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ARTICLE *in* ANALYTICAL CHEMISTRY · MARCH 2013

Impact Factor: 5.64

analytical chemistry

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Bacteria Screening, Viability, And Confirmation Assays Using Bacteriophage-Impedimetric/Loop-Mediated Isothermal Amplification Dual-Response Biosensors

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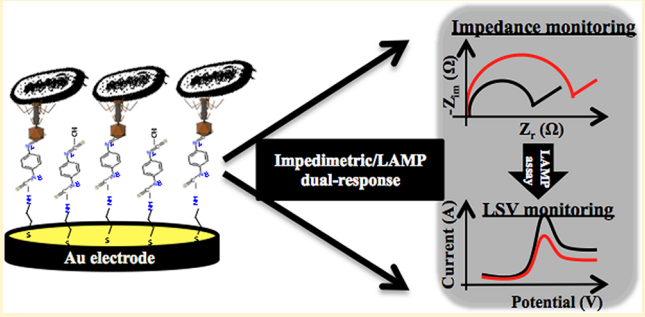
Supporting Information

ABSTRACT: Here, we integrate two complementary detection strategies for the identification and quantification of *Escherichia coli* based on bacteriophage T4 as a natural bioreceptor for living bacteria cells. The first approach involves screening and viability assays, employing bacteriophage as the recognition element in label-free electrochemical impedance spectroscopy. The complementary approach is a confirmation by loop-mediated isothermal amplification (LAMP) to amplify specifically the *E. coli Tuf* gene after lysis of the bound *E. coli* cells, followed by detection using linear sweep voltammetry. Bacteriophage T4 was cross-linked, in the presence of 1,4-phenylene diisothiocyanate, on a cysteamine-modified gold electrode. The impedimetric biosensor exhibits specific and reproducible detection with sensitivity over the concentration range of 10^3–10^9 cfu/mL, while the linear response of the LAMP approach was determined to be 10^2–10^7 cfu/mL. The limit of detection (LOD) of 8×10^2 cfu/mL in less than 15 min and 10^2 cfu/mL within a response time of 40 min were achieved for the impedimetric and LAMP method, respectively. This work provides evidence that integration of the T4-bacteriophage-modified biosensor and LAMP can achieve screening, viability, and confirmation in less than 1 h.



Bacterial infection has been one of the major threats for public health and food safety for decades and remains the leading causes of outbreaks of diseases in the developing and underdeveloped nations. In the United States, the Centers for Disease Control and Prevention (CDC) estimates that approximately more than 48 million illnesses, 128000 hospitalizations, and 3000 deaths occur each year because of food-borne and waterborne pathogens.[1] Besides, treatment for food-borne and waterborne diseases are extremely costly. The Economic Research Service (ERS) of the United States Department of Agriculture (USDA) indicated that the annual medical cost, productivity loss, and human disease caused by the more common food-borne pathogens ranged from 5.6 to 9.4 billion dollars.[2] Among all pathogens, *E. coli* is one of the highly relevant and commonly targeted indicator for routine analysis of contaminated water and food sources. It is a natural inhabitant in the intestinal tracts of humans and warm-blooded animals, which is characterized by diarrhea, urinary tract infections, inflammations, and peritonitis in immune-suppressed patients.[3]

Over the past decade, the detection and identification of bacteria mainly relied on specific microbiological and biochemical methods, including culture and colony counting of bacteria,[4] immunology-based methods (e.g., ELISA) based on antibody–antigen interactions,[5] and polymerase chain reaction (PCR) which involves DNA amplification.[6] These conventional methods are highly selective and sensitive but the main challenges facing these techniques require not only long incubation times to obtain results but also highly trained personnel or specialized equipment that make them impractical for field conditions, plus they are expensive. Moreover, more modern tests such as immunoassays and PCR do not distinguish between viable and nonviable bacteria cells, which can potentially lead to false-positive results and consequently inaccurate estimation of bacteria concentration in samples.[7] In order to overcome the issues associated with the current diagnostic techniques, there is an increasing demand toward diagnostic technologies that are rapid, reliable, low-cost, and user-friendly. These new technologies need to offer discrimination between closely related pathogenic and nonpathogenic microorganisms, detection of small quantities of a target within a complicated background matrix, a higher degree of stability over time, and the capacity of incorporation into a real-time monitoring system.[8,9]

Within the past few years, various biosensors have been developed as an alternative to the conventional methods for the detection of *E. coli* in food and water. These biosensors include

