Cell-Based Electrochemical Assay for Endotoxin Using a Secreted Alkaline Phosphatase Reporter System

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We developed a cell-based assay device for the detection of endotoxin, the potentially toxic compound that induces septic shock. Genetically-engineered cells that secrete alkaline phosphatase (SEAP) on exposure to endotoxin were cultured in an electrochemical cell device in medium containing p-aminophenyl phosphate and various concentrations of endotoxin. After 24 hr incubation, p-aminophenol (pAP), generated by SEAP-catalyzed hydrolysis, was detected by amperometry at +0.35 V. The amperometric response increased with the concentration of endotoxin in the range of 0.01-1 ng/ml.

Key Words : Cell-based Sensor, Lipopolysaccharide, Amperometer, Secreted Alkaline Phosphatase

1 Introduction

Detection of environmental threats, including dioxin, residual agricultural chemicals and allergens, is indispensable to improving the quality of life. The commonly-used methods of chemical analysis, gas chromatography and immunoassay, rely on the identification of a particular agent. In contrast, cell-based biosensors, which use whole living cells as the sensing element, can detect toxic activity in a physiologically relevant manner. Potentially, these biosensors can detect even unanticipated pollutants or chemicals causing complex reactions. Numerous cell-based biosensors have been developed and reviewed. These sensors detect the analytes by sensing the biological responses of the cell; for example, they detect changes in ion concentrations using an insensitive microelectrode (ISME), a fluorescent probe or a light-addressable potentiometric sensor (LAPS). Among these sensors, electrochemistry-based detection has several advantages, such as being small, field portable, easy to use and inexpensive. We developed an electrochemical cell-based device for endotoxin detection in the present study. Endotoxin, also known as lipopolysaccharide (LPS), is a potentially toxic compound found in the cell wall of Gram-negative bacteria. Because endotoxin provokes innate immune responses, thereby inducing septic shock in mammalian cells, monitoring for the biological activity of endotoxin in potentially contaminated medical supplies is important for safe healthcare. In the present study, we used commercially-available cells that have been genetically engineered to express Toll-like receptor 4 (TLR4; critical receptor and signal transducer in innate immune responses for endotoxin) and co-reporters. These cells induce secretory alkaline phosphatase (SEAP) when stimulated by endotoxin. The cells were placed in a device consisting of an electrode chip fabricated by photolithography and a cell culture well, and incubated with 1.5 mM p-aminophenyl phosphate (pAP; electrochemical substrate for alkaline phosphatase) for 24 hr. The p-aminophenol (pAP) produced by the SEAP-catalyzed hydrolysis was then detected by amperometry. This cell-based assay was found to act as a potentially effective detector not only for endotoxin monitoring but also for environmental, food, and pharmaceutical analyses.

2 Experimental

2.1 Materials and reagents

For standard endotoxin solution, E. coli K12 LPS (InvivoGen, USA) was diluted in endotoxin-free water (distilled water for injection; Otsuka, Japan) and mixed vigorously by vortexing. p-aminophenyl phosphate monosodium salt (pAPP; LKT Lab, USA) and all other chemicals were used as received. Aqueous solutions were prepared using high-purity distilled and deionized water from a Milli-Q filtration system (Millipore Corporation, USA).

2.2 Cell culture and handling procedure

The HEK-Blue™-4 cell line was purchased from InvivoGen. This cell line is HEK293 (human embryonic kidney cell line) stably transfected with multiple plasmids expressing human endotoxin receptor TLR4, coreceptors (myeloid differentiation protein-2; MD2 and cluster of differentiation-14; CD14) and SEAP reporter protein under the control of transcription factors such as nuclear factor kappa B (NF-κB). Therefore, when the cells are exposed to the endotoxin, TLR4 and co-receptors recognize the endotoxin, and the signaling cascade
leads to the activation of NF-κB, resulting in secretion of SEAP. Cells were cultured in 75 cm² flasks (BD Biosciences, USA) with Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (Invitrogen, USA) containing 10% fetal bovine serum (FBS; Hyclone, USA), 1% penicillin-streptomycin (Invitrogen), 1 × Normocin (InvivoGen) and 1 × HEK-Blue™-4 Selection (InvivoGen) in a humidified atmosphere containing 5% CO₂. Cells used in the measurements were cultured until 50-80% confluence, rinsed and detached from the flask using a cell scraper (BD Biosciences). Cells were suspended in DMEM containing 10% FBS and 1.5 mM pAPP without antibiotics at a concentration of 1.0 × 10⁶ cells/ml and used in the endotoxin assay.

