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Voltammetric measurement of *Escherichia coli* concentration through p-APG hydrolysis by endogenous β-galactosidase

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A B S T R A C T

This paper reports a facile electrochemical detection method for *Escherichia coli* (*E. coli*) that does not use DNA amplification or immunoassay. The detection principle is based on the activity of the β-galactosidase (β-gal) endogenous enzyme, which hydrolyzes p-aminophenyl-β-D-galactopyranoside (p-APG) into p-aminophenol. After *E. coli* consumes p-APG within 30 min, the remaining p-APG is oxidized on a gold electrode using cyclic voltammetry and square wave voltammetry. The β-gal expression level is increased through treatment with a β-gal expression inducer (isopropyl-β-D-thiogalactopyranoside), and the hydrolysis reaction of p-APG is facilitated through permeabilization treatment with sodium dodecyl sulfate. The calibration curve for *E. coli* has a working range of 10^2–10^4 colony-forming units per mL in nutrient broth buffer. The total assay time is less than 100 min. The successful application of this approach indicates the possibility of rapid detection.

1. Introduction

Microorganisms such as bacteria and pathogens are a major health risk worldwide because they can survive, reproduce, and disperse in water systems [1]. According to a World Health Organization report, there are approximately 1.7 billion cases of childhood diarrhoeal disease. In addition, 525,000 children under the age of five in developing countries die globally each year because of poor water quality and sanitation, mainly through infectious diarrhea [2, 3]. *Escherichia coli* (*E. coli*) is the best-known coliform indicating fecal contamination because it is found almost exclusively in human and animal feces [2, 4]. Human and animal waste in water resources can cause water pollution [5]. Measurement of *E. coli* bacteria in water resources is thus critical.

To date, no approaches have been reported that can numerate or isolate all of the noteworthy pathotypes of *E. coli*. Because of the multiple pathogenic factors in water other than dangerous bacteria, the direct testing of water using a wide variety of pathogens will be difficult, expensive, and time-consuming [3].

Conventional approaches have been developed to specifically quantify or isolate the pathogenic type of *E. coli*, for example by enumerating the levels of bacteria in water or other substances through multiple tube fermentation [6] and the plate count enumeration method and membrane filter technique [7]. Although these methods, developed in the 19th century, are highly accurate, they are impractical for the assessment of water resources because they require several days of culturing.

Several approaches to rapidly detecting *E. coli* concentration in water have been proposed, such as immunoassays [8–10], polymerase chain reaction (PCR) [11–13], flow cytometry [14, 15], and the measurement of *β*-D-glucuronidase activity [16–25]. Immunoassays such as the enzyme-linked immunosorbent assay (ELISA) can detect *E. coli* within 24 h because they employ a pre-enrichment culture [26]. ELISA outperforms conventional methods, which require at least 2 days to obtain results. Another approach employed is the use of PCR to clone a specific gene fragment for signal amplification. The PCR technique is common in biological laboratories because of its high sensitivity and high throughput detection in low-concentration samples within several hours. However, the process of amplifying exogenous DNA can easily result in contamination, and professional technicians are required to conduct the experiment and interpret the results. The need for a large facility, expansive chemicals, and considerable time and the generation of biological waste also make PCR inconvenient for the rapid screening of *E. coli* in water resources. Detection of both viable and dead cells is a barrier to using the PCR technique for sensing *E. coli*. Flow cytometry is another method of *E. coli* detection. When a preconcentration step is employed, a limit of detection of 1×10^2 cfu/mL can be achieved. However, this method requires a large facility and fluorescent dye labeling.

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The direct measurement of *E. coli* through its specific β-galactosidase (β-gal) activity has enabled the performance of rapid assays. A well-known enzyme [7,8], β-gal, is encoded by the lacZ gene of the bacteria chromosome and composed of four noncovalently bound subunits of mass 116 kDa each [9]. When lactose is the only carbon source, *E. coli* produce β-gal to hydrolyze lactose into galactose and glucose. To induce *β*-gal expression in *E. coli*, isopropyl β-D-1-thiogalactopyranoside (IPTG) can be used, which is a molecular mimic of allolactose and not metabolized by *E. coli*. The *β*-gal activity can be amperometrically detected using 4-aminophenyl *β*-dgalactopyranoside (p-APG) as the substrate, which is hydrolyzed by the enzyme into p-aminophenol (p-AP). Limits of detection of 7×10^4 cfu/mL for *E. coli* after 2 h of incubation and 10 cfu/mL after 7 h of incubation were demonstrated [25].