Received: September 20, 2012
Accepted: March 20, 2013
Published: March 20, 2013

   dx.doi.org/10.1021/ac302699x | *Anal. Chem.* 2013, 85, 4893–4901

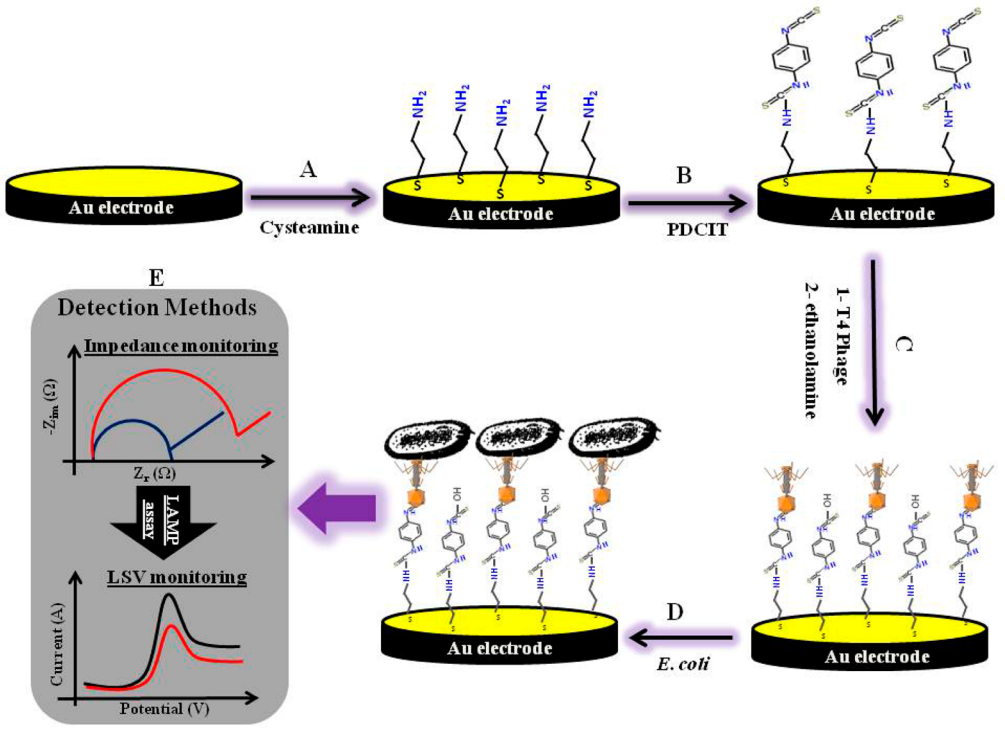


Figure 1. Schematic representation of the T4-bacteriophage biosensors. (A) Cysteamine-assembly on gold electrode. (B) Activation with 1,4-dithiocyanate (PDICT) cross-linker. (C) Immobilization of the T4 phage and blocking with ethanolamine. (D) Capturing of the *E. coli* cells. (E) Detection method for *E. coli* based on the impedimetric/LAMP dual-response.

surface plasmon resonanance (SPR),[10,11] optical fiber, surface enhanced Raman spectroscopy (SERS),[12] cantilever sensors,[13] fluorescent-based methods,[14] and quartz crystal microbalance (QCM).[15] Each of these approaches features some combination of excellent sensitivity, good selectivity, fast time-to-result, and demonstrated clinical or biological applicability. However, they are time and labor intensive and require expensive instrumentation, which limits their use in laboratory and point-of-care applications.[16]

In this regard, electrochemical biosensors hold great potential as the next-generation detection strategy due to their high sensitivity, low cost, and ease of integration into miniaturized devices. Among them, electrochemical impedance spectroscopy (EIS) is a very powerful tool for the analysis of interfacial properties related to biorecognition events occurring at the modified surfaces. One of the advantages of EIS is the small amplitude perturbation from steady state, which makes it a nondestructive technique.[17] In this method, the immobilization of the bioreceptor is the key process in the construction of impedimetric biosensors, since the efficiency of bioreceptor immobilization on the electrode surface can profoundly affect the analytical performance of impedance biosensors. For this, antibody-based biosensing systems were widely used along with immobilization methods such as physical adsorption,[18] the biotin−avidin system,[19] covalent attachment through self-assembled monolayers (SAMs),[20] and a covalently linked antibody on a conducting polyaniline (PANI) film surface.[21] One of the major limitations of antibody-based immunosensors is the lack of discrimination between viable and nonviable bacteria cells. It is because antibodies can bind to the antigen regardless of whether the bacteria was dead or alive.[22,23] Moreover, environmental factors or stress may affect antibody-specific antigen expression of bacterial cells, perturbing the capturing efficiency of the biosensors.[24] Yet, the development of impedimetric biosensors with an excellent selectivity against bacteria strains and dead bacteria still remains a challenge. Among the various bioreceptors that have been investigated, bacteriophage is emerging as a promising alternative due to its sensitivity, selectivity, discrimination between dead and alive cells, low-cost production, and higher thermostability than antibodies.[25−27] More recently, Mejri et al.[28] developed a bacteriophage-based impedimetric biosensor for the detection of *E. coli*; their results show that 10^4 cfu/mL can be detected, and minimal interference was observed upon incubation with nontarget *Lactobacillus*.

To address this challenge, in this work we demonstrate for the first time a novel label-free impedimetric biosensor, integrating screening assay, viability testing, and confirmation assay using loop-mediated isothermal amplification (LAMP). This biosensor can be packaged within a portable micro-bioanalytical system for on-the-spot bacterial monitoring. The general approach (Figure 1) can be extended to develop biosensors for a wide variety of bacteria, and the methodology can easily be applied in a relatively short period of time at low cost, with higher sensitivity and specificity.

EXPERIMENTAL SECTION

Chemical and Materials. Cysteamine hydrochloride (Cyst), 1,4-phenylene diisothiocyanate (PDITC), *N*,*N*-dimethyl formamide (DMF), sulphuric acid, ethanolamine (EA), pyridine, potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), disodium hydrogen orthophosphate, and potassium dihydrogen orthophosphate were obtained from Sigma-Aldrich (Ontario, Canada). Hoechst 33258 {[20(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,50-bi (1*H*-benzimidazole)]} was purchased from Sigma-

dx.doi.org/10.1021/ac302699x | *Anal. Chem.* 2013, 85, 4893−4901

Aldrich (St. Louis, MO). Potassium chloride, sodium hydroxide, sodium chloride, hydrochloric acid, and Luria–Bertani (LB) Broth (Miller) and LB Agar (Miller) were purchased from Bioshop (Ontario, Canada). Bacteriophage T4 (HER27) and its host *E. coli* B were obtained from The Felix d'Herelle Reference Center for Bacterial Viruses (Laval University, Quebec, Canada) and molecular granulated agar was purchased from Fisher Scientific (Ontario, Canada). All chemical were of extra pure analytical grade and used as received without further purification.

Gold disk electrodes (2 mm diameter Au disk), Ag/AgCl reference electrodes, and a platinum wire counter electrode were purchased from Bioanalytical Systems (BAS, West Lafayette, IN, USA). Alumina pounder (1.0, 0.3, and 0.05 μm) and polishing cloth were purchased from Buehler (Ontario, Canada).

A 10 mM phosphate buffer saline (PBS) stock solution (pHs 7.4 and 8.5) was prepared using 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 137 mM NaCl, and 2.7 mM KCl. All aqueous solutions were prepared with deionized distilled water obtained from a Milli-Q water purifying system (18 MΩcm).

