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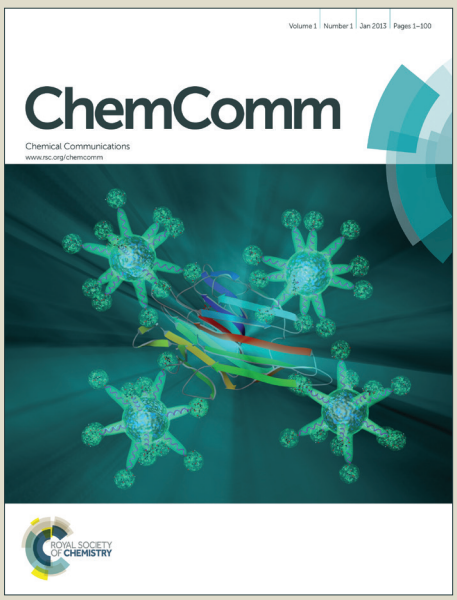

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Target-Aptamer Binding Trigged Quadratic Recycling Amplification for Highly Specific and Ultrasensitive Detection of Antibiotics at Attomole Level

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Hongzhi Wang[a], Yu Wang[b], Su Liu[c], Jinghua Yu[a], Wei Xu[a], Yuna Guo[a], Jiadong Huang*[a,b]

A novel electrochemical aptasensor for ultrasensitive detection of antibiotics by combination polymerase-assisted target recycling amplification with strand displacement amplification with the help of polymerase and nicking endonuclease has been reported. This work is the first time that target-aptamer binding trigged quadratic recycling amplification has been utilized for electrochemical detection of antibiotics.

Antibiotics, as prophylactic agents, are widely used to kill or inhibit microorganisms in modern medicine.[1] However, accumulation of antibiotics in food-producing animals and foods has been implicated in the occurrence of resistant bacterial strains, resulting in various types of side effects in humans and the appearance of super bacteria with tolerance to antibiotics.[2] In the European Union (EU), it is estimated that 25,000 persons die every year from infections with antibiotic-resistant microorganisms.[3] Therefore, it is of great significance and urgent need to develop highly specific and sensitive methods for the determination of trace amounts of antibiotics in foodstuffs and in environmental sample.

Current available determination methods for residue levels of antibiotics mainly include high-pressure liquid chromatography (HPLC),[4a] gas chromatography (GC),[4b] liquid chromatography-tandem mass spectrometry (LC-MS/MS),[4c] capillary electrophoresis (CE),[4d] and mass spectrometry (MS).[4e]Despite their widespread applications, these methods are usually time-consuming and cumbersome, and require laborious pretreatments of samples, sophisticated instrumentation, and trained technical personnel. To circumvent these problems mentioned above, antibody-based immunoassay methods such as enzyme-linked immunosorbent assay (ELISA) have been reported for antibiotics detection.[5] Although these immunoassays possess the advantages of simplicity and high-throughput screening ability, the requirements for tedious culture and wash steps and heterogeneous reactions in these assays will result in nonuniform antibody-binding properties and decreased antibody activity, thus compromising the analytical sensitivity and reproducibility. Alternatively, aptamers, which exhibit distinct advantages over antibodies involving small size, low cost, high chemically stability, remarkable flexibility and convenience in the design of their structures, have considerable potential for resolving above issues effectively and create great opportunities to develop novel biosensors with high selectivity and sensitivity.[6] By the utilization of aptamer as recognition element, various techniques have been developed to identify and quantify antibiotics, including electrochemical method,[7a] photoelectrochemical method,[7b] and UV/visible[7c] or fluorescence[7d]-based assays. Among these techniques, electrochemical-based assays are particularly attractive for realizing antibiotics determination due to its high portability and affordability, and low power requirement.[8]

To meet the requirement of trace determination of antibiotics in foodstuffs and in environmental samples, it is highly desirable for the development of electrochemical biosensor capable of amplified detection of target analyte. It has been generally recognized that, isothermal enzyme-assisted amplification of recognition events is a powerful component for the improvement of the sensitivity of bioanalysis.[9] Herein, a novel electrochemical biosensing strategy for highly selective and ultrasensitive detection of antibiotics by the combination polymerase-catalyzed target recycling amplification with strand displacement amplification via the property of polymerase and nicking activity of restriction endonuclease has been reported.

