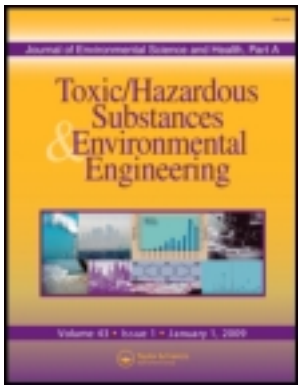

Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lesa20

Real-time label-free affinity biosensors for enumeration of total bacteria based on immobilized concanavalin A

Jongjit Jantra [a b c], Proespichaya Kanatharana [a b c], Punnee Asawatreratanakul [a d], Martin Hedström [e], Bo Mattiasson [e] & Panote Thavarungkul [a b f]

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

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

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

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

[e] Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden

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

Version of record first published: 30 Sep 2011.

To cite this article: Jongjit Jantra, Proespichaya Kanatharana, Punnee Asawatreratanakul, Martin Hedström, Bo Mattiasson & Panote Thavarungkul (2011): Real-time label-free affinity biosensors for enumeration of total bacteria based on immobilized concanavalin A, Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 46:13, 1450-1460

To link to this article: http://dx.doi.org/10.1080/10934529.2011.609022

Journal of Environmental Science and Health, Part A (2011) **46**, 1450–1460

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2011.609022

Real-time label-free affinity biosensors for enumeration of total bacteria based on immobilized concanavalin A

JONGJIT JANTRA[1,2,3], PROESPICHAYA KANATHARANA[1,2,3], PUNNEE ASAWATRERATANAKUL[1,4], MARTIN HEDSTRÖM[5], BO MATTIASSON[5] and PANOTE THAVARUNGKUL[1,2,6]

[1]*Trace Analysis and Biosensor Research Center, Prince of Songkla University, Hat Yai, Songkhla, Thailand*
[2]*Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand*
[3]*Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand*
[4]*Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand*
[5]*Department of Biotechnology,Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden*
[6]*Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand*

This work presents the results of the use of flow injection surface plasmon resonance and impedimetric affinity biosensors for detecting and enumerating total bacteria based on the binding between *E. coli* and Con A, immobilized on a modified gold electrode. The single analysis time for both techniques was less than 20 min. Dissociation between the immobilized Con A and the *E. coli* using 200 mM of glucose in HCl at pH of 2.00 enabling the sensor to be reused for between 29–35 times. Impedimetric detection provided a much lower limit of detection (12 CFU mL^{-1}) than the surface plasmon resonance method (6.1×10^7 CFU mL^{-1}). Using the impedimetric system, real sample analysis was performed and the results were compared to the plate count agar method. Cell concentrations obtained by the biosensor were only slightly different from the result obtained from the plate count agar. The proposed system offers a rapid and useful tool for screening detection and enumeration of total bacteria.

Keywords:: Bacteria, surface plasmon resonance, impedance, biosensor, high sensitivity, real-time.

Introduction

Surface waters, rivers canals and reservoirs are particularly liable to pollution due to sewage, storm water, industrial, urban and agricultural effluents discharges that usually contain a mixture of bacteria. Bottled drinking water can also be contaminated by the bacteria before or during the production process. The consumption of water contaminated by bacteria is a major cause of human morbidity, such as gastrointestinal, upper respiratory tract ailments and meningitis.[1] Therefore, suitable detection methods for the presence of bacteria in water are needed to assist in the prevention of these harmful consequences.

Conventional methods for bacterial detection are based on colony counting, which requires a couple of days for the results. Therefore, a more rapid determination technique would be helpful and the development of such techniques has grown quite quickly over the past 20 years. Among these, biosensors, using various detection principles, are the fastest growing technology for detecting bacteria. Optical transduction ranks first, while electrochemical transduction comes second.[2]

Biosensors for bacterial detection generally employ antibodies, receptors or nucleic acids as the biorecognition elements,[3] however, these methods are relatively expensive. As an alternative, lectins that exhibit strong binding to carbohydrate moieties[4] can be employed based on the affinity of the interactions between the lectins and carbohydrate components on the bacterial cell wall.[5,6] Among the lectins, concanavalin A (Con A) is the most widely used because it is easy to purify from jack beans and also exhibits excellent stability. Moreover, it can interact with saccharides containing α-D-mannose and α-D-glucose,[4,7] which are two of the most common components of bacterial cell walls.[8] The interaction is based on hydrogen bonding and van der Waals forces[9] that can be easily dissociated by a suitable solution. Because Con A can interact with carbohydrate on bacterium cell wall and is not specific to any particular bacteria. Therefore, it can be used as a sensing element for enumeration and screening detection of total bacteria.

The use of Con A as a biorecognition element for detection of total bacteria through affinity binding has

Address correspondence to P. Thavarungkul, Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand; E-mail: panote.t@psu.ac.th
Received March 29, 2011.

been investigated by both direct and indirect assays. One indirect assay was based on the conjugation of Con A with a fluorescent label followed by measurement of the intensity of the fluorescent signal.[10] Another indirect detection was based on a sandwich format using Con A as both a primary and secondary biorecognition element.[8,11] Although indirect detection can provide a low limit of detection of about 60 CFU mL^{-1},[11] a general drawback is the analysis time which needs 1–3 h to complete one assay. Therefore, direct detection using a smaller number of steps would be preferable. Direct assay is based on the detection of a physical property change, for example, mass[12] or an electrochemical property[13,14] after interaction between Con A and the bacterial cells. The limit of detection obtaining from the mass change detection was 10^3 CFU mL^{-1}.[12]

This was still not low enough because the guidelines for drinking-water recommended by most countries is no more than 100 or 500 CFU mL^{-1}.[15] Using electrochemical impedance spectroscopy to measure faradaic impedance spectra to determine bacteria, Wan and coworkers could obtain a very low limit of detection of 2 CFU mL^{-1}, however, with a 2 h incubation time.[13] Although, some of the aforementioned direct and indirect assays could provide a limit of detection low enough for the recommended bacteria level in drinking water, the method is not in real-time and the analysis time is relatively long. Therefore, we became interested in developing a more rapid and sensitive affinity biosensor for bacteria with the capability of real-time detection within a short analysis time.

In this work two real-time detection systems using immobilized Con A as the sensing element were investigated. One is the surface plasmon resonance (SPR), a well known real-time optical detection system.[16–18] The other is the impedimetric system that monitors the imaginary impedance component at a single frequency. This real-time impedimetric detection is a highly sensitive method for label-free affinity detection;[19–21] however, its application has not been widely explored.

To the best of our knowledge, the detection and enumeration of total bacteria using Con A as a biorecognition element together with SPR and impedimetric real-time measurement has not been reported. In both cases Con A was immobilized on a gold electrode surface via a self-assembled monolayer of 11-mercaptoundecanoic acid. Heat-killed *E. coli* were initially used for the optimization and calibration of the system. The interaction between immobilized Con A and *E. coli* caused the change of the SPR angle and the impedance property. Although the use of SPR to detect whole bacteria achieved a relatively high detection limit, 5–7 $\times$ 10^7 CFU mL^{-1} of *E. coli*,[22] it was suggested that further optimization of the system might enhance this detection limit. Therefore, the operating conditions of the SPR system were optimized and the performances of the two systems were investigated under the same conditions. The better system was then used to analyze bacteria in real water samples. The biosensor results were then compared to the conventional non real-time plate count agar method (PCA).

