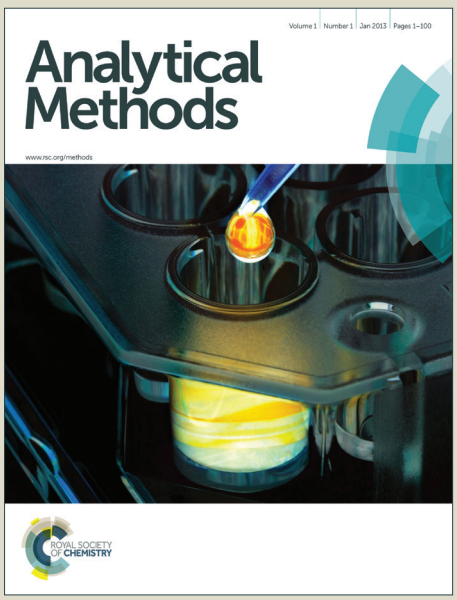
Analytical Methods

Accepted Manuscript



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Ultrasensitive and rapid detection of *Escherichia coli* O157:H7 in beef juice using immunoassay based on field-effect enzymatic detection

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Abstract

Ultrasensitive and rapid detection of *E. coli* O157:H7 in beef juice was achieved using a novel immuno-detection approach. The detection system was realized by incorporating the recently-developed technique of field-effect enzymatic detection with the immunosensing method. The detection of unprocessed samples resulted in a detection limit of 19 CFU/mL with an assay time of 67 min. The ultrasensitive detection of the original sample was due to the intrinsic amplification provided by system. The direct detection without performing pre-enrichment significantly shortened the assay time. The voltage-controlled signal amplification of the detection system also facilitated the transduction of electrical signal through the bulky immune complex so that the detection did not rely on the use of mediators or other diffusional substances. The novel detection approach can be used as a detection platform for ultrasensitive, specific and rapid detection of microorganisms and other types of analytes that can be detected using immunoassay.

1. Introduction

Escherichia coli (*E. coli*) is a bacterium usually found in the intestines of healthy humans. *E. coli* O157:H7 is a strain of *E. coli* that causes severe intestinal infection in humans. Foodborne outbreaks due to *E. coli* O157:H7 have been caused by consumption of undercooked ground beef, raw milk, unpasteurized apple juice, water, and contaminated produce[1]. The low infectious dose of 2 to 2000 of ingested cells makes the detection and control of *E. coli* O157:H7 a challenging task for food safety [1].

Currently, the mostly used detection technologies for microorganism pathogens are polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), in which the required sample pre-enrichment results in long assay times [2]. For example, a PCR-ELISA technique has been used to detect *E. coli* in milk with a detection limit of 100 CFU/mL and an assay time of 5 hours [3]. The current limit of *E. coli* O157:H7 detection using the USDAFSIS method is less than 1 CFU per 65 g sample of raw or ready-to-eat meat product. The enrichment-based method requires 20–24 h to identify a potential positive sample[4]. Electrochemical immunosensors are an attractive technology for the detection of microorganisms due to its potential of providing cost-effective, ease-of-use and portable detection[5]. However, several disadvantages limit the performance of immunosensors. Because of the bulky size of the immune complex, electron transfer through the complex is significantly impeded. Therefore, electrochemical immunosensing of microorganisms is performed with mediators or by amperometric detection of the product of an enzyme-catalyzed reaction, in which the enzyme is used to label the antibody[6, 7]. Since the mediators diffuse through a solution to the detecting electrode, the detection selectivity can be reduced by interference due to electroactive contaminants in the solution[4]. The use of mediators adds additional steps to the detection procedure and may increase the assay time.

Further, since detecting electrodes are generally blocked with proteins such as bovine serum albumin (BSA), the redox reaction of mediators or products at the electrodes can be hampered, leading to suppressed sensitivity. Finally, the detection system of conventional immunosensors does not provide intrinsic signal amplification. Amplification in immunosensors relies on labeling the antibodies with enzymes, whose reaction products diffuse to the blocked surface of electrodes, which limits the sensitivity. Note that immunosensors still need sample pre-enrichment for microorganisms[4, 8].