Pretreatment of the Gold Electrode and Surface Modification. Prior to cysteamine modification, the gold disc electrodes were mechanically polished with 1.0, 0.3, and 0.05 μm alumina/water slurry separately on a polishing cloth to a mirror finish, followed by washing thoroughly with deionized distilled water and acetone, respectively. Then, the polished electrodes were cleaned for 5 min with fresh piranha solution (7:3 v/v concentrated H_2SO_4 and 30% H_2O_2; CAUTION: piranha solution reacts violently with most organic materials and must be handled with extreme care) to remove hydrocarbon contamination and were rinsed intensively with distilled water. Subsequently, the electrodes were electrochemically cleaned by cycling the potential between −0.2 and 1.5 V (vs Ag/AgCl) at 100 mV/s for 25 cycles in a 100 mM H_2SO_4 solution and extensively rinsed with distilled water and dried with N_2 stream. The gold electrodes were then immersed in a 10 mM cysteamine solution overnight at room temperature to form a self-assembled monolayer (SAM), followed by rinsing with distilled water and ethanol to remove unbounded cysteamine molecules. The activation was done by an incubation of the cysteamine-modified gold electrode in a freshly prepared solution of PDICT in DMF/pyridine (9:1 v/v) at room temperature for 2 h. The activated surfaces were washed successively with DMF and distilled water to remove unbound PDICT molecules and then dried under a stream of nitrogen. Next, the covalent immobilization of the T4 bacteriophage was accomplished via coupling their primary amines to the thiocyanate group in the surface of the modified gold electrode. The electrode was immersed in a 10 mM PBS solution (pH 8.5) containing 10^{10} pfu/mL T4 phage overnight at ambient temperature. Finally, the remaining thiocyanate groups were blocked with 0.1 M ethanolamine (pH 8.5) for 30 min. After the rinsing step with distilled water and PBS, the biosensor was installed in the electrochemical cell for the detection experiments.

Culturing of *E. coli* Cells. An inoculum from frozen glycerol stock (kept at −80 °C) was streaked onto a LB agar plate and incubated at 37 °C overnight. A single colony from the plate was inoculated into 5 mL of LB broth and incubated overnight at 37 °C with shaking at 200 rpm. The viable bacterial number was calculated by preparing serial dilutions from the overnight culture and plating on LB agar plates. In order to prepare bacterial suspensions with different concentrations, the overnight culture was centrifuged at 10000 rpm for 5 min, and the pellet was resuspended and diluted in sterile PBS (pH 7.4). The biosensor was tested with skim milk, which was bought from a local store, and it was diluted 1:10 in PBS. The biosensor was also tested with a filtered and sterilized water sample from a local lake and spiked with various concentrations of bacteria. Further information about experimental protocols is available in the Supporting Information.

Apparatus. Cyclic voltammetry (CV), linear sweep voltammetry (LSV), and electrochemical impedance spectroscopy (EIS) were performed using a model 660D potentiostat/galvonastat (CH Instrument Inc.), controlled by a personal computer via CH Instruments. The electrochemical measurements were measured in a three-electrodes electrochemical cell configuration with a gold disc (2 mm) working electrode, a platinum wire as the counter electrode, and an Ag/AgCl (filled with 3 M KCl) reference electrode. Cyclic voltammetry was recorded between −0.2 and 0.5 V with a scan rate of 100 mV/s, in order to determine the midpoint between the oxidation and the reduction of the redox couple, which can be used as an applied DC potential for further EIS measurements. The impedance spectra were carried out in the frequency range of 1 to 10^5 Hz, using an alternative voltage with amplitude of 10 mV, superimposed on the DC formal potential of the redox couple. The impedance data were plotted in the form of a complex plane diagram (Nyquist plot) with a sampling rate of 5 points per decade. The obtained spectra were fitted using the CH instrument fitting program. The CV and EIS measurements were performed in 10 mM of PBS buffer (pH 7.4) in the presence of 10 mM of $Fe(CN)_6^{3-/4-}$. In order to minimize the electrical noise, all the electrochemical measurements were done in a Faraday cage.

RESULTS AND DISCUSSION

The most critical step in biosensors is the immobilization of the biomolecules on the solid support, a process where the orientation of the probe toward the analyte in solution plays a key role in the performance of the biosensors. SAMs on a solid surface have been considered as one of the most suitable functional interlayers for the immobilization of biomolecules, since it can easily be prepared by spontaneous chemisorption of molecules, on a metallic surface with a high degree of orientation, organization, packing, and stability.[29] Alkanethiols with various terminal groups can be used without affecting the self-assembly process or the SAM stability.[30] Cysteamine has been frequently employed as bifunctional building blocks, where the sulfur atoms of the molecules bind to the gold surface, while the amino groups can be used for the attachment of biological recognition agents.[31] The stepwise assembly of the bacterial biosensors was illustrated in Figure 1 and was monitored using multicharacterization techniques such as cyclic voltammetry and electrochemical impedance spectroscopy (EIS).

Electrochemical Characterization of the Gold Electrode and Surface Modification. Cyclic voltammetry is an efficient, well-accepted analytical method and is commonly used to monitor surface modification, since it provides a rapid and simple method for initial characterization of the modified electrode using a redox couple system.[32] The reversible redox probe $Fe(CN)_6^{3-/4-}$ was used to investigate the different steps of the gold electrode modification. Figure 2A shows the cyclic voltammograms of 10 mM $Fe(CN)_6^{3-/4-}$ at the bare gold

