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Aptamer-Based Impedimetric Sensor for Bacterial Typing

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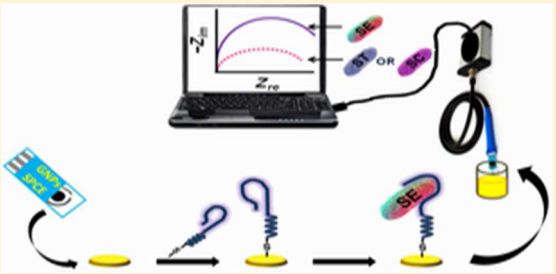
Supporting Information

ABSTRACT: The development of an aptamer-based impedimetric sensor for typing of bacteria (AIST-B) is presented. Highly specific DNA aptamers to *Salmonella enteritidis* were selected via Cell-SELEX technique. Twelve rounds of selection were performed; each comprises a positive selection step against *S. enteritidis* and a negative selection step against a mixture of related pathogens, including *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Citrobacter freundii*, to ensure the species-specificity of the selected aptamers. After sequencing of the pool showing the highest binding affinity to *S. enteritidis*, a DNA sequence of high affinity to the bacteria was integrated into an impedimetric sensor via self-assembly onto a gold nanoparticles-modified screen-printed carbon electrode (GNPs-SPCE). Remarkably, this aptasensor is highly selective and can successfully detect *S. enteritidis* down to 600 CFU mL^{-1} (equivalent to 18 CFU in 30 μL assay volume) in 10 min and distinguish it from other Salmonella species, including *S. typhimurium* and *S. choleraesuis*. This report is envisaged to open a new venue for the aptamer-based typing of a variety of microorganisms using a rapid, economic, and label-free electrochemical platform.



Recent estimates from the U.S. Centers for Disease Control and Prevention (CDC) suggest that approximately 1.4 million cases of salmonellosis occur annually in the United States, resulting in 15 000 hospitalizations, 400 deaths, and annual expenditures up to $2.3 billion per year due to loss of productivity and medical care expenses.[1] Salmonella species are Gram-negative, flagellated, facultative anaerobic bacilli possessing three major antigens: (H) or flagellar antigen, (O) or somatic antigen, and (Vi) antigen, possessed by only a few serotypes.[2] Certain serovars of Salmonella cause variable disease symptoms in different hosts, ranging from gastroenteritis to highly invasive diseases.[3]

Serologic classification of *Salmonella* strains based on the properties of various surface polysaccharide (O) and flagellar (H) antigens is the reference method for epidemiologic surveillance. This method involves the characterization of over 150 unique O and H antigens to produce an antigenic formula which can be scored using the Kauffman-White scheme to determine a serovar of an isolate.[4] *S. enteritidis* is recognized as the first most common serotype of Salmonella found in humans which accounts for the largest number of food poisoning outbreaks, estimating 2751 outbreaks from 1993 to 1997.[5] Although serotyping using the Kauffman-White scheme remains the gold standard for serovar determination, it is not free from significant deficiencies including high cost and inability to serotype between 5% and 8% of the isolates, often taking 3 or more days for a highly trained technician to produce a result. In addition, incorrect serotyping may occur due to atypical expression of surface O or H antigen as in the case of mucoid strains in which the O antigen is obscured or for nonmotile and/or monophasic isolates for which only one flagellar phase antigen can be determined.[6]

A number of recent molecular strategies have been adopted for genovar classification of Salmonella, including PCR-based approaches,[7] ribotyping,[8] pulsed-field gel electrophoresis,[9] IS200 analysis,[10] random amplification of DNA polymorphism,[11] and DNA microarray analysis.[12] Besides involving many steps of DNA processing, these methods require highly skilled personnel, sophisticated instrumentation, and centralized laboratories. Further, both sero- and geno-typing methods require a pre-enrichment step according to the ISO6579 standard, which takes 2−3 days for presumptive results and 7−10 days for confirmation,[13] thus adding to time and complexity of the assays. Therefore, there is a dire need for rapid, simple, sensitive, specific, and affordable techniques for typing of pathogens such as *S. enteritidis*.

