Author's Accepted Manuscript

www.elsevier.com/locate/talanta

Phage-based capacitive biosensor for *Salmonella* detection

Saroh Niyomdecha, Warakorn Limbut, Apon Numnuam, Proespichaya Kanatharana, Ratthaphol Charlermroj, Nitsara Karoonuthaisiri, Panote Thavarungkul

PII: S0039-9140(18)30638-6
DOI: https://doi.org/10.1016/j.talanta.2018.06.033
Reference: TAL18775

To appear in: *Talanta*

Received date: 4 April 2018
Revised date: 9 June 2018
Accepted date: 9 June 2018

Cite this article as: Saroh Niyomdecha, Warakorn Limbut, Apon Numnuam, Proespichaya Kanatharana, Ratthaphol Charlermroj, Nitsara Karoonuthaisiri and Panote Thavarungkul, Phage-based capacitive biosensor for *Salmonella* detection, *Talanta,* https://doi.org/10.1016/j.talanta.2018.06.033

Phage-based capacitive biosensor for *Salmonella* detection

Saroh Niyomdecha[a, b, c], Warakorn Limbut[a, b, d], Apon Numnuam[a, b, c], Proespichaya Kanatharana[a,b, c], Ratthaphol Charlermroj[e], Nitsara Karoonuthaisiri[e*], Panote Thavarungkul[a, b, f*]

[a]Trace Analysis and Biosensor Research Center, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

[b]Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

[c]Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

[d]Department of Applied Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

[e]National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Phahonyothin Road, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand

[f] Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

panote.t@psu.ac.th

panote.t@gmail.com

nitsara.kar@biotec.or.th

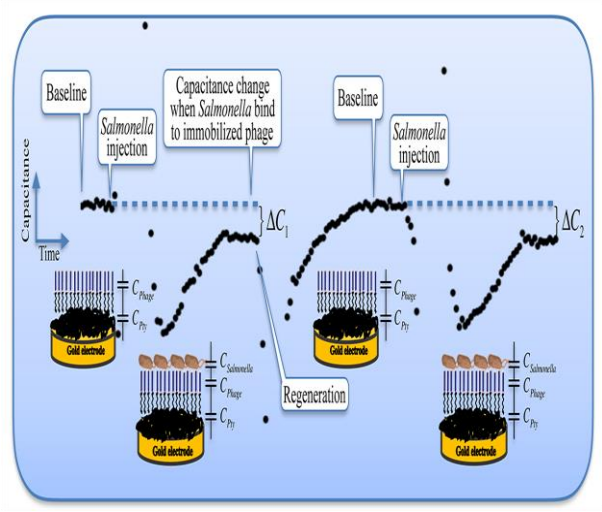
*Corresponding author Nitsara Karoonuthaisiri National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Phahonyothin Road, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand . Tel.: +66 (0) 2564 6700. Fax: +66 (0) 2564 6707.

*Corresponding author Panote Thavarungkul, who will handle correspondence at all stages of refereeing and publication Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand Tel.: +66 (0) 7428 8753. Fax: +66 (0) 7455 8849.

Abstract

This article reports the detection of *Salmonella* spp. based on M13 bacteriophage in a capacitive flow injection system. *Salmonella*-specific M13 bacteriophage was immobilized on a polytyramine/gold surface using glutaraldehyde as a crosslinker. The M13 bacteriophage modified electrode can specifically bind to *Salmonella* spp. via the amino groups on the filamentous phage. An alkaline solution was used to break the binding between the sensing surface and the analyte to allow renewable use up to 40 times. This capacitive system provided good reproducibility with a relative standard deviation (RSD) of 1.1%. A 75 µL min^{-1} flow rate and a 300 µL sample volume provided a wide linear range, from 2.0×10^{2} to 1.0×10^{7} cfu mL^{-1}, with a detection limit of 200 cfu mL^{-1}. Bacteria concentration can be analyzed within 40 min after the sample injection. When applied to test real samples (raw chicken meat) it provided good recoveries (100-111%). An enrichment process was also explored to increase the bacteria concentration, enabling a quantitative detection of S*almonella* spp. This biosensor opens a new opportunity for the detection of pathogenic bacteria using bacteriophage.

Graphical Abstract



Keywords: Capacitive biosensor; M13 bacteriophage; *Salmonella* spp.; Polytyramine; Pathogenic bacteria

1. Introduction

To prevent foodborne illnesses, a rapid, sensitive and reliable method to detect pathogenic bacteria is of importance. Conventional methods based on microbiology are relatively time-consuming and laborious [1], hence various alternatives such as immunology-based assays [2], polymerase chain reaction (PCR) [3, 4] and biosensors [5] had been developed. In all cases, specificity and sensitivity of the detection method are dictated by the choice of biosensing elements that can specifically bind to the target pathogen. The commonly employed biosensing elements for pathogenic bacteria with high binding affinity and specificity are antibodies [6, 7]. However, they are quite expensive and not always stable under strong environmental conditions used in several biosensor platforms. Alternatively, bacteriophage or phage, a virus that can specifically infect host bacteria [8, 9] has gained popularity as an alternative biorecognition element to be employed in various biosensor development [10-12] because it is robust, extremely thermally and chemically stable, specific to target of interest [13, 14], and most importantly can be immobilized on a transducer surface for different sensing platforms. Moreover, specific phages can be screened through a process called phage display biopanning, a technique stemed from an invention by Smith [15].

There are a number of reports using phage to detect pathogenic bacteria, such as *E. coli* [16, 17] *Staphylococcus aureus* [18], *Bacillus cereus, Mycobacterium* smegmatis [19] and *Salmonella* [20]. Among these, *Salmonella* detection had received more attention because it is one of the most common causes of humans foodborne illnesses [21, 22]. Label-free detection techniques of some recent works that employed phage as a specific biosensing element to bind *Salmonella* are magnetoelastic [20, 23-25], surface plasmon resonance (SPR) [18, 26, 27], piezoelectric [28] and microcantilever [29]. Most of these methods have been demonstrated with a batch system where the immobilized phage could not be reused, except for the work based on SPR. However, the SPR

system resulted in high limit of detection (LOD) (1.3×10^{7} cfu mL^{-1}) due to the technical limitation of SPR principle in detecting large molecule like bacteria [27]. Therefore, a method with higher sensitivity and better detection limit would be more applicable to the food testing industry. Notably, the capacitive sensor coupled with a flow injection system is an interesting alternative method that might provide a high sensitivity level of detection [30-33]. The principle of capacitive measurement is based on the electrical double layer on the surface of a metal electrode. A working electrode is immobilized with the biosensing element and has a stable capacitance response. Binding of the target analyte to the biosensing element on the electrode surface causes the capacitance to decrease [31]. This technique had been applied to detect many analytes such as disease markers [30, 34], residual toxin [35-37], bacteria [38-40] and viruses [41]. So far, to the best of our knowledge, no study has been reported on a capacitive flow injection system for the determination of *Salmonella* spp. based on a working electrode modified with *Salmonella*-specific M13 phage.

