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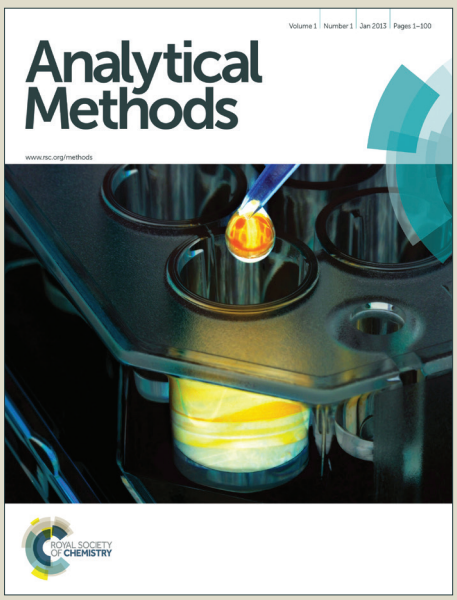

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Cite this: DOI: 10.1039/c0xx00000x

PAPER

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Induction of an electrochemiluminescent sensor for DNA detection of *Clostridium perfringens* based on rolling circle amplification

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Received (in XXX, XXX) Xth XXXXXXXXX 2013, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Clostridium perfringens is one of the predominant pathogens in infectious diseases. This work describes an application of rolling circle amplification (RCA) based electrochemiluminescent sensor for detection of *C. perfringens*. Firstly, the target DNA is captured by the probes on the pretreated electrode. Subsequently, RCA reaction is executed isothermally. The products of RCA are incubated with hemin, resulting to the decrease of the ECL emission which is related to the quantity of the target DNA. The ECL-sensor provides the capability of discriminating the target DNA from non-target sequences even with only one base difference, suggesting an advantageous selectivity. Meanwhile, the lowest concentration of the target DNA is 10^{-15} M, showing a satisfactory sensitivity. Therefore, this strategy combines amplification ability of RCA and high sensitivity of ECL, enables a low fM detection of *C. perfringens* without the bacterial culture.

1. Introduction

C. perfringens is one of the predominant pathogens for infectious diseases. People are at risk of autoinfection if wounded by trauma or major surgery without adequate treatment.[1] *C. perfringens* wound infections are associated with the use of non-sterile elastic outer bandages.[2] Infection by *C. perfringens* can be an unsuspected cause of hemolysis in emergency room patients associated with wound contamination and other tissue infections.[3] *C. perfringens* infection has been reported in a pre-existing hepatic cyst leading to a fatal outcome.[4] *C. perfringens* infection is also found in the brain.[5] A compromised vascular supply may lower the oxidation reduction potential, which allows anaerobic bacteria, such as *C. perfringens*, to cause infection.[6] *C. perfringens* which differed in alpha-toxin plays a key role in systemic intoxication of *clostridium myonecrosis*.[7] Antimicrobials, analgesics and aggressive debridement of necrotic skin and muscle resulted in immediate clinical improvement of the primate.[8] Therefore, the identification and detection of *C. perfringens* is highly important in clinical diagnosis.

Traditional methods for *C. perfringens* determination based on bacteriological culture approaches are considered as the "gold standard", but generally suffer from the disadvantages of time consuming and tedious processes. In recent years, DNA detection has become increasingly important in a wide range of areas. Detection methods of DNA have received particular attention, including real-time PCR,[9] immunochromatographic assay,[10] surface plasmon resonance assay (SPR),[11] and flow-through chemiluminescence microarray.[12] Alpha-toxin is produced by all types of *C. perfringens*. The gene encoding alpha-toxin had been PCR amplified using specific primers.[13] The DNA microarray method is developed to identify the presence of toxin genes in C. *perfringens* too.[14] However, these methods generally require highly precise thermal equipment and suffer from loss of amplification specificity. Thus, it is necessary to develop a precise diagnostic method to provide sensitive and specific *C. perfringens* detection.

Lately improved rolling circle amplification (RCA) shows more improvement, and thus are widely used in biosensor fabrication.[15] Recently, the flourishing electrogenerated chemiluminescence (ECL) based sensors has attracted increasing interest due to its advantages in terms of simplicity, low background noise, high sensitivity and good reproducibility.[16] However, to our knowledge, no reports focus on sensitive detection of RCA products using the ECL-sensor for detection of *C. perfringens*.