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Recently we have developed DNA aptamers specific to oncolytic viruses, including vesicular stomatitis virus[14] and vaccinia virus.[15,16] In this work, DNA aptamers specific to *S. enteritidis* were selected based on Cell-SELEX[17,18] from 80nt DNA library containing a 40nt random region, 5′-CTC CTC TGA CTG TAA CCA CG N40 GC ATA GGT AGT CCA GAA GCC-3′ (Integrated DNA Technologies) as provided in details in the Supporting Information. Aptamer selection consisted of 12 rounds, each comprising alternating negative- and positive-selection steps except the first round where only positive-selection was adopted to enrich the ssDNA pool with binding aptamers (Figure 1). Positive-selection was performed

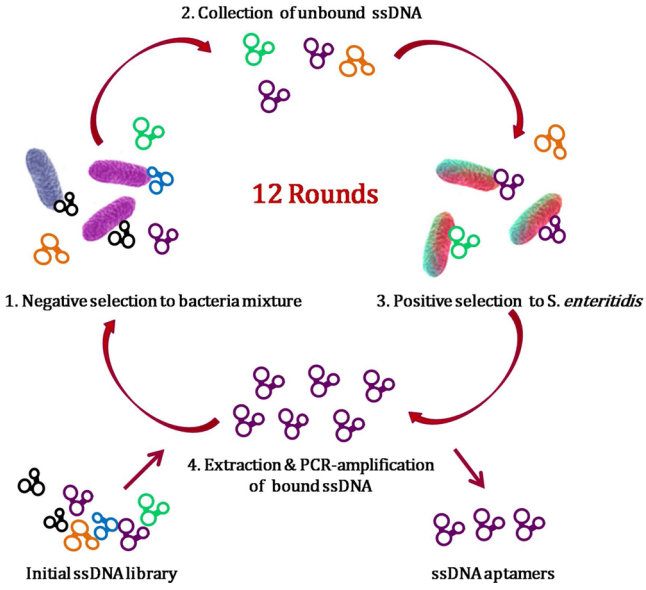

Figure 1. Aptamer selection scheme for *S. enteritidis*. Aptamer selection consists of 12 rounds; each comprises two alternating negative and positive selection steps except the first round where only positive selection was performed. Negative selection was carried out against a mixture of related pathogens, including *S. typhimurium*, *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. freundii*.

against *S. enteritidis* to increase the affinity of the aptamers, whereas negative-selection was carried out against a mixture of related pathogens, including *S. typhimurium*, *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. freundii* to increase the selectivity of the aptamers. Briefly, the first round of selection (only positive) comprised 4 steps, including (1) incubation of the ssDNA library with *S. enteritidis*, (2) removal of unbound aptamers, (3) extraction of bound aptamers, and (4) amplification of extracted aptamers using symmetric and asymmetric PCR. The next 11 rounds of selection were carried out starting from negative-selection and comprised 6 steps, including (1) incubation of the ssDNA sequences obtained from the previous step with a mixture of related pathogens, (2) collection of unbound aptamers, and then this was followed by the previous four steps employed for positive-selection. The affinity of the collected aptamer pools to *S. enteritidis* was analyzed by flow cytometry. This was performed by incubation of 100 nM of Alexa-488-labeled aptamer pools with 50×10^3 CFU of the bacteria followed by flow cytometric analysis. It was observed that the aptamer pool collected at the seventh round of selection had the highest binding affinity to the bacteria (K_D = 7 nM). Subsequently, this pool was cloned and the clones showing the highest affinity to the bacteria were sequenced and the sequences are provided in Table S1 in the Supporting Information. An ssDNA sequence (SENT-9, CTC CTC TGA CTG TAA CCA CGC ACA AAG GCT CGC GCA TGG TGT GTA CGT TCT TAC AGA GGT) showing the highest affinity to *S. enteritidis* was modified at the 5′ position with a 6-hydroxyhexyl disulfide group (Integrated DNA Technologies), and then it was utilized to develop the aptamer-based impedimetric sensor for typing of bacteria (AIST-B), as schematically depicted in Figure 2.

