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Electrochemical detection of *Escherichia coli* from aqueous samples using engineered phages

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ABSTRACT: In this study, an enzyme-based electrochemical method was developed for the detection of *Escherichia coli* (*E. coli*) using the T7 bacteriophages engineered with *lacZ* operon encoding for beta-galactosidase (β-gal). The $T7_{lacZ}$ phages can infect *E. coli*, and have the ability to trigger the overexpression of β-gal during the infection of *E. coli*. The use of the engineered phages resulted in a more sensitive detection of *E. coli* by: (1) overexpression of β-gal in *E. coli* during the specific infection; (2) release of the endogenous intracellular β-gal from *E. coli* following infection. The endogenous and phage-induced β-gal was detected using the electrochemical method with 4-aminophenyl-β-galactopyranoside (PAPG) as a substrate. The β-gal catalyzed PAPG to an electroactive species p-aminophenol (PAP) which could be monitored on an electrode. The electrochemical signal was proportional to the concentration of *E. coli* in the original sample. We demonstrated the application of our strategy in aqueous samples (drinking water, apple juice, and skim milk). Using this method, we were able to detect *E. coli* at the concentration of approximately 10^5 CFU/mL in these aqueous samples in 3 hours, and 10^2 CFU/mL after 7 hours. This strategy has the potential to be extended to detect different bacteria using specific bacteriophages engineered with gene encoding for appropriate enzymes.

Food safety remains an important issue for public health protection. Foodborne illnesses resulting from the bacterial pathogens pose an increasing threat to the human health worldwide.[1] The ability to more rapidly and sensitively detect bacteria in food and water samples is critical to ensure the food safety and minimize the risk of human exposure to potential hazzards.[2,3] *Escherichia. coli* (*E. coli*) has been among the most studied bacteria, and significant research efforts have been placed on improving the ability to detect these bacteria in food and water supplies. The United States Food and Drug Administration (FDA) has suggested to use coliforms or "generic" *E. coli* as indicators for the bacterial contamination level in fresh produce.[4,5] Additionally, the Environmental Protection Agency (EPA) identified *E. coli* as an indicator of the fecal contamination.[6] The most commonly used methods to determine the presence of *E. coli* involve culturing and plate counting using selective medium. Although these methods are accurate and reliable, they require long incubation periods which are time-consuming and laborious.[3,7] Nucleic-based method such as PCR is an alternative for the rapid identification and quantification of bacteria. Unfortunately, it requires significant equipment and sample preparation, and also cannot distinguish viable and nonviable cells.[8,9] Therefore, significant efforts are continuously being invested in the development of new strategies to improve the detection of bacteria with better sensitivity, specificity, and less time. One alternative used for rapid and sensitive bacterial detection is based on the measurement of the activity of a specific enzyme which reflects the contamination of the target bacteria.[10-12]

In *E. coli*, the activity of intracellular enzyme β-galactosidase (β-gal) is able to reflect the concentration of indicator strain *E. coli*.[13-15] Beta-gal is a well-known bacteria-enclosed enzyme of *E. coli*, encoded by *lacZ* operon. It has been widely used as a reporter enzyme to determine the concentration of *E. coli* in food and aqueous samples. There are many colorimetric, electrochemical, and fluorescent substrates that can be catalyzed by β-gal which allows for various methods to measure the activity of this enzyme.[11,16,17]

Here, an electrochemical method was used to specifically measure the activity of β-gal in order to estimate the *E. coli* contamination. Electrochemical methods measure the free electron generation of an electroactive species when it is oxidized and reduced on the electrode. The electroactive compound is usually produced via the catalysis from an enzyme. Electrochemical methods offer a promising alternative to conventional analytical methods, providing a convenient detection by obtaining instantly quantitative signals with minimal equipment required. Moreover, electrochemical detection has some advantages over the other rapid methods, for example it is not affected by the turbidity of sample solution which can be problematic in colorimetric assays for the sample such as juice or milk.[18-20] The detection of these samples could benefit from the development of an electrochemical method. The low-cost nature of electrochemical detection can best be exemplified by the common blood glucose meter which is available for under $10.

However, the concentration of endogenous enzyme β-gal in *E. coli* may not be at high enough for a rapid detection method.[11,17] In order to solve this problem, engineered phages were used in this strategy to overexpress β-gal in order to achieve a more sensitive detection. Bacteriophages (phages) are viruses which specifically recognize, attach to, and infect target bacteria.[21,22] Phages replicate using the molecular machinery of the host bacteria. At the final stages of the infection, lytic phages can induce the disruption of the host cells, releasing of the

intracellular components and replicated cells to the surrounding environment.[23] Genetic engineering techniques allow

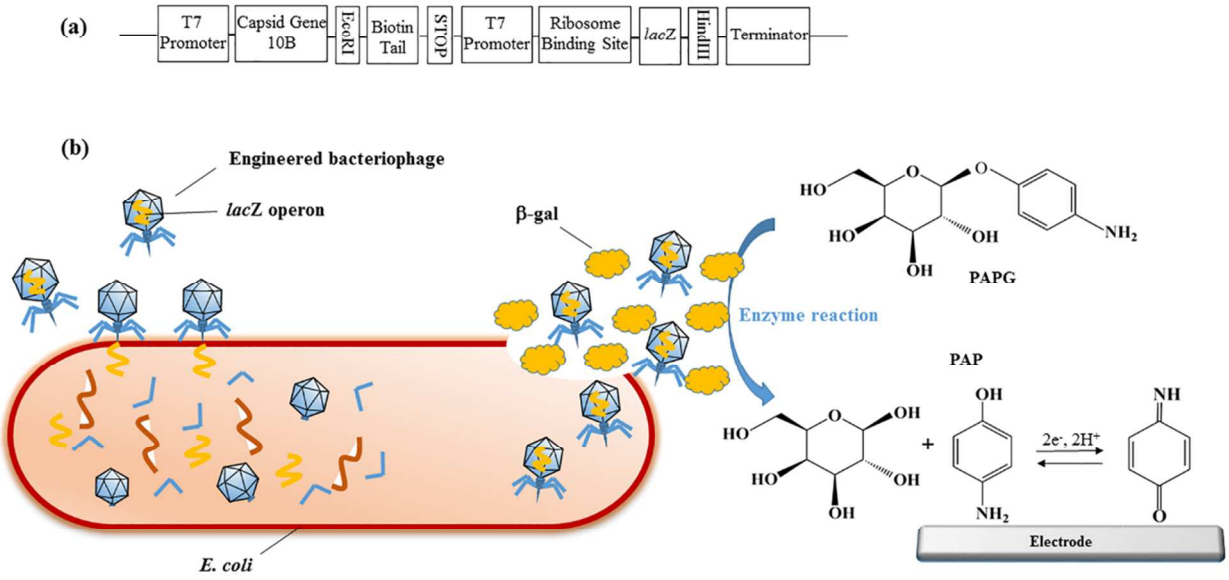

Figure 1. The scheme representation of electrochemical detection of *E. coli* using engineered phage. (a) The designed construct of genome of $T7_{lacZ}$ phage. (b) Specific capture and infection of *E. coli* by $T7_{lacZ}$ phage resulted in the release and overexpression of enzyme β-gal. PAPG was catalyzed by β-gal into an electroactive species PAP that can be quantified by electrochemical device.

the insertion of genes encoding for marker enzyme such as alkaline phosphatase (ALP), green fluorescent protein (GFP) and luciferase (luc) into phage genome, and these enzymes can be expressed in the host cells during the phage life cycle and measured by various methods.[24-26] This expression is under a stronger promoter resulting in increased transcription. Therefore, in addition to intracellular β-gal produced by *E. coli* itself, the bacteriophages engineered with *lacZ* operon encoding for β-gal are able to induce the expression of this enzyme in *E. coli* during the infection, resulting the high production of β-gal which can facilitate to achieve a more sensitive detection. Because phages can only replicate in a live bacterial cell, phage-based detection is able to distinguish viable and non-viable host cells.[27,28] This reduces the possibility of false-positive results caused by non-viable bacteria which may have already succumb to mitigation steps such as antibiotics, chlorine, cleaners, or other antimicrobials.