In this article, we demonstrate the ultrasensitive and rapid detection of *E. coli* O157:H7 in beef juice using a novel immuno-detection approach, which incorporates field-effect enzymatic detection (FEED) [9], a newly developed ultrasensitive detection method, with the conventional immunosensing method. We first show that the gating voltage V_G of FEED facilitated the transduction of detection signal through the bulky immune complex so that the detection did not rely on the use of mediators. The voltage-controlled intrinsic amplification provided by the detection system allowed us to detect *E. coli* O157:H7 in beef juice samples with an estimated detection limit of 19 CFU/mL without pre-enrichment, leading to an assay time of 67 min.

2. Experimental

2.1 FEED principle

The principle of FEED was explained and the detection system described previously[9]. A description of the principle of FEED is also given in Supplementary Information. Briefly, the detection system consists of a conventional three-electrode electrochemical cell modified with additional gating electrodes for applying a gating voltage V_G to the detecting (working) electrode,

on which a redox enzyme, the sensing element, is immobilized. V_G rearranges ions in the sample solution at the electrode-solution interface, inducing an electric field that penetrates the enzyme. The field reduces the effective height of the energy barrier between the active site of the enzyme and the electrode, therefore increasing the transfer rate of electrons and resulting in intrinsic amplification of the signal current. In the present application of FEED, the enzyme is immobilized on the electrode via the immune complex formed by antibodies (Ab) and *E. coli* O157:H7.

2.2 Detecting electrodes

Mortalized *E. coli* O157:H7 (used for safety reasons), positive control (Catalog no. 50-95-90; Lot no. 090922), primary and horseradish peroxidase (HRP) labeled secondary antibodies (Ab) specific to *E. coli* O157:H7 (Catalog nos. 01-95-90 and 04-95-90) were purchased from KPL (Gaithersburg, MD). The positive control gave an *E. coli* O157:H7 concentration of 1.15 x 10^9 CFU/mL. Disks of pyrolytic graphite (PG) (1 cm x 1 cm) were used as electrodes. A layer of the conducting polymer, polyaniline (PANI), was deposited on PG electrodes to host the primary antibody on the electrodes[10] and to electrically connect the antibody to the electrode. The PANI layer was synthesized on the electrodes using electrochemical polymerization of aniline (Sigma Aldrich, CAS # 142-04-1) in a solution of 0.1 M aniline and 1 M HCl at a potential of 1.2 V for 100s[11]. The PANI layer prepared using this method was used previously in a HRP-based biosensor to detect H_2O_2 at pH 6.8[12]. Alternatively, sulfonated PANI was prepared by adding poly(vinylsulfonic acid) (Aldrich, cat. # 27,842-4) to aniline and HCl during electrochemical polymerization as described previously to increase the redox activity of PANI at pH 7[13]. A plastic mask with a 1 mm x 1 mm opening was used to define a window on the PANI layer. The

window was further modified by depositing 2μL of glutaraldehyde (25% with water, diluted 100 times with water) on PANI until dry. Glutaraldehyde (Sigma Aldrich, CAS # 111-30-8) was used as a cross-linker, coupling the primary antibody to the highly porous PANI so that the Ab-*E.coli*-Ab(HRP) structure was firmly entrapped in PANI.

E. coli-detecting electrodes were formed by incubating the modified PG electrodes with 50 μL of 0.1mg/mL primary antibody dissolved in phosphate buffered saline (PBS) for 4 hr at room temperature followed by rinsing with de-ionized water. To carry out the detection, *E. coli*-detecting electrodes were incubated with 10 μL of an *E. coli* O157:H7 sample for 35 min. at 37 °C. After rinsing using de-ionized water and blocking possible open areas on the electrodes with BSA, the electrodes were incubated with 10 μL of 0.1 mg/mL HRP-labeled secondary antibody for 30 min at 37 °C and then rinsed with de-ionized water. The Ab-*E.coli*-Ab(HRP) complex now was formed on the electrodes.

