Analytical Methods

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A chronopotentiometric flow injection system for aptasensing of *E. coli O157*†

Jiahong Lei, Jiawang Ding* and Wei Qin*

In this research, we demonstrate a simple flow injection analysis system for chronopotentiometric aptasensing of *E. coli O157*. The sensing protocol is based on using an aptamer as a bioreceptor and the current-driven-release of protamine from a polyion-sensitive membrane electrode as a signal reporter.

E. coli O157, a notorious pathogen with the production of verocytotoxin, can cause a number of severe diseases that pose a deadly threat to human health.[1] Therefore, the identification and detection of *E. coli O157* have received considerable attention, which involve an array of techniques such as cell culture, DNA/RNA hybridization,[2] luminescence,[3] enzyme-linked immunosorbent assays,[4] polymerase chain reactions,[5] surface plasmon resonance,[6] and inductively coupled plasma mass spectrometry.[7] In recent years, many efforts have been devoted to develop biosensors for the rapid and sensitive detection of bacterial cells. Electrochemical biosensors based on various transduction modes, such as amperometry, potentiometry, and impedimetry, are among the most promising. To date, a number of electrochemical biosensors coupled with a range of biological recognition elements, including antibodies, peptides and aptamers, have been reported for the detection of *E. coli O157*.[8]

Aptamers are single-stranded DNA or RNA molecules that can bind to a myriad of targets with high affinity that is comparable to an antibody.[9] Aptasensors utilizing aptamers as bioreceptors have appeared as promising methods for the detection of pathogenic bacteria. Researchers have summarized and evaluated the current applications of electrochemical aptasensors specific for microbial and viral pathogens.[10] Recently, we developed a label-free potentiometric aptasensor

to monitor bacteria (*e.g.*, *Listeria monocytogenes*) in a homogeneous solution where the addition of protamine as a signal reporter is involved.[11] In such sensing protocol, protamine-sensitive membrane electrodes are for single-use and cannot monitor the analytes continuously. Flow-injection analysis (FIA) offers high-throughput measurements with rapid and highly reproducible results.[12] To achieve potentiometric aptasensing with a high sample throughput and low consumption of reagents, FIA can be coupled with the polyion-sensitive membrane electrode. However, to date, no FIA aptasensors for bacteria have been reported. Therefore, potentiometric aptasensors that allow a sensitive and selective detection of *E. coli O157* in FIA are highly desired.

In recent years, great progress in ion-selective polymeric membrane electrodes has been made by tuning the ion-fluxes across the electrode *via* instrumental control.[13] Chronopotentiometric sensors, in which ion fluxes are controlled by an applied current, represent an exciting new class of dynamic ion-selective sensors. Bakker *et al.* explored ion-selective chronopotentiometry to detect alkalinity by imposing an outward flux of hydrogen ions from an ion selective membrane to the sample solution[14] Recently, polyion-selective pulstrodes have been used as detectors in an FIA measurement configuration for heparin.[15] In our previous research, we have shown that the indicator ions for potentiometric biosensing could be precisely controlled *via* current-controlled reagent delivery and could be used for the determination of small analytes and enzymes.[16] These developed polymeric membrane ion-selective electrodes are reversible and could be utilized as a detector in a FIA system. The FIA systems integrated with those electrodes have been applied successfully to monitor small analytes. In this work, we explore for the first time this avenue by a simple FIA arrangement for chronopotentiometric aptasensing of *E. coli O157*. It will be shown that *E. coli O157* can be sensitively and selectively detected *via* FIA.