In this study, we proposed a new strategy to rapidly and specifically detect *E. coli* in aqueous samples based on electrochemical quantification of β-gal overexpressed by engineered phages. This is an attempt to make a phage-based detection assay field deployable. The *lacZ* operon encoding for β-gal was inserted into the genome of T7 phages to form engineered bacteriophages. The engineered phages were first used to specifically infect the target *E. coli*. Following the infection cycle, β-gal, both endogenous and phage-enabled, was released into the sample solution. Then the enzyme β-gal hydrolyzed the electrochemical substrate to generate an electroactive product which was quantified by the subsequent electrochemical detection (Fig. 1). The use of engineered T7 phages enables this strategy detect live *E. coli* cells and overexpress reporter enzyme into solution without the need of adding lysozyme to lyse bacteria cells. Additionally, the engineered phages coupled with an electrochemical method offers a simple, low-cost and sensitive quantification of *E. coli* with minimal equipment.

EXPERIMENTAL SESSION

Chemical, materials and instrument. 4-aminophenyl-β-galactopyranoside (PAPG) was purchased from Sigma-Aldrich (St. Loius, MO). All other analytical grade chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). The Milli-Q water with 18 MΩ/cm resistivity (EMD Millipore, Billerica, MA) was used for the preparation of all solution.

The Potentiostat/Galvanostat used in the electrochemical detection was from PalmSens (Utrecht, Netherlands). The Drop-cell connector which connected the potentiostat and electrode and thin-film single platinum electrodes were obtained from MicruX Technologies (Austurias, Spain). Platinum is a commonly used standard material for the electrode in the electrochemical detection and has been widely used in many studies. It has high corrosion resistance and high conductivity.[29]

Bacterial culture samples. *E. coli* BL21 (ATCC 25922) was grown overnight (18-20 hours) in 50 mL of Luria-Bertani broth (LB broth, 10 g tryptone, 5.0 g yeast extract, 10.0 g sodium chloride in 1 L distilled water, pH 7.2) under 200 rpm at 37℃. The overnight culture was then centrifuged at 7,000x g for 2 minutes, washed two times with phosphate buffered saline (PBS buffer, 137.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.2) and resuspended in 1x PBS buffer. The concentration of *E. coli* BL21 were determined by enumeration after plating on the LB agar plates and incubation for overnight. And *E. coli* BL21 was serially diluted into the desired concentration (10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 CFU/mL) in LB broth for the subsequent experiments.

Construction, propagation and purification of engineered phage. The method to construct engineered phages

for overexpression of β-gal in *E. coli* was based on the strategy previously described for $T7_{ALP}$ phage and $T7_{TEV}$ phages.[30,31] Briefly, the *lacZ* construct was synthesized in a pUC57 plasmid containing *lacZ* operon from GenScript (Piscataway, NJ). The *lacZ* construct was then amplified using a Phusion PCR kit (Ipswich, MA). Next, all PCR products were purified and digested by PCR Purification Kit (Qiagen, Valencia, CA) and EcoRI and HindIII, respectively. After T4 DNA ligase (Promega, Madison, WI) facilitated the insertion of the construct into the T7Select415 (EMD Millipore, Billerica, MA) genome vector arms, $T7_{lacZ}$ phage was formed by packing the construct using the T7Select packing kit (Fig. 1(a)). In order to prove the effectiveness of the $T7_{lacZ}$ phages on the overexpression of β-gal, a control DNA which contains S-tag was also packaged using T7Select kit to create $T7_{control}$. After $T7_{lacZ}$ and $T7_{control}$ were propagated and plated, the individual colonies were confirmed with the appropriate size insert by the T7Select up and down primers using Phusion PCR kit.

The engineered phages, $T7_{lacZ}$ and $T7_{control}$, were amplified and purified prior to the use in the detection assay. An aliquot of 100 μL *E. coli* BL21 overnight culture was inoculated into a 150 mL of sterile flask with 35 mL LB broth and then incubated at 37℃ with 200 rpm until the OD_{600} of the culture reached 0.6. Then engineered phage stock (15 μL) was inoculated in the culture and incubated for 2 hours with the same conditions to allow infection and replication. The phage lysate was then centrifuged at 8,000x g for 10 min, followed by filtration of the supernatant through a 0.22 μm sterile filter (Corning Life Science, Corning, NY). The lysates were then ultracentrifuged at 35,000x g for 2 hours. The phage pellets were finally resuspended in 4 mL of 1x PBS buffer at 4 ℃. The phage titer (PFU/mL) was determined by plaque assay using a double agar overlay. Briefly, the phages (100 μL) were added into the melt top LB agar (3-4 mL) containing the overnight *E. coli* culture (200 μL) and then placed on solid LB agar, followed by the counting of plaques after an incubation of 3-4 hours at 37℃.

Electrochemical detection of *E. coli* using engineered phage. Aliquots (1 mL) of *E. coli* with concentration of 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 CFU/mL in LB broth were infected with 100 μL of engineered phages (10^4 PFU/mL). The substrate PAPG (100 μL, 15 mM) was also added into the sample prior to the incubation, allowing for the enzyme reaction at the same time with phage infection. The simultaneous phage infection and enzymatic reaction shortened the assay time. Next, the samples were incubated in 37℃ for various times (2, 3, 4, 5, 6 and 7 hours) at 150 rpm to allow for the phage infection of *E. coli* and overexpression of β-gal. An aliquot of 1 mL LB broth without *E. coli* was also incubated with substrate PAPG and engineered phage under the same conditions above as a negative control. All the samples after incubation were used for the following electrochemical detection.

The electrochemical detection system consisted of a Palmsens Potentiostat/Galvanostat, Drop-cell connector and a MicruX thin-film single platinum. Following the incubation steps, 20 μL of sample solution was deposited on the platinum electrode, covering all the three electrodes. So the surface area of the working electrode for each electrochemical detection was consistent. Differential pulse voltammetry (DPV), a commonly used model to measure the electric signal, was used to perform the electrochemical detection for all the measurements with the following conditions: Time for equilibration: 2s; Potential range: -0.1 V – 0.4 V; E step: 0.01 V; E pulse: 0.05 V; t pulse: 0.05 s; Scan rate: 0.05 V/s. The electroactive compound PAP was produced by catalyze of β-gal from the electro-inactive substrate PAPG. DPV allowed a relatively short analysis of the sample solution. The current was measured prior to and after the applied potential and the change in current was plotted against the applied potential.[32,33] The response was determined to be the peak current of the DPV curve which was proportional to the concentration of PAP generated on the electrode. Therefore, the peak current was recorded as the signal of the electrochemical detection.

Detection in the real aqueous samples. Drinking water was obtained from the drinking water fountain at University of Massachusetts, Amherst. Apple juice and skim milk were purchased in a local supermarket. The safety of drinking water is a critical health issue, and is especially a problem for the people in developing countries. In many areas drinking water is obtained from private sources which can become contaminated with bacteria. Many of these areas also suffer from a lack of water safety monitoring.[34] Foods such as juice and raw milk are often associated with the foodborne outbreaks. Apple juice and milk which are not compatible with colorimetric detection methods can be measured by electrochemical methods. The pH of drinking water is 7.01, the pH of skim milk is 6.70 and the pH of apple juice is 3.61. The apple juice was adjusted to approximately pH 7.2 prior to the detection in order to maintain an optimal condition for phage infection and enzymatic reaction. *E. coli* BL21 was inoculated in the drinking water, apple juice, and skim milk, respectively, to final concentration of 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 CFU/mL. Next, a 200 μL aliquot of five times concentrated LB broth supplemented with PAPG with final concentration of 15 mM and $T7_{lacZ}$ phages with final concentration of 10^4 PFU/mL was added into 1mL of each sample (drinking water, apple juice, and skim milk) in a 2 mL microcentrifuge tube (Eppendorf, Hauppauge, NY, USA). An aliquot of samples without *E. coli* were used as a negative control. The microcentrifuge tubes were incubated under 150 rpm agitation for 3 hours and 7 hours at 37℃, respectively. Then the samples were analyzed using the electrochemical assay following the steps described in the previous section.

Statistical analysis. SAS software was used to analyze data with ANOVA (SAS Institute, Inc., Cary, NC). The mean values among different groups were determined significantly different ($P<0.05$) with Tukey's test. The data presented represent a mean of a minimum of three independent samples and error bars represent the standard deviation of the replicates.