In this work, we report the development of a capacitive flow injection system using *Salmonella*-specific M13 phage for *Salmonella* detection. This M13 phage, previously screened from a commercial phage display library for specificity to *Salmonella* spp. [42]. The phage library is based on a M13 phage vector, containing 10^{9} independent clones. Each phage clone displays five copies of linear dodecapeptides, and the phage library encodes 4.1×10^{15} possible 12-mer sequences. The *Salmonella*-specific phage clone was selected from the library through biopanning process. The selected phage usually catches to its bacteria target by binding between the displayed amino acid sequences and the bacterial surface protein [43]. In this study, the phage was immobilized on a gold electrode surface via an electrodeposited polytyramine (Pty) layer, using glutaraldehyde as a crosslinker. The immobilized phage binds to *Salmonella* in the sample solution via the amino acid groups of its filament. After the measurement, the binding could be broken by alkaline solution in a regeneration process. The binding and regenerating cycles were operated continuously in the flow injection system. The selectivity of the sensor was tested against other relevant bacteria species. This capacitive biosensor was tested for its linear range, limit of detection, reproducibility, and reusability. Chicken meat was used to test its application in real sample testing.

2. Materials and methods

2.1 Materials

Tyramine, glutaraldehyde, and 1-Dodecanethiol were from Sigma–Aldrich (Steinheim, Germany). Bacteria used in this study were a mixture of eight commonly found *Salmonella* serovars (Enteritidis, Typhimurium, Dublin, Infantis, Senftenberg, Hadar, Mbandaka, and Virchow), *L. monocytogenes*, and *Escherichia coli* K12. They were cultured and subjected to a 10-kGy dose of gamma radiation for inactivation purpose as previously described [29]. The bacteria cells were diluted to a designated concentration in a citric acid–disodium hydrogen phosphate buffer pH 3.0 before testing with a capacitive biosensor. Other chemicals used were analytical grade.

2.2 *Salmonella*-specific biosensing element

Salmonella-specific biosensing element, a whole M13 phage (MSal020417, displaying a peptide sequence of NRPDSAQFWLHHGGGSC, 2.0×10^{12} pfu mL^{-1}), was obtained from a previous study [42]. Briefly, a cocktail of *Salmonella enterica* subspecies enterica serovars (*S.* Enteriditis, *S.* Typhimurium, *S.* Dublin, *S.* Infantis, *S.* Senftenberg, *S.* Hadar *S.* Mbandaka, *S.* Virchow) was coated on a Petri dish as target for phage display biopanning. A phage library displaying dodecapeptides (Ph.D.-12, New England Biolabs, UK) was added to the *Salmonella* coated dish, the unbound phage clones were subsequently washed off from the dish. The bound phage clones were eluted out and amplified according to the manufacturer's instruction. This was considered one round of biopanning. A total of four biopanning rounds were performed. After the third and fourth biopanning rounds, 18 phage clones were randomly selected for individual amplification, DNA extraction, and sequencing. Corresponding 12-amino acid displayed peptide sequences were deciphered using FinchTV software (http://www.geospiza.com/Products/finchtv.shtml) and ExPASy translate tool (http://web.expasy.org/translate/), respectively. Phage clones were screened for their ability to bind to *Salmonella* spp. and three heat-killed non-target bacteria (*Escherichia coli* K12, *Listeria monocytogenes*, and *Campylobacter jejuni*) using a phage-binding enzyme-linked immunosorbent

assay (ELISA) following the manufacturer's instructions. From the phage-binding ELISA [44] and surface plasmon resonance method [27], MSal020417 phage clone was found to be specific to *Salmonella* spp.

2.3 Preparation of electrode

Gold electrodes (diameter 3 mm, 99.99% purity, custom made) were polished with alumina slurries (5, 1 and 0.3 μm) followed by electrochemical etching in 0.50 M H_2SO_4 between 0.0 to 1.5 V *vs.* an Ag/AgCl reference electrode (Metrohm, Switzerland) at a scan rate of 100 mVs^{-1} for 25 scans and dried with nitrogen gas. Tyramine monomer, 50 mM in 2.0 mM phosphate buffer pH 7.00 and ethanol (3:1 volume ratio), was electropolymerized on a gold electrode by cyclic voltammetry using a microautolab type III (Metrohm Autolab B.V., KM Utrecht, Netherlands) for 15 scans, between 0.00 and 0.80 V (*vs*. Ag/AgCl) with a scan rate of 50 mV s^{-1} [45], and rinsed with distilled water. It was then treated with 5.0% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer pH 7.00 for 20 min to activate the N-aldehyde groups of the polytyramine (Pty) that would bind with the primary amino groups of biosensing elements, 20 μL of 1.0×10^{12} pfu mL^{-1} whole phage in citric acid–disodium hydrogen phosphate buffer pH 3.00. This pH has previously been shown to provide the highest immobilization efficiency [27]. The electrode was kept at 4°C overnight and then treated with 1.0 mM ethanolamine pH 8.00 for 7 min to deactivate all the remaining aldehyde groups not coupled to the immobilized phage. The modified electrode was immersed in 10 mM 1-dodecanethiol ethanolic solution for 20 min to block any pinholes on the electrode surface.

The electrochemical behavior of each immobilization step was studied by cyclic voltammetry coupled to an electrochemical cell containing the modified working electrode, an Ag/AgCl reference electrode and a platinum wire auxiliary electrode.

2.4 Capacitance measurement

The capacitive flow cell (10 μL) consists of a M13 phage/Pty modified gold electrode, a custom-made Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. The three

electrodes were connected to a potentiostat (EA161, EDAQ, Australia). The detection principle has been reported in detail elsewhere [40]. In brief, 50 mV potential step pulses (pulse width 6.4 ms), one pulse per min, were applied on the working electrode. The totally insulated electrode surface acted like a simple RC circuit [31] and the current response, i(t), decay exponentially with time (equation 1).

$$i(t) = \frac{u}{R_S} \exp\left[\frac{-t}{R_S\, C_{Total}}\right] \quad \text{.....(1)}$$

Where, *i(t)* is the current in the circuit as a function of time, u is the potential step pulses, R_s is the dynamic resistance of the recognition layer, t is the time elapsed after the potential step was applied, and C_{Total} is the total capacitance measured at the working electrode/solution interface. Taking the logarithm of equation 1 the relationship becomes

$$In\, i(t) = ln\frac{u}{R_S} - \frac{t}{R_S\, C_{Total}} \quad \text{.....(2)}$$