Herein, we demonstrate the use of the current-controlled-release protamine for monitoring bacteria in a FIA system. The sensing mechanism is illustrated in Scheme 1. When an

Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research (YIC), Chinese Academy of Sciences (CAS), Shandong Provincial Key Laboratory of Coastal Environmental Processes, YICCAS, Yantai, Shandong 264003, P. R. China. E-mail: jwding@yic.ac.cn; wqin@yic.ac.cn; Fax: +86 535 2109000; Tel: +86 535 2109156

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anodic current is applied to the electrode, protamine ions are transported across the membrane from the inner solution to the sample, which results in a potential response. The potential response was used as the readout signal. In contrast to conventional ion-selective electrodes, which exhibit Nernstian responses, the present polycation-selective electrode shows the sigmoidal relationship between the potential measured and protamine concentration. In this work, parameters were optimized to ensure the electrode performed in the most sensitive manner.[16c] In the absence of the target, ion pairing between the protamine and aptamer is favored due to the strong electrostatical interactions between the aptamer with negatively charged phosphate groups and protamine with positively charged guanidinium groups (Scheme 1A). Protamine ions expelled from the membrane electrostatically interact with the aptamer to form a biocomplex. The consumption of protamine would facilitate the stripping of protamine out of the membrane surface *via* an ion-exchange process with sodium ions from the sample solution, which can significantly decrease the EMF value.[16c] This potential change is correlated to the concentration of aptamer in the solution. When the target bacterium binds to the aptamer, the formation of the biocomplex is disrupted, therefore yielding a potential change, which can be used for the potentiometric detection of *E. coli O157* (Scheme 1B).

As a proof of concept, a polymeric polycation-sensitive membrane electrode was constructed. The membranes contained 3.0 wt% dinonylnaphthalene sulfonic (DNNS), 6.0 wt% tetradodecylammonium tetrakis (4-chlorophenyl) borate (ETH 500), 61.0 wt% 2-nitrophenyl octyl ether (o-NPOE), and 30.0 wt% poly(vinyl chloride) (PVC) and were prepared as described before.[16a] 0.05 mg mL^{-1} protamine solution containing 0.12 M NaCl in 50 mM Tris–HCl buffer (pH 7.4) was used as the inner filling solution. The protamine-conditioned polymeric membrane ion-selective electrode enables the current-controlled release of protamine from the inner filling solution to the sample solution.[11] The protamine released from the

polyion-sensitive membrane electrode can be utilized as an indicator for potentiometric aptasensing of *E. coli O157*. The FIA system contains a peristaltic pump, an injection valve and a wall-jet flow cell (8 μL) (as shown in Scheme 1).[16d] The detection chamber, which was constructed in-house from a single block of Perspex, has a three-electrode system (Fig. S1 in the ESI†). A high performance liquid chromatography injection valve (Waters E2695, USA) was used for sample injection. A CHI-660C electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China) was used to perform potentiometric measurements using a conventional three-electrode system with a polycation-sensitive membrane electrode, a platinum auxiliary electrode, and an AgCl/Ag reference electrode. Measurements of electromotive force (EMF) were performed in the cell: Ag/AgCl/3 M KCl/ sample solution/polymeric membrane/inner filling solution/ AgCl/Ag. A 0.05 M Tris–HCl buffer (pH 7.4) containing 1 mM NaCl was used as the carrier solution for sample injection and incubation medium for the formation of the bacteria-aptamer complex. The difference in the potential peak height (ΔE_h) was used for quantification.

The aptamers for *E. coli O157* were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) and their sequences are shown in Table 1. Aptamer solutions were heated at 95 °C and cooled to room temperature prior to use to obtain the structure flexibilities of the aptamers for binding their targets.[17]

Current pulses or a constant current have been used to control transmembrane ion fluxes of polymeric membrane ion-selective electrodes.[20] In the present work, a constant anodic current was applied across the polymeric membrane polycation-sensitive electrode to release protamine from the inner filling solution into the sample solution to obtain a stable baseline. Moreover, the applied current would facilitate the rapid recovery of the membrane electrode. Therefore, the electrode performance is strongly influenced by the applied current. An increase in the applied current can increase the concentration of protamine released at the sample-membrane interface and widen the potential response range, but would decrease the sensitivity for aptamer detection. As a compromise, an anodic current of 10 nA was applied for the controlled release of protamine (Fig. 1A).