RESULTS AND DISCUSSION

Optimization of condition for enzyme reaction. There are various factors (e.g. temperature, pH, and concentration of substrate) that may affect the phage infection and enzyme reaction. It is therefore necessary to optimize the reaction condition in order to increase the sensitivity of our proposed approach. The effect of subtract (PAPG) concentration as well as the effect of incubation temperature (37℃ and 23℃) were investigated. The pH range of this method was determined by activity of phages and β-gal. In general, phages are usually stable at a large pH range (pH 4.0-10.0). Phages show the optimum for physical stability and infection at pH 6.0-8.0.[35,36] Beta-gal is a neutral enzyme which has the optimal activity at pH 6.7 to 7.2 and is stable from pH 6.0 to 9.0. The product PAP is also more stable at a neutral pH and it is oxidized and reduced on the electrode at pH 7.5.[37,38] Therefore, this method

can be applied in a relatively large pH range and is optimal at neutral pH. The pH of the enzyme reaction was then selected at pH 7.2. The concentration of phage used here was based on our previous studies on the phage amplification matrix.[39] There is a dynamic interaction between the bacteria growth and phage replication. The total enzyme finally expressed are related to the total number of cells infected and the initial phage concentration. The more phages used for the infection there are more gene encoding for β-gal, resulting in more production of β-gal. On the other hand, high initial phage concentrations cause the fast lysis of *E. coli* cells and the uninfected cells do not have time for doubling to provide more cells available for infection. Therefore, based on the previous research a concentration of 10^4 PFU/mL engineered phages were used for this study based on the previous research.

Then the concentration of substrate PAPG was investigated to determine the optimal enzymatic activity. An aliquot of 100 µL engineered phage (10^4 PFU/mL) and 100 µL PAPG with varying concentration (0, 5, 10, 12, 15, 18, and 20 mM) in PBS buffer were used for the phage infection and enzyme reaction, respectively, to detect 10^6 CFU/mL of *E. coli* after incubation for 4 hours at 37℃. The DPV peak current model was used to compare the signal obtained for samples with increasing concentration of PAPG. Fig. 2(a) shows that the concentration of PAPG significantly affected the peak current obtained from the DPV curve. The results demonstrate that the peak current increased gradually with the increase of concentration of substrate PAPG and leveled off at approximately 15 mM, after which the peak current increased only slightly. Therefore, 15 mM was used for the concentration of substrate PAPG for the subsequent experiments in order to allow the optimal conversion of PAPG to PAP.

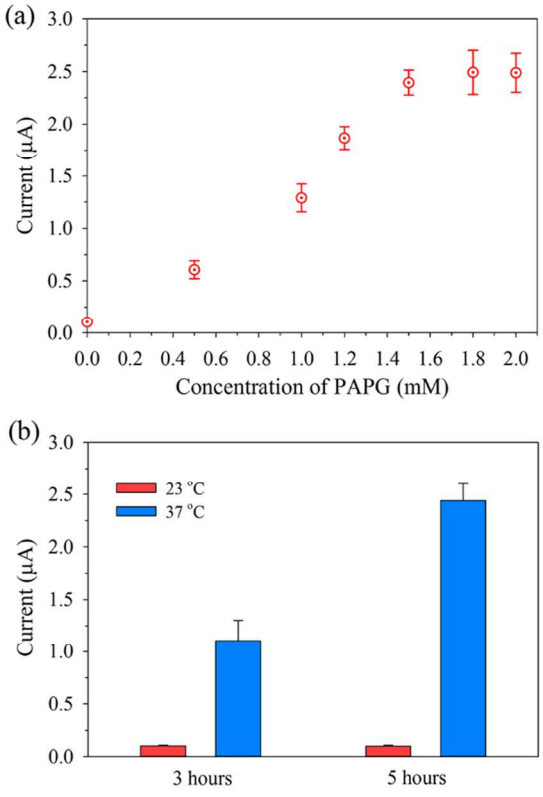

Figure 2. Optimization of condition of enzyme reaction. (a) Dependence of the current on varying concentration of substrate PAPG (0, 5, 10, 12, 15, 18, and 20 mM) detecting 10^6 CFU/mL *E. coli* after incubation of 4 hours at 37℃. (b) Comparison of incubation temperature at room temperature (23℃) and 37℃. Peak current obtained for detecting 10^6 CFU/mL of *E. coli* at 23℃ (red bar) and 37℃ (blue bar), respectively after 3 or 5 hours of incubation.

Incubation temperatures of 37℃ and room temperature (23℃) were also compared to investigate their effect on detection limits. Although the optimal temperature for phage replication and growth of *E. coli* is 37℃, room temperature is more convenient for assays in low-resource settings. Aliquots containing 100 µL engineered phage (10^4 CFU/mL) and 100 µL of 15 mM PAPG were used to detect 10^6 CFU/mL *E. coli* after 3 hours or 5 hours of incubation at 37℃ and room temperature (23℃), respectively. Fig. 2(b) shows the peak current recorded after 3 hours and 5 hours of incubation in the presence of PAPG and T7$_{lacZ}$ phage at either 37℃ or 23℃. It can be observed that the signal obtained after incubation at 37℃ was significantly higher than that at 23℃ for incubation time of both 3 hours and 5 hours as expected. As incubation time increased from 3 hours to 5 hours, the signal of reaction at 23℃ did not show a significant increase while the peak current obtained for reaction at 37℃ increased steadily. Therefore, in order to obtain a high signal, it was necessary to incubate the sample at 37℃.

Comparison of engineered phages (T7$_{lacZ}$ phages), control phages (T7$_{control}$ phages) and no phage for *E. coli* detection. One of the functions of engineered phages was to release endogenous and phage-enabled β-gal into the sample solution during the phage infection. T7 phage is a lytic phage which can lyse the host cell at the end of the infection cycle. This results in a release of the intracellular proteins such as β-gal. Additionally, the T7$_{lacZ}$ phages we used here were engineered with a *lacZ* operon for β-gal, so the enzyme β-gal would be expressed during the phage infection. To prove the effectiveness of engineered phages, we conducted an investigation to compare the signal obtained using the engineered phages, control phages (no operon for β-gal), and without phage. Aliquots containing 100 µL of engineered phages, control phages, respectively were used to infect 10^6 CFU/mL of *E. coli*. The same concentration of *E. coli* was also incubated with substrate PAPG in the absence of phage. Following the incubation for 3, 4 or 5 hours at 37℃, an electrochemical detection was performed and the peak current from each DPV curve was recorded as the signal.

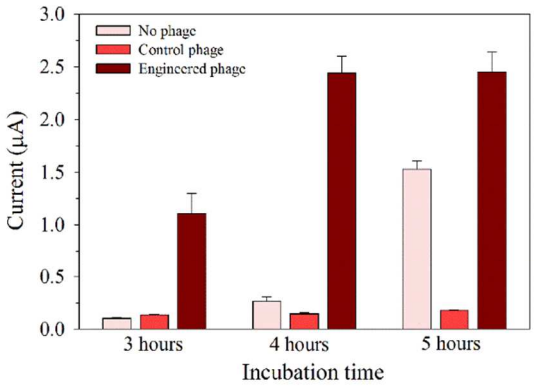

Figure 3. The signal obtained for detection of 10^6 CFU/mL *E. coli* without phage (pink bar), with control phages (red bar) and with engineered phages (dark red bar), respectively, after incubation of 3, 4 or 5 hours at 37℃.

The results were shown in Fig. 3, demonstrating that with the same incubation conditions, the signal obtained using engineered phages was significantly higher than that using control phages or no phage. These data suggest that the concentration of β-gal generated using $T7_{lacZ}$ phages was significantly higher than the other two groups. The enzyme β-gal is an intracellular enzyme which can be produced by *E. coli*. The results also demonstrated that when using engineered phages, more β-gal was expressed in *E. coli* during the infection in addition to the intracellular β-gal produced by *E. coli*, and released into the sample solution, resulting in the signal amplification. The $T7_{control}$ phage without *lacZ* gene did not facilitate the expression of β-gal but did lysed the *E. coli* cells allowing endogenous enzymes to react. In absence of phages, the intracellular enzyme β-gal was not able to be released from *E. coli* cells. The free β-gal in the sample solution, mostly from the natural lysis of the bacterial cells and the diffusion of PAPG across the cell membrane was limited.[11,40,41] The negative control sample consisting of *E. coli* (no phage) also resulted in a high signal after 5 hours. This is most likely due to *E. coli* cells which were able to produce higher concentrations of β-gal with time increased and raised the enzyme to a high concentration during the incubation. Conversely, the *E. coli* infected with the control phage were lysed and could not produce additional enzyme. Overall, these results demonstrated that the engineered phage was more effective for obtaining a higher signal in order to achieve a lower limit of detection.

