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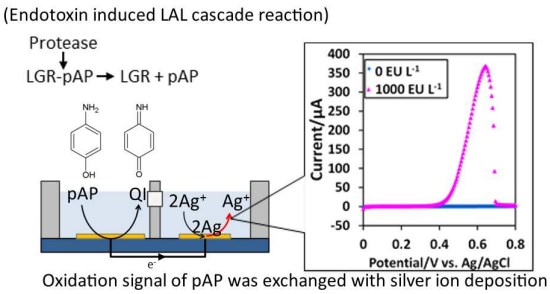

Oxidation signal of pAP was exchanged with silver ion deposition

An extra-highly sensitive sensor for detection of endotoxin was developed. In this sensor, *p*-aminophenole (pAP) was generated with endotoxin induced enzyme reaction and detected with substitutinal stripping voltammetry.

Title

Electrochemical sensor with substitutional stripping voltammetry for highly sensitive endotoxin assay

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Abstract

We have developed a novel method for detection of endotoxin with extra-high sensitivity by introducing substitutional stripping voltammetry (SSV). In this method, *p*-aminophenol (pAP) conjugated peptide (Boc-Leu-Gly-Arg-pAP; LGR-pAP) was used as a substrate for a protease, which is activated at the last step of the endotoxin-induced *Limulus* amebocyte lysate (LAL) cascade reaction. Extra-highly sensitive detection of pAP liberated by the endotoxin-induced LAL reaction was successfully realized with SSV based on the accumulation of an amperometric signal owing to exchanging the oxidation current of pAP generated at an electrode in a reaction cell with silver deposition on another electrode in a deposition cell. This reaction is driven by the difference in the redox potential between pAP/quinoneimine and silver/silver ion. The amount of the deposited silver is quantified by anodic stripping voltammetry (ASV). This SSV-based endotoxin assay was performed with a chip device comprising two cells, each of which was connected via a liquid junction made of Vycor® glass. The reaction cell and the deposition cell contained a standard endotoxin sample with LAL regents containing LGR-pAP and $AgNO_3$ solution, respectively. After the cells were electrically connected for 60 min, ASV was conducted in the deposition cell to quantify the total electrical charge derived by the oxidation of free pAP in the reaction cell. The ASV

signal increased with the increase of the endotoxin concentration in the sample solution in the range 0.5–1000 EU L^{-1}.

Keywords

electrochemical biosensor, endotoxin, lipopolysaccharide, *Limulus* amebocyte lysate assay, protease, substitutional stripping voltammetry

1. Introduction

Endotoxin is a lipopolysaccharide (LPS) existing on the outer membrane of Gram-negative bacteria.[1] Although endotoxin itself is chemically stable and thermally durable, it induces secretion of proinflammatory cytokines leading to septic shock.[2] Therefore, contamination by endotoxin has been a serious problem for the safety of medical treatments and medical supplies. The *Limulus* amoebocyte lysate (LAL) assay, based on the coagulation reaction of hematocytes lysate of horseshoe crabs induced by endotoxin, is a widely used method to test for endotoxin.[3] The conventional LAL assay is classified into three major categories called the gel-clotting method, the turbidimetric method, and the colorimetric method, all of which are based on photometrical detections. Although many types of test kits are commercially available, conventional LAL assay still requires a skilled technician to perform the test. Another problem for the conventional LAL test is the cost for equipment and reagents. Owing to these problems, most clinics cannot substantially control endotoxin by *in situ* monitoring of endotoxin levels in medical supplies at appropriate intervals. Actually, dialysis clinics in Japan typically check endotoxin levels in dialysis fluid by ordering weekly endotoxin tests by an external analytical centre. However, it is too late if the endotoxin contamination is discovered after the dialysis treatment. This is a serious problem for safe medical

treatment, particularly in developed countries with increasing incidence of diabetic nephropathy. To overcome the problem of the conventional LAL test, electrochemical techniques have been proposed as promising candidates because they provide easy-to-use, low-cost, and highly sensitive endotoxin sensors.[4] We have previously developed two types of electrochemical sensor based on the LAL reaction.[5-7] One is a LAL-based voltammetric assay for detecting *p*-nitroaniline (pNA) produced from pNA-conjugated peptide (Boc-Leu-Gly-Arg-pNA; LGR-pNA) using differential pulse voltammetry.[5,6] The other is LAL-based amperometric assay using a novel substrate (Boc-Leu-Gly-Arg-*p*-aminophenol; LGR-pAP) specially developed for electrochemical detection.[7] These methods have achieved detection of 10 endotoxin units (EU) per litre within 60 min with easy-to-use chip devices. The sensitivity of these methods is equal to or higher than that of conventional LAL assay; however, there are growing demands for an extra-highly sensitive sensor, particularly among haemodialysis treatment sites.