However, determining the concentration of endogenous enzyme β-gal in *E. coli* by using IPTG is not sufficiently quick for achieving rapid detection. To enhance the sensitivity, bacteriophages (phages) were engineered to encode the gene for inducing β-gal expression. Phages can specifically recognize, attach to, and infect target bacteria. The lacZ operon was inserted into the genome of T7 phages to form engineered bacteriophages. When the engineered phages target *E. coli*, the infected *E. coli* overexpress β-gal. The p-APG hydrolysis process was used to indicate the number of β-gal molecules. This approach could detect 10^5 cfu/mL *E. coli* after 3 h and 10^2 cfu/mL *E. coli* after 7 h from aqueous samples (drinking water, apple juice, and skim milk). Detection was rapid—(within 2 to 3 h)—within a concentration of 10^4–10^5 cfu/mL [23,24].

The transmission rate of p-APG into *E. coli* is low, and the currents based on p-APG hydrolysis by β-gal are weak; thus, the sensitivity of amperometry, which is employed to directly measure the currents from p-APG hydrolysis by β-gal, is still too low to detect low *E. coli* concentrations. Another approach by using electrochemical magneto immunosensor to measure oxidation of p-AP, which is the production of hydrolysis of p-APG through β-gal endogenous enzyme [27]. Through magnetic beads labeling, the bacteria can be efficiently attracted to electrode surface, so that the reduction-oxidation current can be measured even very small. The limit of detection of 33 cfu/mL in less than 2 h and linear range of 5×10^1 to 5×10^3 cfu/mL can be achieved. Although the method is able to overcome weak current, use of antibody in sensing technique still make the cost become high.

We introduced three major improvements to enhance the sensitivity of *E. coli* detection in low concentrations within a short period (Fig. 1). First, sodium dodecyl sulfate (SDS), a surfactant, was used to lyse bacteria for p-APG rapidly reaching β-gal. Second, after p-APG hydrolysis lasting 30 min, the remaining concentration of p-APG, which was oxidized on a gold electrode using voltammetry, was determined. Third, to enhance sensitivity, the remaining p-APG was quantified by integrating the area of anodic peak. In this study, two techniques of electrochemical voltammetry—cyclic voltammetry (CV) and square wave voltammetry (SWV)—were implemented to investigate p-APG hydrolyzed by endogenous β-gal into p-AP. An electrochemical chip consisting of a gold working electrode, carbon counter electrode, and Ag/AgCl reference electrode was used. The electrochemical chip used in this experiment is a disposable and one-time-use device. Consider to cost, we select carbon electrode as counter electrode in this experiment. We employed IPTG for inducing β-gal expression by *E. coli* and added p-APG to indicate the β-gal activity. To increase the transmission rate of p-APG into *E. coli* and thus catalytically hydrolyzed by β-gal, SDS was added to destroy the cell wall of *E. coli* [28–30]. After this destruction, the p-APG hydrolysis process was measured using electrochemical voltammetry within 30 min. To quantify the *E. coli* concentration, the areas of anodic peaks obtained using CV and SWV were calculated. In our experiments, the sensitivity achieved using SWV was almost 2 times that obtained using CV. Therefore, the anodic peaks of p-AP can be observed using SWV but not be found using CV.