The resistance value (R_s) was first obtained from the intercept of the regression line of the plot of *ln i(t) versus t* (equation 2) and used to calculate the total capacitance (C_{Total}) from the slope of the same equation. The capacitance (nF Cm^{-2}) versus time (min) was then plotted and displayed on the monitor (Fig. 1). The total capacitance at the working electrode/solution interface is given by the relationship of capacitors connected in series, representing the different capacitive components on the electrode surface where C_{Pty} is the capacitance of the Pty layer, C_{phage} the phage layer and $C_{Salmonella}$ the *Salmonella* layer, the final total capacitance (C_{Total}) can be calculated as:

$$\frac{1}{C_{Total}} = \frac{1}{C_{Pty}} + \frac{1}{C_{phage}} + \frac{1}{C_{Salmonella}} \quad \text{.....(3)}$$

For the analysis, a 10 mM phosphate buffer pH 7.00 was firstly flowed through at an initial flow rate of 50 μL min^{-1} to obtain the baseline capacitance (Fig. 1). Then 250 μL of *Salmonella* spp. in citric acid–disodium hydrogen phosphate buffer pH 3.00, the pH that gave the maximum binding

signal between the M13 bacteriophage and bacteria [27], was injected into the continuous flow of the carrier buffer. The capacitance initially increased due to the different buffer of the injected bacteria sample. The continuous flow of the carrier buffer then brought the capacitance back down, revealing the new lower capacitance level. This was due to the binding between *Salmonella* spp. and the immobilized phage that produced a thicker dielectric layer on the electrode/electrolyte interface, similar to adding another capacitor in series, and the capacitance decreased (ΔC). Under these initial conditions, 50 min was allowed after ectionthe bacteria inj before the ΔC was taken. The regeneration solution, 250 μL of 5.0 mM of NaOH, was then applied to break the binding between the *Salmonella* spp. and the M13 phage [27]. An elapse time of 50 min was allowed for the capacitance to return to its original baseline before a new analytical cycle. The effect of flow rate and sample volume were also investigated.

2.5 Performance of the capacitive sensor

The performance of the sensor using the immobilized whole phage was studied for the linearity and limit of detection by measuring *Salmonella* spp. in the concentration range of 1.0 - 1.0×10^{8} cfu mL^{-1}. The calibration curve has an s-shape, the LOD was determined by following IUPAC recommendation 1994 where it is taken as the concentration of an analyte at the point of intersection of the extrapolated linear range and the final lowest concentration level segment of the calibration plot [46]. The reproducibility of the electrode preparation was investigated by preparing six electrodes at the same time. The reusability of an electrode was studied by repeatedly injecting *Salmonella* spp. (1.0×10^{5} cfu mL^{-1}) in the flow injection system and at the end of each cycle, 5.0 mM of NaOH was injected to break the binding between *Salmonella* and the phage-immobilized electrode. The response from the first cycle was taken as 100%. The specificity of the sensor was also studied by comparing the signal of the target *Salmonella* spp. and non-target bacteria, *L. monocytogenes* and *E. coli,* as well as the mixture of these bacteria at the same concentration (1.0×10^{7} cfu mL^{-1}).

This capacitive flow injection system was applied to detect *Salmonella* spp. in 3 minced chicken meat samples from 3 local markets, Hat Yai, Songkhla, Thailand. The samples were prepared followed a previous report [47]. Briefly, 50 g of minced chicken meat samples and 50 mL of 10 mM phosphate buffer pH 7.00 were thoroughly mixed and filtered by a cellulose acetate membrane (pore size of 11 μm, Whatman, England). The liquid samples were then tested. The matrix effect was first evaluated with various dilutions (0, 2.5, 5 times) by comparing the slopes of the standard curves and the spiked curves (200, 400, 600, 800 and 1000 cfu mL^{-1}) using two-way ANOVA. To study the accuracy of the fabricated sensor, the recovery was investigated by spiking *Salmonella* spp. in the minced chicken meat samples before the aforementioned preparation of the liquid samples. The responses obtained from spiked samples were used to calculate the concentration from the matrix matched calibration curve. Recovery percentage was evaluated by the following equation:

$$\%\text{Recovery} = \frac{C_1 - C_2}{C_3} \times 100 \quad \text{……...……..(4)}$$

where C_1 is the concentration of analyte measured in the spike sample, C_2 is the concentration of analyte measured in the blank and C_3 is the concentration of analyte spiked into the sample.

According to the zero tolerance policy for *Salmonella* contamination, any viable *Salmonella* must not be found [1] in poultry food sample. However, the limit of detection of a sensor is generally higher than this regulation, thus a bacterial enrichment process is required before analysis. Viable *Salmonella* serovars (Typhimurium, Enteritidis, Mbandaka, Corvallis, Senftenberg, Albany, Dublin, Infantis) in different concentrations (1.0×10^2, 1.0×10^3, 1.0×10^4 cfu mL^{-1}) were spiked in 50 g of chicken samples and pre-enriched in 50 mL of 0.10% w/v peptone for 24 h before separated into 2 equal portions. One portion was tested with the capacitive sensor after the sample was heated at 90°C for 20 min to kill the bacteria [29]. Medium without the spiked *Salmonella* spp. and buffer solution were used as a control and treated the same way as those spiked with the bacteria. The

other portion was enriched further in a selective medium and cultured at 35°C for 24 h before testing with the capacitive sensor.

3. Results and Discussion

3.1 Electrochemical characterization of the modified electrode

For the capacitive system, insulation of the electrode surface is an important condition. The degree of insulation of the modified gold electrode after each immobilization step was examined by cyclic voltammetry. A voltammogram of the cleaned gold surface showed clearly the oxidation and reduction peaks of ferricyanide (Supplementary material Fig. S1). Both redox peaks decreased when the Pty was formed on the gold surface due to its non-conducting property. The insulating property of the electrode surface increased when glutaraldehyde was used to activate the surface to crosslink with phage which could be observed by the further reduction of the redox peaks. The modified electrode surface was then reacted with ethanolamine pH 8.50 to occupy all the remaining aldehyde groups that had not coupled to the immobilized phage. The disappearance of the redox peaks after blocking with 1-dodecanethiol indicated that the electrode surface was completely insulated.

3.2 Performances of the capacitive system

Performances of the capacitive sensor based on the whole phage were studied in a flow injection system.

3.2.1 Linear dynamic range and limit of detection

The plot in Fig. 2 shows the linear response from 2.5×10^2 to 1.0×10^7 cfu mL^{-1}. The LOD is low as 250 cfu mL^{-1} based on IUPAC Recommendation 1994 [46]. The linearity and LOD of this sensor were much better than other works using the whole phage for *Salmonella* spp. detection [21, 40]. With such a low LOD, the time to enrich the sample for the detection of *Salmonella* could be lessen.