In this study, we successfully developed a chip-type endotoxin sensor with extra-high sensitivity by introducing substitutional stripping voltammetry (SSV) [8-11] and using LGR-pAP as a substrate. SSV is based on the accumulation of an amperometric signal by exchanging the redox current to metal deposition. Figure 1A shows the overall scheme of our detection system using SSV. Endotoxins induce the cascade activation of

zymogens in LAL (Factor C, Factor B and proclotting enzyme), which results in the hydrolysis of LGR-pAP. The enzymatically generated free pAP is oxidized to *p*-quinoneimine (QI) on the reaction electrode, which is electrically connected to the deposition electrode in another cell filled with silver nitrate ($AgNO_3$) solution. The resulting oxidation current is exchanged to silver deposition on the deposition electrode (Fig. S1). Similar to a battery cell, this exchange is forced by the difference in the redox potential between pAP/QI and silver/silver ions in separated cells connected by a liquid junction. Unlike pAP, LGR-pAP has no effect of silver deposition, because the potential owing to LGR-pAP oxidation is higher than that from silver ion reduction. Therefore, the amount of the silver deposition corresponds to the concentration of free pAP (Fig S2), which also corresponds to the concentration of endotoxin. The amount of accumulated metallic silver was quantified by high-sensitivity stripping voltammetry. Using this method, 0.5 EU L^{-1} of endotoxin was detected within 60 min.

An additional advantage of this method is the exchange of unstable pAP to stable silver. It is known that pAP loses its electrochemical activity due to air oxidation under neutral and alkaline conditions.[12] The signal exchange from pAP to silver has a potential advantage for extended-time measurement to achieve highly sensitive

detection. In the future, the combination of SSV with redox cycling of pAP will also increase the sensitivity of the assay.

In the present study, we first characterized the redox potentials of LGR-pAP, pAP and silver to confirm the strategy to use SSV for endotoxin assay. Then we fabricated a chip device for SSV and demonstrated it to monitor silver deposition by the LAL reaction with and without endotoxin. Silver deposition was observed by monitoring the current flow from the reaction cell and to the deposition cell during silver deposition. Finally, we performed quantitative endotoxin assays with the fabricated device.

2. Material and Methods

2.1. Chemicals and Apparatus

An Endospecy® ES-24S set was purchased from Seikagaku Co. (Japan). This set comprised lyophilized LAL reagents divided into individual test vials and assay buffer. United States Pharmacopeia reference standard endotoxin (USP-RSE) was purchased from Seikagaku Co. (Japan). The USP-RSE was diluted with endotoxin-free water (water for injection, Otsuka Pharmaceutical, Japan) to obtain standard endotoxin solutions. Primary stock solutions prepared in 2×10^6 EU L^{-1} were stored at −80°C.

Note that EU is a unit expressing endotoxin activity. A secondary stock solution prepared in 1×10^5 EU L^{-1} was stored at 4°C. According to the protocol for USP-RSE, the stock solutions were mixed by vigorous vortexing for more than 30 min just prior to further dilution. Standard endotoxin solutions for the experiments were prepared immediately before use. LGR-pAP (Watanabe Chemical Industries, Ltd., Japan) and pAP (Wako Pure Chemical Industries, Ltd., Japan) were dissolved in endotoxin-free water to obtain 10 mM stock solutions which were then stored at −20°C. Aqueous solutions were prepared with water for injection. Toxipet endotoxin-free pipet tips (Seikagaku Co., Japan) were used for all endotoxin assays. $AgNO_3$ was purchased from Wako Pure Chemical Ind., Ltd. (Japan). Poly (dimethylsiloxane) (PDMS, SILPOT 184) was purchased from Dow Corning Toray Co., Ltd. (Japan).