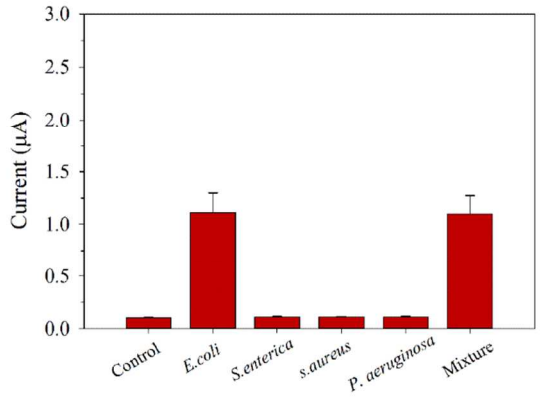

Figure 4. The signal obtained for detecting 10^6 CFU/mL *E. coli*, *S. enterica*, *S. aureus*, *P. aeruginosa* and a mixture of all the strains, respectively, after incubation of 3 hours at 37℃. A sample without any bacteria was used as the negative control.

Specificity of engineered phages. Another use of engineered phages in this strategy was to specifically target *E. coli* using the natural specificity of phages towards its host. A bacteriophage is known to specifically infect a subset of bacteria strains. The specificity of phages is based on the nature and structure of receptors on the surface of bacterial cells. The receptors which are recognized by the tail fibers of bacteriophages are dependent on the different taxonomic groups. T7 phages attach the tail fibers specifically to the lipopolysaccharides (LPS) on the outer membrane of *E. coli*.[42] An investigation on the specificity of this strategy was conducted by using four different single strains including *E. coli* BL 21, *Salmonella enterica* (*S. enterica*), *Staphylococcus aureus* (*S. aureus*), and *Pseudomonas aeruginosa* (*P. aeruginosa*), as well as a mixture of all these four different bacteria strains. A sample without any bacteria was used as a negative control. Engineered phages (100 μL) with concentration of 10^4 PFU/mL and 100 μL of 15 mM PAPG were used to detect the samples. The concentration of each bacteria strain inoculated into the sample was approximately 10^6 CFU/mL. After incubation for 3 hours at 37℃, the signal was obtained from the peak current of the DPV curve. The results shown in Fig. 4 demonstrated that only samples containing *E. coli* resulted in a significant signal. The electrochemical signal obtained for other bacteria had no significant difference with the control (no bacteria). The mixture of all the four strains gave the similar signal as the sample inoculated with only *E. coli*. These results suggest that engineered phages are specifically target to *E. coli*. Therefore, bacteriophages could be used both for their specificity as well as their ability to express reporter enzymes during infection.

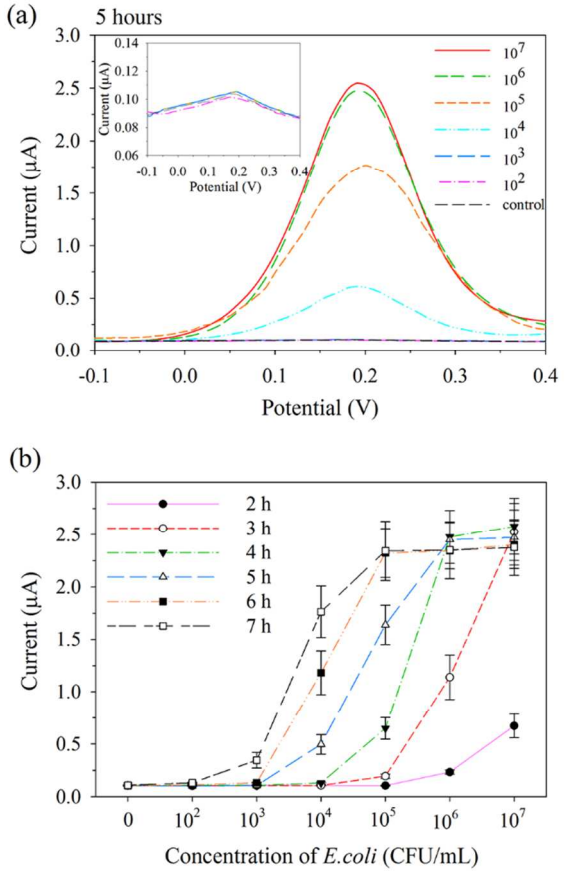

Figure 5. Results of electrochemical detection of *E. coli*. (a) Differential pulse voltammetry (DPV) curve for increasing concentration of *E. coli* after 5 hours of incubation at 37℃. The inset is the amplification of the plot of control and low concentrations (10^2, 10^3 CFU/mL). (b) Dependence of peak current obtained from DPV curve on varying concentration of *E. coli* for different incubation time.

Electrochemical detection of *E. coli* after varying incubation time. After determining the condition of enzyme reaction and proving the effectiveness and specificity of the engineered phages, the detection of *E. coli* with varying concentrations (0, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 CFU/mL) using electrochemical method was performed. The detection of *E. coli* was based on the measurement of activity of β-gal generated during the infection by $T7_{lacZ}$ phages. The simultaneous addition of engineered phages and the substrate PAPG allowed simultaneous phage replication and enzyme reactions. The expressed β-gal converted the substrate PAPG into an

electroactive product PAP which was reflected by the electrochemical signal. The current was measured immediately before each potential change by DPV model, and the current difference is plotted as a function of potential. The peak potential is proportional to the concentration of the electroactive product. The DPV curve was obtained for the detection of each concentration of *E. coli* BL21. Fig. 5(a) shows one of the electrochemical results - the DPV curves obtained when detected *E. coli* with concentration of 0, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 CFU/mL after incubation for 5 hours. We can observe from the results that the negative control had a background signal because the substrate has a formal potential. It is necessary to maintain the background signal at a relatively low level. PAPG has been shown to be an ideal and commonly used substrate of β-gal for electrochemical detection [43,44] and the product PAP is reversibly oxidized on the electrode at a mildly positive potential. Therefore, there is no need for additional reagent to form the redox cycling. Additionally, PAP causes very low level of electrode fouling, allowing the reuse of the electrode.[11] The results also demonstrate that the signal increased with the increasing concentration of *E. coli*. The working concentration range of detection after 5 hours is between 10^3 and 10^6 CFU/mL of *E. coli* and the method is able to detect approximately 5×10^3 CFU/mL *E. coli*. The detection was also performed for each concentration of *E. coli* after varying incubation times (2, 3, 4, 5, 6 and 7 hours) and the peak current obtained from each DPV curve dependent on concentration of *E. coli* for different incubation time were shown in Fig. 5(b). The DPV result for each incubation time was respectively shown in Fig. S1(a)-(f). A limit of detection was obtained for each incubation time and the limit of detection decreased with the time increasing. After 7 hours, we were able to detect the sample with the lowest concentration (10^2 CFU/mL). As the incubation time was increased, the signal for each concentration of *E. coli* was also increased due to the utilization of substrate. A longer incubation time allowed a more sufficient phage infection and allowed for additional bacterial doubling.

Electrochemical detection in real aqueous samples. The electrochemical detection was then conducted in the real aqueous samples including drinking water, apple juice, and skim milk. We incorporated engineered phages into an electrochemical method, and thereby enable the detection of *E. coli* from aqueous samples by measuring the activity of β-gal generated. Fig. 6 shows the signal obtained for these three samples inoculated with varying concentration of *E. coli* after 3 hours and 7 hours. After 3 hours of incubation, the limit of detection was approximately 10^5 CFU/mL for all the three samples. The limit of detection was able to reach 10^2 CFU/mL after 7 hours of incubation. There were no significant differences among the signal obtained for the three aqueous samples (drinking water, apple juice and skim milk). This demonstrates the feasibility of this enzyme-based electrochemical assay with engineered phages in beverages. Bacteriophages are not only able to specific capture target bacteria, but also can be engineered to overexpress enzyme that produce electrical, visual and fluorescent signal.