Materials and methods

Chemicals

Concanavalin A (Con A) was from Sigma (St. Louis, MO, USA). 11-mercaptoundecanoic acid (11-MUA), dimethylamino-propyl-*N'*-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Sigma-Aldrich (Steinheim, Germany). 1-dodecanethiol was obtained from Aldrich (Milwaukee, Wisconsin, USA). Bovine serum albumin (BSA) was from Fluka (Buchs, Switzerland). Nutrient agar and nutrient broth were from Difco (Sparks, MD, USA). All other chemicals were analytical grade. Buffer solutions used in the SPR and the impedimetric systems were prepared with deionized water. Before use, these were filtered through an Albet® nylon membrane filter (Albet, Spain), pore size 0.2 μm, with subsequent degassing.

Sample preparation of E. coli

The stock culture of *E. coli* was kindly provided by the Faculty of Agro Industry, Prince of Songkla University, Thailand and resuscitated by inoculating into 100 mL of sterile nutrient broth and incubated at 37°C for 24 h in a shake incubator. The culture was enumerated using 10-fold serial dilutions plated on nutrient agar and the incubation was carried out with the same conditions.

E. coli in liquid nutrient broth was harvested by centrifugation at 7,000 $\times$ g for 15 min and the supernatant was discarded. The untreated *E. coli* sample was washed and resuspended in 10 mM Tris-HCl buffer pH 7.00. *E. coli* cells were heat-killed by exposing the cells at 90°C for 1 h and sedimented at 7,000 $\times$ g for 15 min. Heat-killed cells were used in this work because at this level of heat their ability to bind to Con A is not affected,[12,23] and it could also help to improve the limit of detection. [24] The cells were resuspended in 10 mM Tris-HCl buffer pH 7.00. Serial 10-fold dilutions were carried out when an *E. coli* sample was analyzed in the label-free affinity biosensor system.

Immobilization of Con A

Con A was immobilized on a gold disk for the SPR and on a gold rod for the impedimetric system, both with an active area of 7.1 mm^2 (diameter = 3.0 mm). The immobilization procedures followed the optimized conditions of Suwansaard et al[25] and are described as follows.

On gold disk for SPR system

A gold disk (diameter 2.5 cm; 50 nm thick gold-coated BK-7 glass plate, Metrohm Autolab B.V., The Netherlands) was cleaned with piranha solution (3:1 v/v H_2SO_4 and H_2O_2) before rinsing with deionized water and drying with nitrogen gas. The glass side of the gold disk was attached to the prism of the SPR equipment (AutoLab Spirit®, Metrohm Autolab B.V., The Netherlands) via matching oil with the same refractive index ($n = 1.518$). Then the gold disk was placed inside the SPR holding block with the gold surface facing upwards. An SPR flow cell was screwed on top of the gold disk leaving a volume of 10 μL for the solution to flow through. Con A immobilization was carried out on the gold surface inside the flow cell (diameter = 3 mm). The immobilization steps were monitored by detecting the change in the SPR signal (AutoLab SPR version 4.2.1 software).

The gold surface was first cleaned by ethanol (1.0 mL) and then exposed to 150 mM of 11-MUA for 5 h to form a self-assembled monolayer (SAM). The surface of the SAM was washed with ethanol (1.0 mL) and 10 μL min^{-1} of deionized water for 5 min, respectively. The formation of SAM caused an increase of the SPR angle. Then the C-terminals of SAM were activated with 0.2 M EDC/0.05 M NHS for 50 min and washed with 10 μL min^{-1} of deionized water for 5 min.[25] Further incubation with EDC/NHS activated the carboxylic acids of the 11-MUA towards ester and an SPR angle change was observed. Then 2.0 mg mL^{-1} Con A in 10 mM sodium acetate buffer pH 4.00 was introduced and left for the immobilization to occur for 2 h. This step caused a further increase of the SPR angle. Finally, 1.0 M ethanolamine pH 8.50 was used to react with the excess carbonyl group resulting in a slight decrease of the SPR angle. Finally, non-specific binding groups were blocked with 1%BSA (w/v) for 40 min, before the unbound molecules were washed away with carrier buffer. This step produced a slight increase of the SPR angle.

On gold electrode for impedimetric system

A gold rod electrode (diameter 3 mm, 99.99% purity) was cleaned with piranha solution and subsequently polished with alumina slurries (5, 1 and 0.3 μm). The electrode was then cleaned through sonication in ethanol for 1 min followed by deionized water for 10 min. The electrochemical etching was carried out in 0.5 M H_2SO_4 by cycling the electrode from 0 V to 1.5 V versus a Ag/AgCl reference electrode with a scan rate of 0.1V s^{-1} for 25 scans. The cleaned gold electrode was then modified with 11-MUA (150 mM) dissolved in ethanol. SAM was allowed to form at room temperature for 30 min. The activation of SAM was carried out using 0.2 M EDC/0.05 M NHS for 50 min before 2.0 mg mL^{-1} of Con A in 10 mM sodium acetate buffer pH 4.00 was placed on the electrode surface. The immobilization took place at 4°C for 24 h. Finally, the electrode was immersed in 1.0 M ethanolamine pH 8.50 for 7 min followed by 10 mM 1-dodecanethiol ethanolic for 20 min to make the insulation complete.

The immobilization steps were monitored by observing the electrochemical behavior through cyclic voltammetry using a potentiostat (Microautolab type III, Metrohm Autolab B.V., The Netherlands) coupled to an electrochemical cell containing the modified electrode (working electrode), a Ag/AgCl reference electrode (Metrohm, Switzerland) and a platinum rod auxiliary electrode (Metrohm, Switzerland). Cyclic voltammograms of the modified electrode were obtained with 10 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl solution at a scan rate of 0.1 V s^{-1} vs. a Ag/AgCl reference electrode. The voltage range was -0.3 to 0.8 V. Voltammograms were obtained after each immobilization steps where the reduction of redox peaks were observed after each step. A flat voltammogram was obtained after the last step indicating total insulation of the surface.

Detection of *E. coli* using label-free affinity biosensors

SPR system

The SPR experiment was performed using the SPR AutoLab (AutoLab Spirit®, Metrohm Autolab B.V., The Netherlands). The carrier buffer solution was delivered using a microsyringe pump (Kd Scientific, USA) into a flow injection SPR system. The *E. coli* standard in buffer solution, real samples and regeneration solution were introduced via an injection valve (Valco, USA). The temperature of the flow cell (10 μL volume) was maintained at 25°C with a circulating water bath (Grant Instrument, UK). A monochromatic *p*-polarized laser ($\lambda = 670$ nm) was directed through the prism onto the gold disk (Fig. 1a). When the carrier buffer was passed through the flow cell, the SPR angle ($\theta_{\text{Con A}}$) was recorded as a baseline. The interaction between immobilized Con A on the gold disk and *E. coli* caused the change of the refractive index and this was measured as the SPR angle shift ($\theta_{\text{Con A}/\textit{E. coli}}$) by a photodiode detector among the dynamic range of 4,000 millidegree. The SPR angle change ($\Delta\theta = \theta_{\text{Con A}/\textit{E. coli}} - \theta_{\text{Con A}}$) was obtained and this was proportional to the concentration of *E. coli*.