The cyclic voltammetry (CV) and anodic stripping voltammetry (ASV) were performed with a potentiostat (Compact Stat; Ivium Technologies B.V., The Netherlands). An Ag/AgCl electrode and a Pt plate were used as a reference electrode and a counter electrode, respectively. The CV and the ASV were conducted with a fabricated device using a reaction electrode (5.0 mm in diameter, Au) or a deposition electrode (3.0 mm in diameter, Au) as a working electrode. The reference and counter electrodes were inserted into the solution from above. The deposition current was

measured with a current amplifier (428-MAN; TFF Corporation Keithley Instruments Inc., Ohio, USA). A data acquisition device (DAQ; NI USB-62ll; National Instruments Japan Co., Japan) was inserted between the current amplifier and a monitoring computer in order to control the measurement and data acquisition using a program written in LabVIEW ver. 2010 (National Instruments Japan Co., Japan).

2.2. Fabrication of the chip device

The chip device comprised a glass substrate with patterned electrodes and a PDMS block with two wells to accommodate the solutions (Fig. 1B, C). A glass substrate with patterned electrodes was fabricated by conventional photolithography to make a reaction electrode (Au, 5.0 mm in diameter) and a deposition electrode (Au, 3.0 mm in diameter). Titanium, platinum, and gold were successively sputter-deposited (L-332S-FH; Anelva Co., Japan) onto a glass slide (Matsunami Glass Ind., Ltd., Japan) patterned with S1818 positive photoresist (The Dow Chemical Company, USA). After removing the photoresist with acetone (lift-off), an insulation layer was fabricated using a negative photoresist (SU-8 3005; Nippon Kayaku Co., Ltd., Japan). A PDMS block with two wells (7.0 mm in diameter, 5.0 mm in depth) was made as follows. The PDMS pre-polymer was poured into a mould made of acrylic resin and cured in an oven at

90°C for 30 min. After peeling off the PDMS from the mould, we inserted a Vycor® glass rod (3.0 mm in diameter, 3.0 mm in length; Corning, Inc., USA) into a furrow made between the wells. To embed the Vycor® glass into the PDMS, PDMS pre-polymer was poured into the furrow, both ends of which were covered with cellophane adhesive tape (Cellotape; Nichiban Co. Ltd., Japan) to prevent the leaking of PDMS into undesired areas. After the PDMS was cured in the oven at 90°C for 30 min, the adhesive tape was removed. The electrode-patterned substrate and the PMDS block were then joined with PDMS.

2.3. Endotoxin assay using the fabricated device

To prepare LAL assay solution containing 1.0 mM LGR-pAP, we put 180 µL of assay buffer of Endospecy® ES-24S set and 20 µL of 10 mM LGR-pAP into one test vial of Endospecy® ES-24S set containing lyophilized LAL reagents. Just before the endotoxin assay, the fabricated chip device was treated with O_2 Plasma Asher (LTA-101; Yanaco, Ltd., Japan) at 100 W for 6 min in order to remove the contaminated endotoxin. Next, 130 µL of a 1:1 mixture of the LAL assay solution and endotoxin standard solution was placed in the reaction cell of the device. Then, 130 µL of 10 mM $AgNO_3$ with 0.1 M KNO_3 solution was placed in the deposition cell. After the reaction

cell and the deposition cell were electrically connected, the chip device was incubated at 37°C for 60 min. During the incubation, the PDMS well was covered with Parafilm (Pechiney Plastic Packaging, Inc., Chicago, USA) to avoid contamination of the endotoxin and evaporation of the solvent. After the electrical connection was cut, the solution in the deposition cell was exchanged to 130 μL of 0.1 M KNO_3 solution. The reference electrode and the counter electrode were inserted into the deposition cell to perform ASV using the deposition electrode as the working electrode. The scan rate for ASV was set to 20 mV s^{-1} to improve its productivity and accuracy.[9] From the ASV voltammogram, the total electrical charge (Q) was calculated with following equation:

$$Q = \int I dt \,, \tag{1}$$

where I is the current observed on ASV, t is the elapsed time from the start of the ASV measurement, and Q is the total electrical charge produced by oxidation at the reaction electrode while it was connected to the deposition electrode.