The influences of the flow rate and the injected sample volume on the sensitivity of the polycation-sensitive electrode were investigated. As shown in Fig. 1B, with an increase in the flow rate, the potential response is gradually increased, which is probably due to the more efficient electrostatical interactions between the aptamer and protamine in the flow cell induced by
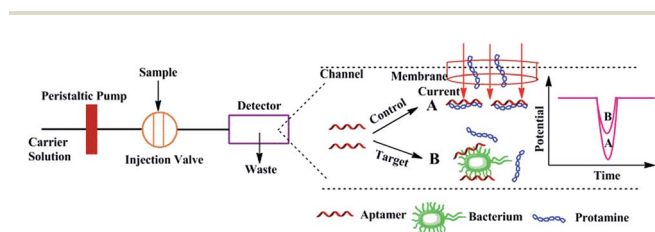
Scheme 1 Schematic illustration of the flow injection chronopotentiometric detection of *E. coli O157* using a protamine-sensitive membrane electrode.

Table 1 Sequences of the aptamers for *E. coli O157*

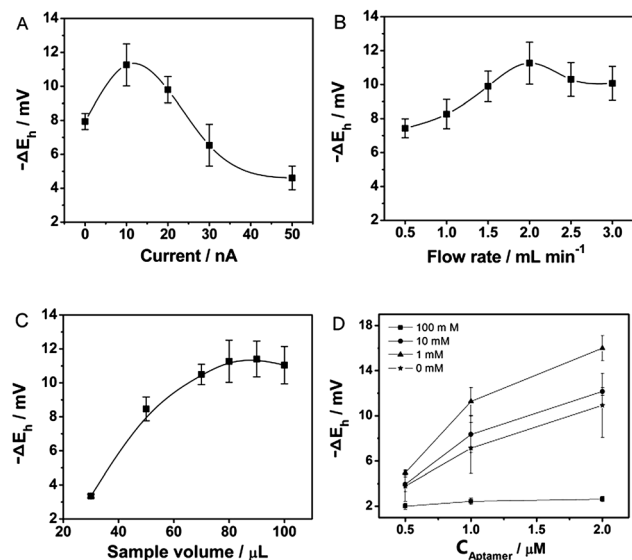| Aptamer	Sequence	References
EcO 4 Rev	5′-ACGGCGCTCCCAACAGGCCTCTCCTTACGGCATATTA-3′	18
EcO 3 Rev	5′-GTCTGCGAGCGGGGCGCGGGCCCGGCGGGGGGATGCG-3′	18
E17F-37	5′-ATCAAATGTGCAGATATCAAGACGATTTGTACAAGAT-3′	19
E18R-42	5′-CCGGACGCTTATGCCTTGCCATCTACAGAGCAGGTGTGACGG-3′	19

Fig. 1 Effects of (A) current, (B) flow rate, (C) sample volume and (D) buffer concentration on the potential response to the aptamer Eco 3 Rev of 1 μM in the FIA system. Unless stated otherwise, the experiments were performed under the following conditions: current, 10 nA; flow rate, 2.0 mL min^{-1}; sample volume, 80 μL; buffer solution, 50 mM pH 7.4 Tris–HCl buffer containing 1 mM NaCl. Each error bar represents one standard deviation of three measurements.
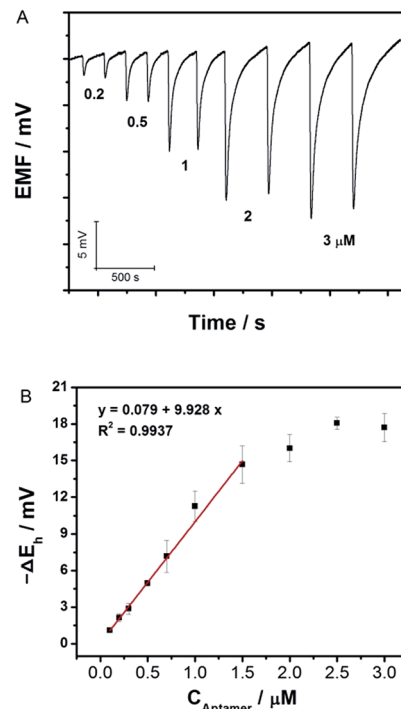