2.3 Fabrication of the cell device

The cell device consisted of an electrode chip and a cell culture well made of poly-dimethyl siloxane (PDMS; SILPOT W/C, Dow Corning Toray, Japan). Figures 1A and B show the fabricated device and its structure, respectively. An electrode chip with three gold electrodes was fabricated by conventional photolithography using a previously described method. Briefly, a gold film (200 nm thickness) with a Ti adhesive layer was sputter-deposited (L-3328-FI; Avelva Corp., Japan) on a glass slide with the electrode pattern of a positive photoresist (S1805, Shipley Corporation, USA). After removal of the photoresist with acetone (lift-off), an insulation layer (~50 μm thickness) was fabricated using a negative photoresist (SU-8 3050, MicroChem Corp., USA) to define the electrode area exposed to the solution. The effective size of the working electrode was 200 × 200 μm. The cell culture well was formed by casting PDMS pre-polymer against an acrylic master plate, curing in an oven at 90°C for 30 min and peeling from the master. The flat bottom of the PDMS well and the SU-8 insulator surface of the electrode chip were bonded naturally.

2.4 96-well format assay

According to the protocol in the HEK-Blue™ LPS detection kit (InvivoGen), endotoxin sample solution (20 μl) was added to a 96-well tissue culture plate (BD Biosciences). A cell suspension containing 1.5 mM pAPP (200 μl) was then added to each well containing the endotoxin sample solution. After incubation at 37°C in 5% CO₂ for 24 hr, a working electrode (ϕ300 μm Au disc electrode), a reference electrode (Ag/AgCl-sat. KCl) and a counter electrode were inserted together into the well for electrochemical detection.

2.5 Cell device assay

Endotoxin sample solution (10 μl) was added into the PDMS well of the cell device in a laboratory dish containing drops of endotoxin-free water to prevent dryness. The chip was first warmed at 37°C in an incubator and then cell suspension containing 1.5 mM pAPP (100 μl) was added to the well and the whole device was incubated at 37°C in 5% CO₂ for 24 hr. Figure 1C shows an optical image of the working electrode after 24 hr incubation. The electrochemical measurement was performed using an Au pseudo reference electrode as reported previously.

2.6 Equipment and methods for electrochemical measurement

Cyclic voltammetry (CV) was performed using a potentiostat (HA1010mNR; Hokuto Denko Corp., Japan) in order to characterize the electrochemical behavior and basic performance of the cell device. The potential of the working electrode of the chip was scanned at 20 mV/sec between -0.6 V to +0.2 V vs. the Au pseudo-reference electrode.

Potential step chronoamperometry (PSCA) was used for the quantitative endotoxin assay. Figure 1D shows the principle of this detection system. When HEK-Blue cells are exposed to endotoxin, the cells secrete SEAP, which catalyzes the hydrolysis of pAPP to pAP. The enzymatically-generated pAP can be oxidized at an electrode held at +0.35 V. The resulting oxidation current is monitored to quantify the endotoxin in the solution.

3 Results and Discussion

3.1 Electrochemical optimization of medium pH based on 96-well format assay

First, we estimated the optimum pH of the medium using 96-well format assay in order to produce efficient electrochemical endotoxin detection. The pH of the DMEM with 10% FBS and 1.5 mM pAPP was adjusted to pH 5.5, 6.5, 7.5 and 8.5 using 1N NaOH or 1N HCl. HEK-Blue cells were suspended in these media and added to the well containing the endotoxin solution. After 24 hr incubation, pAP was detected at the Au disc electrode (300 μm in diameter). Figure 2A shows the PSCA responses at pH 6.5 after the potential was stepped from the open circuit voltage to +0.35 V vs. Ag/AgCl. The spike-like responses of the capacitive currents were observed immediately after the potential step, after which the oxidation currents decreased gradually. We used average currents of between 25 and 35 sec after the potential step as the amperometric responses for calibration. Figure 2B shows the calibration curves at various medium pH. The responses depended on the endotoxin concentration and the pH of the media.
um. A clear correlation between endotoxin concentration and the amperometric response was observed at pH 6.5 under the present conditions. SEAP is active at higher pH, which is not suitable for cell culture (data not shown). In addition, at higher pH, pAP is not sufficiently stable to retain enough electrochemical activity during 24 hr incubation (data not shown). Therefore, we used pH 6.5 medium in the subsequent experiment.