Impedimetric system

The modified gold electrode with immobilized Con A was used as a working electrode for the impedimetric detection. The modified gold working electrode, a custom made Ag/AgCl reference electrode and a stainless steel tube auxiliary electrode were attached to a flow cell (10 μL) connected to the impedance analyzer (Fig. 1b). Impedimetric measurement was carried out using the AutoLab PGSTAT30 electrochemical impedance analyzer and potentiostat/galvanostat (Metrohm Autolab B.V., The Netherlands) connected to a computer. Impedance was

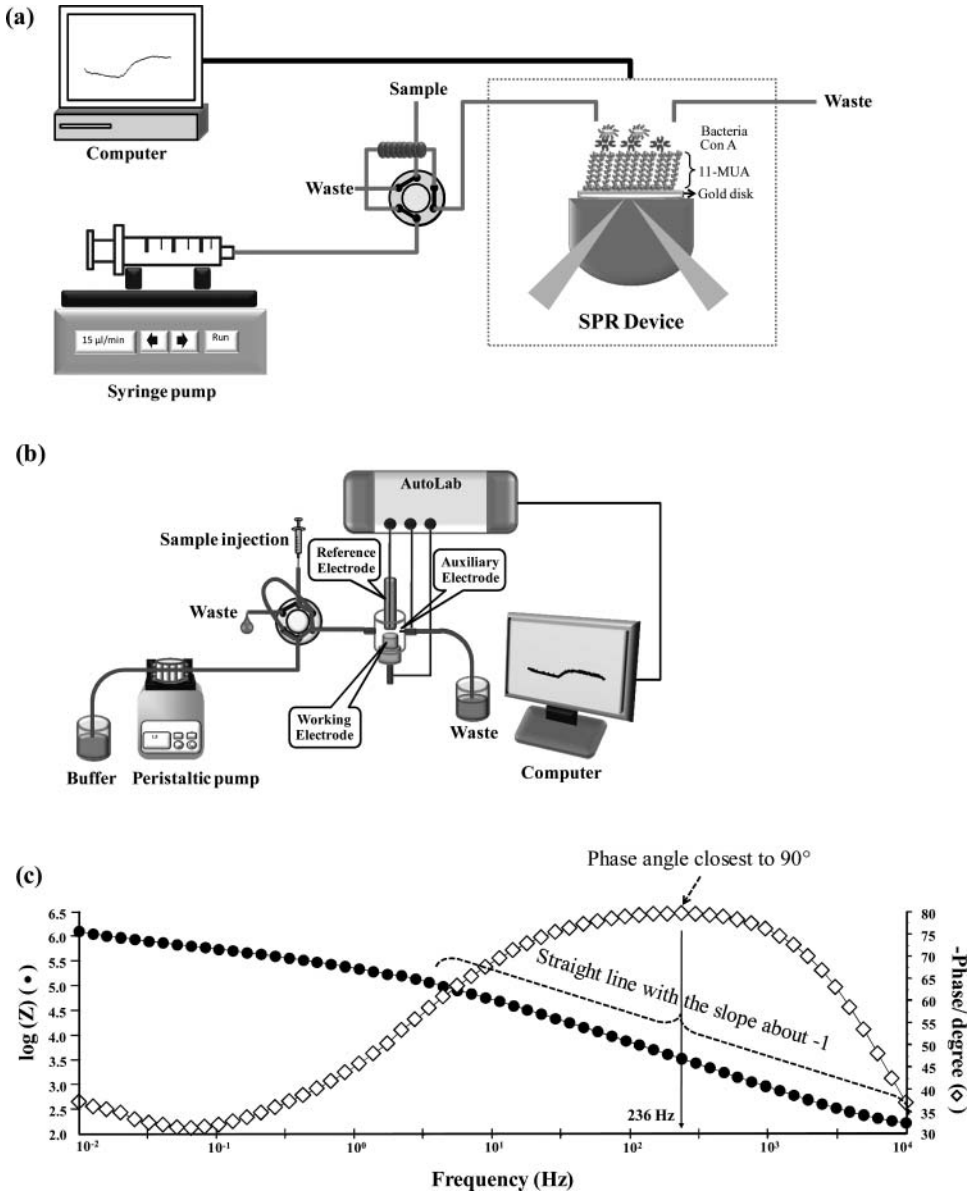

Fig. 1. Schematic diagram showing (a) the flow injection surface plasmon resonance biosensor and (b) the impedimetric biosensor for detecting total bacteria. (c) A Bode plot, log of amplitude (absolute) of the impedance and phase angle are plotted against the log of the frequency used to determine the optimum frequency.

recorded at +200 mV DC potential with the AC amplitude of ± 10 mV. The impedance response was monitored by the Frequency Response Analyzer (FRA 4.9.005).

Quantitative analysis was carried out by measuring the impedance at a single frequency obtained from a Bode plot, i.e., a plot of the phase angle (y-axis) and the log of the impedance amplitude (y-axis) vs the log of the frequency (x-axis) (Fig. 1c). This was carried out by applying an AC voltage with an amplitude of ± 10 mV at a frequency of between 0.01 and 10,000 Hz. The optimum frequency was determined based on the phase curve in the region where the impedance was a straight line with the slope about −1 and the phase angle closest to 90°, i.e., when the system exhibits the behavior of an ideal capacitor. The imaginary part of the impedance (Z″) was chosen for the monitoring of impedance when the binding event occurred, since the change of Z″ provides a better correlation to the analyte concentration than the real part of the impedance (Z′).[21] Each layer, i.e., insulating layer, biorecognition layer and analyte layer, was considered to contribute to the total capacitance (Fig. 2). The inverse of the total capacitance is the sum of the inverse of each layer's capacitance[26] according to the equation:

$$\frac{1}{C_{total}} = \frac{1}{C_{insulation}} + \frac{1}{C_{biorecognition}} + \frac{1}{C_{analyte}} \qquad (1)$$

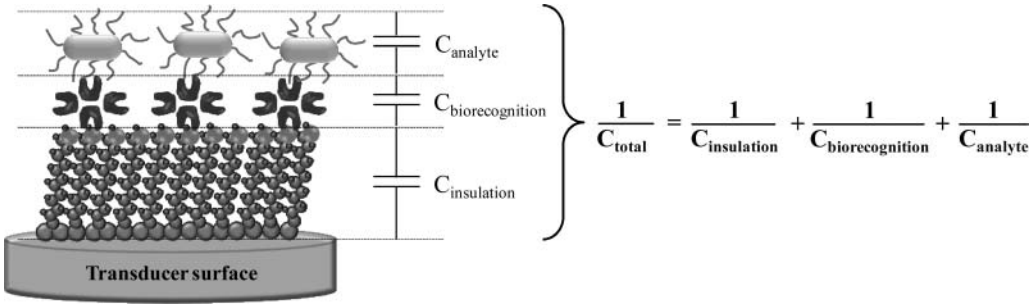

Fig. 2. Schematic diagram showing the capacitive layers on the electrode surface. The total capacitance measured at the working electrode /solution interface (C_{total}) is from a number of capacitors connected in series. $C_{insulation}$ is the capacitance of the self-assembled monolayer (11-MUA), $C_{biorecognition}$ is the capacitance of the Con A layer and the $C_{analyte}$ is the capacitance of the attached bacteria.