Fig. 2 (A) Potentiometric responses of the electrode to the aptamer Eco 3 Rev. (B) Calibration curve for the aptamer Eco 3 Rev. Each error bar represents one standard deviation of three measurements. The other experimental conditions are as given in Fig. 1.

more vigorous mixing. However, at a flow rate higher than 2.0 mL min^{-1}, the electrode response is decreased probably due to a sample dilution effect and/or the short duration for the interaction between the protamine and the aptamer.[16e] Fig. 1C shows that the potential response increases rapidly with increase in the sample volume up to 80 μL and then tends to be constant. Therefore, a sample volume of 80 μL and flow rate of 2.0 mL min^{-1} were chosen for the present system.

The interfering ions in the buffer solution also have an impact on the potential response due to ion exchange between the interfering ions in the sample solution and protamine in the membrane. Fig. 1D indicates that higher sodium concentrations facilitate the stripping of protamine from the membrane surface and result in a lower potential change. On the other hand, lower sodium concentrations lead to larger noise in potential signal. Therefore, 50 mM pH 7.4 Tris–HCl buffer containing 1 mM NaCl was used in the subsequent experiments.

Under the optimal experimental conditions, the potentiometric response to the aptamer is shown in Fig. 2. The potential responses decrease in the presence of the aptamer Eco 3 Rev due to electrostatical interaction between the protamine released at the sample-membrane interface and the aptamer in solution. Such decreases will be larger with an increase in aptamer concentration. Moreover, there is a linear relationship between the potential change and the aptamer concentration in the range of 0.1–1.5 μM. Although higher concentrations of the aptamer cause larger potential changes, it would induce lower sensitivities for the *E. coli O157* detection. Therefore, 1.0 μM of the aptamer was employed for the wide response range and high sensitivity.

In recent years, a number of aptamers for *E. coli O157* have been available.[18,19] In this work, four aptamers with different sequences were compared in terms of their analytical performance. As shown in Fig. 3, all these aptamers can be applied for chronopotentiometric aptasensing of *E. coli O157* based on the proposed method. However, a larger potential response can be obtained with the aptamer Eco 3 Rev, which may due to the higher affinity of the aptamer to the *E. coli O157*. Therefore, the aptamer Eco 3 Rev was applied for further experiments.

The *E. coli O157* cells were identified by the chromogenic medium and the concentrations of the bacteria cells were
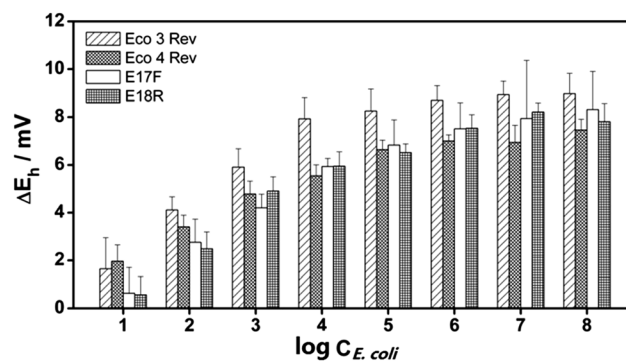

Fig. 3 Determination of *E. coli O157* ATCC35150 with 1.0 μM of the aptamers Eco 3 Rev, Eco 4 Rev, E17F and E18R. Each error bar represents one standard deviation of three measurements. The other experimental conditions are as given in Fig. 1.