There have been a variety of strategies developed for rapid and sensitive bacterial detection. Our presented research showed advantages based on the obtained results over the other reported electrochemical methods. First, compared with the electrochemical methods commonly using antibodies,[19,45,46] bacteriophages have the ability to distinguish the live cells and have a large range of stability in non-biological matrices with the same capture efficiency for bacteria with antibodies.[36] Antibody preparation is laborious and expensive. Conversely, phages can easily grow in the lab which is cost-effective. In addition, compared with methods using phages without gene encoding for reporter enzyme,[17] our results have demonstrated that engineered phages not only have high specificity, but also facilitate the overexpression of reporter enzymes and lyse the bacterial cells, resulting in a higher signal. Furthermore, since β-gal is originally intracellular enzyme of *E. coli*, it is relatively easy to trigger the overexpression of β-gal in *E. coli*. Thus, our strategy has shown the improvement for the current electrochemical detection of *E. coli*.

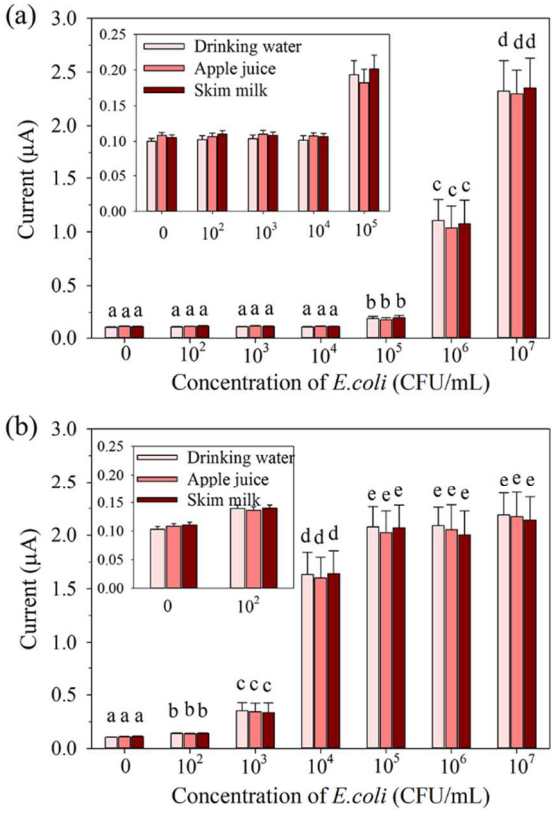

Figure 6. Peak current obtained for varying concentration of *E. coli* after (a) 3 hours and (b) 7 hours of incubation in drinking water (pink bars), apple juice (red bars) and skim milk (dark red bars), respectively. Bars with different letters (a, b, c, d or e) are significantly different ($P<0.05$).

When compared with other methods using bacteriophages,[26,47] the engineered T7 phages and electrochemical methods also demonstrated benefits. For example, the study of Derda[47] used filter-based colorimetric detection of *E. coli* coupled with phages amplification. The bacteriophages used in their study was a nonlytic M13 phage. While we used T7 phage is a lytic phage, thereby eliminating the need for the addition of lysozyme, and the engineered T7 phages can facilitate the overexpression of enzyme directly. When detecting in turbid or colored samples, they required a pretreatment step (filtration) for the sample to obtain a relatively clean sample for the detection. Electrochemical methods are able to detect such samples directly, thus simplify the detection procedure. Additionally, electrochemical methods offer the advantage of instant quantification of bacteria with minimal equipment. In their research, filtration was also used as a pre-concentration step. If our detection is coupled with pre-concentration, we have the potential to reach a lower limit of detection with less time. Therefore, the use of engineered phages coupled with

electrochemical detection made this method sensitive, easy and cost-effective for live bacteria detection.

CONCLUSION

There is increasing need for the rapid quantification of bacteria in the food and water sample in order to ensure the health of the public. Here, an alternative strategy based on electrochemical methods using engineered bacteriophages was developed to detect *E. coli* in aqueous samples. Following the infection of *E. coli* with the engineered phages, overexpressed β -gal was released into sample solution that allows for the electrochemical detection. This approach was able to detect 10^5 CFU/mL *E. coli* after 3 hours and 10^2 CFU/mL *E. coli* after 7 hours from aqueous samples (drinking water, apple juice, and skim milk). The standards and regulations for monitoring bacterial contamination is very stringent. EPA requires a public water supply to maintain less than one CFU of coliform bacteria in 100 mL of water. To achieve this low limit of detection, the EPA-approved analytical method using membrane filtration for pre-concentration needs incubation of the plate for 24 hours at 35 ℃, followed by the fluorescent measurement.[48] Compared with this method, our approach is able to detect 10^2 CFU/mL of *E. coli* in 7 hours without pre-concentration. When coupled with pre-concentration (such as filtration or immunomagnetic beads) and pre-enrichment steps, we have the potential to detect one CFU *E. coli* per 100 mL sample while still requiring much less time than the EPA-approved method. Moreover, our presented strategy is more easy-of-use due to the simplicity of the equipment.

In this assay, engineered phages were used to 1) provide specificity, 2) overexpress a reporter enzyme during infection, and 3) lyse the host cell, allowing the release of reporter enzymes. The sensitivity of this assay benefits from signal amplification through the overexpression of the marker enzyme β-gal by the engineered phages. This method is simple and economical, showing the potential to be applied into detection for other bacteria using their relative specific phage. The phages can be engineered with gene encoding for other enzymes which can be used in different detection methods with appropriate substrate. Further work to improve the sensitivity by enhancing the signal may focus on the improvement of the turnover-rate of the enzyme and use of interdigitated array microelectrodes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Differential pulse voltammetry (DPV) curve for the detection of *E. coli* with increasing concentration after 2, 3, 4, 5, 6 and 7 hours of incubation at 37℃, respectively. (PDF)

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Notes

The authors declare no competing financial interest.

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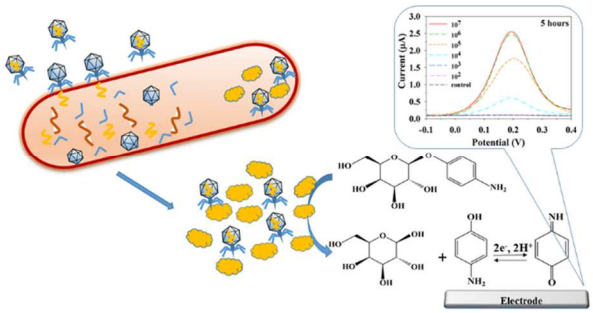
REFERENCES