dx.doi.org/10.1021/ac302699x | *Anal. Chem.* 2013, 85, 4893–4901

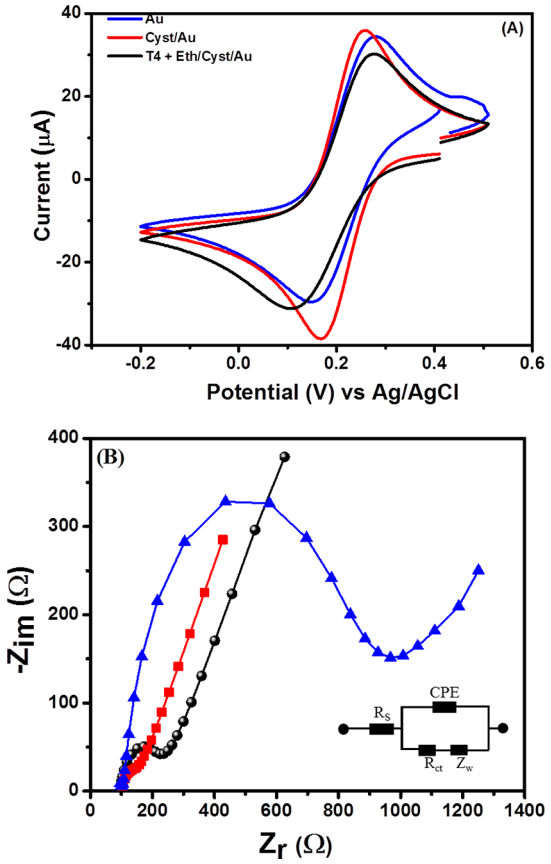


Figure 2. (A) Cyclic voltammetry (CV) showing the steps of the T4-bacteriophage biosensors construction: (blue) bare gold electrode (Au), (red) cysteamine-modified gold electrode (blue ▲), and (black) immobilization of the T4 bacteriophage and blocking with ethanolamine in 10 mM PBS (pH 7.4) solution (T4+Eth/Cyst/Au), containing 10 mM $Fe(CN)_6^{3-/4-}$ at a scan rate of 100 mV/s. (B) Nyquist plot of the impedance spectra for bare gold electrode (Au) (●), cysteamine-modified gold electrode (Cyst/Au) (red ■), and immobilization of the T4 bacteriophage and blocking with ethanolamine (blue ▲), in the presence of millimeters of $Fe(CN)_6^{3-/4-}$ as a redox probe and a 10 mM PBS (pH 7.4) solution, over the frequency range from 10^5 to 1 Hz, a bias potential of +0.23 V vs Ag/AgCl (3M), and an ac signal of 10 mV. The inset is the Randle's equivalent circuit used to fit the impedance data.

electrode, cysteamine-modified gold electrode, immobilization of T4 bacteriophage, and afterward, blocking with ethanolamine. As expected, the $Fe(CN)_6^{3-/4-}$ redox probe exhibited a reversible behavior on the bare gold electrode with a peak-to-peak separation of 80 mV. The formal potential of 0.23 V was estimated from the mean of the anodic and cathodic peaks of the cyclic voltammogram of the bare gold electrode. This value can be used as the fixed DC potential for all the electrochemical impedance spectroscopy experiments. On the cysteamine-modified gold electrode, the peak current was slightly increased and the peak-to-peak separation decreased to 62 mV, due to the electrostatic attraction between the positively (surface pK_a 7.6) charged amine groups of the cysteamine-modified gold electrode and the negatively charged of the redox probe.[33] However, the peak current of the redox probe was considerably decreased, and the peak-to-peak separation was increased after each step of the electrode modification: immersion in PDICT solution, immobilization of T4 bacteriophage, and ethanolamine blocking. The decreases in the peak currents might be attributed to the fact that the T4 bacteriophage and ethanolamine insulated the surface and effectively enhanced the electron transfer barriers.

Electrochemical impedance spectroscopy (EIS) was also used to further characterize the stepwise assembly of the bacterial biosensors, since it was reported as a nondestructive and an effective method to monitor the surface features, allowing the understanding of chemical transformation and the interfacial properties of the modified electrode.[34] Figure 2B illustrates the results of the Nyquist plot ($-Z_{im}$ vs Z_r) of the bare gold electrode, cysteamine-modified gold electrode, activation with PDCIT, and immobilization of the T4 bacteriophage in the presence of a 10 mM $Fe(CN)_6^{3-/4-}$ solution as a redox probe in 10 mM of PBS (pH 7.4). As shown in Figure 2B, an obvious semicircle was observed at high frequencies, corresponding to the electron transfer-limited process and followed by a straight line with a slope close to unity at lower frequencies, resulting from the diffusion limiting of the redox species from the electrolyte to the electrode interface. However, after cysteamine self-assembly on the gold electrode, the semicircle diameter was decreased because of the electrostatic interaction between the positive amino group of the cysteamine and the negatively charged redox probe. When the cysteamine-modified gold electrode was activated with PDCIT, the diameter of the semicircle was increased as expected. This is due to the chain length increase of the SAMs and the generation of neutral charge after the association of the amino groups of the cysteamine with the thiocyanate group. This increased chain length may introduce a certain hinderance to the flow of the redox probe to the surface of the gold electrode, as represented by a slight increase in the diameter of the semicircle. After the T4-bacteriophage molecules were attached to the activated electrode, the diameter was significantly increased in comparison with the previous modified electrode surfaces. The reason is that the T4-bacteriophage molecules on the electrode acted as an insulting layer because they were negatively charged (pI 4−5) at the working pH 7.4.[35] Consequently, they significantly hindered the diffusion of redox probe toward the electrode surface, resulting in a higher electron-transfer barrier and therefore enlarges the diameter of the semicircle.

The impedance spectra showed a semicircle and linear portion. The semicircle portion at higher frequencies corresponds to the electron-transfer limited process, and the linear portion at lower frequencies represents the diffusion-limited process. The equivalent circuit for an electrode undergoing heterogeneous electron transfer is usually described on the basis of the Randles equivalent circuit.[36] Due to the lack of surface regularity of biosensors and the nonhomogeneity of the current distribution, double layer capacitance can be replaced by a constant phase element (CPE). Therefore, the modified Randles equivalent circuit can be used as an ideal equivalent circuit to analyze the impedance spectra in this work.[37] As illustrated in the inset of Figure 2B, the equivalent circuit consists of a background solution resistance, R_s, a charge-transfer resistance, R_{CT}, the Warburg impedance, Z_W, resulting from the diffusion of the redox couple from the bulk of the solution to the electrode interface, and a CPE, representing the electrical double layer capacitance, which leads to a depressed semicircle in the corresponding Nyquist impedance plot. The impedance of the CPE is given by the following equation:

$$Z_{CPE} = Q^{-1}(j\omega)^{\alpha}$$

dx.doi.org/10.1021/ac302699x | Anal. Chem. 2013, 85, 4893−4901

where ω is the electric field frequency, Q^{-1} is the modulus of the constant phase element, measured in Farads cm^{-2} $s^{\alpha-1}$, and the exponent α varies from 0 to 1 and reflects the ideality of the capacitor.[38] The double layer capacitance can be estimated from[39]

$$Q = C_{dl}{}^{\alpha}(R_s{}^{-1} + R_{ct}{}^{-1})^{1-\alpha}$$

R_s and Z_W represent the bulk properties of the electrolyte solution and diffusion features of the redox probe and ions in solution. These two components are not affected by chemical modification occurring at the electrode surface. On the other hand, R_{ct} and CPE depend on the dielectric and insulating features at the electrode/electrolyte interface, respectively, hence they are controlled by the electrode surface modification. For Faradic impedance measurement, the R_{ct} is the most sensitive and straightforward parameter that can be used to characterize the events occurring at the interface between the immobilized probe on the electrode and the analyte in the solution.[40] Moreover, the variation in CPE is almost unnoticeable compared to the change in R_{ct}. R_{ct} for a bare Au electrode was estimated to be 220 Ω and decreased to 90 Ω when the electrode was modified with cysteamine, peripheral amine groups of which produced positive charge that accelerated the electron transfer for the $Fe(CN)_6^{3-/4-}$ redox probe. Subsequently, assembly of T4 bacteriophage on the aminated electrode after activation of the amine groups with the PDCIT cross-linker, generated an insulating layer on the electrode surface that functioned as a barrier to the interfacial electron transfer. This was reflected by the increased R_{ct} to 865 Ω, which was in agreement with the results reported by Yang et al.[41] All these data show that the stepwise modifications, cysteamine, PDCIT, and T4 bacteriophage, were successfully assembled on the gold electrode surface and formed a tunable kinetic barrier. The results obtained from EIS measurements are consistent with those extracted from cyclic voltammograms and confirm the successful immobilization of T4-bacteriophage molecules on the surface of the modified gold electrode.

Detection of *E. coli* Bacteria by Electrochemical Impedance (EIS). The impedance measurement can be performed in the presence or absence of a redox couple, which is referred to as Faradaic and non-Faradaic impedance measurements, respectively.[37] Faradaic and non-Faradaic measurements are widely utilized methods that allow the detection of bacteria cells using immobilized antibodies on the electrode surface.[42,43] The major drawbacks associated with antibodies as a bioreceptor is the low capturing efficiency of the biosensors,[42] cross-reactivity with nontarget bacterial cells, instability due to environmental fluctuations, and high-cost production and long analysis time, which can limit their long-term storage and their field applicability.[22,23] Therefore, these issues need to be addressed by developing Faradaic impedance methods with immobilization of a new bioreceptor on the electrodes. Recently, bacteriophages are employed as bio-recognition elements for the identification of various pathogenic microorganisms.

In order to demonstrate the practical utility of the proposed biosensor, the T4-bacteriophage-modified electrode was exposed to various concentrations of nonpathogenic *E. coli* B. The T4-bacteriophage biosensors were first incubated for 15 min with 50 μL of the appropriate *E. coli* concentration in 10 mM of PBS (pH 7.4), washed three times with PBS, and then the washed biosensor was placed into the conventional electrochemical cell containing a 10 mM of $Fe(CN)_6^{3-/4-}$ (pH 7.4) buffer solution for impedance measurement. The corresponding Nyquist plot of the impedance spectra is shown in Figure 3A. It appears that the diameter of the semicircle

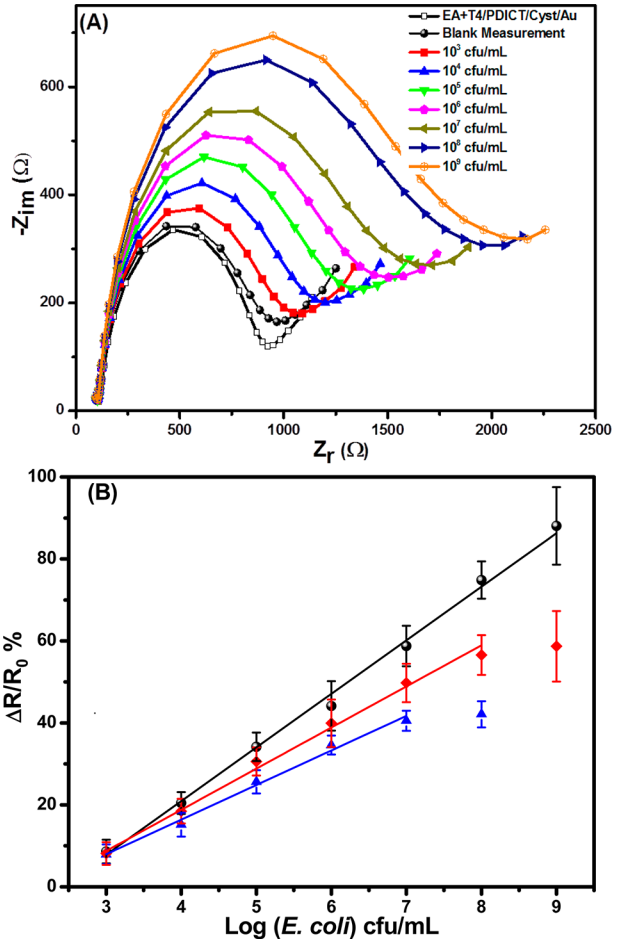


Figure 3. (A) Nyquist impedimetric diagram of T4-bacteriophage-modified gold electrode interface at different concentrations of *E. coli*. Measurements were performed in 10 mM PBS (pH 7.4) containing 10 mM $Fe(CN)_6^{3-/4-}$: 0 (●), 10^3 (■), 10^4 (▲), 10^5 (▼), 10^6 (◆), 10^7 (◀), 10^8 (▶), and 10^9 (⊕) cfu/mL, (B) Normalized response, ΔR_{ct} (%), as a function of varied logarithmic concentration of the *E. coli* bacteria cells in PBS (●), skim milk (blue ▲), and lake water (♦). ΔR_{ct} (%) values obtained from the fitting of three independent impedance data sets.