During the continuous flow of the carrier buffer, a baseline of Z'' was recorded ($Z''_{\text{Con A}}$). When the analyte (*E. coli*) was injected it bound to immobilized Con A, and the decrease of the capacitance causes the imaginary part of the impedance to increase ($Z''_{\text{Con A}/E.\ coli}$). The impedance change ($\Delta Z'' = Z''_{\text{Con A}/E.\ coli} - Z''_{\text{Con A}}$) could then be detected and this corresponded to the concentration of the analyte.

Optimization and performance

Parameters affecting the interaction between Con A and *E. coli* were optimized in the SPR system. The optimization started with the regeneration solution and carrier buffer conditions. Then parameters affecting the flow injection system, i.e., sample flow rate and sample volume, were evaluated. The initial conditions were 15 μL min^{-1} for the sample flow rate, 420 μL min^{-1} for the regeneration solution flow rate, 350 μL of sample volume and 10 mM sodium acetate buffer pH 5.30 as a carrier buffer. Standard heat-killed *E. coli* (1.2×10^8 CFU mL^{-1}) was used for the optimization. Three replications were carried out for each tested value. The optimization was carried out by changing a single parameter and keeping other parameters constant.

The interaction between Con A and *E. coli* can be dissociated using a regeneration solution so that the biosensor can be reused. The residual activity of Con A to *E. coli* after regeneration was used to consider the optimum regeneration solution. The percentage residual activity was calculated from the SPR angle shift before ($\Delta\theta_1$) and after ($\Delta\theta_2$) regeneration as follows (Fig. 3a):

$$\%\,\text{Residual activity} = \frac{\Delta\theta_2}{\Delta\theta_1} \times 100 \qquad (2)$$

The optimum buffer conditions, i.e., sample flow rate and sample volume, were obtained by balancing between the signal change and the analysis time. Under the optimum conditions, the linear range, limit of detection and reproducibility of SPR detection were evaluated. The linear range, limit of detection and reproducibility of the impedimetric system were then investigated based on the optimum conditions obtained from the SPR system.

Detection of total bacteria in real samples

Water sample from six sources were analyzed by the system using the better performing, flow injection impedimetric biosensor, under the determined optimum conditions. Samples included two of locally produced bottled drinking water, water from three waste water ponds and a sample of reservoir water from within the Prince of Songkla University grounds, Hat Yai, Thailand. Bottled drinking water samples were directly injected into the impedimetric system while waste water and reservoir water were simply diluted with carrier buffer to remove the matrix effect. A calibration curve of standard *E. coli* was constructed. The obtained impedimetric signal was calculated as the bacterial concentration using the linear equation of the calibration curve.

The same samples analyzed by the flow injection impedimetric system were tested for the number of viable cells by the PCA method. The sample was serially diluted in normal saline solution and 100 μL of each dilution was plated onto duplicate nutrient agar plates. The cultures were incubated at 37°C for 48 h before making a final count of CFU mL^{-1} and calculating the average CFU taking into account the dilutions used.[27]

Results and discussion

The label-free affinity biosensor response

SPR system. Figure 3a shows an example of the SPR sensorgram when different concentrations of heat-killed *E. coli* were injected. When the carrier buffer, 10 mM sodium acetate pH 4.00, was passed through the SPR flow cell, a stable baseline was obtained ($\theta_{\text{Con A}}$). *E. coli* was then injected

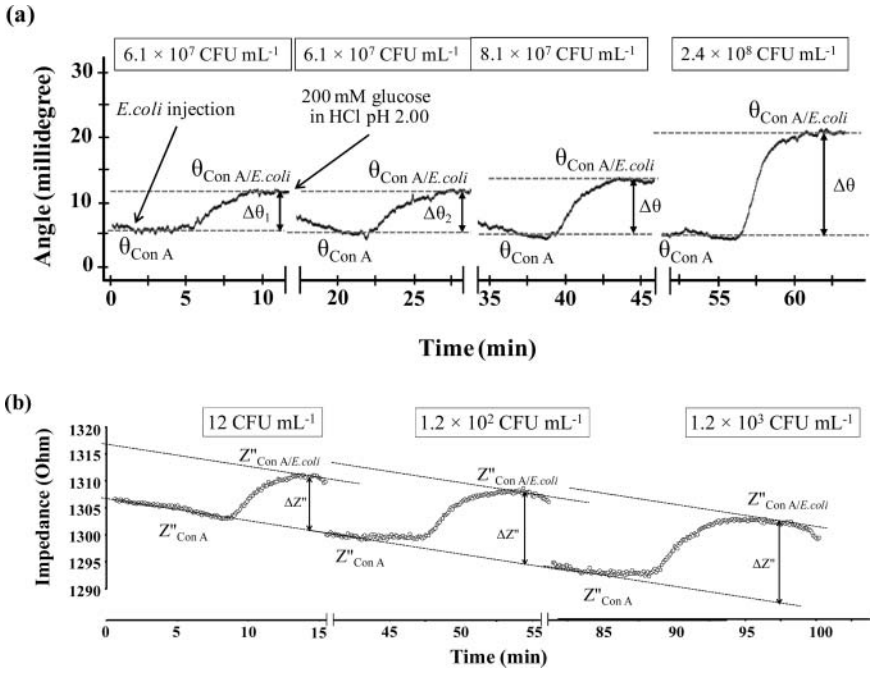

Fig. 3. (a) An example of an SPR sensorgram when heat-killed *E. coli* (6.1×10^7, 8.1×10^7 and 2.4×10^8 CFU mL^{-1}) was injected. The SPR angle shift ($\Delta\theta = \theta_{\text{Con A}/\textit{E. coli}} - \theta_{\text{Con A}}$) was obtained. The regeneration procedure was carried out using the regeneration solution (200 mM glucose in HCl pH 2.00). After the increase of the SPR angle ($\Delta\theta_1$), regeneration solution was injected to remove *E. coli* from the immobilized Con A. The same concentration of *E. coli* was then injected after the baseline was recovered and a new SPR angle ($\Delta\theta_2$) change was obtained. The regeneration step was carried out after each analysis before a new sample of 8.1×10^7 and 2.4×10^8 CFU mL^{-1} were analyzed. (b) An example of a sensorgram obtained from the impedimetric measurement when suspensions of *E. coli* (12, 1.2×10^2 and 1.2×10^3 CFU mL^{-1}) was injected. The binding between the immobilized Con A and the heat-killed *E. coli* caused an increase of impedance ($\Delta Z'' = Z''_{\text{Con A}/\textit{E. coli}} - Z''_{\text{Con A}}$).

into the flow injection SPR biosensor system, it bound to the immobilized Con A on the sensor surface. This caused the refractive index to increase and led to the increase of the SPR angle ($\theta_{\text{Con A}/\textit{E. coli}}$). The signal reached a steady state after 15–20 min and the change of SPR angle was detected as the angle shift ($\Delta\theta = \theta_{\text{Con A}/\textit{E. coli}} - \theta_{\text{Con A}}$). The binding between Con A and *E. coli* was then dissociated with 350 μL of the regeneration solution (see optimization and performance). The SPR angle then returned to the baseline and the system was ready for the next analysis.