3.2.2 Reproducibility and reusability

Six electrodes, prepared at the same time, were used to measure *Salmonella* spp. at 1.0×10^5 cfu mL^{-1}. The results showed good reproducibility with an RSD of 1.1% (Supplementary material Fig. S2). For the reusability, the immobilized M13 phage was evaluated by repeatedly injecting the same concentration of *Salmonella* spp. At 1.0×10^5 cfu mL^{-1} with subsequent regeneration step by injecting 250 μL of 5.0 mM of NaOH as regeneration solution. The change in capacitance signal after regeneration was used to calculate the percentage of relative response of the immobilized M13 phage by comparing it to the initial capacitance change. The electrode could be used up to 40 times with a relative response of 99±2% and an RSD of 2.0% (Supplementary material Fig. S3). The ability to reuse the electrode up to 40 times can reduce the cost/sample significantly ($0.5 per test).

After 40 injections, the relative response suddenly decreased below 90%. The electrode was then tested by cyclic voltammetry. The voltammogram of the electrode after 40 analysis shows much higher redox peaks than before used (Supplementary material Fig. S3 inset), indicating that the surface was more conductive. That is, the decrease of response was probably caused by some part of the polymer was lost from the electrode surface.

3.2.3 Specificity

Fig. 3 shows the response of the capacitive system to 1.0×10^7 cfu mL^{-1} of the target *Salmonella*, the non-target *E. coli* and *L. monocytogenes* and their mixture. The capacitance change for *Salmonella* was about 8 times higher than the capacitive response of the non-target bacteria that were similar to that of buffer solution. The mixture of target and non-target bacteria was also tested, and the results showed no significant difference ($P > 0.05$) between *Salmonella* and *Salmonella* with the other two bacteria. These results indicated that this phage-based capacitive biosensor was highly selective for *Salmonella* detection. The specificity of phage was due to the displayed peptide which forms mimotopes-mimic epitopes [48] that could specifically bind to a target of interest by protein-protein interaction [42, 50]

3.2.4 Improving the limit of detection

Although the detection limit of the capacitive system was low, it was still higher than the zero-tolerance regulation of *Salmonella* in poultry food sample [1]. In any flow injection analysis, a low flow rate or a high sample volume normally provides a high response due to the longer contact time between the cell and the analytes, but as a consequence there is a long response time. To increase the sensitivity and decrease the detection limit, the flow rate (50-150 µL min^{-1}) and sample volume (250-500 µL) were investigated together using 1.0×10^5 cfu mL^{-1} of *Salmonella* spp. (Fig. 4). In order to produce a balance between a short detection time and a high response, a 75 µL min^{-1} flow rate and a 300 µL sample volume resulting in a detection time of 40 min were chosen. These conditions provided a slightly wider linear range (2.0×10^2 to 1.0×10^7 cfu mL^{-1}) with a higher sensitivity (improved from 14.6±0.5 log(x) - 18±2 to 15.3±0.2 log(x) -18±1), a lower LOD of 200 cfu mL^{-1} and a shorter detection time of 40 min. These conditions were subsequently used to test real samples. This detection time of 40 min may be longer than some other works, however, this is still much shorter than conventional methods that use 24-48 h for *Salmonella* spp. detection [3].

3.3 Detection of *Salmonella* spp. in chicken samples

The application of the phage modified electrode was demonstrated by measuring *Salmonella* spp. concentrations in chicken samples. The effect of the matrix was first studied by comparing the response from the standard (*Salmonella* spp. in buffer) and spiked samples at various dilution factors. Without the dilution and at 2.5 times dilution matrix effect existed, indicated by the significantly difference of the sensitivity between the standard and the spiked samples ($P < 0.05$). At a dilution factor of 5 the sensitivities showed no significant difference ($P > 0.05$) (Supplementary material Fig. S4), i.e., there was no matrix effect. Therefore, samples were diluted 5 times before being analyzed by the capacitive biosensor. The concentration of the spiked *Salmonella* spp. samples showed good recovery (100±3-111±6 %) (Table 1).

Since the LOD of this system is 200 cfu mL^{-1}, real samples with a lower amount of *Salmonella* need an enrichment step so the contaminated *Salmonella* (if any) could increase to a

detectable level. Table 2 shows the results of *Salmonella* spp. detection of a sample, with and without enrichment. Without the enrichment, i.e., only buffer was used, *Salmonella* spp. in food sample spiked at 100 cfu mL^{-1} was not detectable, i.e., the concentration was lower than the LOD. Using 0.10% w/v peptone as the enrichment medium, the same result was obtained while the selective medium could increase the concentration of *Salmonella* spp. by 1.9-2.5 times. The most enrichment was obtained from 0.10% w/v peptone in the selective medium where the concentration of *Salmonella* increased by 3.2-3.9 times. Therefore, using a suitable enrichment medium can increase the concentration of *Salmonella* spp. to be detectable by the developed sensor. A longer incubation than 24 h used here would increase the enrichment factor and enable the system to detect at a lower concentration.

Comparing the performances between this capacitive biosensor and other previous biosensors using different phages and their peptides for *Salmonella* spp. specific detection, the developed capacitive biosensor gave a better LOD than most methods using other phages such as E2 phages [20, 24, 25] and C4-22 phage [23] and also better than the work that used the same M13 phage [26, 27] (Table 3). The sensor platforms that have better LOD than our capacitive sensor are the acoustic wave biodetector [28] and the phage imprinted capacitive biosensor [38]. However, the acoustic wave biodetector could only be used once while our capacitive biosensor could be reused for 40 times. For the phage imprinted sensor, though reusable it was not very selective and further investigation was suggested. Although the LOD of this work was still higher than the limit of zero-tolerance regulation for the amount of *Salmonella* spp. in food, using the enrichment processes can increase the amount of *Salmonella* to be in a detectable range of this sensor within 48 h. Therefore, it would be possible to detect *Salmonella* of 1 cfu mL^{-1} in real sample by incubating through the enrichment process for a longer time, possibly not more than 72 h [51]. As far as we know, there is still no method that can achieve the limit of the regulation without enrichment method. The linear range of this capacitive biosensor (2.0×10^{2}-1.0×10^{7} cfu mL^{-1}) was also similar to most sensors. Thus, this phage-based capacitive sensor using a flow system is a simple technique that can detect *Salmonella* spp. and provide good and reliable performance.

4. Conclusions

The biosensor based on *Salmonella*-specific M13 phage/Pty/gold electrode coupled with capacitive technique clearly demonstrated its potential for the detection of *Salmonella* spp. with a wide linear range, a low detection limit and a relatively short detection time. The modified electrode preparation showed good reproducibility. The flow system made it easy to regenerate the electrode surface, allowed it to be reused, thus reduced the analysis cost. When applied to detect *Salmonella* in chicken samples, at a 5 times dilution, the matrix had no effect on the analysis. Good recoveries were also obtained. This biosensor opens new opportunities for pathogenic bacteria detection using bacteriophage as a specific biosensing element.