In this work, we report a highly sensitive strategy for ECL detection of DNA to *C. perfringens* (alpha toxin gene, Genbank

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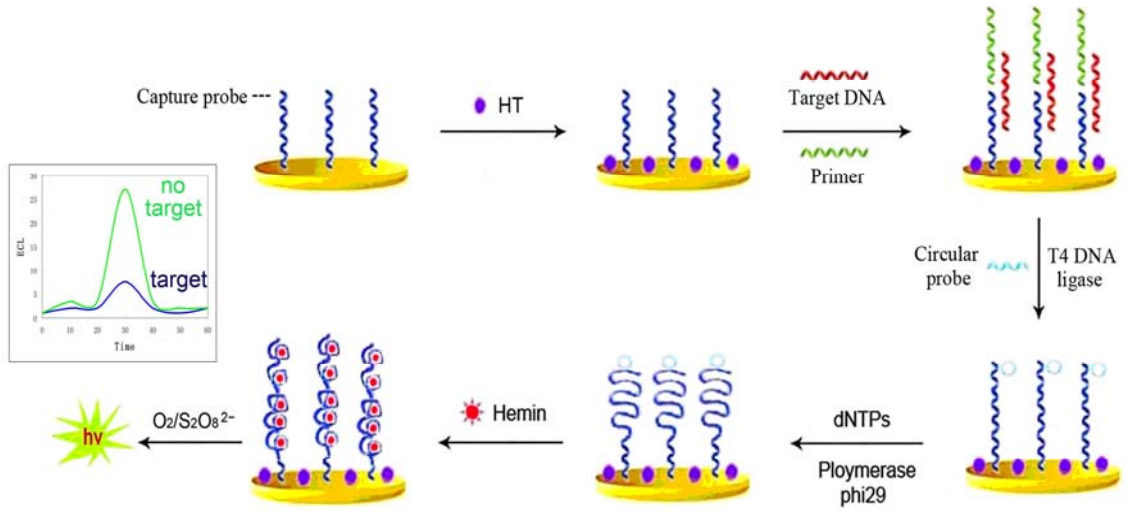

Scheme 1 The scheme of the ECL-sensor for detection of *C. perfringens* DNA based on RCA.

No: AB794298.1). Our intention is to couple the RCA for signal amplification and the ECL for signal detection. Firstly, the capture probe is cast onto the pretreated electrode. Then target DNA is catched by the capture probe on the surface of electrode. Subsequently, RCA reaction is executed isothermally in the presence of primer and circular probe. Then the products of RCA are incubated with hemin to form hemin/G-quadruplex DNAzymes, which lead to the consumption of oxygen and results in the decrease of the ECL emission in detection buffer. At last, the quenching effect in ECL intension toward $O_2/S_2O_8^{2-}$ system is obtained, thus be related to the quantity of the *C. perfringens* target DNA (Scheme 1).

2. Experimental

2.1. Materials and reagents

Hexylthiol (HT), T4 DNA ligase, and phi29 polymerase are purchased from New England (New England Biolabs Co., USA). A 10 mM PBS (pH 7.4) is used as washing buffer. The ECL measurement is performed in 10 mM PBS (pH 7.4) containing 0.1 M $K_2S_2O_8$ and 0.1 M KCl. DNA extraction reagents are provided by Tiangeng (Tiangeng Co., China). The oligonucleotides (listed in Table 1) are synthesized by Takara (Takara Co., China). All other reagents are analytical grade and prepared using Milli-Q Academic water (⩾18 MΩ) (Millipore Co., France).