increased with increasing *E. coli* B concentrations. Gram-negative bacteria such as *E. coli* theoretically have an overall negative charge due to the charges present in their external membrane in the lipopolysaccharide.[44] Thus, their binding to T4 bacteriophage can act as a barrier for the electron transfer between the anionic redox species and the electrode surface. To complete the impedance investigation, the obtained results were fitted with the proposed modified Randle's equivalent circuit (inset of Figure 2B). As expected, we observed a significant change in the charge transfer resistance, R_{ct}, while the changes in the R_s, CPE, and Z_w were very small and irregular upon an increase of the *E. coli* concentration, supporting the use of R_{ct} as a quantitative parameter to evaluate the analytical performance of the developed T4-bacteriophage biosensor. In order to compare the relative responses of the developed biosensor and to suppress the small variation between electrodes, the signal values were normalized

dx.doi.org/10.1021/ac302699x | *Anal. Chem.* 2013, 85, 4893−4901

with respect to the charge transfer resistance (R_{ct}) of the immobilized T4 bacteriophage. The normalized response, ΔR_{ct} (%) = [R_{ct}(phage bacteria) − R_{ct}(phage)]/R_{ct}(phage), was plotted as a function of *E. coli* concentration on a logarithmic scale (Figure 3B). R_{ct}(bacteria phage) and R_{ct}(phage) were the charge transfer resistance in the presence and the absence of *E. coli* cells, respectively. The change in the normalized response was linear with the logarithm of *E. coli* concentration from 10^3 to 10^9 cfu/mL and had a regression equation of ΔR_{ct} (%) = 13 log (*E. coli*) − 30.6, with a correlation coefficient of 0.998. The limit of detection (LOD) was estimated to be 8×10^2 cfu/mL in 15 min time, which was calculated using the $3S_D/m$ equation, where m is the slope of the linear part of the calibration curve, and S_D is the standard deviation of the blank measurement.

In order to verify the ability of the phage biosensor to detect *E. coli* cells in a complex biological matrix, the performance of the biosensor was evaluated with skim milk and lake water spiked with *E. coli* B cells. On the other hand, the T4-bacteriophage biosensor exhibited a linear range between 10^3–10^8 cfu/mL, with LOD of 10^3 cfu/mL. The linear regression equation was ΔR_{ct} (%) = 10 log [*E. coli* (cfu/mL)] − 21.4 with a correlation coefficient of 0.993. The biosensor in lake water was saturated at 10^8 cfu/mL and showed no significant change with higher *E. coli* concentration.

The impedimetric label-free method described here for the detection and identification of nonpathogenic *E. coli* B cells represents a promising method. The dynamic range and the response time of this approach are much wider and extremely shorter, respectively, than those of methods reported previously. A short detection time is the most important demand concerning assays of polluted water sources and even more critical when bioterrorism is suspected. For comparison, Ruan et al.[42] reported an impedimetric immunosensor for *E. coli* O157:H7 detection using horseradish peroxide enzyme-labeled for signal amplification. The corresponding calibration curve of their immunosensors was linear over the range from 6×10^4 to 6×10^7 cfu/mL; the LOD was 6×10^3 cfu/mL, and the overall assay time was 90 min. However, this method has significant drawbacks, such as the high cost of the reagents involved in the immunoreaction and the need of labeled antibodies. Another approach has been reported by Geng et al.[45] for the detection of *E. coli*, by immobilizing anti-*E. coli* antibodies on EDC/NHS-activated carboxyl groups, and then EIS was used to follow the specific binding between the antibodies and the bacteria. The authors showed that there is a linear relationship between the charge transfer resistance (R_{ct}) and the logarithmic of the *E. coli* concentration in the range from 3×10^3 to 6×10^7 cfu/mL, and a concentration as low as 10^3 cfu/mL can be ambiguously detected within 60 min. More recently, the use of lectin as the biorecognition element has been proven as an effective tool for the detection and identification of bacteria. Gamella et al.[46] reported biotinylated lectin-based screen-printed gold electrodes for the impedimetric label-free detection of *E. coli*. The proposed biosensor was able to detect *E. coli* in the range from 5×10^3 to 5×10^7 cfu/mL with a limit of detection of 5×10^3 cfu/mL within 60 min. A novel attempt for the detection of *E. coli* based on the recognition properties of the carbohydrate was reported recently by Guo et al.[47] The sensing layer was first constructed by mixed self-assembly of a long-chain, thiol-terminated α-mannoside and thiol-terminated oligoethylene glycol "spacer" molecule onto a clean gold electrode surface, and then the interaction between the α-mannoside and *E. coli* was monitored by EIS. The authors show that the calibration plot curve exhibits a linear relationship between the charge transfer resistance (R_{ct}) and the logarithmic concentration of the *E. coli* over the range from 10^2 to 10^3 cfu/mL. However, the limit of detection and time of detection were not determined in their work.

Specificity, Reproducibility, Stability, and Viability Test. To further investigate the performance of the T4-bacteriophage-based impedimetric biosensors, a series of experiments was performed on the specificity, reproducibility, stability, and viability test. The specificity of the T4-bacteriophage biosensor was investigated by verifying the normalized response of the proposed biosensors in relation to *Listeria* cells. As expected, no significant change in the normalized response (7.0 ± 2.8%) was observed to 10^8 cfu/mL of *Listeria* cells, which is 10-fold lower than the response to the same concentration of the specific bacteria (Figure 4). An additional experiment was used as a negative control with a T4-bacteriophage-free electrode (i.e., the isothiocyante groups were capped with ethanolamine under similar solution conditions for T4-bacteriophage immobilization). In this case, a 4.5 ± 1.8% change in the normalized response was noticed (Figure 4). These support that the changes of the charge-transfer resistance were induced by the specific binding between the immobilized T4 bacteriophage and the bacteria in solution and not due to the nonspecific adsorption of the bacteria on the modified gold electrode.