2.3 Electrochemical measurements

The electrodes prepared according to the above steps were used as working electrodes for the measurement of the detection signal. A piece of 0.5 mm-diameter copper wire coated with a thin layer of insulator (enamel) was used as the gating electrode for applying V_G. The wire was bent to form a U-shaped structure and was attached on the working electrode using nonconductive epoxy. The detection signal was obtained by measuring the reduction peak current of HRP in cyclic voltammograms. For each data point on the calibration curves, 4 electrodes were tested. Electrochemical measurements were made using a conventional three-electrode electrochemical cell to extract the detection signal. A commercial Ag/AgCl (3 M KCl) electrode was used as the

reference electrode, and a platinum wire was used as the counter electrode. The volume of the electrochemical cell was 1 mL. The cell was driven by a commercial potentiostat (CHI 660C Work Station). A potential scan rate of 20 mV/s was used in recording cyclic voltammograms (CV) and linear voltammograms (LV). PBS (0.1M at pH 7) was prepared using de-ionized water (18.2 Ω-cm). Commercial hydrogen peroxide (Fisher Scientific, 30% concentration) was diluted to the concentrations used in the experiment. All measurements were made with PBS at room temperature. Reproducible results were obtained by repeating each measurement multiple times.

3. Results and discussion

3.1 Control and related measurements

The detection of *E. coli* O157:H7 was achieved by applying FEED to the conventional electric-current immunosensing method, the operation of which requires the sandwich immune complex, Ab-*E.coli*-Ab(horseradish peroxidase, HRP), to be formed on the detecting electrode as shown (not drawn to scale) in Figure 1. The reduction peak current of HRP was used as the detection signal. Control experiments (See Supplementary Information) were performed using electrodes immobilized with different participants of the immune reaction in order to avoid misinterpretation of experimental results.

3.2 Voltage-controlled electron transfer through immune complex

Conventional electrochemical immunosensing of microorganisms is performed with diffusional mediators or by electric-current detection of diffusional product of the enzyme-catalyzed reaction, since transfer of electrons through the bulky immune complex is severely impeded. Nevertheless, the literature shows that electron transport occurs through macroscopic biological structures such

as DNAs[14]. In fact, it is even possible to modulate the conductance of biological structures using electric fields, examples of which include protein transistors[15, 16]. The long-range electron transfer through biological structures is thought to be the hopping mechanism between a series of sites[17]. The electron transfer rate constant between a pair of site is the same as the one described below.

To demonstrate the direct electron transfer via the immune complex, we first carried out the detection of *E. coli*, which was dissolved in a water-glycerol solution. After the Ab-*E.coli*-Ab(HRP) sandwich immune complex was formed, the detecting electrodes were rinsed to remove unstable structures from the electrodes. The CVs of the electrodes showed a pair of redox peaks, which were attributed to the presence of HRP (see the discussion on immobilization of HRP given in the second paragraph following this paragraph). The reduction peaks of the electrodes were used as the detection signal. Figure 2 (a) shows the high concentration (up to $20x10^3$ CFU/mL) *E. coli* O157:H7 calibration curve obtained with the detecting electrodes without applying V_G. Each data point shows the reduction peak current versus the corresponding concentration of the bacterium. The calibration curve shows a linear range starting from 400 CFU/mL to 4000 CFU/mL with the current of 400 CFU/mL being very close to zero. Detection below 400 CFU/mL was unsuccessful since no peaks appeared in CVs. The high-concentration part of the curve shows current saturation. Note that, as the concentration of *E. coli* is increased and more immune complexes are formed on the detecting electrode, the amount of available primary or capture antibody on the detecting electrode becomes limited because the complexes block certain amount of available primary antibody. The saturation of the signal current can be removed by adjusting the size of the detecting electrode.

Electron transfer through the immune complex is an overlooked problem. The length of the non-electroactive antibody-antigen-antibody complex is greater than 100 nm for bacteria. Since the electron transfer rate k_{et} depends exponentially on distance as $k_{et} \propto \exp(-\beta d)$, where d is the distance of the non-electroactive region (the energy barrier) between the active site of the enzyme and the electrode and β is the attenuation coefficient, which is proportional to the square root of the energy barrier height ($\beta \propto (\Phi_o)^{1/2}$)[18], the length of the complex is long enough to diminish k_{et} and, therefore, reduce detection sensitivity. For example, using the expression for k_{et}, at 100 nm away from the electrode surface, the value of k_{et} is 3.7 x10^{-44} times smaller than that at the electrode's surface. The gating voltage V_G of the FEED technique modifies the energy profile of the immune complex so that Φ_o is reduced and hence k_{et} is enhanced[9, 19], resulting in signal amplification and significantly lowered detection limit. A previous work shows that the reduction-peak current increases as V_G is increased[9].

