analysis was carried out using autodisplayed Adx before and after the reconstitution process for incorporating the iron–sulfur cluster. As shown in Fig. 3(B), CV diagrams at the 1st and 5th cycles of the intact *E. coli* (10 mM HEPES buffer, pH 7.0) showed nearly no significant changes in shape, with no redox peaks in the potential ranges of −0.6 to +0.6 V (vs. Ag/AgCl). *E. coli* (UT5600) with autodisplayed Adx before the reconstitution process for the incorporation of the iron–sulfur cluster also showed no significant changes in shape, with no redox peaks in the same potential range, as shown in Fig. 3(C). The *E. coli* cells (UT5600) with autodisplayed Adx after the reconstitution process for the incorporation of the iron–sulfur cluster was immobilized on the MUA-modified gold electrode. As shown in Fig. 3(D), CV diagrams at the 1st and 5th cycles of the *E. coli* (10 mM HEPES buffer, pH 7.0) with autodisplayed Adx showed significant changes in shape with an oxidation peak at +0.4 V (vs. Ag/AgCl) and a reduction peak at −0.3 V (vs. Ag/AgCl) after the reconstitution process for the incorporation of the iron–sulfur cluster. In the previous work, the purified Adx or the outer membrane layer with Adx was immobilized onto the electrode and the redox potential was monitored using CV analysis. These results showed that the immobilized *E. coli* with autodisplayed Adx could also carry out the electron transfer to the MUA-modified gold electrode.

After the reconstitution process, the iron–sulfur cluster of the autodisplayed Adx is known to be in an oxidized state [3,4]. When the reductive potential from 0 to −0.6 V (vs. Ag/AgCl) was applied to the Adx just after the reconstitution process, the reduction current was observed in the 1st cycle, which decreased as the reduction cycle was repeated, as shown in Fig. 4(A). These results showed that the autodisplayed Adx just after the reconstitution process had the iron–sulfur cluster in an oxidized state. After these reduction CV cycles, the autodisplayed Adx was applied to the oxidative potential from 0 to +0.6 V (vs. Ag/AgCl) and the oxidative current was observed at the 1st cycle, which decreased as the reduction cycle was repeated, as shown in Fig. 4(B). These results showed that the iron–sulfur cluster of the autodisplayed Adx could reversibly perform a redox reaction via direct electron transfer to the MUA-modified gold electrode.

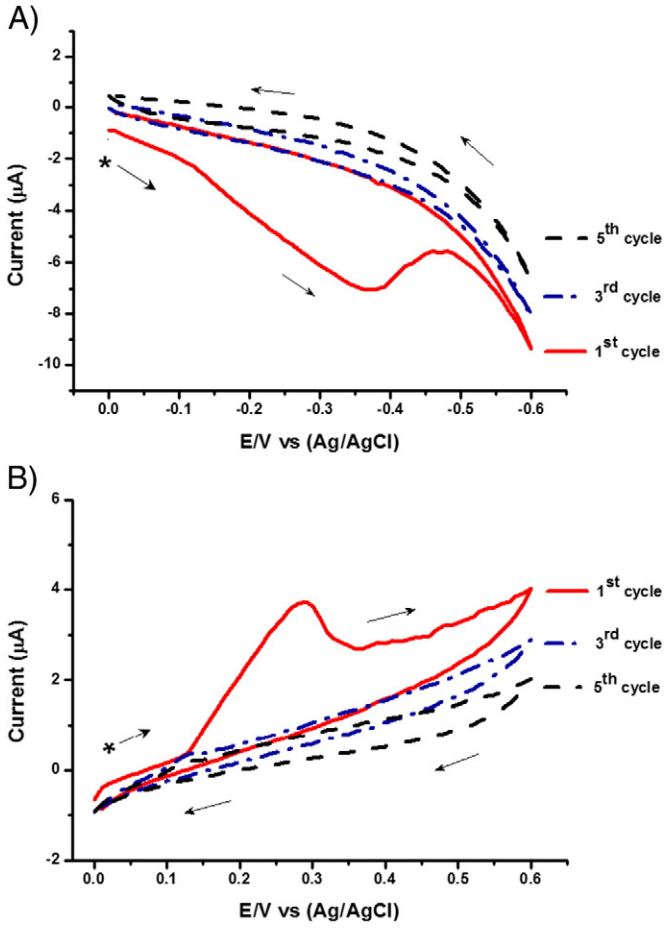


**Fig. 4.** Cyclic voltammetry (CV) analysis of reversible electron transfer of the autodisplayed Adx. (A) CV analysis of *E. coli* with the autodisplayed Adx just after the reconstitution process in the range of reduction potentials. (B) Sequential CV analysis in the range of oxidation potentials just after the reduction process of (A). The scan rate was controlled to be 50 mV/s.

## 4. Conclusions

In this work, adrenodoxin (Adx) was expressed on the outer membrane of *E. coli* by autodisplay with a surface density of 4.8 molecules/*E. coli* cell. The iron–sulfur cluster was incorporated into the apo-Adx by an anaerobic reconstitution process, and analysis of the EDX spectrum clearly showed iron species peaks (FeKα E = 6.4 eV) in the autodisplayed Adx. For the determination of the redox potentials of the iron–sulfur cluster of the autodisplayed Adx, the *E. coli* cells with the autodisplayed Adx were immobilized on a gold electrode modified with a self-assembled monolayer of MUA. From the repeated CV analysis, *E. coli* (10 mM HEPES buffer, pH 7.0) with autodisplayed Adx showed significant changes in shape, with an oxidation peak at +0.4 V (vs. Ag/AgCl) and a reduction peak at −0.3 V (vs. Ag/AgCl) after the reconstitution process for the incorporation of the iron–sulfur cluster. After the reconstitution process, the iron–sulfur cluster of the autodisplayed Adx was shown to be in an oxidized state. From the repeated CV analysis in the reduction (or oxidation) potential ranges, the reduction (oxidation) current was observed in the 1st cycle, which decreased as the reduction cycle was repeated. These results showed that the iron–sulfur cluster of the autodisplayed Adx could reversibly carry out a redox reaction via direct electron transfer to the MUA-modified gold electrode.

## Conflict of interest

Here, I (Jae-Chul Pyun, corresponding author on behalf of the other co-authors) declare that there is no conflict of interest related to the submitted paper.

## Acknowledgements

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