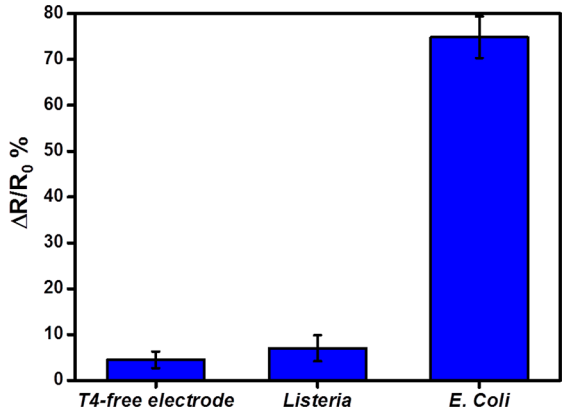


Figure 4. T4-bacteriophage biosensors selectivity against *Listeria* cells.

Repeatability and reproducibility of a proposed biosensor is always of great interest, and it appears to be a challenge. It depends mainly on the stability of the biological recognition element and the preparation of the biosensor itself. The repeatability and reproducibility of the present biosensor were investigated with intra- and interassay coefficient of variation (% CV). The intra-assay precision of the biosensor was evaluated, by measuring five times the *E. coli* B with a concentration of 10^8 cfu/mL. The interassay precision was assessed, by assaying the *E. coli* at the same concentration (10^8 cfu/mL) with three different biosensors prepared independently at the same experimental conditions. The coefficient of variation of intra- and interassay obtained from 10^8 cfu/mL *E. coli* B were 4.3% and 10.7%, respectively. Besides repeatability and reproducibility, stability of the biosensors was also investigated. As expected, no obvious change was observed after 7 days of biosensors storage in PBS solution at 4 °C. These results demonstrate the acceptable repeatability,

dx.doi.org/10.1021/ac302699x | *Anal. Chem.* 2013, 85, 4893−4901

reproducibility, and stability of the proposed T4-bacteriophage biosensors.

Real-time detection of alive bacteria using a portable instrument remains a challenge to many scientists and engineers, since the presence of dead bacteria is not considered a health risk. The impedemitric response for alive and dead *E. coli* B cells was tested after incubation of 10^8 cfu/mL of a mixture of alive and dead cells on the T4-bacteriophage-modified electrode for 15 min, followed by washing with PBS. The biosensors were transferred to the electrochemical cell, and the Faradaic impedance was consecutively measured over 1 h to determine the viability of the bacteria. Figure 5A depicts the

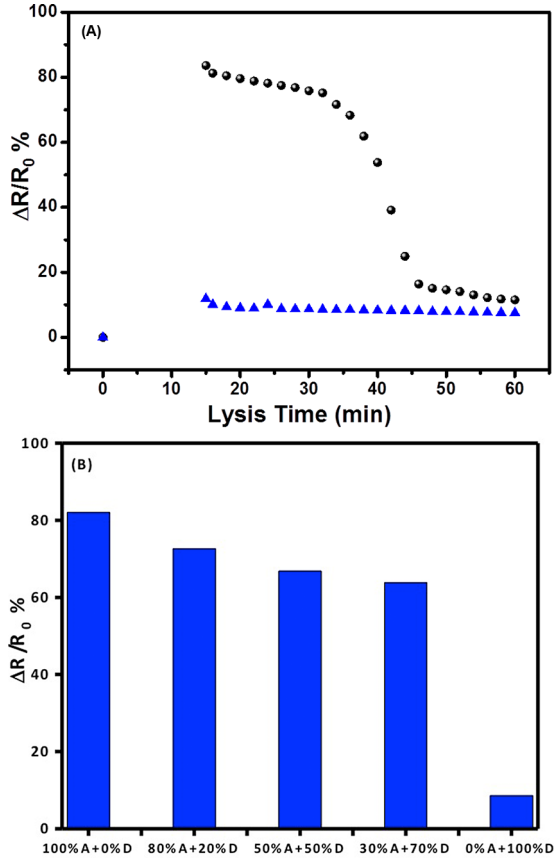


Figure 5. (A) Viability test of live (●) and dead (blue ▲) *E. coli* bacteria cells (B) shows the sensor response for different ratios of alive/dead cells (alive:dead, 100:0, 80:20, 50:50, 30:70, and 0:100); the final concentration was 10^8 cfu/mL.

normalized response change versus time for both cells. The normalized response of the biosensors with an alive cell was increased by 82% and reached a steady-state condition within 15 min, indicating the binding of the alive cells on the T4 bacteriophage. This response then started to decrease gradually, confirming the infection and lysis of the alive bacteria cells by the immobilized T4 bacteriophage. In contrast, a slight increase of 8.6% was observed in the case of the biosensors with dead cells, as can be seen from the curve (b) in Figure 5A, revealing that the T4 bacteriophage cannot recognize the dead *E. coli* B cells. These results are in good agreement with the previously reported data, where the author has shown that the T4 bacteriophage cannot attach to the acetone-ether-treated cells because the adsorption was inhibited by the lipopolysaccharides hydrolysis.[48,49] Moreover, it was reported a long time ago that the adsorption rate constant of bacteriophage to bacterium is found to change between wide limits, depending on the physiological state of the bacterium.[50] Recently, it was found that the adsorption rate in alive host cells was much larger than that in dead host cells.[51] In our case, the small variation in the normalized response might be due to a short incubation time of 15 min and/or to the denaturation of the bacteriophage attachment receptors on the bacteria cell surface. This clearly confirms that our developed T4-bacteriophage biosensor can be used for excellent discrimination against the dead *E. coli* cells. Furthermore, more experiments have been performed for different ratios of alive/dead cells (alive:dead, 100:0, 80:20, 50:50, 30:70, and 0:100); the final concentration was 10^8 cfu/mL bacteria cells. As shown in Figure 5B, there is a small variation in the normalized response (ΔR_{ct} (%)) between the 100% of alive cells and the other mixed concentrations (80%, 50%, and 30%). This result is expected since the different ratios of alive/dead cells present high concentration of live cells (more than 3×10^7 cfu/mL).