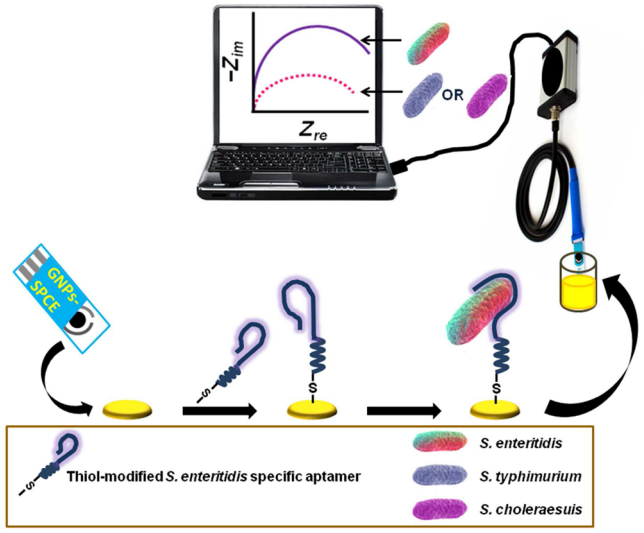

Figure 2. Schematic diagram of an aptamer-based impedimetric sensor for typing of bacteria (AIST-B). A thiolated *Salmonella enteritidis*-specific DNA aptamer was self-assembled onto a gold nanoparticles-modified screen-printed carbon electrode (GNPs-SPCE). Binding of *S. enteritidis* bacteria to the immobilized aptamer causes an increase in interfacial resistance, measured via electrochemical impedance spectroscopy. On the other hand, incubation with other Salmonella species (*S. typhimurium* and *S. choleraesuis*) produces negligible changes in impedance.

The electrochemical characteristics of the developed aptasensor were investigated by cyclic voltammetry (CV), as shown in Figure 3A. The pretreated electrode surface presents a quasi-reversible voltammogram indicating that the redox reactions easily occurred on the bare gold surface, evidenced by very large redox currents (curve a). Formation of SAM of the thiolated aptamer onto the electrode surface substantially reduced the electrode current, indicating the formation of a highly compact layer (curve b). Final treatment with 2-mercaptoethanol significantly reduced the redox currents because they could penetrate down to the electrode surface, thereby blocking the direct access of the conducting ions (curve c). The thiol group of the backfilling agent could effectively displace the weaker adsorption contacts between the aptamer nucleotides and the gold surface, leaving the capture probe primarily tethered through the thiol end group. Such a conformation improved the flexibility of the probe and rendered it more accessible to the target molecules. Moreover, displacement of the nonspecific adsorption provided free volume into the film, which enhanced the transport of counterions and solvent molecules through the modified film.[19] Importantly, Steel et al. have demonstrated that hexanethiol can be also utilized as a backfilling agent for the development of an electrochemical DNA sensor.[20]

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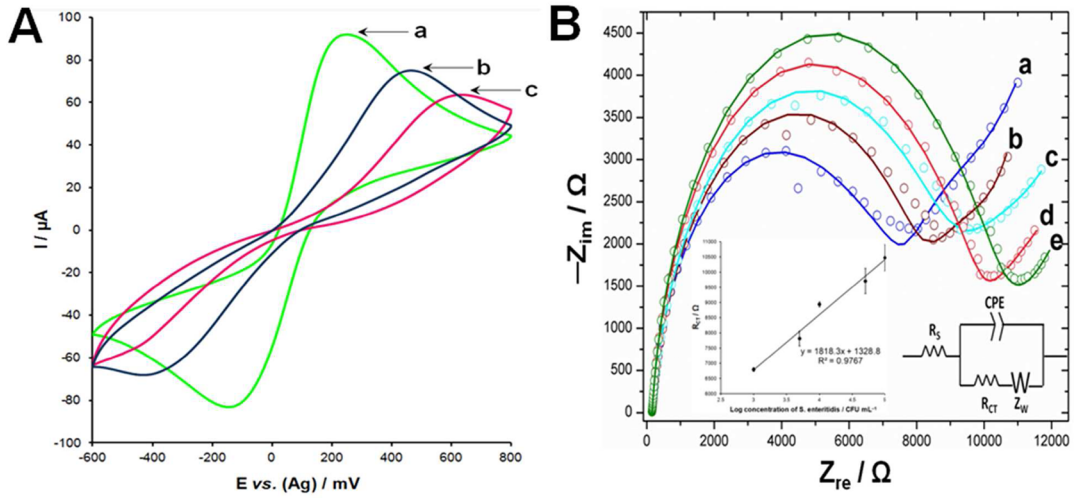