2. Experiments

2.1. E. coli culture and sample preparation

Nutrient agar (NA) and nutrient broth (NB) were purchased from Merck. Sodium dodecyl sulfate polyacrylamide (SDS), isopropyl-β-D-thiogalactopyranoside (IPTG), and p-aminophenyl-β-D-galactopyranoside (p-APG) were purchased from Sigma. Cefsulodin was obtained from Gold Biotechnology, and *E. coli* BCRC 11634 was used in this experiment. NA and NB are commercially available in powder form. We added 4 g of NA powder to 200 mL of distilled deionized water, and after sterilization for 30 min, the NA was placed in a refrigerator at 4 °C for storage and microwaved before use. Subsequently, 1.6 g of NB powder was added to 200 mL of distilled deionized water, and the pH was adjusted to 7.0 ± 0.2. After sterilization for 30 min and addition of 5 mL of cefsulodin with a concentration of 0.2 mg/mL, the NB was placed in a refrigerator at 4 °C for storage.

The *E. coli* stored at −80 °C were cultured on an NA plate in an incubator at 37 °C for 24 h and then stored in a 4 °C refrigerator for at most 1 month. To suspend the *E. coli*, one colony was scratched out

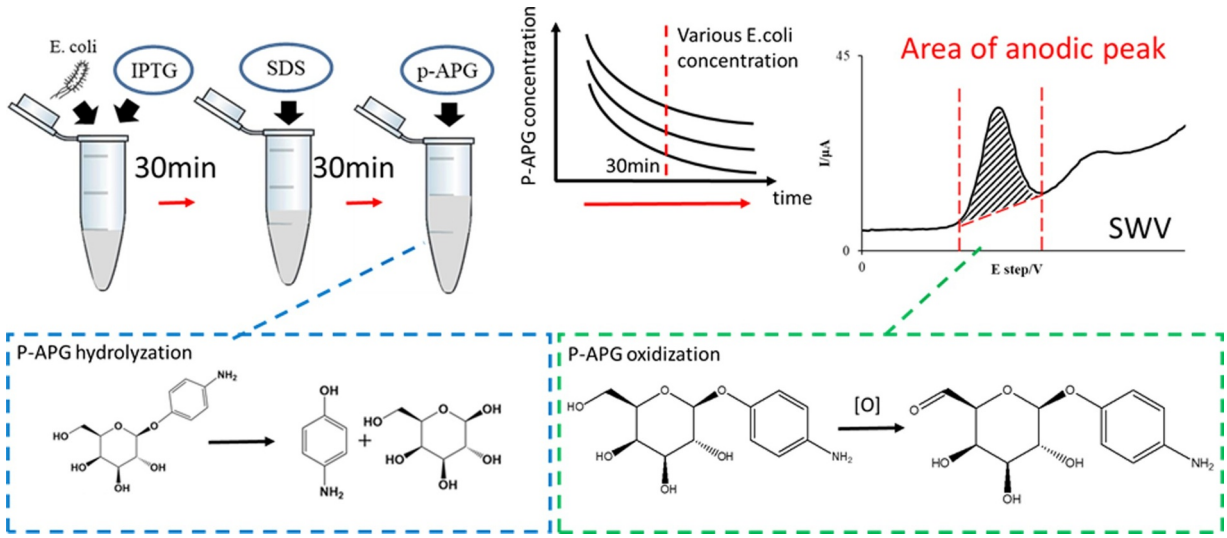

Fig. 1. Schematic of the experiments. *E. coli* was sequentially added IPTG, SDS, and p-APG, and the p-APG concentration, which is related to *E. coli* concentration, was determined by using voltammetry.

from the *E. coli* culture plate, dropped in 5 mL of NB medium, and placed in a 37 °C incubator with continuous shaking for 24 h. This procedure was repeated several times to ensure the activity of the *E. coli*. The number of active *E. coli* was determined using both spectrophotometry and the plate count method. *E. coli* concentrations of 10^2–10^4 cfu/mL were prepared for further experiments.

To detect *E. coli*, referring to the previous effort by Cheng et al., 0.1 mL of 5 mM IPTG was added to 0.9 mL of *E. coli* sample, achieving a final concentration of 0.5 mM IPTG [31]. The sample was incubated at 37 °C with shacking for 30 min to stimulate endogenous β-gal expression in the *E. coli*. Subsequently, according to a review study by Alakomi, A total of 10 μL of 1% SDS was used and added to the sample and left for 30 min to accelerate p-APG catalytically hydrolyzed by endogenous β-gal [32]. Then, 500 μL of p-APG with concentration 1 mg/mL was added to the sample and left for 30 min for p-APG hydrolysis by β-gal. The 150 μL prepared sample was used for further *E. coli* detection.