(1) Scharff, R. L. *Journal of Food Protection®* **2012**, *75*, 123-131.
(2) Nugen, S. R.; Baeumner, A. J. *Analytical and Bioanalytical Chemistry* **2008**, *391*, 451-454.
(3) Zhao, X.; Lin, C.-W.; Wang, J.; Oh, D. H. *J. Microbiol. Biotechnol* **2014**, *24*, 297-312.
(4) U. S. Food and Drug Administration. Analysis and evaluation of protective control measures for the control and reduction/elimination of microbial hazards on fresh and fresh-cut produce: Chaper VII. The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. Silver Spring, MD, 2001.
(5) U. S. Food and Drug Administration. Guidance for industry: Bottled water: Total coliform and *E. coli*; Small entity compliance guide. Silver Spring, MD, 2010.
(6) U.S. Environmenal Protection Agency. 40 CFR 141 Analytical Methods Approved for Compliance Monitoring under the Ground Water Rule. Washington D.C., 2008.
(7) Lazcka, O.; Campo, F. J. D.; Muñoz, F. X. *Biosensors and Bioelectronics* **2007**, *22*, 1205-1217.
(8) Khan, I. U. H.; Gannon, V.; Kent, R.; Koning, W.; Lapen, D. R.; Miller, J.; Neumann, N.; Phillips, R.; Robertson, W.; Topp, E.; van Bochove, E.; Edge, T. A. *Journal of Microbiological Methods* **2007**, *69*, 480-488.
(9) Law, J. W.-F.; Ab Mutalib, N.-S.; Chan, K.-G.; Lee, L.-H. *Frontiers in Microbiology* **2015**, *5*.
(10) Rompré, A.; Servais, P.; Baudart, J.; de-Roubin, M.-R.; Laurent, P. *Journal of Microbiological Methods* **2002**, *49*, 31-54.
(11) Laczka, O.; García-Aljaro, C.; Del Campo, F. J.; Pascual, F. X. M.; Mas-Gordi, J.; Baldrich, E. *Analytica chimica acta* **2010**, *677*, 156-161.
(12) Yagi, K. *Applied microbiology and biotechnology* **2007**, *73*, 1251-1258.
(13) Mittelmann, A. S.; Ron, E. Z.; Rishpon, J. *Analytical Chemistry* **2002**, *74*, 903-907.
(14) Burnham, S.; Hu, J.; Anany, H.; Brovko, L.; Deiss, F.; Derda, R.; Griffiths, M. W. *Analytical and Bioanalytical Chemistry* **2014**, *406*, 5685-5693.
(15) Tryland, I.; Fiksdal, L. *Applied and Environmental Microbiology* **1998**, *64*, 1018-1023.
(16) Chen, J.; Alcaine, S. D.; Jiang, Z.; Rotello, V. M.; Nugen, S. R. *Analytical chemistry* **2015**, *87*, 8977-8984.
(17) Neufeld, T.; Schwartz-Mittelmann, A.; Biran, D.; Ron, E.; Rishpon, J. *Analytical chemistry* **2003**, *75*, 580-585.
(18) Yang, H. *Current opinion in chemical biology* **2012**, *16*, 422-428.
(19) Wang, D.; Wang, Z.; Chen, J.; Kinchla, A. J.; Nugen, S. R. *Food Control* **2016**, *62*, 81-88.
(20) Ronkainen, N. J.; Halsall, H. B.; Heineman, W. R. *Chemical Society Reviews* **2010**, *39*, 1747-1763.
(21) Hatfull, G. F.; Hendrix, R. W. *Current Opinion in Virology* **2011**, *1*, 298-303.
(22) Singh, A.; Arutyunov, D.; Szymanski, C. M.; Evoy, S. *Analyst* **2012**, *137*, 3405-3421.
(23) Tawil, N.; Sacher, E.; Mandeville, R.; Meunier, M. *Analyst* **2014**, *139*, 1224-1236.
(24) Pires, D. P.; Cleto, S.; Sillankorva, S.; Azeredo, J.; Lu, T. K. *Microbiology and Molecular Biology Reviews* **2016**, *80*, 523-543.
(25) Jackson, A. A.; Hinkley, T. C.; Talbert, J. N.; Nugen, S. R.; Sela, D. A. *Analyst* **2016**, *141*, 5543-5548.

(26) Oda, M.; Morita, M.; Unno, H.; Tanji, Y. *Applied and environmental microbiology* **2004**, *70*, 527-534.
(27) Chen, J.; Duncan, B.; Wang, Z.; Wang, L.-S.; Rotello, V. M.; Nugen, S. R. *Nanoscale* **2015**, *7*, 16230-16236.
(28) Wang, Z.; Wang, D.; Chen, J.; Sela, D. A.; Nugen, S. R. *Analyst* **2016**, *141*, 1009-1016.
(29) Wang, J. *Talanta* **2002**, *56*, 223-231.
(30) Alcaine, S.; Pacitto, D.; Sela, D.; Nugen, S. *Analyst* **2015**, *140*, 7629-7636.
(31) Alcaine, S.; Tilton, L.; Serrano, M.; Wang, M.; Vachet, R.; Nugen, S. *Applied microbiology and biotechnology* **2015**, *99*, 8177-8185.
(32) Romani, A.; Minunni, M.; Mulinacci, N.; Pinelli, P.; Vincieri, F. F.; Del Carlo, M.; Mascini, M. *Journal of Agricultural and Food Chemistry* **2000**, *48*, 1197-1203.
(33) Wang, H. Y.; Pan, M. L.; Oliver Su, Y. L.; Tsai, S. C.; Kao, C. H.; Sun, S. S.; Lin, W. Y. *Journal of Analytical Chemistry* **2011**, *66*, 415-420.
(34) Marino, D. D. *Journal of the American Dietetic Association* **2007**, *107*, 1930-1934.
(35) Jończyk, E.; Kłak, M.; Międzybrodzki, R.; Górski, A. *Folia microbiologica* **2011**, *56*, 191-200.
(36) Wang, Z.; Wang, D.; Kinchla, A. J.; Sela, D. A.; Nugen, S. R. *Analytical and bioanalytical chemistry* **2016**, 1-10.
(37) Tenu, J. P.; Viratelle, O. M.; Garnier, J.; Yon, J. *European Journal of Biochemistry* **1971**, *20*, 363-370.
(38) Juers, D. H.; Matthews, B. W.; Huber, R. E. *Protein Science* **2012**, *21*, 1792-1807.
(39) Alcaine, S. D.; Law, K.; Ho, S.; Kinchla, A. J.; Sela, D. A.; Nugen, S. R. *Biosensors and Bioelectronics* **2016**, *82*, 14-19.
(40) Shin, H. D.; Chen, R. R. *Biotechnology and bioengineering* **2008**, *101*, 1288-1296.
(41) Mergulhão, F. J. M.; Summers, D. K.; Monteiro, G. A. *Biotechnology Advances* **2005**, *23*, 177-202.
(42) Rakhuba, D.; Kolomiets, E.; Dey, E. S.; Novik, G. *Pol. J. Microbiol* **2010**, *59*, 145-155.
(43) Mássen, M.; Liu, Z.; Haruyama, T.; Kobatake, E.; Ikariyama, Y.; Aizawa, M. *Analytica chimica acta* **1995**, *304*, 353-359.
(44) Kaya, T.; Nagamine, K.; Matsui, N.; Yasukawa, T.; Shiku, H.; Matsue, T. *Chemical communications* **2004**, 248-249.
(45) Radke, S. M.; Alocilja, E. C. *Biosensors and Bioelectronics* **2005**, *20*, 1662-1667.
(46) Abu-Rabeah, K.; Ashkenazi, A.; Atias, D.; Amir, L.; Marks, R. S. *Biosensors and Bioelectronics* **2009**, *24*, 3461-3466.
(47) Derda, R.; Lockett, M. R.; Tang, S. K.; Fuller, R. C.; Maxwell, E. J.; Breiten, B.; Cuddemi, C. A.; Ozdogan, A.; Whitesides, G. M. *Analytical chemistry* **2013**, *85*, 7213-7220.
(48) U.S. Environmental Protection Agency. Method 1604: Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium). Washington D.C., 2002.

Figure for TOC



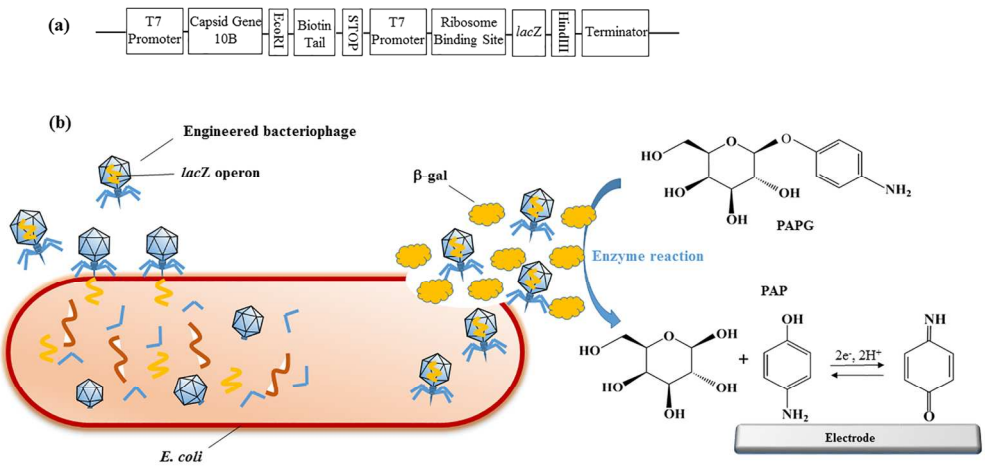


Figure 1. The scheme representation of electrochemical detection of E. coli using engineered phage. (a) The designed construct of genome of T7lacZ phage. (b) Specific capture and infection of E. coli by T7lacZ phage resulted in the release and overexpres-sion of enzyme β-gal. PAPG was catalyzed by β-gal into an electroactive species PAP that can be quantified by electrochemical device.