Fig. 1 illustrates the analytical principle of the electrochemical biosensing strategy for sensitive detection of antibiotics based on target-aptamer binding trigged quadratic recycling amplification,[10] and ampicillin (AMP) is selected as a model analyte. Our reaction system consists of a programmed hairpin probe (PHP), a methylene blue-labelled probe (MBP, denoted in green in Fig. 1), phi29 DNA

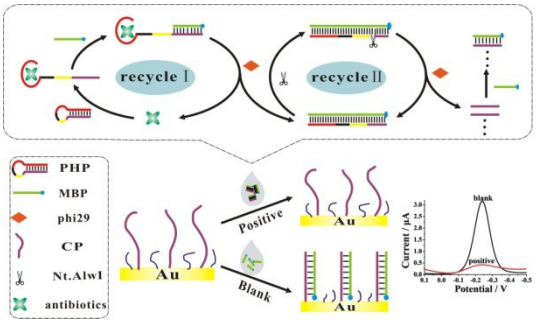

Fig. 1 Schematic illustration of antibiotics assay using target-aptamer binding triggered quadratic recycling amplification strategy.

polymerase and nicking endonuclease *Nt.AlwI*. The PHP contains an anti-AMP aptamer (denoted in red in Fig. 1), a specific recognition sequence of *Nt.AlwI* (denoted in yellow in Fig. 1), and a nucleic acid segment complementary to 5' end of aptamer (denoted in violet in Fig. 1). When target AMP is added to the reaction solution, the specific binding of PHP to AMP triggers its conformational change. Then MBP associates with the opened PHP and functions as a primer to initiate an extension reaction and replicates the template part in the PHP in the presence of polymerase and dNTPs, in which AMP is displaced. A new such cycle is initiated when the displaced AMP combines to a second PHP. As a result, AMP is recycled and a new PHP-templated DNA duplex is synthesized at each new cycle (named recycle Ⅰ). Meanwhile, as a recognition sequence for *Nt.AlwI* is generated in DNA duplex produced from the first cycle, *Nt.AlwI* can nick the DNA duplex to release a short ssDNA fragment, which initiates a new extension cycle. Cleavage reaction, replication reaction and strand-displacement reaction are carried out repeatedly, generating a large amount of short ssDNA products (named recycle Ⅱ). These products then hybridize with MBP to form complementary DNA, which eliminates the probability of being captured on electrode, thus resulting in only a negligible electrochemical signal. In contrast, in the absence of target AMP, the PHP is in a stabilized stem-loop structure and coexisted with MBP in solution. Following its capture on electrode, a remarkably strong electrochemical signal can be obtained. In general, each target molecule can successfully afford a high quadratic amplified signal via polymerase-assisted target recycling amplification and strand displacement amplification with the help of polymerase and nicking

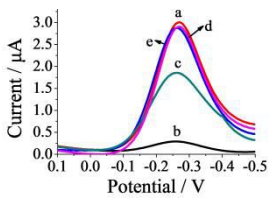

Fig. 2 Typical DPV responses of target-triggered quadratic recycling amplification via the exposure of the modified electrode to blank sample (a), positive sample (10 nM AMP, b), 10 nM AMP without *Nt.AlwI* (c), 10 nM AMP without phi29 (d), 1μM penicillin in place of AMP (e).

endonuclease. Hence, the proposed cascade enzymatic amplification approach holds great potential for offering an ultrahigh sensitivity for the determination of antibiotics.