calculated following the standard plate count method (Fig. S2 and S3 in the ESI†). 1.0 μM *E. coli O157* aptamer was mixed with the target cells at various concentrations in 1.0 mL Tris buffer. After incubation at room temperature for 1 h, the sample solution was injected into the detection cell. *E. coli O157* at different concentrations induced different decreases in the potential peak height. Under the optimized conditions, the system shows a good linear relationship between the potential response and the cell concentration in the range of 10–10^4 cfu mL^{-1}. The detection limit was calculated to be 10 cfu mL^{-1} (3σ) (Fig. S4 in the ESI†). The detection limit is at least one order of magnitude lower than those obtained by the immuno-sensors.[8a,d,21] The comparison between the present chro-nopotentiometric aptasensing protocol and other detection methods is listed in Table S1.† It should be noted that the aptamer's 3D structure plays a crucial role in its interactions and the buffer environment is very important.[22] The selection of buffer for the EcO 3 Rev aptamer was originally 0.5 M NaCl, 10 mM Tris, 1 mM MgCl$_2$.[18] For the present work, because the interfering ions in the buffer solution have an impact on the potential response due to the ion exchange between the inter-fering ions in the sample solution and protamine in the membrane, 50 mM pH 7.4 Tris–HCl buffer containing 1 mM NaCl was used. However, the employed buffer solution has a considerably lower ionic strength and could make the binding toward the *E. coli O157* target considerably weaker, thus leading to the low potential changes observed in Fig. 4.

Further optimization of the membrane composition and other parameters may enhance the sensitivity.

To demonstrate the specificity of the method, the aptasensor was challenged with non-specific bacteria such as *Listeria monocytogenes* and *Salmonella typhimurium*. As shown in Fig. S5,† the proposed sensing system does not respond to high concentrations of nonspecific bacteria but did respond to a low concentration of *E. coli O157*. Our previous research has shown that there is no significant potential change for a scramble DNA sequence in the presence of the target cells, which indicates that the measured potential changes are caused exclusively by the specific interaction between the bacteria and the aptamer.[11] Similar phenomenon has also been observed in this work (please see Fig. 3 for aptamer selection). Indeed, the DNA segment of E18R shows considerably less response to *E. Coli O157* when compared to the aptamer Eco 3 Rev. The good selectivity bodes well for future use of this sensing system in real samples.

Conclusions

In summary, a flow-injection analysis system for chro-nopotentiometric aptasensing of *E. coli O157* has been demon-strated. The use of a simple current-controlled reagent delivery strategy enables the integration of the polycation-sensitive elec-trode with flow-injection analysis to achieve a rapid, reproducible and continuous detection of whole cells. The system is readily adaptable to detect other bacteria by changing the aptamers. In addition, coupled to an online filtration system, the FIA system can be used to detect bacteria in real samples.

Acknowledgements

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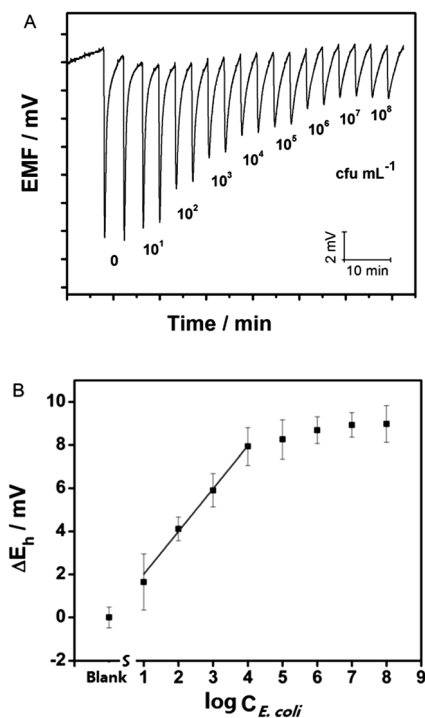7 F. Li, Q. Zhao, C. Wang, X. F. Lu, X. F. Li and X. C. Le, *Anal. Chem.*, 2010, **82**, 3399–3403.

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