LAMP-Based *E. coli* Biosensors. The concentration of the bacteria allowed by the Environmental Protection Agency regulation in treated drinking water should contain no (zero) bacteria in 100 mL.[52] Hence, a biosensor must be able to provide LOD as low as a single bacteria in 100 mL of potable water.[53] In this regard, the combination of the LAMP-based method and the related release of intrinsic substances following lysis of the cell by the specific bacteriophage can provide a powerful confirmation technology for highly specific and sensitive detection of *E. coli* and allowing the LOD to be pushed down. To demonstrate the signal amplification of the LAMP method, the binding of *E. coli* B to the T4-bacteriophage-modified electrode was removed from the electrochemical cell after finishing the impedance experiments, followed by washing with PBS and water, and then it was incubated in 200 μL of the LAMP master mix at 65 °C for 40 min before being subject to a LSV scan; the LAMP reaction protocol is described in the Supporting Information. The time was already optimized in our previous work, since it plays a major role in bacteria quantification.[54] After that, the electrochemical signal (LSV) of the intercalating agent Hoechst was measured in a mixture containing 6 μL of the amplicon, 12 μL of Hoechst redox, and 12 μL of PBS (10 mM) buffer and then was plotted against the *E. coli* concentration. As shown in Figure 6A, a gradual decrease in the redox peak of the Hoechst was clearly observed with increasing *E. coli* concentrations. This can be attributed to the intercalation of the Hoechst molecules within the double-stranded LAMP reaction products.[55] From Figure 6B, it can be seen that the normalized response [ΔI (%) = $(I_0 - I\ (\mathrm{LAMP}))/I_0$, where I_0 and I(LAMP) are the current peak at 0.5 V vs Ag/AgCl in the absence and the presence of LAMP product, respectively] has a linear relationship with the logarithmic scale of *E. coli* concentration over the range from 10^2 to 10^7 cfu/mL and had a regression equation ΔI (%) = 4 log (*E. coli*) + 7.36 with a correlation coefficient of 0.998. The LOD was 100 cfu/mL and with total assay time less than 45 min. With this strategy, LOD was enhanced by at least 1 order of magnitude.

CONCLUSION

A robust and convenient biosensing platform could provide a promising tool for rapid detection of pathogenic infection. Toward this goal, we have demonstrated for the first time a fast, selective, and highly sensitive assay for the detection of *E. coli* B,

dx.doi.org/10.1021/ac302699x | *Anal. Chem.* 2013, 85, 4893−4901

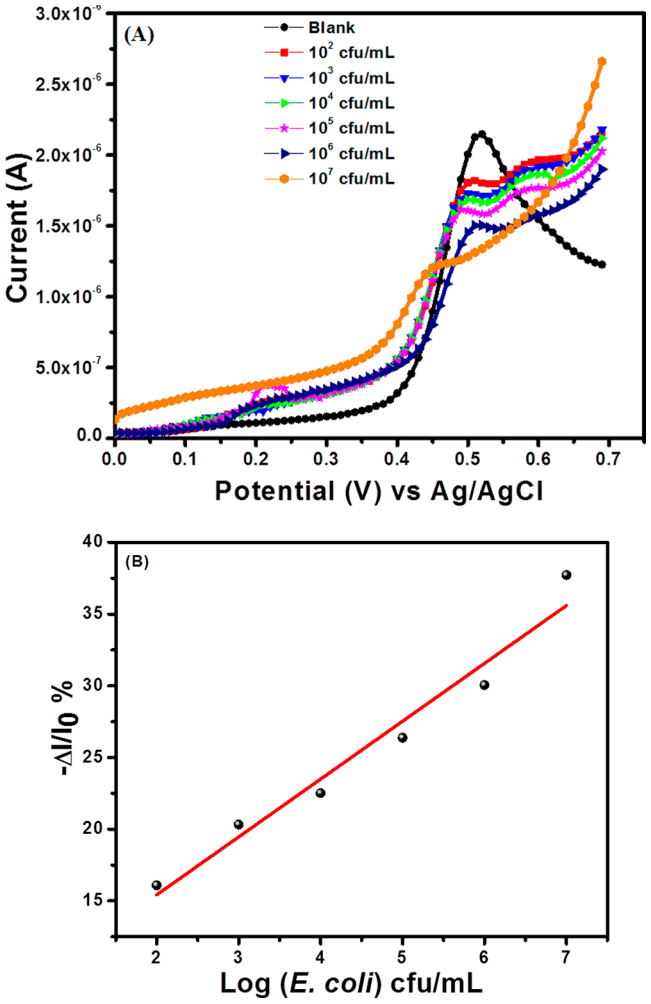


Figure 6. (A) Linear Sweep voltammetry (LSV) monitoring the LAMP amplification of the *Tuf* gene target. (B) Calibration curve of the LAMP assay.

using bacteriophage-based EIS/LAMP dual-response biosensors. The impedimetric assay incorporates a bacteriophage, a virus that can be used as a "capturing" agent, identifies, and lysis only one bacterial species among mixed populations, thereby releasing the intracellular *Tuf* gene that can be amplified by LAMP method and monitored by the LSV technique. Our electrochemical impedance results reveal that the *E. coli* bacteria can be detected quickly and accurately without preconcentration or enrichment steps at the level of 800 cfu/mL with excellent discrimination against other bacteria or even dead E. coli bacteria. The limit of detection corresponding to the LAMP assay was almost 1 order of magnitude lower. The time required for detection was approximately 15 min for the screening assay using EIS and 40 min for the LAMP confirmation assay. Given the advantages arising from the specificity, stability, and capture efficiency of the bacteriophage over antibodies, we expect that this assay could form a generic platform for advanced bacteria sensing, with a high promise in practical applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Dr. Andy Ng for proofreading the manuscript and the useful discussions.

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