Impedimetric system. The frequency of 236 Hz was selected from the Bode plot in the region where the system behaved as an ideal capacitor. This single frequency was used to monitor the imaginary part (Z'') of the impedance in real-time. During the flow of the carrier buffer, the recorded Z'' was the baseline ($Z''_{\text{Con A}}$) (Fig. 3b). When *E. coli* was injected, it bound to the immobilized Con A and Z'' increased ($Z''_{\text{Con A}/\textit{E. coli}}$). A plateau of the signal was obtained after 15 min. The impedance change ($\Delta Z'' = Z''_{\text{Con A}/\textit{E. coli}} - Z''_{\text{Con A}}$) was detected and this was proportional to the concentration of *E. coli*. The surface was regenerated with the regeneration solution.

Optimization

Regeneration solution. Affinity binding can generally be dissociated with acidic and alkaline solution or ionic strength shock.[28] In the case of the Con A-carbohydrate interaction, both low pH (<3) and high pH (>7) were not suitable for the binding. In addition, sugar can inhibit the binding of Con A and carbohydrate on the cell surface of *E. coli*[29] and urea can interfere with the stabilizing of the intramolecular interaction of the non-covalent bonds, hydrogen bonds and van der Waals forces.[30] Therefore, solutions with low or high pH, sugar and urea may be used as a regeneration solution to remove *E. coli* from Con A so the electrode surface could be reused.

The type of regeneration solution was first investigated, i.e., HCl pH 1.80 (low pH), 250 mM urea, 125 mM glucose (sugar) and 125 mM glucose in HCl pH 1.80. The efficiency of each solution type was determined by the residual activity of Con A to the *E. coli* using the SPR response (Equation 2). The low pH solution (HCl pH 1.80) provided a lower percentage of residual activity (88 ± 1%) than the 250 mM urea (93 ± 1%) and 125 mM glucose (94 ± 3%) solutions. The highest percentage residual activity (98 ± 3%) was obtained using 125 mM glucose in HCl with a

final pH of 1.80. Therefore, glucose in HCl was used as the regeneration solution.

The optimum pH of the HCl containing glucose was then studied between 1.80 and 2.80. The maximum percentage residual activity was obtained with a pH of 1.80 and 2.00, i.e., 104 ± 6% and 101 ± 4%, respectively. Any increase of pH did cause a decrease of the residual activity reaching 78 ± 4% at a pH of 2.80. Between pH 1.80 and 2.00, the pH of 2.00 was selected to help lessen the damage of SAM from the low pH.[31]

The concentration of added glucose was also evaluated from 100 mM to 300 mM. At 100 mM the percentage residual activity was 89 ± 5%. This increased to 100 ± 3% at 200 mM. Any higher concentration of glucose also caused complete regeneration. Therefore, 200 mM glucose was chosen as the optimum concentration.

Carrier buffer solution. The carrier buffer solution used initially was 10 mM Tris-HCl buffer pH 7.00 with 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$.[32] At this pH Con A exists in a tetrameric form that has a molecular weight of 110 kDa. Due to its large dimension, only a small amount of Con A could be immobilized on the transducer surface leading to a short operational stability (7 cycles of regeneration). To reduce the size of the Con A, the carrier buffer was changed to 10 mM sodium acetate buffer pH 5.30 with 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. At this pH Con A exists as a dimer (molecular weight of 55 kDa) which is smaller than the tetrameric Con A and results in the orientation of a dimeric Con A as a closed-packed monolayer that were mostly oriented in an upright position.[33] This would help the Con A to be more accessible to the analyte. Therefore, a much longer operational stability (29 cycles of regeneration) was obtained.

Because the interaction between Con A and bacteria is based on hydrogen bonding, this can be affected by a high ionic strength solution, and result in a low affinity for binding. Therefore, the concentration of carrier buffer was optimized by varying the concentration of sodium acetate buffer between 10 and 40 mM. The SPR angle shift decreased from 8.8 ± 0.5 millidegree at 10 mM to 6.1 ± 0.2 millidegree at 40 mM. Therefore, 10 mM of sodium acetate buffer was used for further studies. A concentration lower than 10 mM was not studied because of its low buffering capacity.

The effect of pH on the Con A-bacteria interaction was studied from pH 3.60 to 5.60. The SPR angle shift ($\Delta\theta$) increased from 15.3 ± 1.2 millidegree at pH 3.60 to 20.8 ± 0.9 millidegree at pH 4.00 and then decreased to 10.8 ± 0.7 millidegree at pH 5.60. Therefore, pH 4.00 was chosen as the optimum carrier buffer pH.

Flow rate

The interaction between the immobilized Con A and *E. coli* could be affected by the flow rate. The increase of flow rate resulting in a shorter interaction time between *E. coli* in the sample and the immobilized Con A leading to a decrease of the SPR angle shift. The response of the SPR biosensor was evaluated between 10 and 30 μL min^{-1}. A flow rate of 10 μL min^{-1} (18.5 ± 1.1 millidegree) and 15 μL min^{-1} (18.9 ± 0.6 millidegree) produced the maximum SPR angle shift while a higher flow rate caused a reduction of the SPR angle shift, down to 7.9 ± 0.2 millidegree at 30 μL min^{-1}. Therefore, 15 μL min^{-1} was chosen as the optimum flow rate to shorten the analysis time.

Sample volume

The effect of sample volume in the SPR system was studied between 100 μL and 350 μL. A small sample volume (100 μL) caused a small SPR angle shift (10.68 ± 0.08 millidegree). The biosensor response ($\Delta\theta$) increased with the sample volume and reached a plateau at 300 μL (15.2 ± 0.3 millidegree). Therefore, 300 μL was the optimum sample volume for detecting *E. coli*.

In summary the optimum conditions were 10 mM sodium acetate buffer pH 4.00 as a carrier buffer, 200 mM glucose in HCl pH 2.00 of regeneration solution, 15 μL min^{-1} of sample flow rate, 300 μL of sample volume for SPR system and 450 μL of sample volume of impedimetric system.

Performance

Linearity and limit of detection

Under the optimum conditions, the linear range of the SPR affinity biosensor for detection of heat-killed *E. coli* was tested at 6.1×10^7, 6.9×10^7, 8.1×10^7, 1.2×10^8, 2.4×10^8 and 4.9×10^8 CFU mL^{-1}. Each concentration was analyzed 3 times. The calibration plot between the *E. coli* concentration (CFU mL^{-1}) vs. SPR angle (millidegree) produced linearity between 6.1×10^7 CFU mL^{-1} and 2.4×10^8 CFU mL^{-1} with a linear equation of $\Delta\theta$ (millidegree) = $((4.7 \pm 0.2) \times 10^{-8})$x (CFU mL^{-1}) + (2.2 ± 0.2), r = 0.997. No SPR angle shift signal was observed when the concentration of *E. coli* was lower than 6.1×10^7 CFU mL^{-1}.