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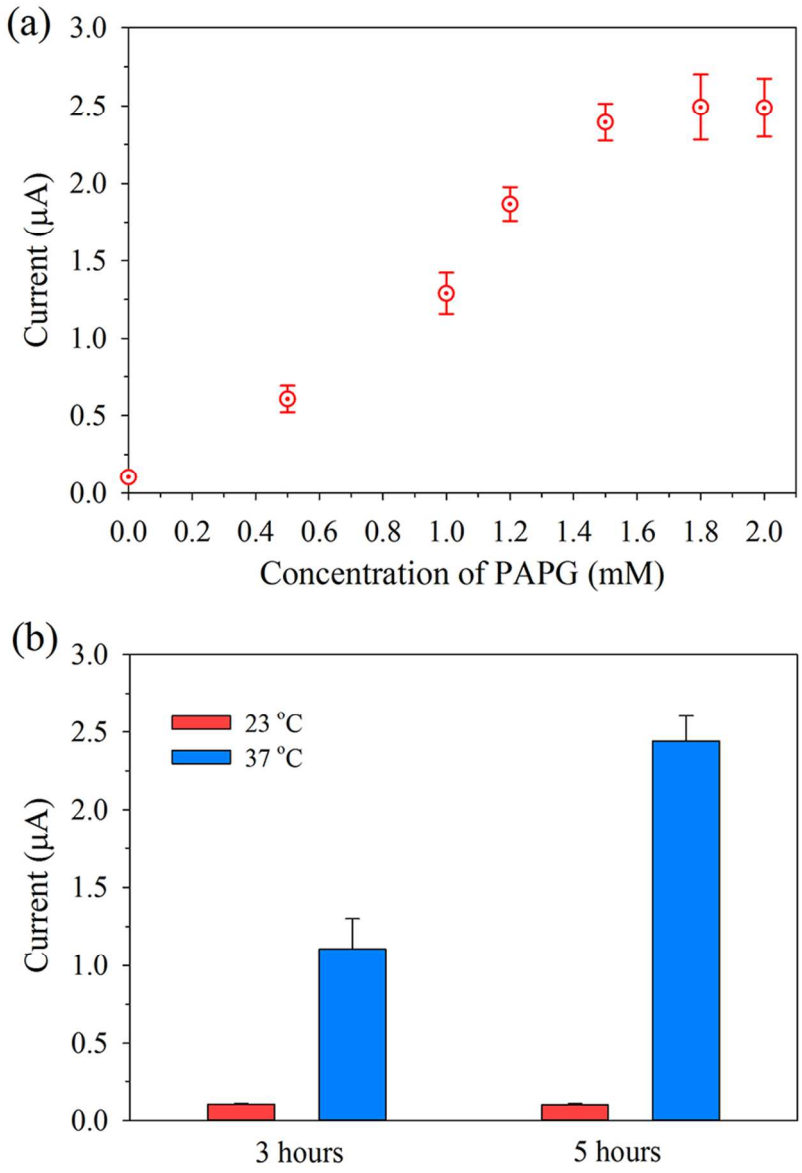

Figure 2. Optimization of condition of enzyme reaction. (a) Dependence of the current on varying concentration of sub-strate PAPG (0, 5, 10, 12, 15, 18, and 20 mM) detecting 106 CFU/mL E. coli after incubation of 4 hours at 37℃. (b) Comparison of incubation temperature at room temperature (23℃) and 37℃. Peak current obtained for detecting 106 CFU/mL of E. coli at 23℃ (red bar) and 37℃ (blue bar), respectively after 3 or 5 hours of incubation.

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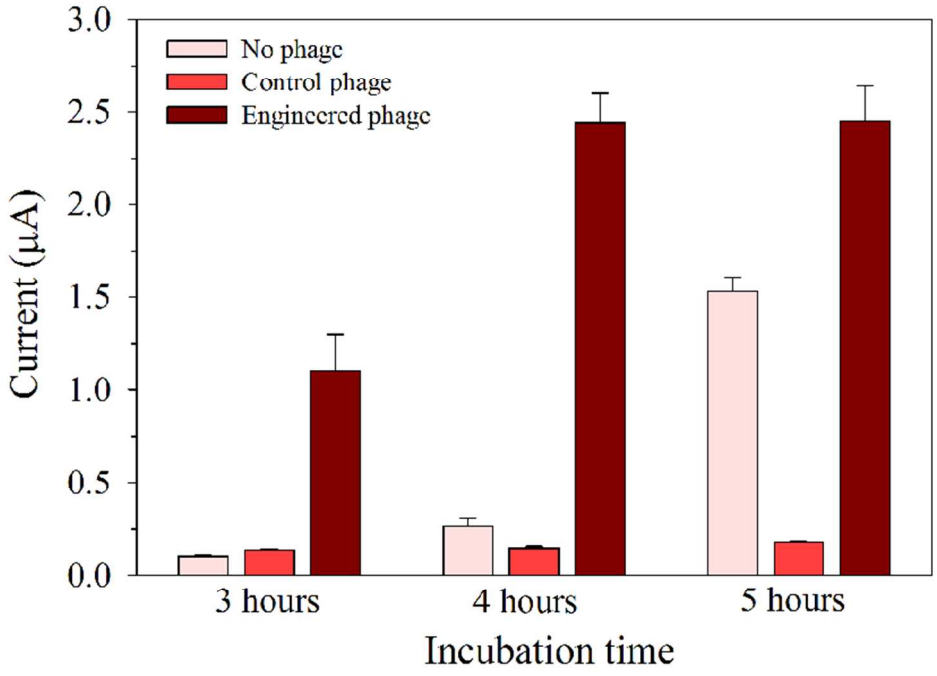

Figure 3. The signal obtained for detection of 106 CFU/mL E. coli without phage (pink bar), with control phages (red bar) and with engineered phages (dark red bar), respectively, after incubation of 3, 4 or 5 hours at 37℃.

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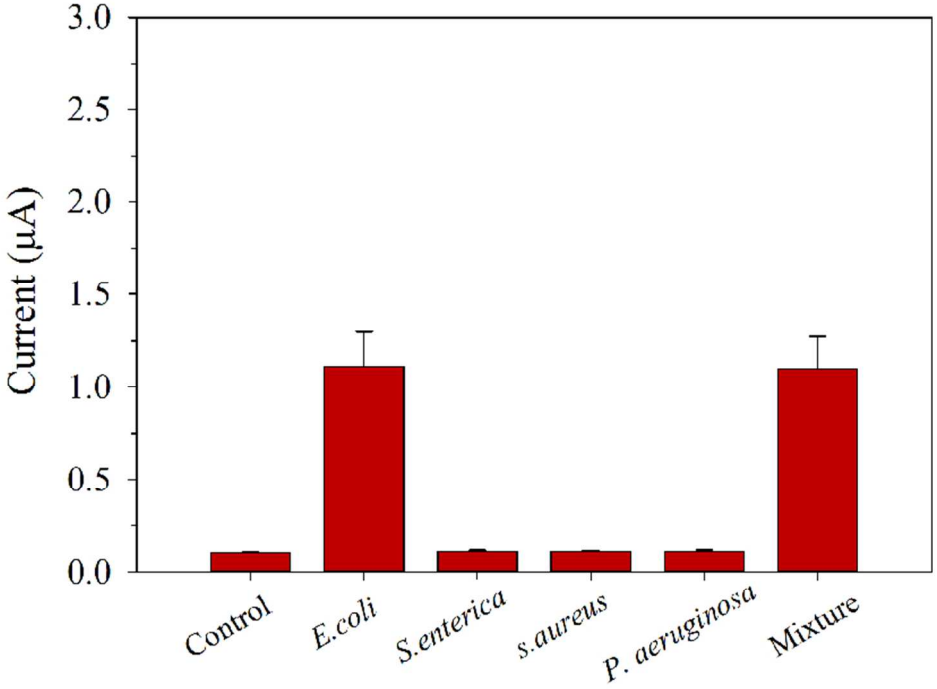

Figure 4. The signal obtained for detecting 106 CFU/mL E. coli, S. enterica, S. aureus, P. aeruginosa and a mixture of all the strains, respectively, after incubation of 3 hours at 37℃. A sample without any bacteria was used as the negative control.