To verify the feasibility of designed target-aptamer binding trigged quadratic recycling amplification strategy for AMP assay, different differential pulse voltammograms (DPV) obtained upon quantifying AMP,and those obtained in a series of control experiments were depicted in Fig. 2. As shown in Fig. 2a, a significantly strong DPV peak at around -0.25 V was obtained for blank sample, indicating a substantial number of MBP combined to the capture probe (CP) on electrode. In contrast, it was found that a negligible DPV peak was achieved in the presence of AMP, suggesting almost no MBP was captured on electrode surface (curve b). This clearly revealed that AMP triggered enzymatic amplification reaction with the aid of phi29 and *Nt.AlwI*,and produced abundant ssDNA fragments complementary to MBP, thus very few MBP was captured on electrode. For the proof-of-concept experiment, we performed other control experiments differing in enzymes. In the presence of MBP, phi29 and AMP, a moderate DPV peak appeared in the DPV curves, which was attributed that each AMP could interact with many of PHP by phi29-induced target recycling (curve c). This implied the signal amplification obviously relied on nicking endonuclease, which confirmed the amplification mode of our proposed assay is quadratic. In the presence of MBP, *Nt.AlwI* and AMP, there was an extremely strong DPV peak similar to that of blank sample in the DPV curves (curve d). This signified that the target-recycling amplification was dependent on the phi29-catalyzed extension reaction, whereas the greater extent amplification relied on strand displacement amplification with the help of phi29 and *Nt.AlwI*. Furthermore, a very strong DPV peak was observed in the presence of phi29, *Nt.AlwI*, and a nontarget antibiotic penicillin instead of AMP, demonstrating the significantly decreased DPV signal was induced by the specific recognition of AMP and PHP (curve e). On the basis of these results, it was reasonably concluded that our designed route should be feasible for AMP assay.

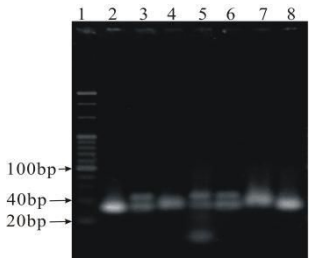

Fig. 3 Gel electrophoresis image of target-triggered quadratic recycling amplification. Lane 1, DNA marker; Lane 2, PHP; Lane 3, PHP incubated with MBP and AMP; Lane 4, blank sample; Lane 5, positive sample; Lane 6, PHP incubated with MBP, phi29, and AMP; Lane 7, PHP incubated with MBP and phi29; Lane 8, PHP incubated with MBP, phi29, *Nt.AlwI* and penicillin.

More direct proof of the biosensor mechanism could be acquired through gel electrophoresis analysis, as shown in Fig. 3. Lane 2 displayed a bright band of our designed PHP on the image. After incubation with AMP and MBP, a new band with high molecular

weight appeared except for PHP, indicating the binding of AMP and PHP resulted in its structure switch and MBP could associate with the opened PHP (lane 3). As seen from lane 4, the PHP gave a band of the same mobility with that of lane 1 for blank sample. In contrast, the brightness of the band at the position of PHP was significantly weaker, and two new bands were observed on the lane 5 for positive sample. The top band demonstrated the hybridized MBP with PHP could prime the polymerization reaction in the presence of phi29, while the band at the bottom indicated the repeated process of cleavage reaction, replication reaction and strand-displacement reaction could produce numerous short ssDNA fragments which immediately formed DNA duplex via association with MBP. In addition, after incubation with AMP, MBP and phi29, we observed two bands similar to the upper two bands at lane 5 appeared on the image (lane 6). However, there was only a band characteristic for PHP after the treatment of MBP and phi29 on the lane 7. This further revealed not only the combination of free MBP with PHP but phi29-catalyzed polymerization reaction was induced by AMP-mediated structure change of PHP. Furthermore, an additional control test was performed by replacing AMP with penicillin in the presence of PHP, MBP, phi29 and *Nt.AlwI*. It was found that a bright band of the same mobility with that of PHP appeared on the lane 8. These results suggested only the addition of target AMP could result in the structure switch of PHP and trigger the quadratic recycling amplification reaction with the help of phi29 and *Nt.AlwI*. The corresponding fluorescence spectra analyzed by fluorescence measurements could further verify the above results (Fig. S1, ESI).

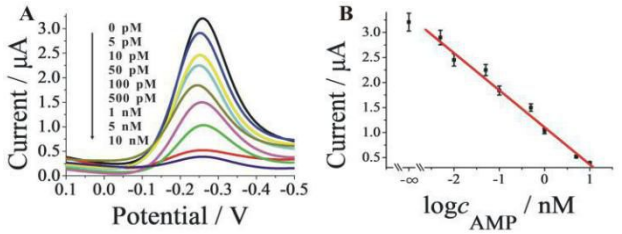

Fig. 4 (A) Typical DPV responses of the biosensor to different concentrations of AMP; (B) The calibration curve of DPV peak current versus the logarithm of AMP with various concentrations. Error bars are standard deviations across four repetitive experiments.