Acknowledgements

This project was co-supported by The Thailand Research Fund (TRF), Faculty of Science and Prince of Songkla University (BRG6080009). Thanks also to the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Trace Analysis and Biosensor Research Center (TAB-RC) and Graduate School, Prince of Songkla University, Thailand. The financial support for Saroh Niyomdecha from Prince of Songkla University Ph.D. Scholarship is also gratefully acknowledged. We would like to thank Dr Irene Grant, Dr Josephine Morton and Prof Chris Elliott at Queen's University Belfast (UK) for the phage display bio-panning techniques.

References

[1] ISO, International Organization for Standardization, (2002).

[2] C. Wiuff, E.S. Jauho, H. Stryhn, L.O. Andresen, K. Thaulov, U. Boas, M.H. Jakobsen, P.M.H. Heegaard, Evaluation of a Novel Enzyme-Linked Immunosorbent Assay for Detection of Antibodies against Salmonella, Employing a Stable Coating of Lipopolysaccharide-Derived Antigens Covalently Attached to Polystyrene Microwells, Journal of Veterinary Diagnostic Investigation 12(2) (2000) 130-135.

[3] P.F.G. Wolffs, K. Glencross, R. Thibaudeau, M.W. Griffiths, Direct quantitation and detection of salmonellae in biological samples without enrichment, using two-step filtration and real-time PCR, Applied and Environmental Microbiology 72(6) (2006) 3896-3900.

[4] A. R. Bennett, D. Greenwood, C. Tennant, J.G. Banks, R. P. Betts, Rapid and definitive detection of Salmonella in foods by PCR, Letters in Applied Microbiology 26(6) (1998) 437-441.

[5] D. Zhang, Y. Yan, Q. Li, T. Yu, W. Cheng, L. Wang, H. Ju, S. Ding, Label-free and high-sensitive detection of Salmonella using a surface plasmon resonance DNA-based biosensor, Journal of Biotechnology 160(3) (2012) 123-128.

[6] Q. Sun, G. Zhao, W. Dou, A nonenzymatic optical immunoassay strategy for detection of Salmonella infection based on blue silica nanoparticles, Analytica Chimica Acta 898 (2015) 109-115.

[7] S.D. Aribam, Y. Ogawa, H. Matsui, J. Hirota, M. Okamura, M. Akiba, Y. Shimoji, M. Eguchi, Monoclonal antibody-based competitive enzyme-linked immunosorbent assay to detect antibodies to O:4 Salmonella in the sera of livestock and poultry, Journal of Microbiological Methods 108 (2015) 1-3.

[8] F.W. Twort, An investigation on the nature of ultra-microscopic viruses, The Lancet 186(4814) (1915) 1241-1243.

[9] F. d'Herelle, An invisible microbe that is antagonistic to the dysentery bacillus. Comptes Rendus Academie Sciences Paris, 165 (1917), 373–375.

[10] F. Wang, S. Horikawa, J. Hu, H. Wikle, I.-H. Chen, S. Du, Y. Liu, B. Chin, Detection of Salmonella Typhimurium on Spinach Using Phage-Based Magnetoelastic Biosensors, Sensors 17(2) (2017) 386.

[11] J.D. Mack, T. Yehualaeshet, M.K. Park, B. Tameru, T. Samuel, B.A. Chin, Phage-Based Biosensor and Optimization of Surface Blocking Agents to Detect Salmonella Typhimurium on Romaine Lettuce, Journal of Food Safety 37(2) (2017).

[12] M.K. Park, B.A. Chin, Novel approach of a phage-based magnetoelastic biosensor for the detection of Salmonella enterica serovar typhimurium in soil, Journal of Microbiology and Biotechnology 26(12) (2016) 2051-2059.

[13] G.P. Smith, Surface presentation of protein epitopes using bacteriophage expression systems, Current Opinion in Biotechnology 2(5) (1991) 668-673.

[14] K. Farrokhzad, C. Rosenfield, B. Applegate, In High Thorughput Screening for Food Safety Assessment (2015) pp81-121.

[15] G.P. Smith, Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface, Science 228(4705) (1985) 1315-1317.

[16] C. Xiao, F. Jiang, B. Zhou, R. Li, Y. Liu, Immobilization of Escherichia coli for detection of phage T4 using surface plasmon resonance, Science China Chemistry 55(9) (2012) 1931-1939.

[17] A. Shabani, C.A. Marquette, R. Mandeville, M.F. Lawrence, Magnetically-assisted impedimetric detection of bacteria using phage-modified carbon microarrays, Talanta 116 (2013) 1047-1053.

[18] S. Balasubramanian, I.B. Sorokulova, V.J. Vodyanoy, A.L. Simonian, Lytic phage as a specific and selective probe for detection of Staphylococcus aureus—A surface plasmon resonance spectroscopic study, Biosensors and Bioelectronics 22(6) (2007) 948-955.

[19] M. Yemini, Y. Levi, E. Yagil, J. Rishpon, Specific electrochemical phage sensing for Bacillus cereus and Mycobacterium smegmatis, Bioelectrochemistry 70(1) (2007) 180-184.

[20] R.S. Lakshmanan, R. Guntupalli, J. Hu, V.A. Petrenko, J.M. Barbaree, B.A. Chin, Detection of Salmonella typhimurium in fat free milk using a phage immobilized magnetoelastic sensor, Sensors and Actuators B: Chemical 126(2) (2007) 544-550.

[21] FAD, Food and Drug Administration (2009) 1-7.

[22] WHO, World Health Organization Report on Infectious Diseases: Removing Obstacles to Healthy Development, World Health Organization, Switzerlland (2005).

[23] I.H. Chen, S. Horikawa, K. Bryant, R. Riggs, B.A. Chin, J.M. Barbaree, Bacterial assessment of phage magnetoelastic sensors for Salmonella enterica Typhimurium detection in chicken meat, Food Control 71 (2017) 273-278.

[24] S. Li, Y. Li, H. Chen, S. Horikawa, W. Shen, A. Simonian, B.A. Chin, Direct detection of Salmonella typhimurium on fresh produce using phage-based magnetoelastic biosensors, Biosensors and Bioelectronics 26(4) (2010) 1313-1319.

[25] M.-K. Park, H.C. Wikle, Y. Chai, S. Horikawa, W. Shen, B.A. Chin, The effect of incubation time for Salmonella Typhimurium binding to phage-based magnetoelastic biosensors, Food Control 26(2) (2012) 539-545.

[26] S. Agrawal, P.K. Kulabhusan, M. Joshi, D. Bodas, K.M. Paknikar, A high affinity phage-displayed peptide as a recognition probe for the detection of Salmonella Typhimurium, Journal of Biotechnology 231 (2016) 40-45.