2.2. Fabrication and preliminary test of the ECL-sensor

Before the coating, the CHI 101 gold electrode (CH Instruments Inc., China) is polished with 0.3 and 0.05 μm aluminum slurry and sonicated sequentially in ethanol and ultrapure water for 5 min each. Then the electrodes are electrochemically cleaned in 0.5 M H_2SO_4 with potential scanning from 0.2 to 1.6 V until a remarkable voltammetry peak is obtained, followed by sonication again and drying with nitrogen. A droplet of 10 μL capture probe (1 μM) is modified onto the pretreated electrode and incubated overnight at room temperature in humidity. Then the surface of the electrode is rinsed with deionized water and blocked with 1.0 $mg.ml^{-1}$ HT for 2 h to block the residual gold activesites. Electrochemical impedance spectroscopy (EIS) is measured after every processing step to check the effect of the ECL-sensor preparation. EIS experiment is performed under an oscillation potential of 5mV over the frequency range of 0.1Hz to 100 KHz and in the solution of 5 mM $K_4[Fe(CN)_6]$ /$K_3[Fe(CN)_6]$, 10 mM PBS (pH 7.4) .

After washing with PBS (pH 7.4), the modified ECL-sensor is soaked in 50 μL target DNA at 37 °C for 20 min. Next, the ligation reaction is performed in the condition of 1 μM primer, 1 μM circular probe and 5 U T4 DNA ligase in 1× ligation buffer at 37 °C for 1 h. After that, a droplet of 10 μL RCA reaction mixture (0.5 U phi29 DNA polymerase and 500 μM dNTP) is placed on the ECL-sensor and incubated for 2 h at 37 °C. Then, a droplet of 10 μL hemin (1 mM) is casted onto the ECL-sensor and incubated for 1 h at 37 °C. Finally, the ECL emission is

Table 1. Sequences of oligonucleotides used in the assay

Oligonucleotide	Sequence (5'→3')
capture probe	SH-$(CH_2)_3$ -TTTTTTTTAGTTTCCTCTTTG
primer	CCATTCTAATCTAGTTTTTCCTCCGTCCTCCTACGATGCG
circular probe	CCATTCTAATCTAGCCGTAGTAGAATGAAGATAGCGCATCGTAGGAGGACGG AGGATGATGGGTATGGGAATACAGG
target DNA (*C. perfringens* alpha-toxin gene)	CTAGATATGAATGGCAAAGAGGAAACTA
single-base mismatch sequence (sDNA)*	CTAGATATGAATGGTAAAGAGGAAACTA
non-complementary sequence (nDNA)	GCCATGTCAGCACTGGCCAGTCGTCGCGC

* Only one base difference compares to target DNA (C→T)

monitored by a MPI-A electrochemiluminescence analyzer (Xi'an Remax Electronic Science and Technology Co., China).

2.3. Optimizing conditions of the ECL-sensor

Temperature of hybridization between capture probe and target DNA has greatly influence on the test results. So we focus on the hybridization temperature in this paper. The hybridization temperature (37, 40, 43, 46, 49, 52 and 55 °C) of capture probe and target DNA is investigated to ensure successful amplification. RCA reaction conditions, including temperature, pH and ionic strength, had been deep studied and multiple optimized. The appropriate parameters are cited from reference.[17] The primer and circular probe are 1 μM, and *C. perfringens* alpha-toxin target DNA is 1.0×10^{-12} M. Different RCA durations (20, 40, 60, 80 and 100 min) are tested for the effects on signal amplification. The emission of ECL is monitored by a MPI-A electrochemiluminescence analyzer.

2.4. Selectivity of the E-sensor

The selectivity of the proposed gene sensor is evaluated by using the complementary, single-base mismatched (sDNA) and non-complementary sequence (nDNA) as initiators, respectively. The effect of base mismatch is studied by 1.0×10^{-12} M *C. perfringens* alpha-toxin target DNA, compared with 1.0×10^{-10} M sDNA and nDNA (100 fold of target DNA). Corresponding control experiments (in the absence of target DNA) are performed under the same detection conditions as negative control.

2.5. Sensitivity of the ECL-sensor

The solution of *C. perfringens* alpha-toxin gene target DNA is diluted into series solutions (from 1×10^{-15} to 1×10^{-9} M). Then 10 μL of every diluted solution is subjected to probe catch, RCA amplification and ECL analysis. ECL measurements are performed in the electrolyte of phosphate buffer solution (pH 7.4) containing 100 mM $S_2O_8^{2-}$.