2.2. Electrochemical measurements

In this experiment, we employed a potentiostat (SP-150, Bio-Logic Science Instruments) and disposable high quality electrochemical biochips (G3, Vida Bio technology) with three electrodes: a gold working electrode, carbon counter electrode, and Ag/AgCl reference electrode. CV and SWV were implemented. For CV, the sweep voltage range was −1.0 to 1.0 V, the sweep rate was 50 mV /s, and the initial voltage was −0.99 V. For SWV, the sweep voltage range was −0.6 to 1.0 V, the waiting time was 10 s, the pulse height was 25 mV, the pulse width was 100 ms, and the step height was 10 mV. The electrodes were cleaned using deionized water and then blow-dried. In each experiment, 150 μL of prepared sample was dropped on the electrodes for measurement.

3. Results and discussion

3.1. Determination of E. coli concentration

One *E. coli* colony from an NA culture plate, suspended in 5 mL of NB solution and placed in an incubator for 24 h, was used as the sample. The optical density (OD) of the sample at 600 nm was measured. After 10^6 times dilution, the sample was cultured for 24 h on an NA plate by using the pour plate method, and then the number of colonies was counted to estimate the original concentration of the *E. coli* in the NB solution. After this experiment was conducted four times, OD versus *E. coli* concentration was plotted, as displayed in Fig. 2(a). Using linear regression analysis, the linear regression function $y = 112.41x - 1.368$ with an R^2 of 0.9957 was obtained.

To study how the number of *E. coli* increased with time, the growth curve of *E. coli* was investigated. The sample of *E. coli* in NB medium with an initial OD of 0.1 was cultured in an incubator, and the *E. coli* concentration was determined by measuring the OD every hour. The results plotted in Fig. 2(b) reveal that in the first 3 hours, the concentration of *E. coli* slowly increased (lag phase); during hours 3–6, the concentration dramatically increased (exponential phase); and after culturing for 6 hours, the concentration reached saturation and the growth rate was almost zero (stationary phase). The *E. coli* growth curve indicates that the entire *E. coli* detection process is best completed within 3 hours for accuracy.

3.2. Electrochemical properties obtained using CV and SWV

CV can be used to determine the peak value of the oxidation and reduction potential in a chemical reaction. In CV, the effect of scan rate on the peak current i_{pc} is described using the Randles–Sevcik equation:

$$i_{pc} = 2.69 \times 10^5 (n^{3/2} D^{1/2} v^{1/2} A c),$$

where n is number of electrons transferred in the redox event, D is the diffusion coefficient, v is the scanning rate, A is the electrode area, and c is the concentration of the sample. For simple redox events, when the substance of the sample, electrode area of the device, and scanning rate are kept constant, the sample concentration c is proportional to the current i_{pc}.

The electrochemical properties of *E. coli* in NB medium at various concentrations were investigated using CV, and the current at the work electrode versus the applied voltage is plotted in Fig. 3(a). The anodic peak was observed in the range of 0.7–0.8 V. The anodic peak currents for the samples with concentrations 10^3 and 10^5 cfu/mL were 32.453 and 30.154 μA, respectively. The cathodic peak currents for the samples with concentrations 0, 10^3, and 10^5 cfu/mL were −7.807, −9.284, and −8.891 μA, respectively. Neither the anodic peak current nor cathodic peak increased with increasing concentration of *E. coli*.

To study the electrochemical properties of *E. coli* with additives, 0.1 mL 5 mM IPTG, 10 μL 1% SDS, and 500 μL 1 mg/mL p-APG were individually added to three *E. coli* NB medium samples with 1 mL. The samples were incubated at 37 °C with shaking for 30 min and their currents at the work electrode were then measured using CV. The results stack-plotted in Fig. 3(b). The anodic peak for *E. coli* with p-APG (black line) was observed in the range of 0.4–0.5 V but was not found for the *E. coli* sample with either IPTG (blue line) or SDS (red line). This indicates that the anodic peak may result from p-APG contribution.