This limit of detection was comparable to the one obtained by Fratamico et al.[22] 5–7 $\times 10^7$ CFU mL^{-1}. However, in their work this limit of detection was obtained by a sandwich like assay, i.e., after the injection of bacteria, an injection of polyclonal antibodies followed. This indicated that the system employed in this work could perform better since the limit of detection was obtained directly from the binding of bacteria to the detection surface. The limitation of the SPR method is due to the penetration depth of the evanescent field being only 300–400 nm.[34]

This means that only the changes within the 400 nm distance from the surface will cause a change in the generated

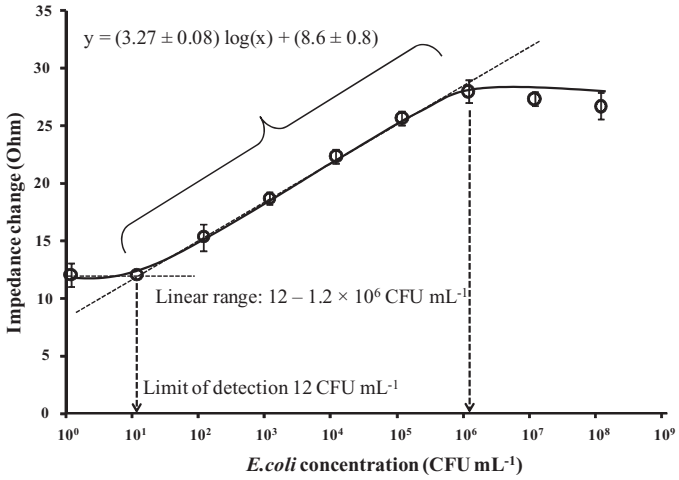

Fig. 4. The calibration curve of *E. coli* detection obtained from the impedimetric system under optimum conditions.

SPR signal. Therefore, bacteria that are typically larger than 400 nm cannot be measured totally. Only a small part of the bacterial cell that is close to the surface contributes to the response.[22] This factor can limit the sensitivity of the direct cell capture assay by the SPR. When interferences in the real sample need to be removed by dilution, the system that provides a low limit of detection is needed. Since the limit of detection was high, at 6.1×10^7 CFU mL^{-1}, the proposed SPR system would not be suitable for the analysis of *E. coli* in the real samples.

The impedimetric detection study was carried out under the optimum conditions obtained for the SPR system. The performances of the system were tested between 1.2 CFU mL^{-1} and 1.2×10^8 CFU mL^{-1} of *E. coli*. The limit of detection was determined following IUPAC recommendation 1994[34] and was found to be 55 CFU mL^{-1}. That is, for a large analyte like a whole cell of *E. coli*, the real-time impedimetric detection provided a much lower limit of detection than the SPR.

To obtain a lower limit of detection for the impedimetric system, a larger sample volume could be employed and this was tested between 300 μL and 500 μL. The sensor response increased with the increase of sample volume between 9.0 ± 1 Ω (300 μL) and 15.3 ± 0.6 Ω (500 μL) (concentration of *E. coli* = 1.2×10^3 CFU mL^{-1}). However, a sample volume of 450 μL (15.0 ± 0.6 Ω) and 500 μL gave nearly the same sensor response. Therefore, 450 μL was chosen as the new sample volume for impedimetric detection. The calibration curve obtained with this sample volume is shown in Figure 4. The limit of detection could be improved to 12 CFU mL^{-1} with linearity of the calibration curve between 12 CFU mL^{-1} and 1.2×10^6 CFU mL^{-1} (Fig. 4).

The obtained limit of detection by this impedimetric system was extremely low. This is partly due to the optimization of the experimental set up to obtain the most effective conditions, i.e., buffer and regeneration conditions, flow rate and sample volume. The operation in a flow system with a low flow rate also ensured that the bacteria were slowly transferred through the small electrochemical confined flow cell (10 μL dead volume) and this helped to increase the binding prospect between the bacteria and the Con A on the electrode surface. The obtained good limit of detection was also the result of the measurement of the imaginary part of the impedance, Z″ that can provide higher sensitivity than the real part of impedance, Z′.[21,36] This system was then investigated further for real sample analysis.

Reproducibility

Reproducibility was tested for the impedimetric biosensor by analyzing the same concentration of heat-killed *E. coli* (1.2×10^4 CFU mL^{-1}) 9–10 times per day for 4 days. The regeneration step was included after each injection of *E. coli*. For the first 35 regeneration cycles, the average percentage of residual activity was 95 ± 3% (RSD = 3.2%). After 35 regeneration cycles, the residual activity of immobilized Con A rapidly reduced (85%). The electrode surface was then tested by cyclic voltammetry. A flat voltammogram similar to the one obtained after electrode preparation was observed. This confirmed that the SAM on the electrode surface was not destroyed by the frequent regeneration. The results indicated that the decrease of residual activity was more likely due to the loss of Con A and/or its activity after being used several times.

Detection of total bacteria in real sample

Because Con A can bind to several type of bacteria, it is suitable for detecting and enumerating total bacteria. Six water samples were tested by the flow injection impedimetric label-free affinity biosensor. The matrix effect was studied by spiking standard heat-killed *E. coli* into each sample between 50 and 2,000 CFU mL^{-1}. The calibration curve of the spiked sample (spiked curve) and the standard in carrier buffer (standard curve) were compared using two way ANOVA (analysis of variance), calculated by R software.[37] For bottled drinking water (2 samples), there was no difference between the slope of the two curves ($P > 0.05$) indicating that there is no matrix effect.[38]

Therefore, the standard curve could be used to calculate total bacteria in the bottled water. The bottled drinking water was analyzed without any dilution and total bacteria were detected in the bottled drinking water brand I (98 ± 11 CFU mL^{-1}) and the bottle drinking water brand II (67 ± 16 CFU mL^{-1}). However, the detected concentration of the total bacteria in the drinking water was lower than the guideline of 500 CFU mL^{-1}.[39]

When the reservoir and waste water I were tested after dilution 100 times there was no matrix effect or when waste water II and III were diluted 1,000 times. Therefore, these

Table 1. Comparison of the total concentration of bacteria in water samples obtained from the flow injection impedimetric biosensor (mean ± SD, n = 3) with plate count agar.

	Concentration of total bacteria (CFU mL^{-1})	
Samples	*Impedimetric*	*Plate count agar*
Drinking water		
Brand I	98 ± 11	100 ± 28
Brand II	67 ± 16	60 ± 28
Reservoir water	$(63.7 \pm 1.7) \times 10^3$	$(71.0 \pm 1.4) \times 10^3$
Waste water I	$(42.9 \pm 1.9) \times 10^3$	$(45.3 \pm 2.5) \times 10^3$
Water water II	$(26.1 \pm 0.3) \times 10^4$	$(29.2 \pm 0.8) \times 10^4$
Waste water III	$(93.2 \pm 5.1) \times 10^3$	$(100.5 \pm 0.7) \times 10^3$

Table 2. Recoveries of bacteria from spiked water samples obtained from the flow injection impedimetric label-free affinity biosensor (mean ± SD, n = 3).