3. Results and Discussion

3.1 Comparison of formal redox potential of pAP, LGR-pAP, and $AgNO_3$ solution

As mentioned in the introduction, the driving force of the deposition step of SSV is the potential difference between the reaction electrode and the deposition

electrode. Therefore, we first checked the formal redox potential of LGR-pAP, pAP and silver in the deposition system using cyclic voltammetry. Figure 2A shows cyclic voltammograms of 1.0 mM LGR-pAP and 1.0 mM pAP solutions in ES-24S assay buffer using the reaction electrode as a working electrode. The scan rate was 20 mVs^{-1}. As a reference, a cyclic voltammogram of the assay buffer is also shown in the same graph. Peaks shown around −0.30 V indicate the reduction of dissolved oxygen in the solutions. When the potential of the Au working electrode was scanned upward from 0.0 V, LGR-pAP began to be oxidized at +0.35 V. The oxidation current reached a peak at +0.47 V, and no obvious reduction current of the oxidized LGR-pAP was observed on the reversed potential sweep from +0.80 V to −0.60 V. The reason for missing the reduction current, which was clearly observed in our previous paper[7], was due to the difference in reactivity of the substrate. The reduction of the oxidized LGR-pAP in the reversed scan was difficult at the Au surface in the present potential range compared with that at the carbon surface in the previous paper. On the other hands, pAP showed clear oxidation and reduction peaks at +0.12 V and −0.06 V, respectively. This indicates a reversible reaction of pAP/QI redox couple. The formal redox potential of pAP/QI was estimated to be +0.03 V from this result. Figure 2B shows a cyclic voltammogram of 10 mM $AgNO_3$ in 100 mM KNO_3 solution using the deposition electrode as a

working electrode. When the potential of the working electrode was scanned downward from +0.80 V, silver ions began to be reduced to silver at +0.39 V. On the reverse sweep, the deposited silver on the electrode was oxidized back to silver ions at +0.39 V. No obvious redox current was observed above +0.65 V. This means that the deposited silver was completely stripped away during the potential scan from +0.39 V to +0.65 V. Importantly, Fig. 2B indicates that the deposition of silver begins at +0.39 V. Therefore, LGR-pAP, of which oxidation begins at +0.35 V, cannot promote silver deposition in the deposition step, while pAP, of which oxidation begins above +0.03 V, promotes the silver deposition. These results indicate the possibility to use LGR-pAP as an LAL substrate for endotoxin detection with SSV.

3.2 Measurement of deposition current

To confirm the strategy of LAL-based SSV endotoxin detection using LGR-pAP, the current in the deposition process was measured using the current amplifier connected to the reaction electrode and the deposition electrode. The mixture solution containing the LAL reagent, 0.5 mM LGR-pAP, and endotoxin was put into the reaction cell, and 10 mM of $AgNO_3$ was put into the deposition cell. The recording of the current was started a few minutes before the electrical connection between the

reaction and deposition cell. After putting the device covered with the Parafilm into the incubator which was maintained at 37°C, we connected the cells through the current amplifier. It takes less than 3 min to start the recording from the mixing of the endotoxin and reagents. Figure 3A shows a comparison of the deposition current with and without endotoxin. The arrow shows the time to connect the cells. When the reaction mixture contained 1000 EU L^{-1} endotoxin, the deposition current increased rapidly about 20 min after mixing the endotoxin and reagents. In contrast, no obvious increase of the deposition current was observed without endotoxin. These results indicate that the most of the deposition current is induced by endotoxin activating the LAL reaction to release free pAP. After measuring the deposition currents, the amount of silver deposited on the deposition electrode was measured with ASV (Fig. 3B) at a scan rate was 20 mV s^{-1}. A characteristic peak of silver stripping was observed when the sample containing 1000 EU L^{-1} endotoxin was tested. The stripped amount of silver was calculated to be 3616 μC, which was consistent of the deposited amount of silver ions calculated from the result shown in Fig. 3A (4126 μC). In contrast, only a small stripping peak was observed when the reaction mixture without endotoxin was tested. These results also indicate the consistency of our strategy of LAL-based SSV endotoxin detection using LGR-pAP.