3.2 Electrochemical characterization of the chip

Because an Au thin layer was employed as the pseudo-reference electrode, it was important to characterize the electrochemical behavior of the chip. For this purpose the HEK-Blue cells were incubated in the cell device with 1.5 mM pAPP with various concentrations of endotoxin for 24 hr and CV was conducted. Figure 3 shows typical cyclic voltammograms of cell culture medi-

![Figure 2](image)

**Fig. 2** (A) Typical amperometric responses using 300 μm-diameter gold electrode with a 96-well microplate. Amperometric measurements were carried out at +0.35 V vs. Ag/AgCl reference electrode 24 hr after incubation in 200 μl DMEM with 10% FBS, 1.5 mM pAPP, 1 × 10⁶ cells/ml HEK-Blue cells and 20 μl endotoxin solution, pH 6.5. The concentration of endotoxin: (×) 0, (○) 0.01, (□) 0.1, (◇) 1, (△) 10, (+) 100 ng/ml. (B) Calibration curves for the average amperometric responses of 25-35 sec in figure 2A against the concentration of endotoxin, pH of the medium: 5.5 (○), 6.5 (●), 7.5 (□) and 8.5 (■).

![Figure 3](image)

**Fig. 3** Typical cyclic voltammograms 24 hr after incubation observed on the cell device with 100 μl DMEM with 10% FBS, 1.5 mM pAPP, 1 × 10⁶ cells/ml HEK-Blue cells and 10 μl endotoxin solution, pH 6.5. The size of the Au working electrode was 200 × 200 μm. Potential was applied against the Au pseudo-reference electrode (500 × 400 μm) integrated on the chip. Scan rate: 20 mV/s.

3.3 Detection of endotoxin using the cell device

Analytical calibration of endotoxin was conducted in order to estimate the sensitivity and preciseness of the cell device assay. After 24 hr incubation of HEK-Blue cells in the cell device with 1.5 mM pAPP and different concentrations of endotoxin (0, 0.01-100 ng/ml) at pH 6.5, PSCA was performed with the step potential from the open circuit voltage to +0.35 V. Figure 4A shows the current responses observed at the cell device. Similar to the 96-well format protocol, the average currents between 25 and 35 sec were used as the responses for the calibration curve and plotted against the concentration of endotoxin in Fig. 4B. Each point of the calibration graph corresponds to the mean value, and error bars indicate the standard deviation obtained from seven independent measurements. The amperometric responses increased with increasing endotoxin concentration in the ranges of 0.01-1 ng/ml. This range nearly covers the concentration levels required for monitoring endotoxin contamination of medicinal instruments and drugs for injection. For example, the upper limit of endotoxin concentration in an ultrapure dialysate, one of the most severely-controlled medical liquids with a very low endotoxin concentration tolerance, is set at 0.03 EU/ml (0.006 ng/ml of Reference Endotoxin Standard, EC-2) by the Association for the Advancement of Medical Instrumentation (AAMI). This concentration level is
identical to the detection limit provided by the commonly used Limulus amebocyte lysate (LAL) assay.\(^{20}\) However, ultra-sensitive detection methods to monitor trace endotoxin are required to secure the quality of medicine for long-term dialytic treatment. Further improvement is also necessary to minimize signal errors. We are presently in the process of developing a highly sensitive, precise and quick assay using this cell device. This cell-based assay system will provide highly sensitive endotoxin monitoring in the end-stream dialysate at bed-side and will also provide a model of cell-based biosensors for detection of hazardous materials in environmental, food and pharmaceutical monitoring.

4 Conclusion

In this study we developed a cell-based electrochemical device for endotoxin detection. When the cells in the device are exposed to endotoxin, the cells secrete alkaline phosphatase, which produces amperometric responses. The response increases with the concentration of endotoxin in the range of 0.01-1 ng/ml. The present method will be used for an on-site, rapid, easy to use and portable detection system for endotoxin and can be applied to detection of other hazardous materials.

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References