Furthermore, the *I–V* curves of the samples containing p-APG in concentrations of 0, 1, 2, or 3 mg/mL were obtained using CV. Fig. 3(c) shows the results and reveals that the anodic peak of pure p-APG is in the range 0.4–0.5 V. Thus, p-APG can be catalyzed by a gold working electrode and oxidized when a voltage is applied. Another small anodic peak located in the range 0–0.1 V was observed, which was a p-AP anodic peak [33].

To demonstrate the sensing mechanism, SWV was employed to study the electrochemical properties of the mixture of IPTG, SDS, p-

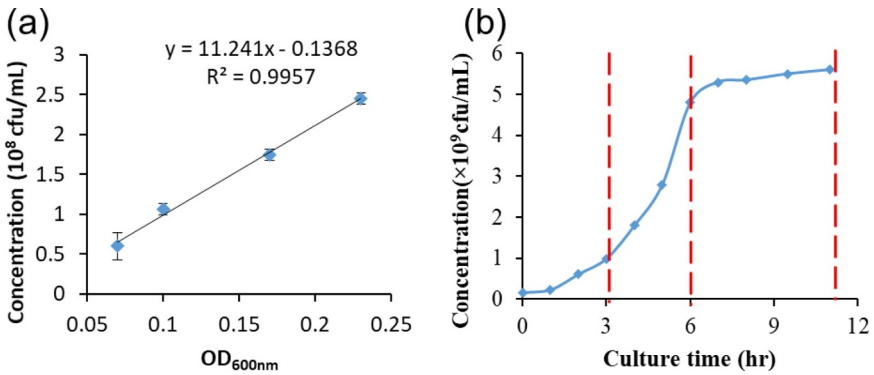

Fig. 2. (a) OD versus *E. coli* concentration, estimated by cell counting, has a highly linear relationship, indicating the reliability of the linear regression. (b) Growth curve of *E. coli*. When the incubation time is more than 3 h, the number of *E. coli* dramatically increases.