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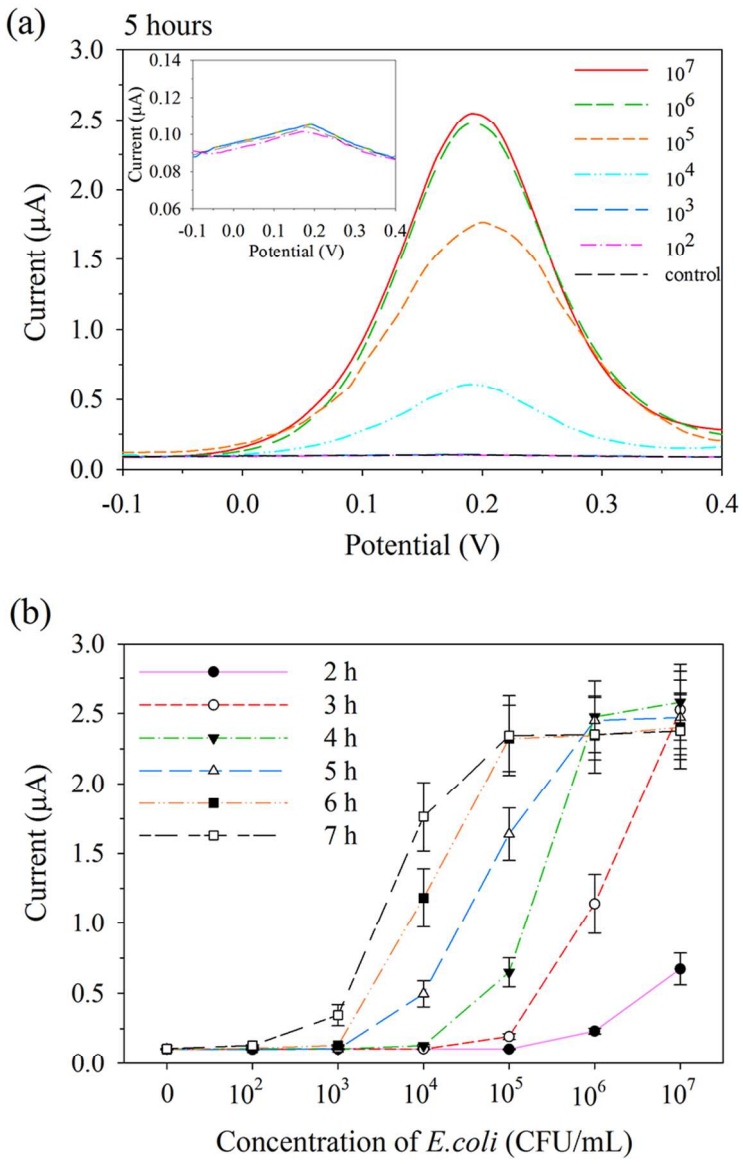

Figure 5. Results of electrochemical detection of E. coli. (a) Differential pulse voltammetry (DPV) curve for increasing concentration of E. coli after 5 hours of incubation at 37℃. The inset is the amplification of the plot of control and low concentrations (102, 103 CFU/mL) (b) Dependence of peak current obtained from DPV curve on varying concentration of E. coli for different incubation time.

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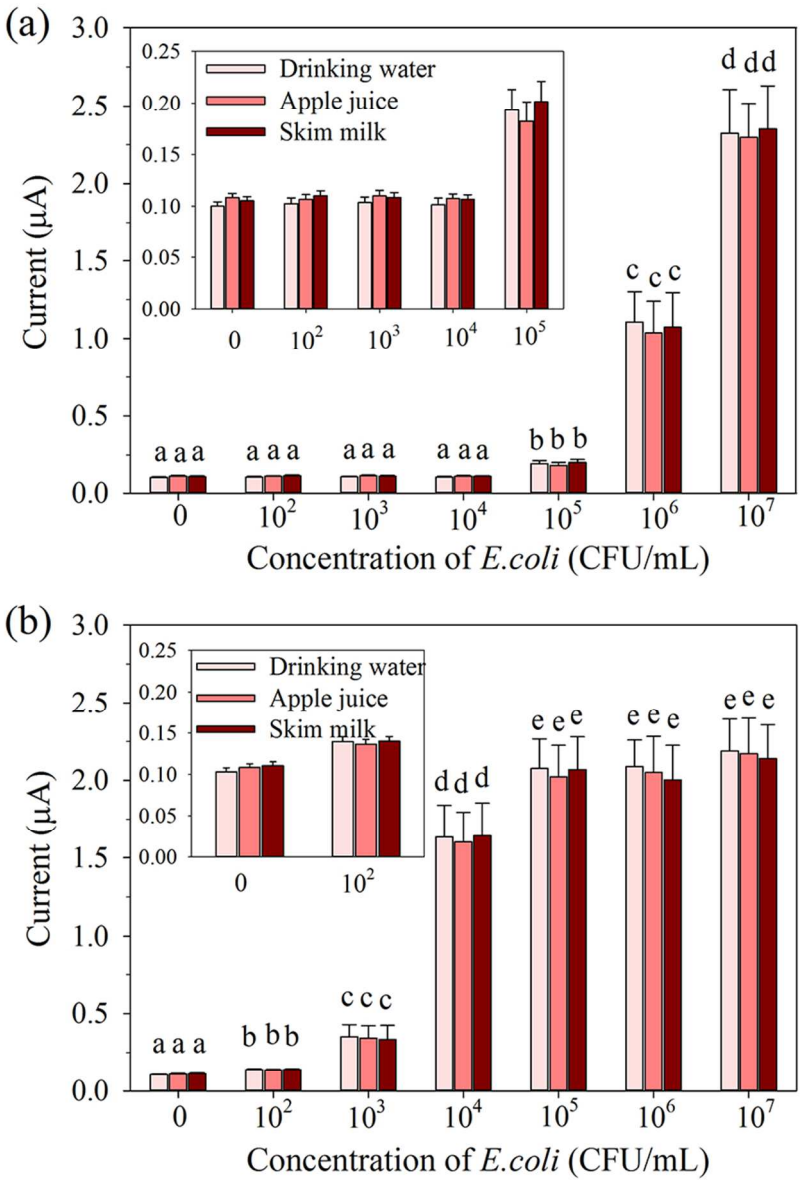

Figure 6. Peak current obtained for varying concentration of E. coli after (a) 3 hours and (b) 7 hours of incubation in drinking water (pink bars), apple juice (red bars) and skim milk (dark red bars), respectively. Bars with different letters (a, b, c, d or e) are significantly different ($P<0.05$).

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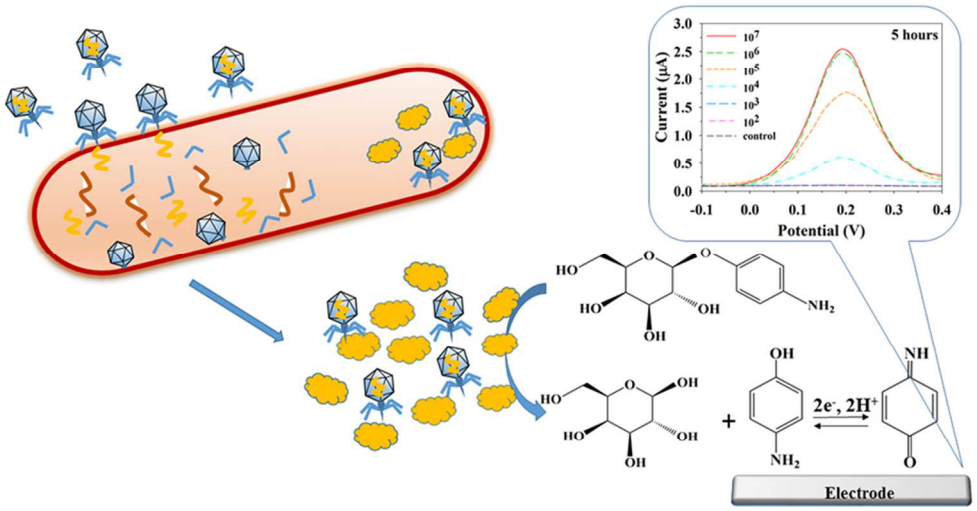

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