	Recovery (%)			
	Spiked concentration (CFU mL^{-1})			
Samples	50	500	1,000	2,000
Drinking water				
Brand I	92 ± 22	98 ± 5	102 ± 9	103 ± 2
Brand II	102 ± 19	94 ± 5	103 ± 12	101 ± 3
	Spiked concentration (CFU mL^{-1})			
	5,000	*50,000*	*100,000*	*200,000*
Waste water I	98 ± 5	99 ± 5	97 ± 3	95 ± 1
Reservoir water	99 ± 10	117 ± 7	106 ± 6	101 ± 1
	Spiked concentration (CFU mL^{-1})			
	50,000	*500,000*	*1,000,000*	*2,000,000*
Waste water II	102 ± 9	102 ± 4	98 ± 4	101 ± 3
Waste water III	102 ± 1	109 ± 5	107 ± 7	98 ± 2

samples were diluted as indicated before injecting them directly into the impedimetric system. The obtained signals were used to calculate the number of bacteria from the standard calibration curve and multiplied by the dilution factor of each sample. Total bacteria were detectable for all four samples, i.e., reservoir water $(63.7 \pm 1.7) \times 10^3$ CFU mL^{-1}, waste water I $(42.9 \pm 1.9) \times 10^3$ CFU mL^{-1}, waste water II $(26.1 \pm 0.3) \times 10^4$ CFU mL^{-1} and waste water III $(93.2 \pm 5.1) \times 10^3$ CFU mL^{-1}.

The same samples were tested by the PCA method. The results are shown in Table 1. The cell concentrations obtained from the impedimetric analysis were generally lower than that from the PCA method by about 2–10%. This may be because the calibration curve was constructed using *E. coli*. However, in real water samples mixtures of microorganisms exist and these can bind to Con A with different selectivities.[10,14] Furthermore, microbial cells differ in size and may therefore give different responses in the sensor analyses, even if the PCA does not react to this fact. Because the cell concentrations obtained from the impedimetric system were only slightly different from the results obtained by the PCA method, the method would be a useful tool for a rapid screening detection and enumeration of total bacteria.

One point that needs to be considered before applying this system for the analysis is the presence of sugar and starch in the sample. Since Con A binds to carbohydrate, the existence of these compounds may interfere with the analysis. For drinking water, or any sample without carbohydrate, this should not be a problem. Other types of real sample, for example waste water, may contain these interferences. If the number of bacteria in the sample is large, as shown in the case of the waste water, dilution of the sample before analysis helped to reduce the interference effect. On the other hand, this system would also be useful for the screening detection of sugar and/or starch in a sample that contains no bacteria.

Recovery

To further validate the system, a recovery test was carried out by spiking different concentration of heat-killed *E. coli* into the water samples. Bottled drinking water was spiked between 50 and 2,000 CFU mL^{-1} and directly injected into the impedimetric system. For reservoir and waste water, the spiking was carried out to obtain a final concentration after dilution of between 50 and 2,000 CFU mL^{-1}. Waste water II and waste water III were spiked with heat-killed *E. coli* from 5×10^4 to 2×10^6 CFU mL^{-1} while waste water I and the reservoir water were spiked from 5×10^3 to 2×10^5 CFU mL^{-1}. The dilutions were carried out as described in the real sample detection section. The spiked samples were analyzed by the impedimetric biosensor. The percentage of recovery was calculated using Equation 3.

$$\%\text{Recovery} = \frac{C_1 - C_2}{C_3} \times 100 \qquad (3)$$

Where C_1 = the concentration determined in the fortified sample, C_2 = the concentration determined in the unfortified sample and C_3 = the concentration of fortification.[38] The results are shown in Table 2. The average of the percentage recovery was between 92% and 117% with an RSD of between 1.1% and 24%.

Conclusions

In this work we have presented alternative methods by: monitoring the SPR angle shift and by impedance measurement for enumerating total bacteria based on detection of the binding event between immobilized Con A on a

sensor surface and suspensions of *E. coli*. The sensors can be reused by breaking the affinity binding using a regeneration solution (200 mM glucose in HCl pH 2.00). Both the SPR and impedimetric detections took less than 20 min to complete one assay.

Under optimum conditions, the SPR system had a high limit of detection (6.1×10^7 CFU mL^{-1}), while the impedimetric system showed a much lower limit of detection of 12 CFU mL^{-1}. The proposed impedimetric detection system also provided a much lower limit of detection than the direct mass detection (LOD = 10^3 CFU mL^{-1}) of Safina et al.[12] Although the impedimetric detection system reported by Wan et al.[13] could provide a lower limit of detection (2 CFU mL^{-1}) than this work, their detection required 2 h incubation time and was based on a Nyquist plot that cannot be performed in real-time. In contrast, the proposed impedimetric detection is a real-time measurement that is simple and rapid.

Real samples of bottled drinking water were directly analyzed by the impedimetric system while reservoir water and waste water only required a simple dilution with the carrier buffer before the analysis and this also abolished the matrix effect. The comparison between the impedimetric detection and the PCA method found that the cell concentration obtained from the impedance measurement was slightly lower than the PCA method. This may be caused by the different selectivity and size of microorganisms in real samples to bind to Con A. However, the proposed technique could be a useful tool for the screening and enumeration of total bacteria in water sources.

Acknowledgments

The authors are grateful for the grant for Jongjit Jantra under The Royal Golden Jubilee Ph.D. Program (RGJ:PHD/0191/2548) supported by The Thailand Research Fund. Financial support from the National Research University Project of Thailand, Office of the Higher Education Commission; the Center for Innovation in Chemistry (PERCH-CIC); Trace Analysis and Biosensor Research Center (TAB-RC); Graduate School and Faculty of Science, Prince of Songkla University, Hat Yai, Thailand were gratefully acknowledged. The authors also thank Dr. Brian Hodgson, Prince of Songkla University, Hat Yai, Songkhla, Thailand for assistance with the manuscript.

References

[1] Mansilha, C.R.; Coelho, C.A.; Reinas, A.; Moutinho, A.; Ferreira, S.; Pizarro, C.; Tavares, A. Salmonella: The forgotten pathogen: health hazards of compliance with European Bathing Water Legislation. Mar. Pollut. Bull. **2010**, *60*, 819–826.

[2] Lazcka, O.; Campo, F.J.D.; Muñoz, F.X. Pathogen detection: A perspective of traditional methods and biosensors. Biosens. Bioelectrons. **2007**, *22*, 1205–1217.

[3] Ivnitski, D.; Abdel-Hamid, I.; Atanasov, P.; Wilkins, E. Biosensors for detection of pathogenic bacteria. Biosens. Bioelectrons. **1999**, *14*, 599–624.

[4] Bittiger, H.; Schnebli, H.P. Concanavalin A: The tool, the techniques and the problems. In *Concanavalin A as a tool*; John Wiley & Sons: London & New York, **1976**; 3–7.

[5] Chambers, J.P.; Arulanandam, B.P.; Matta, L.L.; Weis, A.; Valdes, J.J. Biosensor recognition elements. Curr. Issues. Mol. Biol. **2008**, *10*, 1–12.

[6] Ni, Y.; Tizard, I. Lectin-carbohydrate interaction in the immune system. Vet. Immunol. Immunop. **1996**, *55*, 205–223.

[7] Ballerstadt, R.; Evans, C.; McNichols, R.; Gowda, A. Concanavalin A for in vivo glucose sensing: a biotoxicity review. Biosens. Bioelectrons. **2006**, *22*, 275–284.

[8] Shen, Z.; Huang, M.; Xiao, C.; Zhang, Y.; Zen, X.; Wang, P.G. Nonlabeled quartz crystal microbalance biosensor for bacterial detection using carbohydrate and lectin recognitions. Anal. Chem. **2007**, *79*, 2312–2319.