Figure 3. (A) Cyclic voltammograms of the typing impedimetric sensor after each immobilization or binding step. Cyclic voltammograms were recorded at a scan rate of 100 mV s^{-1} (a) with bare SPGE, (b) after self-assembly of the thiolated *S. enteritidis*-specific aptamer, and (c) after backfilling with 0.1 mM 2-mercaptoethanol. (B) Nyquist plot (—Z_{im} vs Z_{re}) of impedance spectra obtained using (a) 1×10^3, (b) 0.5×10^4, (c) 1×10^4, (d) 0.5×10^5, and (e) 1×10^5 CFU mL^{-1} of *S. enteritidis* in Dulbecco's phosphate buffered saline (DPBS). The right inset represents a modified Randles circuit applied to fit to the measured data and consists of the ohmic resistance, R_S, of the electrolyte solution, the electronic charge transfer resistance, R_{CT}, in series with the finite length Warburg *W*, and in parallel with a constant phase element, CPE. The left inset represents a calibration plot of the resistance to charge transfer (R_{CT}) vs log concentration of *S. enteritidis*. The impedance spectra are recorded from 100 kHz to 0.1 Hz, and the amplitude is 0.25 V vs pseudo Ag reference using 20 mM Tris-ClO_4 buffer (pH 8.6), containing 2.5 mM $K_4Fe(CN)_6$ and 2.5 mM $K_3Fe(CN)_6$.

Prior to titration experiments, aliquots containing different concentrations of *S. enteritidis* (1×10^3, 0.5×10^4, 1×10^4, 0.5×10^5, and 1×10^5 CFU mL^{-1}) in 30 μL of Dulbecco's phosphate buffered saline (DPBS, no. D8662, Sigma-Aldrich) were incubated with the aptasensor at 25 °C for 1 h. Electrochemical impedance spectroscopy (EIS) was performed at each concentration (Figure 3B). The complex impedance was presented as the sum of the real *Z*, Z_{re}, and imaginary *Z*, Z_{im}, components that originate mainly from the resistance and capacitance of the cell, respectively. A suitable equivalent circuit, shown in the inset of Figure 3B, was carefully selected to reflect the real electrochemical process and to enable fit producing accurate values. A modified Randles circuit consists of the ohmic resistanc,; R_S, of the electrolyte solution, the electronic charge transfer resistance, R_{CT}, in series with the finite length Warburg *W*, and in parallel with a constant phase element, CPE, associated with the double layer and reflects the interface between the assembled film and the electrolyte solution. The solution resistance, R_S, is the resistance between the aptamer-modified electrode and the reference electrode. The high frequency semicircle of the Nyquist diagram corresponds to the charge transfer resistance, R_{CT}, in parallel with the CPE. The former represents the electron-transfer kinetics of the redox probe at the electrode surface, whereas the latter corresponds to a nonlinear capacitor accounting for the inhomogeneity of the formed film.[21] The diameter of the semicircle corresponds to the interfacial resistance at the electrode surface, the value of which depends on the dielectric and insulating features of the surface layer. On the other hand, the Warburg impedance, Z_W, accounts for a diffusion-limited electrochemical process, presumably due to molecular motions within the film caused by conducting ions penetration.[22] The rationale behind this electrochemical approach is that the binding between the target bacteria and the respective aptamers will further block the charge transfer from a solution-based redox probe to the electrode surface. This could be attributed to the steric hindrance created by the bound bulky bacterial cells.[23] Also, the repulsion between the negatively charged bacterial cell surface and the redox probe anions, $[Fe(CN)_6]^{3-/4-}$, might contribute to the increase of interfacial resistance observed upon bacterial binding to their respective aptamers.[24] Consequently, R_{CT} will become increasingly high and can be used to monitor the binding event.[25,26] It was observed that the R_{CT} value increases linearly with increasing the concentration of *S. enteritidis*, in the range from 1×10^3 to 1×10^5 CFU mL^{-1}, with the regression equation of $y = 1818.3x + 1328.8$ ($R^2 = 0.97670$), where *y* is the R_{CT} value in Ω and *x* is the log concentration of *S. enteritidis* in CFU mL^{-1}, as shown in the inset of Figure 3B and Table S2 in the Supporting Information. The relative standard deviation (RSD) values were between 0.9 and 4.1%. Beyond the upper bacterial concentration, the response became nonlinear, indicating the saturation of the surface with bacteria. The limit of detection (LOD) was 600 CFU mL^{-1}, estimated from $3(S_b/m)$, where S_b is the standard deviation of the measurement signal for the blank and *m* is the slope of the analytical curve in the linear region.[27] In addition, incubation of the developed sensor with 500 CFU mL^{-1} of the bacteria did not cause a significant change in the R_{CT} value compared to the blank.