2.6. Statistical analysis

The curves are drawing in OriginPro 7.5 software (OriginLab Co., USA). Data regarding the selectivity and sensitivity of the ECL-sensor are compared with respect to the control group using a t-test and regression analysis, which is performed by SPSS 16.0 software (SPSS Inc., USA).

3. Results and discussion

3.1. Feasibility of the ECL-sensor

The feasibility of the ECL-sensor successfully proceeds of RCA, and generation of hemin/G-quadruplex DNAzymes is verified by EIS and ECL detection (Fig. 1). The effects of the ECL-sensor preparation are checked by EIS after every processing step. The EIS of bare SPE gold electrode is shown in Fig. 1a. The capture probe is modified on the surface of the electrode. Due to the depressing of electrochemical diffusion on the surface of electrode, the impedance is increased. After the blocking of HT, the impedance increases furthermore. The preliminary test of the ECL-sensor is shown in Fig. 1b. After hybridization of target DNA and reaction of RCA, the ECL intensity decreases significantly due to the production of numerous tandem repeats containing G-quadruplex sequences, the intercalation of abundant hemin into the DNA strand to form hemin/G-quadruplex DNAzymes which competitively consume the dissolved oxygen in detection buffer and thus quench the ECL emission which originated from the reaction between oxygen and $S_2O_8^{2-}$. Based on the observation of changes in EIS and ECL, we preliminary believe that modify of the ECL-sensor is successful.

3.2. Optimization condition of the ECL-sensor

Hybridization temperature mainly has great influence on the effect of DNA capture. Similarly, temperature of RCA reaction has mainly affects on the efficiency of DNA amplification. Hybrid time is usually slightly longer than requirement, so as to complete the hybridization effectively. The sensitivity of amplitude and the strength of signal depend on the time of RCA

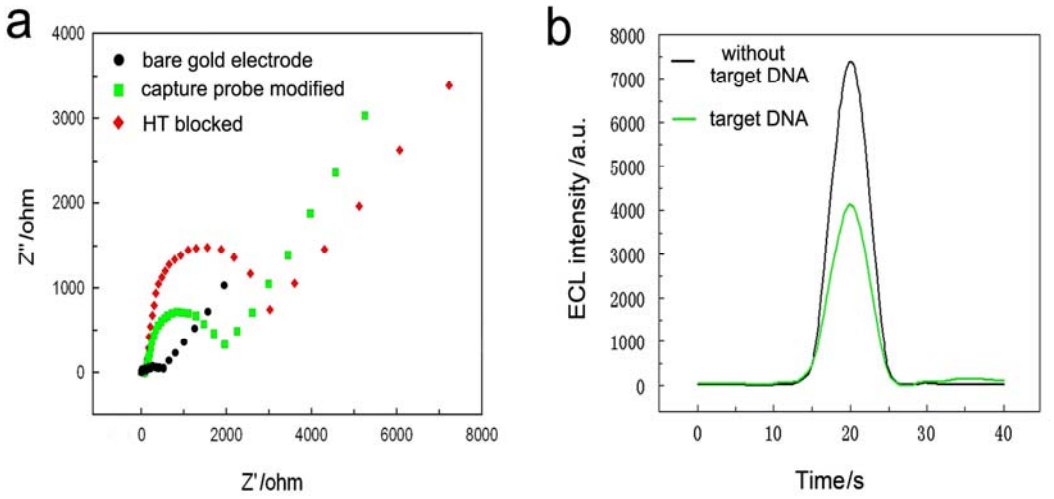

Fig. 1 Feasibility of the ECL-sensor. a: The EIS measurements of the modified gold electrode. b: The preliminary test of the ECL-sensor to *C. perfringens* target DNA (1.0×10^{-12} M)