3.3 Quantitative endotoxin assay with SSV using the fabricated device

Quantitative endotoxin detection with SSV was performed using the fabricated chip device. The reaction cell containing LAL reagent, 0.5 mM LGR-pAP, and 0–1000 EU L^{-1} endotoxin was electrically connected to the deposition cell containing 10 mM $AgNO_3$ for 60 min. After disconnecting from the reaction cell, the deposition cell was filled with new 0.1 M KNO_3 solution for ASV. Figure 4A shows the results of ASV with different concentrations of endotoxin. The oxidation currents in ASV increased with the concentration of endotoxin, indicating that this oxidation response resulted from stripping the silver deposited via oxidation of pAP released by the endotoxin-induced LAL reaction. Multiple peaks observed in the voltammograms of 0.1–100 EU L^{-1} were caused by the difference in the bonding force "between the electrode and deposited metals (E-M bond)" and "between atoms of deposited metals (M-M bond)".[13, 14] The stronger E-M bond dissociates at a higher potential than does the weaker M-M bond. Figure 4B shows the calibration plot for the quantitative endotoxin assay based on the total electrical charge of the ASV response calculated by Eq. (1). Each point corresponds to the mean value, and error bars indicate the standard deviation obtained from independent measurements. Electrical charges increased with the increase in

endotoxin concentration in the range of 0.1–1000 EU L^{-1}. The shape of calibration curve was not linear because the LAL reaction is a cascade reaction constructed with multiple proenzyme activated in order. The cascade reaction makes the progress of LAL reaction to follow the sigmoidal curve with time and also causes the nonlinear relationship between the current values and LPS concentrations at a given time point. The detection limits (adapting 3σ of the blank) and minimum limit of determination (adapting 10σ of the blank) of this study were 0.5 EU L^{-1} and 100 EU L^{-1}, respectively, calculated by the IUPAC recommended methods[15]. The limit of determination would have been improved if more detailed test had been performed at the range of 10 EU L^{-1} and 100 EU L^{-1}. The measurement range was 0.5-1000 EU L^{-1}. Compared with our previous study[7], the detection limit was improved from 10 EU L^{-1} to 0.5 EU L^{-1} on using the chip type device. With the improvement of the detection limit, the measurement range was widened from 10-1000 EU L^{-1} to 0.5-1000 EU L^{-1}. This range satisfactorily covers endotoxin levels of less than 30 EU L^{-1}, which is defined as ultrapure dialysis water by the Associated for the Advancement of Medical Instrumentation (AAMI). Considering the fact that the detection limit of commonly used LAL reagent is 30 EU L^{-1},[16] our novel method innovatively improves endotoxin assay. We should emphasize that our system ensures the safety of long-term dialysis

patients by meeting the stricter standard (less than 1 EU L^{-1}) for ultrapure dialysis fluid defined by The Japanese Society for Dialysis Therapy[17] within 60 min with a small device applicable to the onsite monitoring. Higher sensitivity will be available by taking longer connection time because the longer connecting time is, the more LAL reaction progresses as well as the more Ag deposits (Fig. S3), resulting in the larger ASV signal. As the presented method involves a trade-off between the sensitivity and detection time, the selection of the optimal detection time should be considered in the practical use by taking the required detection limit and allowed detection time into account.

4. Conclusions

In this study, we developed a novel endotoxin detection method based on LAL assay and SSV. In this method, the generation of free pAP by endotoxin-induced LAL reaction is substituted to the integration of silver deposition which is then quantified by ASV. First, we checked the redox potential of LGR-pAP, pAP and silver using cyclic voltammetry in order to estimate the driving force of the deposition step in SSV. The result showed that pAP, of which oxidation begins above +0.03 V, promotes the silver deposition beginning below +0.39 V, while LGR-pAP, of which oxidation begins above +0.35 V, does not promote the silver deposition in the deposition step. Then, we

measured the current in the deposition process on the fabricated chip device to ensure the strategy of LAL-based SSV endotoxin detection. The deposition current was observed only when the reaction mixture contained endotoxin. The total charge observed in the detection process was consistent of the charge observed in the subsequent stripping process.

Finally, we performed quantitative endotoxin assays using the fabricated chip device. The electrical charges obtained from the silver stripping current increased with the concentration of endotoxin in the solution. The detection limit was 0.5 EU L^{-1} for a LAL reaction time of 60 min. This method will provide simple quantitative analysis tools for extra-low-concentration endotoxin in dialysis water and on medical instruments.