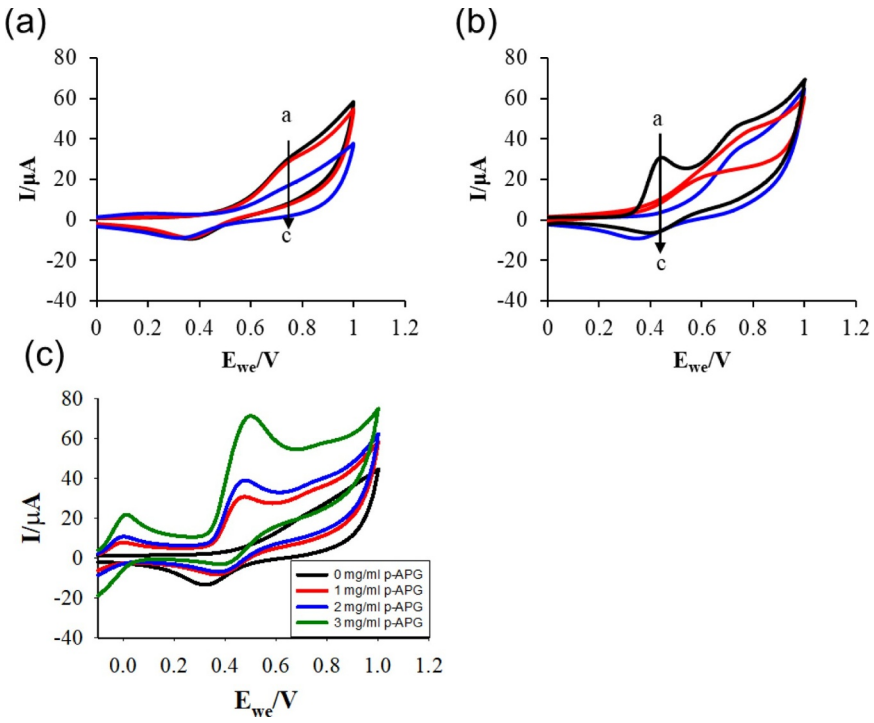

Fig. 3. (a) *I–V* curve of *E. coli* suspended in NB with concentrations of 10^3 (red line), 10^5 (black line), and 0 (blue line) cfu/mL, obtained using CV and showing no anodic peak. (b) When IPTG (blue line), SDS (red line), and p-APG (black line) were added to *E. coli* samples, only the *I–V* curve of the sample with p-APG has an anodic peak. (c) *I–V* curve of different concentrations of p-APG, obtained using CV, indicates that the p-APG concentration can be determined using gold working electrode–based electrochemical chip and voltammetry. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

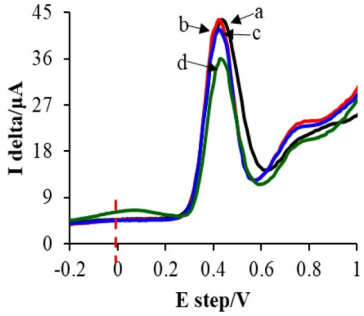

Fig. 4. Effect of permeabilization of cell membranes for p-APG oxidation peak current, as determined using SWV. The *E. coli* concentration was 2.01×10^3 cfu/mL. After *E. coli* were incubated with additives for 30 min, the *I–V* curve of (a) pure p-APG and (b) *E. coli* + p-APG exhibited the same high anodic peak at 0.4–0.5 V, indicating that the p-APG was not consumed by the *E. coli*. The anodic peak of (c) *E. coli* + IPTG + p-APG and (d) *E. coli* + IPTG + SDS + p-APG was slightly smaller and much smaller, respectively, than that of pure p-APG, showing that the p-APG was consumed by the *E. coli*. Therefore, the *I–V* curve exhibited another small anodic peak at 0.05–0.15 V (red dashed line), which represents the oxidized current of p-AP. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

APG, and *E. coli* after 30 min of reaction (Fig. 4). There four samples were prepared including (1) 500 μL 1 mg/mL p-APG adding to 1 mL DD water, (2) 500 μL 1 mg/mL p-APG adding to 1 mL *E. coli* sample with concentration of 2.01×10^3 cfu/mL and incubating at 37 °C with shaking for 30 min, (3) 0.1 mL of 5 mM IPTG adding to 0.9 mL of *E. coli* sample and incubating at 37 °C with shaking for 30 min, after that, adding 500 μL 1 mg/mL p-APG and incubating at 37 °C with shaking for another 30 min, (4) 0.1 mL of 5 mM IPTG adding to 0.9 mL of *E. coli* sample and incubating at 37 °C with shaking for 30 min, after that, adding 10 μL 1% SDS and incubating at 37 °C with shaking for another 30 min, following, adding 500 μL 1 mg/mL p-APG and incubating at 37 °C with shaking for final 30 min.

When p-APG was hydrolyzed by β-gal in *E. coli*, the anodic peak value of the voltammogram decreased. The anodic peak value of the 1 mg/mL p-APG and mixture of 1 mg/mL p-APG and *E. coli* was 43.487 and 43.574 μA, respectively, which are extremely similar. Thus, the p-APG was not consumed by the *E. coli*. This may be because the *E. coli* did not secrete β-gal, the p-APG did not reach the β-gal in the *E. coli*, or both. ITPG was added to induce β-gal secretion by *E. coli*, and the anodic peak value of the mixture of ITPG, p-APG, and *E. coli* was 40.808 μA, slightly less than that of the sample of p-APG and *E. coli*. This indicated that an increase in β-gal resulted in a decrease in p-APG.

To enhance the hydrolysis rate of p-APG, SDS was added to the mixture of ITPG, p-APG, and *E. coli* to increase the p-APG permeability into *E. coli*. The anodic peak value of the mixture of SDS, ITPG, p-APG, and *E. coli* was 30.875 μA, clearly lower than the aforementioned results. Thus, SDS helps enhance the penetration by p-APG of *E. coli* cell walls, and the p-APG is then hydrolyzed by β-gal. Furthermore, a small anodic peak current of 6.452 μA at 0.05–0.15 V was obtained (dashed line in Fig. 4), which represents the oxidized current of p-AP, the product of p-APG hydrolysis.