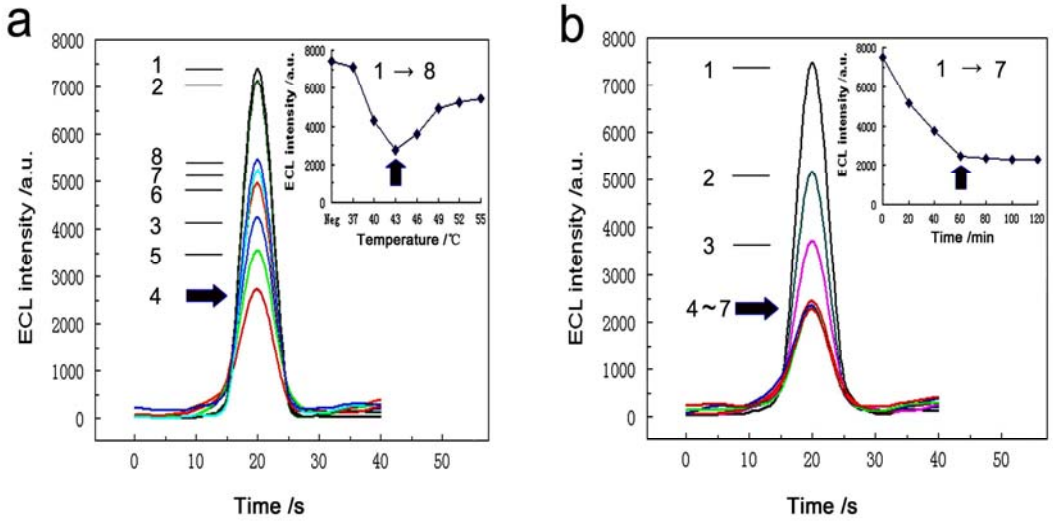

Fig. 2 Optimization condition of the ECL-sensor. a: ECL tests of different hybridization temperature between capture probe and target DNA. b: ECL tests of durations for RCA amplification. (*C. perfringens* target DNA: 1.0×10^{-12} M.)

reaction. They are relatively independent, but also influence each other. In this paper, the temperature of hybridization between capture probe and target DNA, reaction time of RCA are considered the key parameters being tested.

Hybridization temperatures and RCA durations are investigated to ensure successful amplification (Fig. 2). A series of hybridization temperatures (37, 40, 43, 46, 49, 52 and 55 °C) are evaluated in Fig. 2a. The results suggested that the decline of ECL signal intensity is most noticeable at 43 °C, while the signal intensity of negative control is changed scarcely. The hybridization and capture reaction are proceeded more completely in 43°C may be the main reason of this phenomenon. So 43 °C is chosen as the optimized hybridization temperature for subsequent experiments.

Different RCA durations (0, 20, 40, 60, 80, 100 and 120 min) are tested for the effects on signal amplification (Fig. 2b). It is found that the ECL readout decreased rapidly with the RCA reaction time up to 60 min. However, the signal exhibited no further significant decrease when the reaction duration went beyond 60 min. It may be attributed to the fact that when the reaction duration increases to more than 60 min, the RCA products are entangled with each other, which can hinder the extension of RCA product. Therefore, 60 min is selected as the optimum RCA time in the succeeding experiments.

3.3. Specificity of the ECL-sensor

The selectivity of the proposed gene sensor is evaluated by using the complementary, sDNA and nDNA as initiators, respectively. As is shown in Fig. 3, the electrochemical signals of single-base mismatched DNA sequences are significantly weaker than those of the complementary sequences ($p<0.01$), indicating the effective selectivity of the improved method. The result demonstrates that the ligation reaction and RCA is highly specific for single-base discrimination. There is no significant difference in the ECL responses between the groups of sDNA, nDNA, and negative control. Thus, the RCA and ECL system has provided the capability of discriminating the complementary target DNA sequences from non-target sequences even with only one base difference upon DNA hybridization, suggesting advantageous selectivity of our protocol.

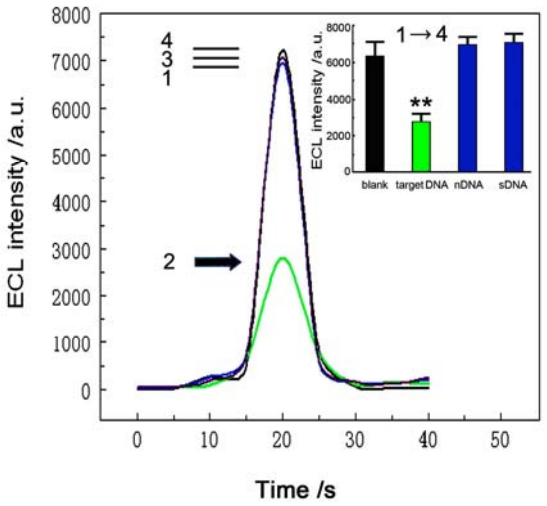

Fig. 3 Selectivity of the ECL-sensor. 1) blank; 2) target DNA (10^{-12} M); 3) nDNA (10^{-10} M); and 4) sDNA (10^{-10} M).