[27] N. Karoonuthaisiri, R. Charlermroj, M.J. Morton, M. Oplatowska-Stachowiak, I.R. Grant, C.T. Elliott, Development of a M13 bacteriophage-based SPR detection using Salmonella as a case study, Sensors and Actuators B: Chemical 190 (2014) 214-220.

[28] E.V. Olsen, I.B. Sorokulova, V.A. Petrenko, I.H. Chen, J.M. Barbaree, V.J. Vodyanoy, Affinity-selected filamentous bacteriophage as a probe for acoustic wave biodetectors of Salmonella typhimurium, Biosensors and Bioelectronics 21(8) (2006) 1434-1442.

[29] J. Wang, M.J. Morton, C.T. Elliott, N. Karoonuthaisiri, L. Segatori, S.L. Biswal, Rapid Detection of Pathogenic Bacteria and Screening of Phage-Derived Peptides Using Microcantilevers, Analytical Chemistry 86(3) (2014) 1671-1678.

[30] S. Suwansa-ard, P. Kanatharana, P. Asawatreratanakul, B. Wongkittisuksa, C. Limsakul, P. Thavarungkul, Comparison of surface plasmon resonance and capacitive immunosensors for cancer antigen 125 detection in human serum samples, Biosensors and Bioelectronics 24(12) (2009) 3436-3441.

[31] C. Berggren, B. Bjarnason, G. Johansson, Capacitive Biosensors, Electroanalysis 13(3) (2001) 173-180.

[32] S. Dawan, P. Kanatharana, B. Wongkittisuksa, W. Limbut, A. Numnuam, C. Limsakul, P. Thavarungkul, Label-free capacitive immunosensors for ultra-trace detection based on the increase of immobilized antibodies on silver nanoparticles, Analytica Chimica Acta 699(2) (2011) 232-241.

[33] D. Jiang, J. Tang, B. Liu, P. Yang, X. Shen, J. Kong, Covalently coupling the antibody on an amine-self-assembled gold surface to probe hyaluronan-binding protein with capacitance measurement, Biosensors and Bioelectronics 18(9) (2003) 1183-1191.

[34] W. Limbut, P. Kanatharana, B. Mattiasson, P. Asawatreratanakul, P. Thavarungkul, A comparative study of capacitive immunosensors based on self-assembled monolayers formed from thiourea, thioctic acid, and 3-mercaptopropionic acid, Biosensors and Bioelectronics 22(2) (2006) 233-240.

[35] S. Loyprasert, P. Thavarungkul, P. Asawatreratanakul, B. Wongkittisuksa, C. Limsakul, P. Kanatharana, Label-free capacitive immunosensor for microcystin-LR using self-assembled thiourea monolayer incorporated with Ag nanoparticles on gold electrode, Biosensors and Bioelectronics 24(1) (2008) 78-86.

[36] M. Labib, M. Hedström, M. Amin, B. Mattiasson, A capacitive immunosensor for detection of cholera toxin, Analytica Chimica Acta 634(2) (2009) 255-261.

[37] L. Lebogang, J. Jantra, M. Hedström, B. Mattiasson, Electrochemical flow-ELISA for rapid and sensitive determination of microcystin-LR using automated sequential injection system, Sensors (Switzerland) 17(7) (2017).

[38] G. Ertürk, R. Lood, Bacteriophages as biorecognition elements in capacitive biosensors: Phage and host bacteria detection, Sensors and Actuators B: Chemical 258 (2018) 535-543.

[39] N. Idil, M. Hedström, A. Denizli, B. Mattiasson, Whole cell based microcontact imprinted capacitive biosensor for the detection of Escherichia coli, Biosensors and Bioelectronics 87 (2017) 807-815.

[40] R. Wannapob, P. Kanatharana, W. Limbut, A. Numnuam, P. Asawatreratanakul, C. Thammakhet, P. Thavarungkul, Affinity sensor using 3-aminophenylboronic acid for bacteria detection, Biosensors and Bioelectronics 26(2) (2010) 357-364.

[41] S. Samanman, P. Kanatharana, W. Chotigeat, P. Deachamag, P. Thavarungkul, Highly sensitive capacitive biosensor for detecting white spot syndrome virus in shrimp pond water, Journal of Virological Methods 173(1) (2011) 75-84.

[42] J. Morton, N. Karoonuthaisiri, L.D. Stewart, M. Oplatowska, C.T. Elliott, I.R. Grant, Production and evaluation of the utility of novel phage display-derived peptide ligands to Salmonella spp. for magnetic separation, Journal of Applied Microbiology 115(1) (2013) 271-281.

[43] S.S. Rao, K.V.K. Mohan, Y. Gao, C.D. Atreya, Identification and evaluation of a novel peptide binding to the cell surface of Staphylococcus aureus, Microbiological Research 168(2) (2013) 106-112.

[44] J. Morton, N. Karoonuthaisiri, L.D. Stewart, M. Oplatowska, C.T. Elliott, I.R. Grant, Production and evaluation of the utility of novel phage display-derived peptide ligands to Salmonella spp. for magnetic separation, J Appl Microbiol 115(1) (2013) 271-81.

[45] S. Loyprasert, M. Hedström, P. Thavarungkul, P. Kanatharana, B. Mattiasson, Sub-attomolar detection of cholera toxin using a label-free capacitive immunosensor, Biosensors and Bioelectronics 25(8) (2010) 1977-1983.

[46] P. Buck Richard, E. Lindner, Recommendations for Nomenclature of Ion-Selective Electrodes, Pure and Applied Chemistry, 1994, p. 2527.

[47] N. Karoonuthaisiri, R. Charlermroj, J. Teerapornpuntakit, M. Kumpoosiri, O. Himananto, I.R. Grant, O. Gajanandana, C.T. Elliott, Bead array for Listeria monocytogenes detection using specific monoclonal antibodies, Food Control 47 (2015) 462-471.

[48] C.-H. Wu, I.J. Liu, R.-M. Lu, H.-C. Wu, Advancement and applications of peptide phage display technology in biomedical science, Journal of Biomedical Science 23(1) (2016) 8.

[49] K.J. James, M.A. Hancock, J.-N. Gagnon, J.W. Coulton, TonB Interacts with BtuF, the Escherichia coli Periplasmic Binding Protein for Cyanocobalamin, Biochemistry 48(39) (2009) 9212-9220.

[50] J. Bazan, I. Całkosiński, A. Gamian, Phage display—A powerful technique for immunotherapy, Human Vaccines & Immunotherapeutics 8(12) (2012) 1817-1828.

[51] S. Taskila, M. Tuomola, H. Ojamo, Enrichment cultivation in detection of food-borne Salmonella, Food Control 26(2) (2012) 369-377.