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Figure Captions

Figure 1. (A) Principle of the LAL-based SSV endotoxin assay. (B) Photograph of the chip device. (C) Diagram of the chip device structure.

Figure 2. (A) Cyclic voltammograms of assay buffer, 1.0 mM LGR-pAP, and 1.0 mM pAP obtained with the reaction electrode on the chip device. LGR-pAP and pAP were dissolved in the assay buffer. (B) Cyclic voltammogram of 10 mM $AgNO_3$ in 100 mM KNO_3 obtained with the deposition electrode on the chip device. Potentials were swept upward from 0.0 V Fig. A and downward from +0.8V in Fig. B at a scan rate 20 mVs^{-1}.

Figure 3. (A) Deposition currents observed with 1000 EU L^{-1} and without endotoxin in sample solutions. (B) Anodic stripping voltammograms of the deposition electrode after observing the deposition current shown in Fig. A.

Figure 4. (A) Anodic stripping voltammograms obtained with the deposition electrode on the chip device after 60 min of deposition with endotoxin concentrations of 0, 0.5, 1, 10, 100 and 1000 EU L^{-1}. (B) Calibration curves for quantitative detection of

SSV-based electrochemical assay with the chip device. Electrical charges calculated from the voltammograms of ASV are plotted versus endotoxin concentration. Error bars indicate ±standard deviations (n = 3–6).

Fig. 1

A

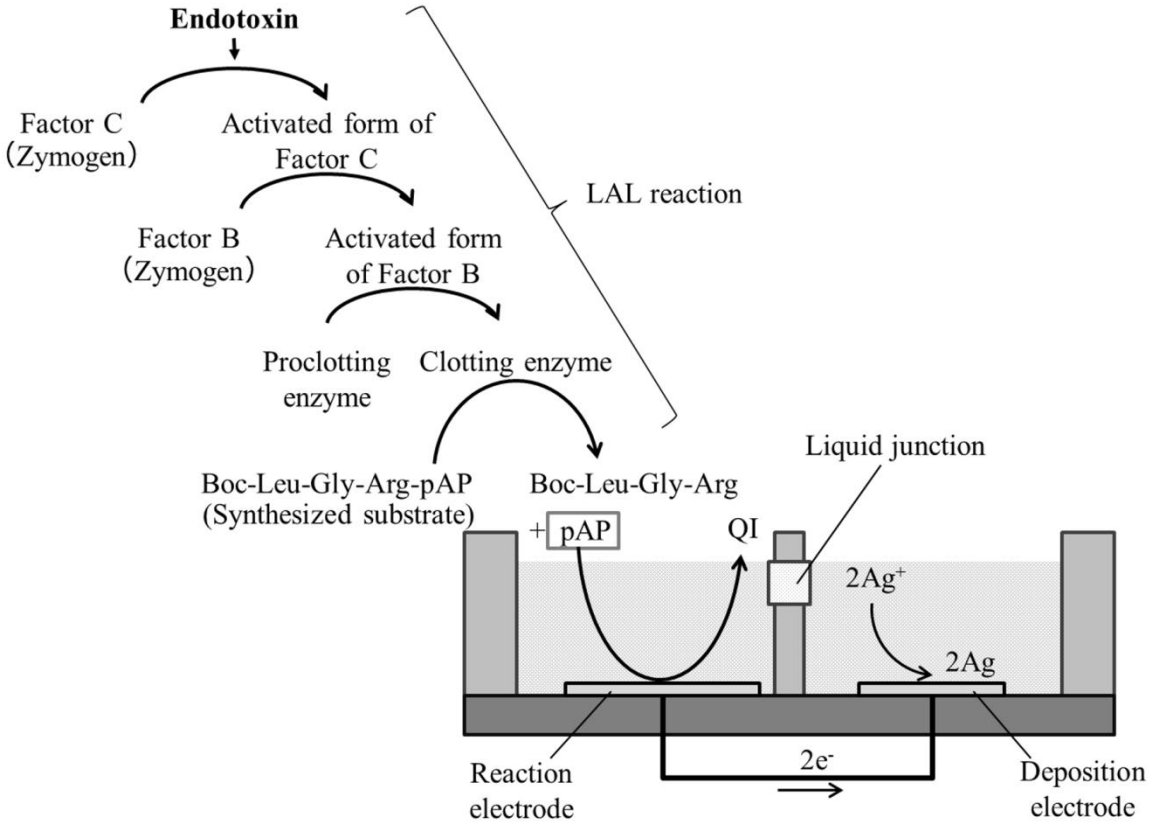

Fig. 1（Continued)