3.4. Sensitivity of the ECL-sensor

With the above optimizations, the relationship between the ECL-sensor response and the concentration of *C. perfringens* target DNA is investigated. As showed in Fig. 4, there is a good linear correlation between the current and the amount of *C. perfringens* target DNA in the concentration range from 10^{-15} to 10^{-9} M. The correlation equation is $y=-939.29 \times \log(x) +7889.3$ (y: ECL intensity; x: concentration of target DNA) with a good correlation coefficient ($R^2=0.9903$). The lowest concentration of *C. perfringens* target DNA which can be successfully amplified is 10^{-15} M, showing a superior sensitivity of the ECL-sensor. Therefore, the ECL-sensor has a high sensitivity, associated with a reliable correlation equation which could be served as the basic standard curve for the quantitative detection of *C. perfringens*

target DNA.

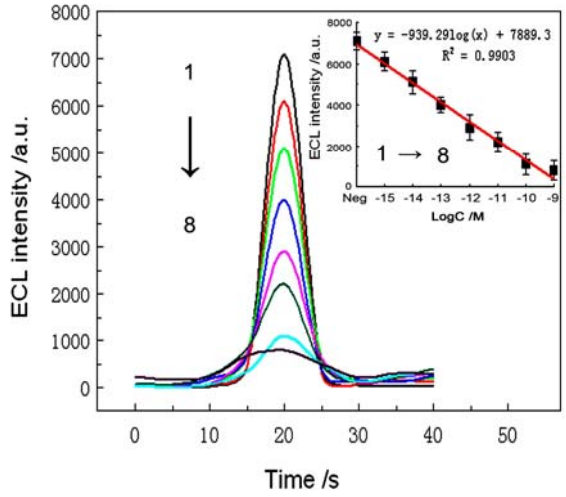

Fig. 4 Sensitivity of the ECL-sensor (error bars=SD, n=5). Target DNA: 1) neg; 2) 10^{-15} M; 3) 10^{-14} M; 4) 10^{-13} M; 5) 10^{-12} M; 6) 10^{-11} M; 7) 10^{-10} M; and 8) 10^{-9} M.

4. Conclusion

In summary, we have demonstrated a highly sensitive ECL-sensor for detecting *C. perfringens* specific DNA. Our approach relies on the RCA reaction for signal amplification and the formation of hemin/G-quadruplex DNAzymes which show a significant ECL quenching effect towards $O_2/S_2O_8^{2-}$ system. The electrochemical signals of target DNA are significantly reduced than the blank group ($p<0.01$), indicating the excellent selectivity of the improved method. There is a good linear correlation between the current and the amount of *C. perfringens* target DNA in the concentration range from 10^{-15} to 10^{-9} M. The lowest concentration of the *C. perfringens* target DNA which can be successfully amplified is 10^{-15} M, showing a good sensitivity of the ECL-sensor. Therefore, the ECL-sensor has a high sensitivity, associates with a fair correlation equation which could be served as the basic standard curve for the quantitative detection of *C. perfringens* target DNA.

Acknowledgments

The authors are grateful for financial support from the National Natural Science Foundation of China (NSFC 81371898). We appreciate the constructive comments from all members of our laboratories.

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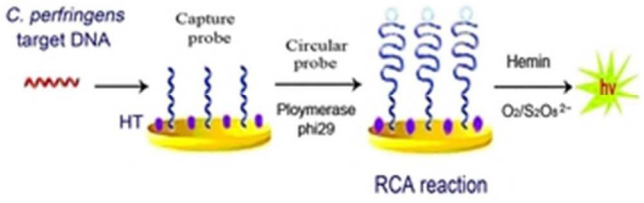

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