[9] Gour, N.; Verma, S. Synthesis and AFM studies of lectin-carbohydrate self-assemblies. Tetrahedron **2008**, *64*, 7331–7337.

[10] Oda, Y.; Kinoshita, M.; Nakayama, K.; Ikeda, S.; Kakehi, K. Flow injection analysis of binding reaction between fluorescent lectin and cells. Anal. Biochem. **1999**, *269*, 230–235.

[11] Lu, Q.; Lin, H.; Ge, S.; Luo, S.; Cai, Q.; Grimes, C. Wireless, remote-query, and high sensitivity *Escherichia coli* O157: H7 biosensor based on the recognition action of concanavalin A. Anal. Chem. **2009**, *81*, 5846–5850.

[12] Safina, G.; van Lier, M.; Danielsson, B. Flow-injection assay of the pathogenic bacteria using lectin-based quartz crystal microbalance biosensor. Talanta **2008**, *77*, 468–472.

[13] Wan, Y.; Zhang, D.; Hou, B. Monitoring microbial populations of sulfate-reducing bacteria using an impedimetric immunosensor based on agglutination assay. Talanta **2009**, *80*, 218–223.

[14] Ertl, P.; Mikkelsen, S. Electrochemical biosensor array for the identification of microorganisms based on lectin-lipopolysaccharide recognition. Anal. Chem. **2001**, *73*, 4241–4248.

[15] Robertson, W.; Brooks, T. Heterotrophic plate count and drinking-water safety. In *The significance of HPCs for water quality and human health*; IWA Publishing; London, **2003**; 233–244.

[16] Subramanian, A.; Irudayaraj, J.; Ryan, T. Mono and dithiol surfaces on surface plasmon resonance biosensors for detection of *Staphylococcus aureus*. Sens. Actuators. B **2006**, *114*, 192–198.

[17] Wei, D.; Oyarzabal, O.A.; Huang, T.S.; Balasubramanian, S.; Sista, S.; Simonian, A.L. Development of surface plasmon biosensor for the identification of *Campylobacter jejuni*. J. Microbiol. Meth. **2007**, *69*, 78–85.

[18] Taylor, A.D.; Ladd, J.; Yu, Q.; Chen, S.; Homola, J.; Jiang, S. Quantitative and simultaneous detection of four foodborne bacterial pathogens with a multi-channel SPR sensor. Biosens. Bioelectrons. **2006**, *22*, 752–758.

[19] Thavarungkul, P.; Dawan, S.; Kanatharana, P.; Asawatreratanakul, P. Detecting penicillin G in milk with impedimetric label-free immunosensor. Biosens. Bioelectrons. **2007**, *23*, 688–694.

[20] Bart, M.; Stigter, E.C.A.; Stapert, H.R.; Jong, G.J.; Bennekom, W.P. On the response of a label-free interferon-γ immunosensor utilizing electrochemical impedance spectroscopy. Biosens. Bioelectrons. **2005**, *21*, 49–59.

[21] Dijksma, M.; Kamp, B.; Hoogvliet, J.C.; Bennekom, W.P. Development of an electrochemical immunosensor for direct detection of interferon-γ at the attomolar level. Anal. Chem. **2001**, *73*, 901–907.

[22] Fratamico, P.M.; Strobaugh, T.P.; Medina, M.B.; Gehring, A.G. Detection of Escherichia coli O157:H7 using a surface plasmon resonance biosensor. Biotechnol. Tech. **1998**, *12*, 571–576.

[23] Wong, K.H.; Skelton, S.K.; Feeley, J.C. Strain characterization and grouping of *Campylobacter jejuni* and *Campylobacter coli* by interaction with lectins. J. Clin. Microbiol. **1986**, *23*, 407–410.

[24] Taylor, A.D.; Yu, Q.; Chen, S.; Homola, J.; Jiang, S. Comparison of E. coli O157:H7 preparation methods used for detection with surface plasmon resonance sensor. Sens. Actuators. B **2005**, *107*, 202–208.
[25] Suwansa-ard, S.; Kanatharana, P.; Asawatreratanakul, P.; Wongkittisuksa, B.; Limsakul, C.; Thavarungkul, P. Comparison of surface plasmon resonance and capacitive immunosensors for cancer antigen 125 detection in human serum samples. Biosens. Bioelectrons. **2009**, *24*, 3436–3441.
[26] Berggren, C.; Johansson, G. Capacitance measurements of antibody-antigen interactions in a flow system. Anal. Chem. **1997**, *69*, 3651–3657.
[27] Olsen, E.V.; Pathirana, S.T.; Samoylov, A.M.; Barbaree, J.M.; Chin, B.A.; Neely, W.C.; Vodyanoy, V. Specific and selective biosensor for Salmonella and its detection in the environment. J. Microbiol. Meth. **2003**, *53*, 273–285.
[28] Luppa, P.B.; Sokoll, L.J.; Chan, D.W. Immunosensors: principles and applications to clinical chemistry. Clin. Chim. Acta. **2001**, *314*, 1–26.
[29] So, L.L.; Goldstein, I.J. Protein-carbohydrate interaction XIII: The interaction of concanavalin A with alfa-mannans from a variety of microorganisms. J. Biol. Chem. **1968**, *243*, 2003–2007.
[30] Andersson, K.; Hämäläinen, M.; Malmqvist, M. Identification and optimization of regeneration conditions for affinity-based biosensor assays. A multivariate cocktail approach. Anal. Biochem. **1999**, *71*, 2475–2481.
[31] Jiang, D.; Tang, J.; Liu, B.; Yang, P.; Shen, X.; Kong, J. Covalently coupling the antibody on an amine-self-assembled gold surface to probe hyaluronan-binding protein with capacitance measurement. Biosens. Bioelectrons. **2003**, *18*, 1183–1191.
[32] Sharon, N.; Lis, H. How proteins bind carbohydrates: lessons from legume lectins. J. Agric. Food Chem. **2002**, *50*, 6586–6591.
[33] Serra, B.; Gamella, M.; Reviejo, A.J.; Pingarron, J.M. Lectin-modified piezoelectric biosensors for bacteria recognition and quantification. Anal. Bioanal. Chem. **2008**, *391*, 1853–1860.
[34] Autolab application note. Surface plasmon resonance with the autolab SPR instruments. Metrohm Autolab B.V. in the Netherlands, **2010**, http://www.metrohm-autolab.com/Applications/
[35] Buck, R.P.; Lindneri, E. Recommendations for nomenclature of ion-selective electrodes. Pure Appl. Chem. **1994**, *66*, 2527–2536.
[36] Daniels, J.S.; Pourmand, N. Label-free impedance biosensors: opportunities and challenges. Electroanalysis **2007**, *12*, 1239–1257.
[37] R Development Core Team. R: A language and environment for statistical computing. R foundation for statistical computing. R development core team, Vienna, Austria, ISBN 3-900051-07-0, **2006**.
[38] Eurachem Working Group, Fitness for purpose of analytical methods: A laboratory guide to method validation and related topics Eurachem Guide, 1st English ed. 1.0. LGC (Teddington) Ltd., **1998**.
[39] Pollution Control Department. Water quality standards. Ministry of Natural Resources and Environmental, Thailand, **2010**, http://www.pcd.go.th/info_serv/reg_std_water01.html#s2