We then applied V_G to the system to achieve detection below 400 CFU/mL. Figure 2 (b) shows three CVs obtained in phosphate buffered saline (PBS) using an electrode incubated with a 400 CFU/mL water-glycerol solution of *E. coli* O157:H7 with different values of V_G. CV1, obtained with V_G = 0 V, shows a pair of very weak current peaks as indicated by the arrows. The weak detection signal can be attributed to the highly impeded transport of electrons across the bulky immune complex. With V_G = 0.2 V, the redox peaks in CV 2 become apparent with a measurable reduction peak current of 5 μA. The peaks in CV 3 become even more apparent and the reduction peak current increases to 7.5 μA as V_G is increased to 0.6 V, demonstrating the voltage-controlled (intrinsic) amplification of detection signal. The formal potentials of the two pairs of enhanced redox peaks are located at -0.4 V and -0.42 V vs. Ag/AgCl, respectively.

Previous works on direct electron transfer associated with immobilized HRP show that the formal potential of this redox process is between -0.5 V and - 0.3 V vs. Ag/AgCl[20, 21]. Thus, the redox peaks in Figure 2 (b) indicate the direct electron transfer between the HRP and the electrode through the Ab-*E. coli*-Ab(HRP) complex. Previously, the increase in the signal current of the FEED system caused by V_G was attributed to the reduction of the height of the energy barrier between the active site of the enzyme and the electrode. V_G induces an electric field at the enzyme-electrode interface, and the field reduces the effective height of the energy barrier, resulting in an increase in the transfer rate of electrons[9, 19]. We believe that the same mechanism is responsible for the observed increase in the signal current in the present work.

Figures 2 (c) shows the detection of *E. coli* O157:H7 below 400 CFU/mL due to the intrinsic amplification of the signal obtained with V_G = 0.6 V. The first concentration, 12 CFU/mL, corresponds to zero current. The current of each additional concentration is clearly separated from that of others. The first two data points indicate a detection resolution of 8 CFU/mL. The data points reveal a nearly linear dependence of the current on the bacterium concentration. The red line is the regression line of the data points, having a correlation coefficient of 0.980. The detection limit is estimated to be 15 CFU/mL, obtained using the signal/noise=3 method. The small error bars indicate reproducible device performance. The sensitivity is 11 nA mL/CFU. The time needed to complete the detection, counting the time starting from the incubation of the bacterium to the time when the signal current was available was 67 min.

3.3 Detection of *E. coli* O157:H7 in beef juice

Detection of *E. coli* O157:H7 was carried out with beef juice samples. Beef juice was obtained by thawing frozen beef at room temperature, slightly squeezing the beef and collecting the liquid appearing on the beef [22]. *E. coli* O157:H7 was spiked in the beef juice. Undiluted juice samples were used in the detection. Figure 3 (a) shows the high-concentration calibration curve of *E. coli* O157:H7 in beef juice obtained with V_G = 0.6 V. The detection range covers 400 - 20,000 CFU/mL. The 4,000 CFU/mL point separates the linear range from the saturation range, which is explained above. Figure 3 (b) shows the low-concentration calibration curve of *E. coli* O157:H7 in beef juice obtained with V_G = 0.6 V. The first concentration, 20 CFU/mL, corresponds to 11 nA. The current of each additional concentration is clearly separated from those of other concentrations, indicating a detection resolution of 8 CFU/mL. The data points reveal a nearly linear dependence of the current on the concentration of the bacterium. The red line is the regression line for the data points, having a correlation coefficient of 0.994 The detection limit of the low-concentration is estimated to be 19 CFU/mL, obtained using the signal/noise=3 method. The small error bars indicate reproducible device performance. The sensitivity is 2.1 nA mL/CFU. The time needed to complete the detection, starting from the incubation of the bacterium to the reading of the output current, was 67 min.

In conclusion, this work reports the ultrasensitive and rapid detection of *E. coli* O157:H7 in beef juice. The detection was achieved due to the intrinsic amplification provided by FEED, which was incorporated with the immunosensing method. Compared with the conventional immunosensors, the novel detection approach offers the advantage of direct electron transfer through the sandwich immune complex without using mediators. The electron transfer through the bulky complex was facilitated using the gating voltage of FEED. The detection of *E. coli*

O157:H7 in beef juice resulted in a detection limit of 19 CFU/mL with an assay time of 67 min. The ultrasensitive and rapid detection was due to the intrinsic amplification of the detection system, which allowed the direct detection of the unprocessed sample without performing sample pre-enrichment. Since *E. coli* is considered a model bacterium in microbiology studies, the present work shows that the FEED-based detection technique potentially provides a platform for ultrasensitive, specific and rapid detection of a range of microorganisms.