Fig. 1. The flow injection capacitive system and the capacitive properties of the sensing surface. A baseline capacitance was obtained with a flow of the running buffer. When Salmonella was injected into the continuous flow of buffer the capacitance increased due to the different ionic strength of the solution. During this time the binding between *Salmonella* and the immobilized phage occurred. When the carrier buffer washed away the sample solution from the detection surface, the binding revealed the decreased capacitance (ΔC_1). The regeneration solution was then injected to break the binding between *Salmonella* cell and phage. The capacitance returned to the original baseline ready for a new analysis cycle of *Salmonella* (ΔC_2).

Fig. 2. The linear response of the capacitive sensor (n=3).

Fig. 3. Specificity study of the capacitive sensor based on *Salmonella*-specific M13 phage. The capacitance change (nF cm^{-2}) was obtained when measuring 1.0×10^7 cfu mL^{-1} of *Salmonella spp., E. coli*, *L. monocytogenes* and their mixture. An error bar represents the standard deviation of the three replicates detection.

Fig. 4. Capacitance response (a) and response time (b) of the biosensor at various flow rates (x axis) and different sample volumes. The *Salmonella*-specific M13 phage-based capacitive sensor was used to detect 1.0×10^5 cfu mL^{-1} of *Salmonella* spp. An error bar represents the standard deviation of the three replicates detection.

Table 1. The recoveries of spiked *Salmonella* spp. in chicken sample (n=3)

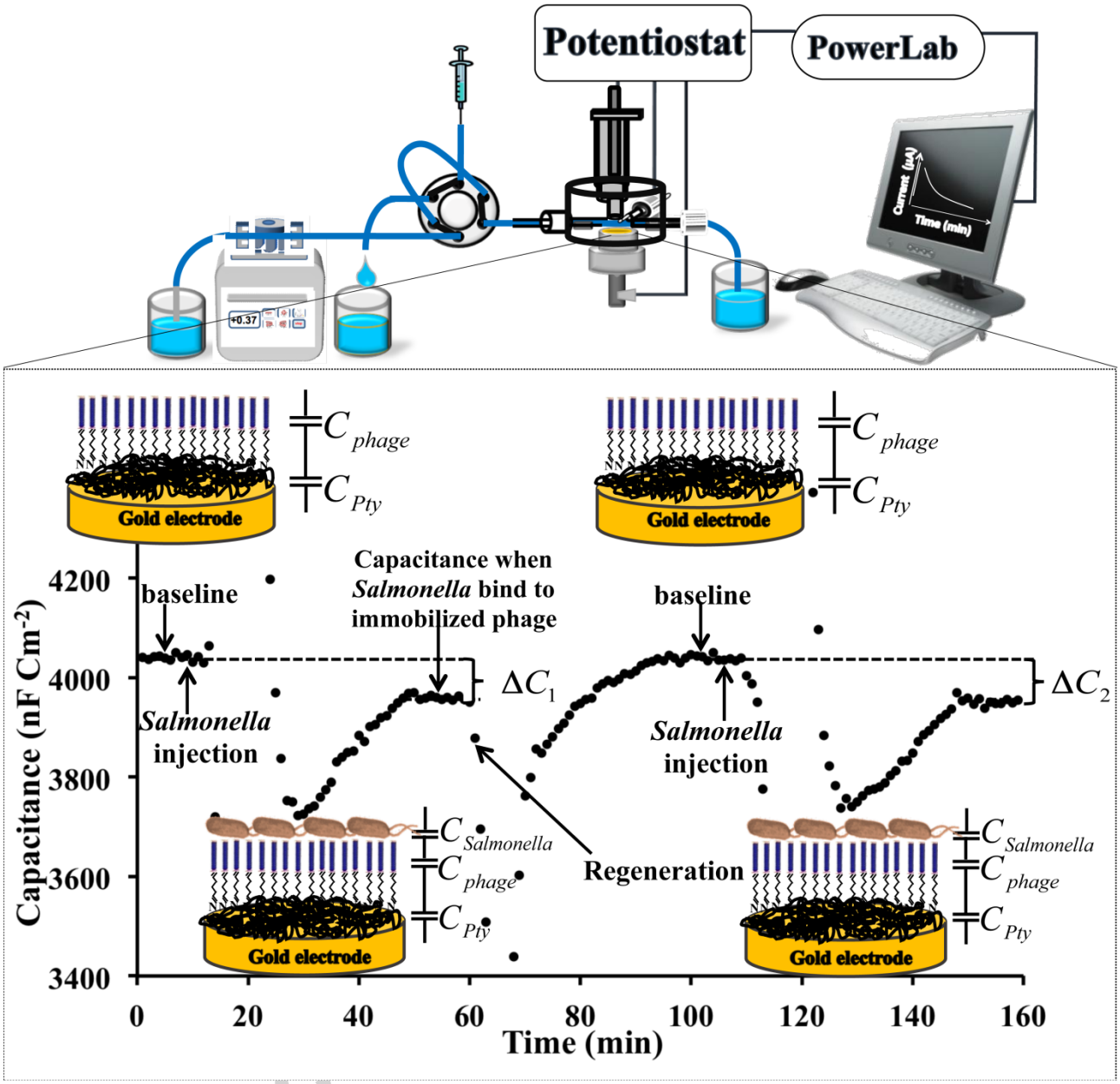
Spiked concentration (cfu mL^{-1})	**Measured concentration (cfu mL^{-1})**	**Recovery (%)**
200	213±12	106±6
400	443±23	111±6
600	624±29	104±5
800	837±31	105±4
1000	1003±27	100±3

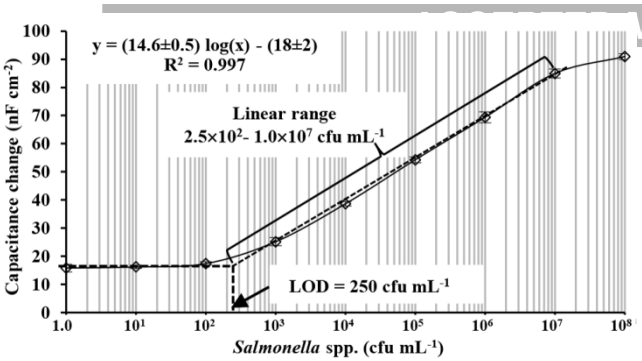
Table 2. Comparison of non-enrichment and enrichment (24-48 h) for *Salmonella* spp. detection. ND represents not detectable.