Fig. 5(a) and (b) presents the CV- and SWV-obtained *I–V* curves, respectively, of various concentrations of *E. coli*, sequentially adding IPTG, SDS, and p-APG, with incubation/reaction time of 30 min for every additive. The procedures of processes are described in Section 2.1.

The p-APG is hydrolyzed by the β-gal of *E. coli*, and its concentration decreases faster when the *E. coli* concentration is higher. The *I–V* curves obtained using CV and SWV both have an anodic peak at 0.4–0.5 V, which corresponds to p-APG oxidation. The samples with higher *E. coli* concentration have weaker anodic peak signals. Different from traditional CV, in which a continuously linearly sweeping voltage is employed, SWV uses a combined square wave and staircase potential applied to a stationary electrode. Increasing the pulse-type voltage can help voltage from the working electrode penetrate the diffusion layer to the liquid sample without too much potential loss. The SWV method results in less potential loss than the CV method, resulting in more current amplification. The anodic peak of the *I–V* curve obtained using SWV has a higher aspect ratio than that obtained using CV. Thus, the sensitivity is improved. A small peak at 0.05–0.15 V, corresponding to p-AP, was also observed when SWV was used, as shown in Fig. 5(b), but not observed using CV.

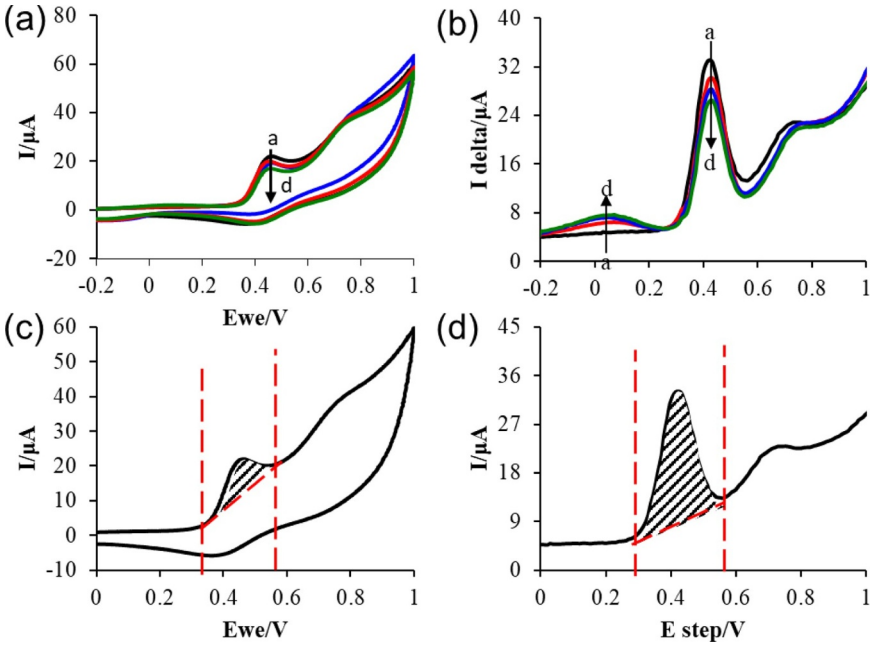

Fig. 5. After IPTG, SDS, and p-APG were sequentially added, with 30 min of incubation for each additive, the *E. coli* samples with concentrations 0, 2.3×10^2, 2.3×10^3, and 2.3×10^4 cfu/mL were measured using (a) CV and (b) SWV. *E. coli* concentration was quantified by calculating the area of anodic peak for (c) CV and (d) SWV.