4. Conclusions

This work reports the detection of *E. coli* O157:H7 in beef juice using a mediator-less immuno-detection approach. For a gating voltage of 0.6 V, a detection limit of 19 CFU/mL was obtained with an assay time of 67 min without using pre-enrichment. Since *E. coli* is considered a model bacterium in microbiology studies, the present work shows that the FEED-based detection technique potentially provides a platform for ultrasensitive, specific and rapid detection of a range of microorganisms.

Acknowledgements

We thank Cleveland State University for providing support to this work.

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Captions

Figure 1 Schematic description (not drawn to scale) of the immunological sandwich structure of *E. coli* O157:H7 formed on an electrode.

Figure 2 Detection in water-based samples. (a) Calibration curve of water-based *E. coli* O157:H7 samples. The curve was obtained without V_G. (b) CVs of a 400 CFU/mL *E.coli*-detecting electrode. The CVs were obtained using different V_G. The CVs show the voltage-controlled intrinsic amplification of signal current. (c) Low-concentration calibration curve of water-based *E. coli* O157:H7 samples. The calibration curve was obtained with $V_G = 0.6$ V.

Figure 3 Detection in beef juice. (a) High-concentration and (b) low-concentration calibration curves of *E. coli* O157:H7 in beef juice. The curves were obtained with $V_G = 0.6$ V.

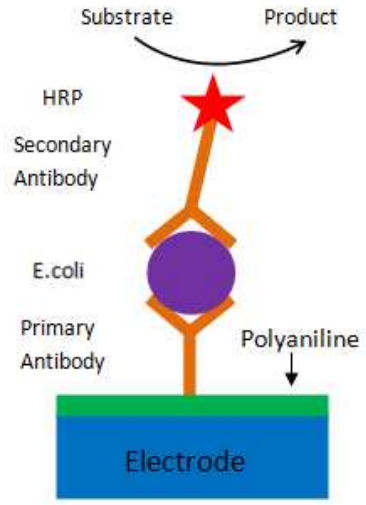
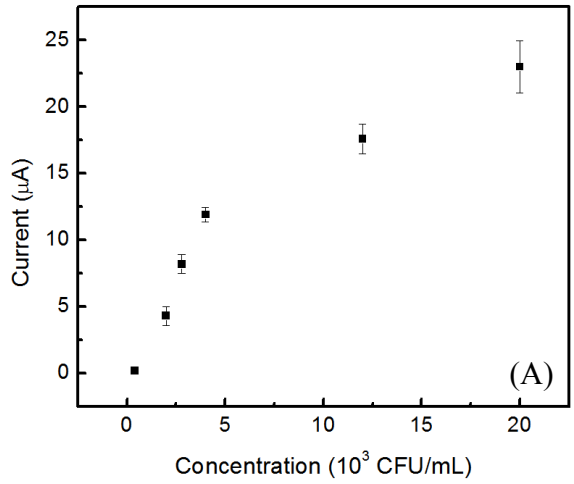
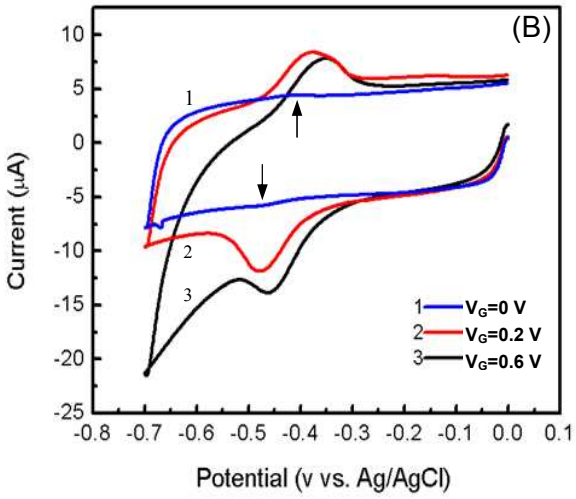