The sensor's ability to distinguish between *S. enteritidis* and other Salmonella species, namely, *S. typhimurium* and *S. choleraesuis* was confirmed by EIS experiments, as shown in Figure 4. It was observed that incubation of the sensor with 1×10^5 CFU mL^{-1} of *S. typhimurium* caused a 26.2% increase in the R_{CT} value (7648 Ω), whereas incubation with 1×10^5 CFU mL^{-1} of *S. choleraesuis* caused only a 18.1% increase in the R_{CT} value (7370 Ω). Percentages were obtained with reference to incubation with buffer alone (0%, 6751 Ω) and with 1×10^5 CFU mL^{-1} of *S. enteritidis* (100%, 10170 Ω). The specificity of the sensor was also tested using 5.1 mg mL^{-1} human serum albumin (HSA, Sigma-Aldrich), which caused a 20.3% increase in the R_{CT} value (7445 Ω).

This work presents the proof of concept for the first aptamer-based impedimetric sensor for typing of bacteria (AIST-B)

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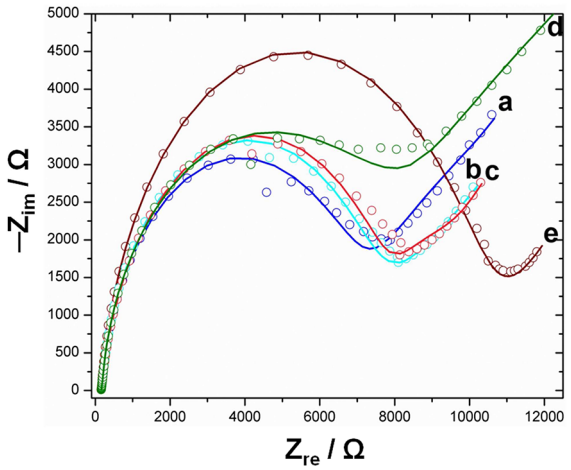

Figure 4. Nyquist plot ($-Z_{im}$ vs Z_{re}) of impedance spectra for selectivity experiments performed using (a) buffer alone, (b) 5.1 mg mL^{-1} HSA, (c) 1×10^5 CFU mL^{-1} of *S. typhimurium*, (d) 1×10^5 CFU mL^{-1} of *S. choleraesuis*, and (e) 1×10^5 CFU mL^{-1} of *S. enteritidis*. The impedance spectra are recorded from 100 kHz to 0.1 Hz, and the amplitude is 0.25 V vs pseudo Ag reference using 20 mM Tris-ClO_4 buffer (pH 8.6), containing 2.5 mM $K_4Fe(CN)_6$ and 2.5 mM $K_3Fe(CN)_6$.

taking advantage of the tunable selectivity of aptamers, which enables the underneath sensor to distinguish between different species of the same bacteria. This work is envisaged to open a new venue for the development of a genus-specific array chip comprising highly specific aptamers for each species, to enable typing of a variety of pathogens. Although there is a long way ahead for establishing such typing format, it is attainable considering the substantial efforts of many researchers to produce highly specific aptamers and the recent advances in selection technologies. Both trends are foreseen to produce a species-specific aptamer with the same ease of analyzing the species genome. Finally, aptamers either alone or coupled with antibiotics were recently reported as highly capable of inhibiting pathogenic bacteria such as *Bacillus cereus*.[28] This trend is recently adopted to overcome the persistent problem of antibiotic resistance. The inhibitory effect of the developed aptamers on *S. enteritidis* is currently under investigation in our laboratories, and further results will be released in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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