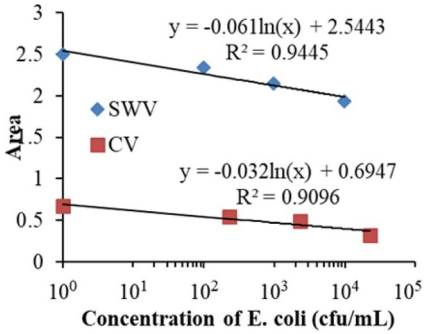

Fig. 6. Calculated areas of anodic peaks obtained using CV and SWV, showing a log-linear function of *E. coli* concentration. The slopes for the SWV and CV methods are −0.061 and −0.032, respectively.

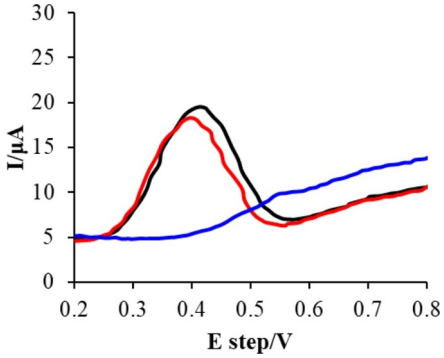

Fig. 7. Spiked sample testing by SWV method. *I–V* curves of pure lake water (blue), p-APG added lake water (black), and 10^2 cfu/mL *E. coli* spiked lake water with adding IPTG, SDS, and p-APG (red). For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

To analyze the peak variation between the different conditions of using CV and SWV, the area of the anodic peaks were first defined, as illustrated in Fig. 5(c) and (d), for use of the CV and SWV methods and were then calculated. Fig. 6 plots the peak area versus *E. coli* concentration. The linear regression of the logarithmic *E. coli* concentration versus anodic peak area for CV and SWV was also analyzed. Linear regression lines of $y = -0.032\ln(x) + 0.6947$ with an R^2 of 0.9096 for the CV method and $y = -0.061\ln(x) + 2.5443$ with an R^2 of 0.9445 for SWV method were obtained. The slope of regression line for SWV was almost 2 times that for CV. Thus, detection using SWV is twice as sensitive as that using CV. The limit of detection of 10^2 cfu/mL and the linear range of 10^2–10^4 cfu/mL can be found in this experiment.

The water form Chung-Hsing lake, Taiwan, was used to spike the *E. coli* detection experiment. Firstly, the *I–V* curve of pure lake water was measured by using SWV. Following, p-APG was added in to lake water and the *I–V* curve was carried out by SWV. Another sample with 10^2 cfu/mL *E. coli* spiked in lake water was prepared for testing. By the procedures revealed in Fig. 1, the *I–V* curve can be obtained. From the results shown in Fig. 7, the *I–V* curve of pure lake water showed no anodic peak (blue line) and both of p-APG added lake water (black line) and *E. coli* spiked lake water with adding IPTG, SDS, and p-APG (red line) showed anodic peak in the range of 0.4–0.5 V. The p-APG added lake water showed higher peak current than that of E. coli spiked lake water with adding IPTG, SDS, and p-APG because the p-APG in *E. coli* spiked lake water was consumed, and the p-APG concentration decreased.

4. Conclusions

In this study, we demonstrated the sensing of *E. coli* concentrations 10^2–10^4 cfu/mL by using CV and SWV. The *I–V* curves obtained using CV and SWV both had anodic peaks at 0.4–0.5 V, corresponding to the p-APG oxidation current. The area of the anodic peaks obtained using CV and SWV was related to the *E. coli* concentration of the sample. In higher *E. coli* concentration samples, more p-APG was consumed and the area of the anodic peaks was smaller. Furthermore, when measuring samples with the same *E. coli* concentration, the use of the SWV method resulted in a higher aspect ratio of anodic peaks than the use of the CV method. The variation in the area of the anodic peaks when SWV was employed was almost twice that when CV was employed. Thus, the SWV method has higher sensitivity for measuring *E. coli* concentrations.

CRediT authorship contribution statement

Yu-Jui Fan: Methodology, Writing - review & editing. **Yu-Chen Hsu:** Investigation, Data curation, Writing - original draft. **Bing-Chen**

Gu: Formal analysis, Validation. **Chia-Che Wu:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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