Figure 1





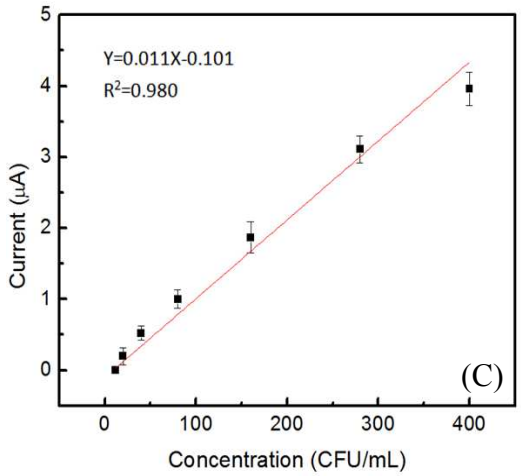
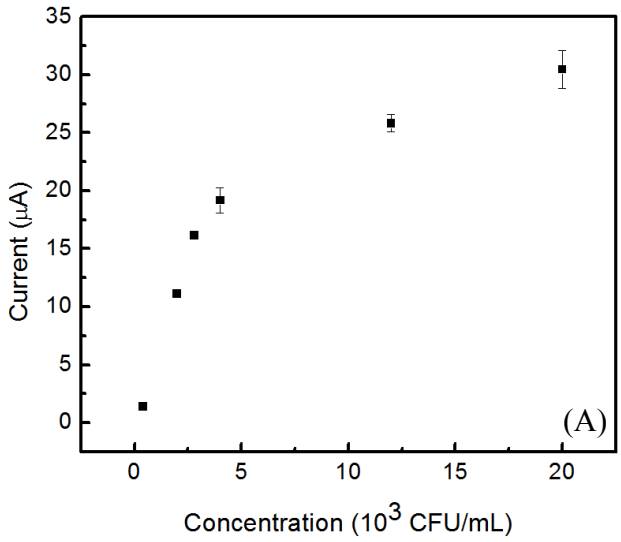


Figure 2



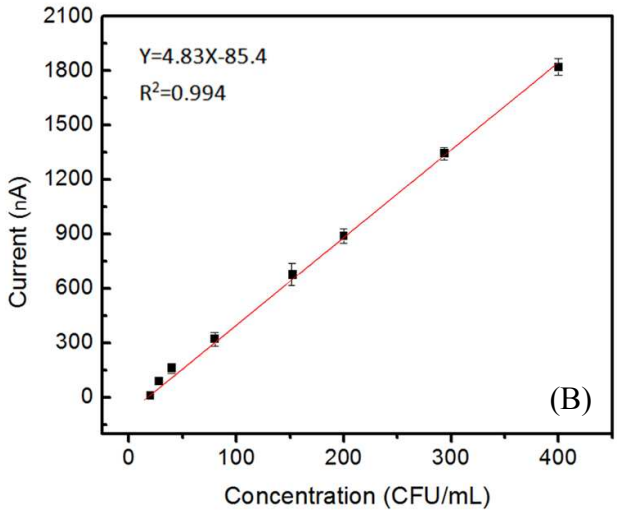
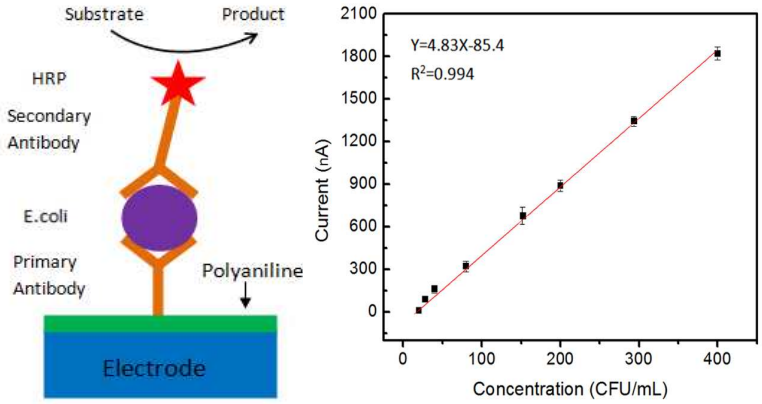


Figure 3



Detection of E. coli O157:H7 in beef juice with a detection limit of 19 CFU/mL. An assay time of 67 min was resulted due to the absence of sample pre-enrichment.