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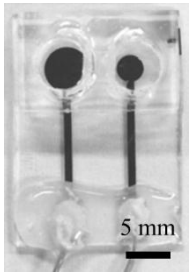

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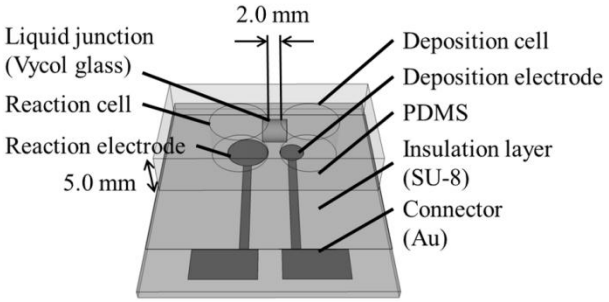


Fig. 2

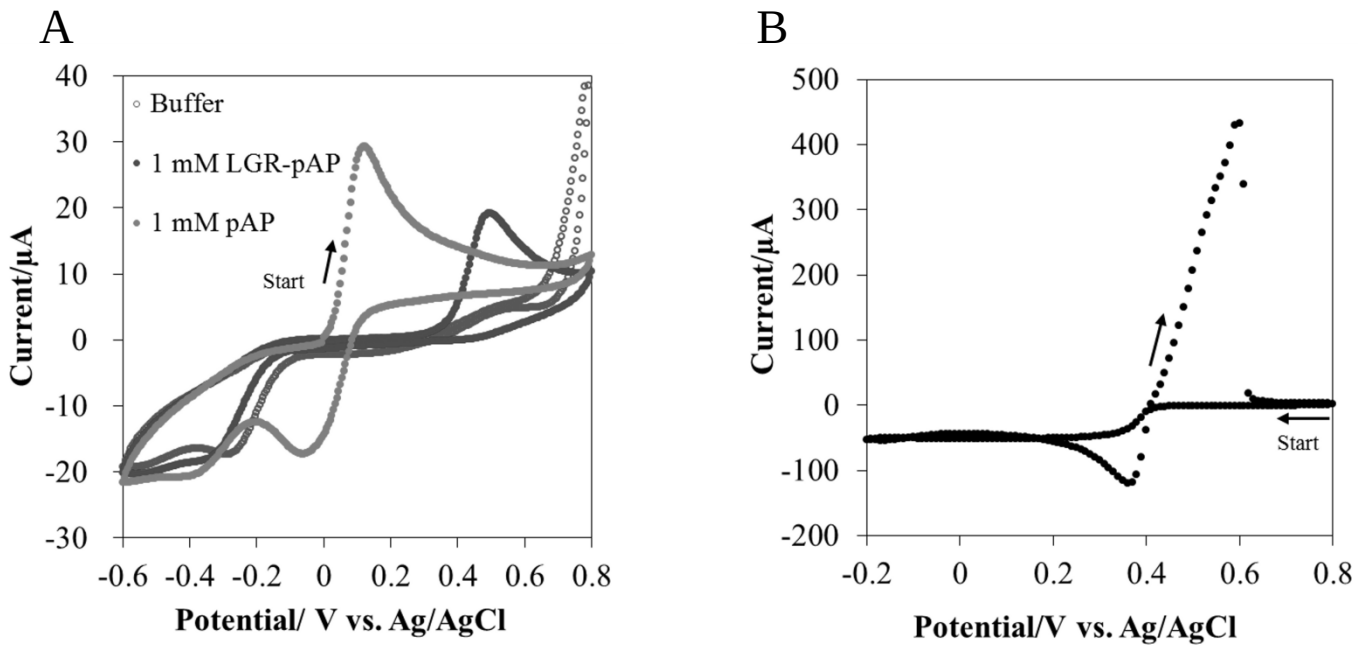


Fig. 3

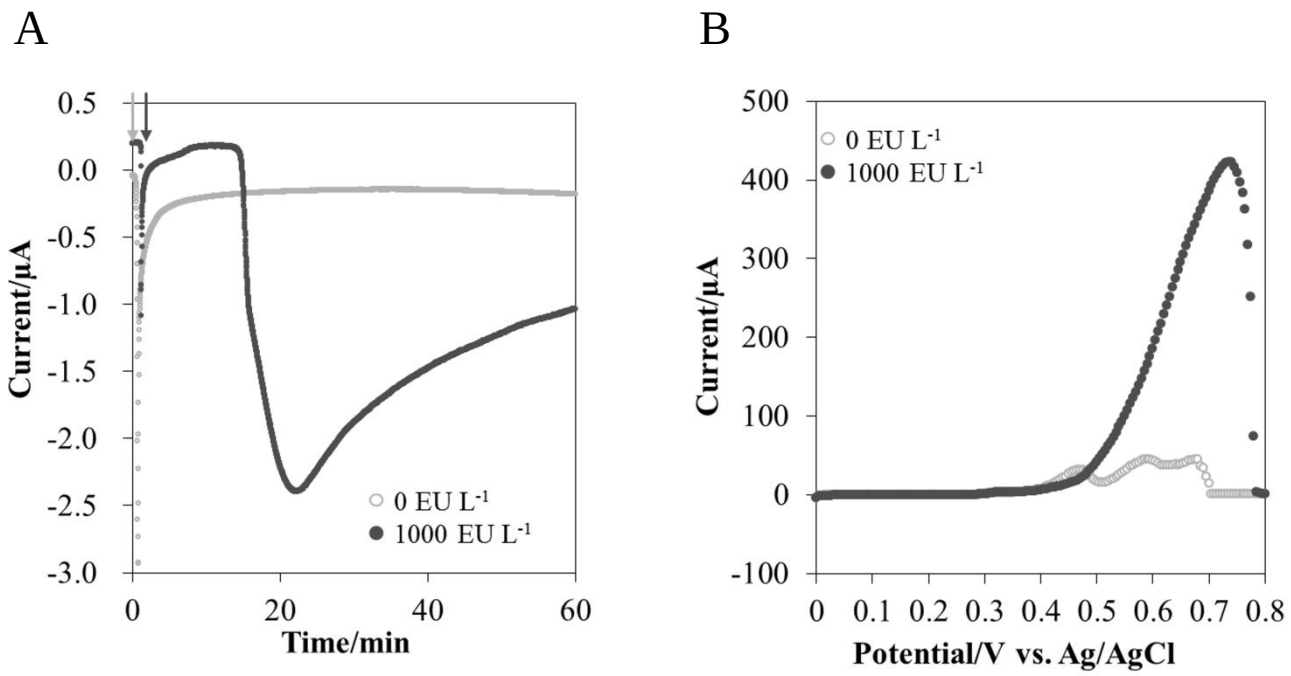
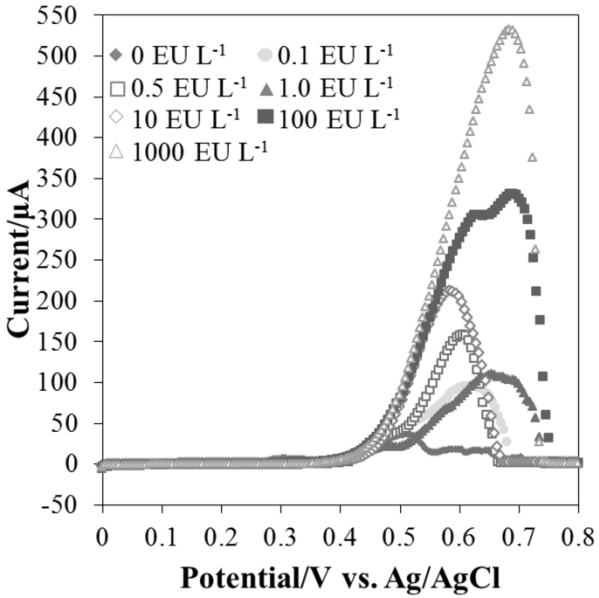


Fig. 4

A



B