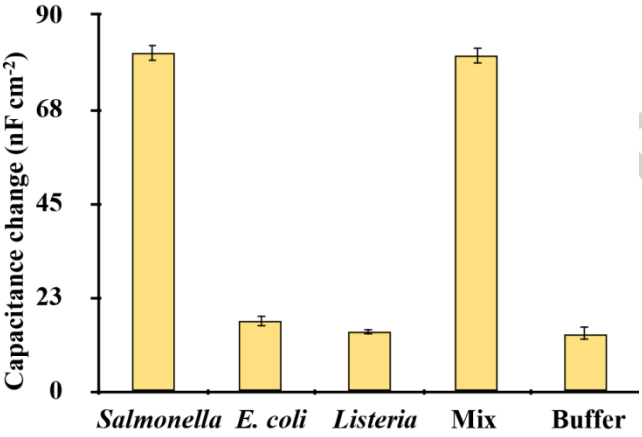
Medium	Spiked concentration (cfu mL^{-1})	Measured concentration (cfu mL^{-1})	Increasing ratio
Non-enrichment			
Buffer	0	ND	-
	$1.0x10^2$	ND	-
	$1.0x10^3$	$(1.0±0.1)x10^3$	-
	$1.0x10^4$	$(1.0±0.1)x10^4$	-
Pre-enrichment with 0.1% peptone (24 h)			
0.1% peptone	0	ND	-
	$1.0x10^2$	ND	-
	$1.0x10^3$	$(1.1±0.1)x10^3$	-
	$1.0x10^4$	$(1.1±0.1)x10^4$	-
Control (buffer)	0	ND	-
	$1.0x10^2$	ND	-
	$1.0x10^3$	$(1.1±0.2)x10^3$	-
	$1.0x10^4$	$(1.1±0.3)x10^4$	-
Enrichment with selective medium (24 h)			
Selective medium	0	ND	-
	$1.0x10^2$	ND	-
	$1.0x10^3$	$(1.9±0.1)x10^3$	1.9
	$1.0x10^4$	$(2.5±0.1)x10^4$	2.5
Control (buffer)	0	ND	-
	$1.0x10^2$	ND	-
	$1.0x10^3$	$(1.1±0.3)x10^3$	-
	$1.0x10^4$	$(1.0±0.2)x10^4$	-
Pre-enrichment with 0.1% peptone + enrichment with selective medium (48 h total)			
0.1% peptone + selective medium	0	ND	-
	$1.0x10^2$	$(3.4 ±0.1)x10^2$	3.4
	$1.0x10^3$	$(3.9±0.2)x10^3$	3.9
	$1.0x10^4$	$(3.2±0.1)x10^4$	3.2
Control (buffer)	0	ND	-
	$1.0x10^2$	ND	-
	$1.0x10^3$	$(1.2±0.1)x10^3$	-
	$1.0x10^4$	$(1.1±0.2)x10^4$	-

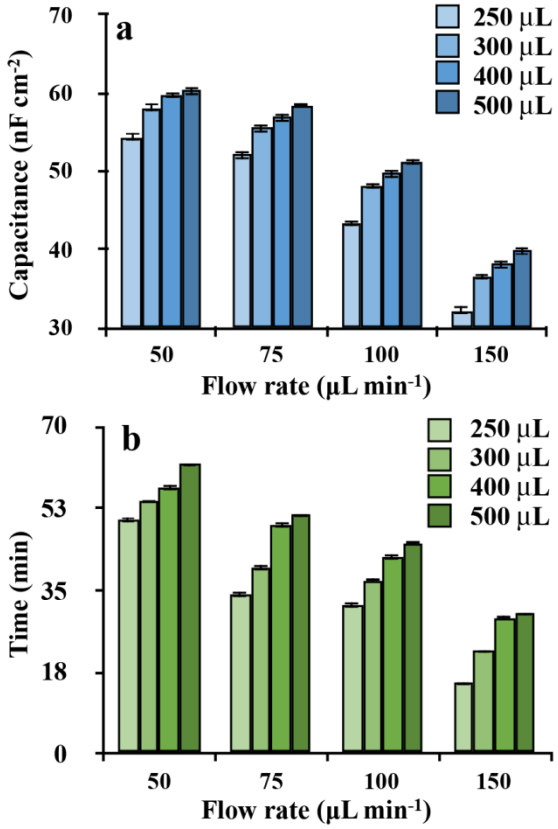
Table 3 Comparison of the performance between the fabricated biosensor and other biosensors.

Phage type	Phage immobilization	Detection technique	Analyte	Real sample	Linear range (cfu mL^{-1})	LOD (cfu mL^{-1})	Reusability	Ref.
E2 phage	Phages / Au layer by physical adsorption	magnetoelastic (batch system)	***S. typhimurium***	fat free milk	-	5×10^3	-	[20]
C4-22 phage	Phage/ Au layer by physical adsorption and cysteine	magnetoelastic (batch system)	***S. typhimurium***	chicken sample	-	7.9×10^3	-	[23]
E2 phage	Phages /Au layer by physical adsorption	magnetoelastic (batch system)	***S. typhimurium***	tomato surface	-	5×10^2	-	[24]
E2 phage	Phages /Au layer by physical adsorption	magnetoelastic (batch system)	*S. typhimurium*	tomato surface	-	-	-	[25]
M13 Phage derived peptide	Phage /Au surface using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimid linker	SPR (flow system)	*S. typhimurium*	-	-	10^3	-	[26]
M13 phages	Phage /Au surface using EDC/NHS linker	SPR (flow system)	***Salmonella*** spp.	-	-	1.3×10^7	-	[27]
Filamentous phage	Phage /Au strip by physical adsorption	maxtek acoustic wave device (batch system)	*S. typhimurium*	-	10^1-10^7	10^1	-	[28]
M13 phage-derived peptides	Phage / Au surface using succinimidyl propionate linker	Microcantilevers (batch system)	*Salmonella* spp.	-	$1x10^6$ - $1x10^8$	1×10^6	-	[29]
Phage imprinted polymer	Bacteriophage imprinted capacitve gold electrode	Capacitive (flow system)	*E.coli*	-	$1.0x10^2$ - $1.0x10^7$	$1.0x10^2$	yes (no data for number of reuse)	[38]
M13 phage clone	phage / Pty/Au elecectrode using glutaraldehyde linker	Capacitive (flow system)	*Sallmonella* spp.	chicken sample	$1.0x10^3$ - $1.0x10^7$	$2.0x10^2$	40 times	This work


Potentiostat
PowerLab
Current (μA)
Time (min)
Gold electrode
C_{phage}
C_{Pty}
Capacitance when
Salmonella bind to
immobilized phage
baseline
Salmonella
injection
ΔC_1
ΔC_2
Regeneration
$C_{Salmonella}$
Capacitance (nF Cm^{-2})
Time (min)
4200
4000
3800
3600
3400
0
20
40
60
80
100
120
140
160







Highlights

- A capacitive flow system for *Salmonella* detection using whole phage
- Immobilized bacteriophage allowed renewable use up to 40 times
- The sensor can detect *Salmonella* as low as 200 cfu mL^{-1}
- Good results were obtained when real samples were tested
- An enrichment process was explored, enabling a quantitative detection of S*almonella*