To acquire an optimal analytical performance of quadratic recycling amplification-based sensing strategy, some experimental conditions including MBP concentration (10 μM), reaction time for the isothermal amplification (2 h), and conjugate time for free MBP to CP on electrode (2 h), were evaluated in detail by DPV measurements (Fig. S1 and S2, ESI). Under the optimal conditions, the sensitivity and the dynamic range of the proposed method was investigated towards AMP at various concentrations. As seen from Fig. 4A, the peak current decreased with increasing AMP concentration from 0 to 10 nM. A linear dependence between the peak current intensity and the logarithm of AMP concentration was obtained in the range from 5 pM to 10 nM, that was, over 3 orders of magnitude (Fig. 4B). The linear regression equation was I (10^{-6}A) = 1.11 - 0.741 × lg (C_{AMP} /nM) with a correlation coefficient of -0.991. The limit of detection (LOD) was calculated to be 1.09 pM (or 21.8 amol) in terms of the rule of 3 times standard deviation over the blank response. Furthermore, the developed approach showed very desirable reproducibility, which was attributed that both the target recognition and signal amplification was performed through the homogeneous assay format. The relative standard deviations (RSDs) of peak intensities at -0.25 V were 3.09%, 3.23%, 3.21%, and 3.15%, respectively, in four repetitive assay of 10 pM, 100 pM, 1 nM, and 10 nM of AMP. These data manifested the developed approach held great potential for quantitative assay of AMP with excellent sensitivity and reproducibility. Besides, the analytical performance of our method for quantitative assay of AMP was compared with that of some earlier reported methods. The results were shown in Tab. 1, ESI. It was found that the sensitivity of our biosensor improved at least by 2 orders of magnitude as compared to the existing methods[5a, 7a, 7c, 7d, 9] for AMP assay.

The selectivity of our method for AMP assay was also examined by the addition of three interfering substances including penicillin, amoxicillin and benzylpencillin. As shown in Fig. S3, ESI, the current variation in the presence of the interferences were all less than 4%, demonstrating the excellent selectivity towards AMP of the proposed method. To further validate the universality of this biosensor, the quantitative assay of spiked milk samples was considered. Different concentrations of AMP were spiked into the milk samples and measured directly without any pretreatment except for dilution with a buffer solution. The typical DPV responses of the biosensor to the different spiked milk samples were shown in Fig. S5, ESI. The spiked samples were also detected by a classic ELISA method and compared with the results obtained by our method. The results were shown in Tab. 2. It was observed that these data obtained using our method was in good agreement with those obtained via the ELISA method, and the discrepancies between the two methods were all smaller that 13%. This clearly revealed the proposed approach might hold a great promise for real sample analysis with great accuracy and reliability.

In summary, we have developed a high specific and ultrasensitive electrochemical aptasensor by coupling target-triggered structure change of programmable hairpin probe with isothermal signal amplification strategy. To the best of our knowledge, this work is the first time that target-aptamer binding trigged quadratic recycling amplification has been utilized for electrochemical detection of antibiotics. With the signal amplification strategy, the resulting electrochemical aptasensor can detect antibiotics AMP with excellent sensitivity, and the LOD can be as low as attomole level, which represents at least 100-fold improvement compared to the existing methods (e.g., electrochemical microfluidic assay[7a]). Moreover, our biosensing strategy offers the advantage of facilitated instrumentation, shortened analysis time, and simplified operations without the need of sample pretreatment and multiple washing steps. Hence, this biosensing strategy might provide a simple, rapid, and cost-effective electrochemical method for the determination of antibiotics in foodstuffs with high specificity and sensitivity. Additionally, the proposed strategy holds the potential of being extended for the detection of aptamer binding molecules and combined with other detection tools such as fluorescent assay. This

biosensor may create a versatile platform in detecting molecules with trace amounts in bioanalysis and molecular diagnosis.

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Notes and references

[a]*Key Laboratory of Chemical Sensing & Analysis in Universities of Shandong, School of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, P.R. China.*
[b]*College of Biological Sciences and Technology, University of Jinan, Jinan 250022, P.R. China*
[c]*College of Resources and Environment, University of Jinan, Jinan 250022, P.R. China*

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