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Electronic Supporting Information

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

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EXPERIMENTAL SECTION

Reagents and Materials. Ampicillin **(**AMP), penicillin, amoxicillin, benzylpencillin and 6-Mercapto-l-hexanol (MCH) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Phi29 DNA polymerase, deoxyribonucleoside triphosphates (dNTPs), and nicking endonuclease *Nt.AlwI* were obtained from New England Biolabs Inc. (Beijing, China). Agarose G-10, 20-bp DNA ladder marker and 10 × TAE buffer were from Sangon Biotech Co., Ltd. (Shanghai, China). SYBR Green Ⅰ was obtained from Aidlab Biotechnologies Co., Ltd. (Beijing, China). All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). All solutions were prepared using ultrapure water with an electric resistance >18.25 MΩ, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA). Oligonucleotides used in this work were synthesized from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The programmed hairpin probe

(PHP) had the sequence: 5' ***GCG GGC GGT TGT ATA GCG G***TT TTT TT**G GAT CTT TT** ↓ G TTC GCC CGC 3', where the short underlined sequences were complementary to each other to make the probe form a hairpin structure. The italic bold letters were the sequence of the anti-AMP aptamer. The GGATC sequence highlighted in red was the recognition site for Nt.AlwI, and the arrow indicated the nicking position. The methylene blue-labelled probe (MBP) had the sequence: 5' MB-GCG GGC GAA C 3'. The capture probe had the sequence: 5' GTT CGC CCG C-SH 3'.

Gold electrode treatment and the capture probe immobilization. Prior to the experiment, the working gold electrodes were cleaned by immersing in freshly-prepared piranha solution (H_2O_2/H_2SO_4 1/3 in volume) for 2 h three times and then rinsed with ultrapure water. Subsequently, the electrodes were polished on a microcloth with 0.05 μm alumina suspension, followed by sonication in ultrapure water, ethanol and ultrapure water, respectively. Finally, the electrodes were again rinsed thoroughly with ultrapure water and dried under nitrogen stream.

The immobilization of the capture probe on gold electrode was performed by dropping 10 μL of the capture probe solution (50 μM) on the surface of gold electrode. The self-assembly was allowed to proceed for 3 h at 37 ℃. Then the electrodes were rinsed using 10 mM PBS (pH 7.4) containing 0.14 M NaCl to remove the weakly adsorption of the capture probe. After that, these electrodes were then immersed into 2 mM MCH solution for 2 h to block the unoccupied sites of the electrode and eliminate the nonspecific adsorption effect. Finally, the resulting electrodes were thoroughly rinsed with PBS and stored at 4 ℃ for ready.

Target-triggered quadratic recycling amplification reaction. A 2 μL aliquot of 10 μM PHP was added in 16 μL reaction buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 μM MBP, 10 U Phi29 DNA polymerase, 10 mM dNTPs, and 10 U *Nt.AlwI*. Sample solution (2 μL) of AMP (the final concentrations ranging from 0 pM to 10 nM) was then added in the buffer followed by incubation at 37 ℃ for 2 h to perform the enzymatic reactions. Then the resulting mixture was immersed in hot water bath at 65 ℃ for 10 min to inactivate the enzyme. Subsequently, 10 μL of the above mixture was dropped on the surface of the capture probe-modified electrode and

incubated in a humid atmosphere at 37 ℃ for 2 h. Finally, the electrode was thoroughly rinsed with PBS for three times. Differential pulse voltammetry (DPV) measurements were carried out using a three-electrode system consisting of a Ag/AgCl reference electrode, a platinum wire auxiliary electrode, and the modified working electrode. DPV was performed on a CHI 660D electrochemical workstation (Shanghai CH Instruments, China). All electrochemical data were recorded in 10 mM PBS (containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4) at room temperature. DPV was recorded within the potential range from 0.1 to -0.5 V under modulation amplitude of 50 mV and a scan rate of 50 mV/s with a step potential of 0.83 mV.

Fluorescence measurements of target-triggered quadratic recycling amplification. A 2 μL aliquot of 10 μM PHP was added in 16 μL reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM MBP, 10 U phi29 DNA polymerase, 10 mM dNTPs, 10 U *Nt.AlwI*, and AMP of a given concentration (0 and 10 nM). The mixture was incubated at 37 ℃ for 2 h to allow complete enzymatic reaction followed by the incubation at 65 ℃ for 10 min to inactivate the enzyme. Then, 1 μL SYBR Green Ⅰ (100×) was added to the mixture and incubated at room temperature for 5 min. The resulting solution was immediately subjected to the fluorescence measurements. The fluorescence spectra were measured in a 200 μL quartz cuvette on an RF-5301PC spectrofluorometer (Japan). The excitation wavelength was 488 nm and the emission wavelength was in the range from 500 nm to 650 nm with excitation slit of 3 nm and emission slit of 5 nm.

Gel electrophoresis analysis of target-triggered quadratic recycling amplification. The gel electrophoresis was performed at 120 V for 45 min using prepared samples (5 μL per well) on a 4% (w/w) agarose containing 1% (w/w) ethidium bromide in 50 mM Tris-borate running buffer (pH 7.9) containing 2 mM EDTA. After electrophoresis, the gel was visualized using using a Bio-Rad Gel imaging system (Bio-Rad, USA).

Real sample analysis. Milk was bought from a local supermarket. 2 mL of the milk was added to 8 mL of 10 mM PBS (pH 7.4) and completely mixed for 10 min. Then different concentrations of AMP were spiked into the

diluted milk samples and detected directly without any pretreatments. The spiked samples were further quantified by a classic ELISA method and compared with results obtained with our electrochemical methods.

RESULTS AND DISCUSSION

Fluorescence measurements of target-triggered quadratic recycling amplification. To further confirm the above results, we carried out the fluorescence measurements under different conditions, as shown in Fig. S1. A relatively weak fluorescence response with the characteristic emission peak of 522 nm in typical fluorescence spectra for PHP was observed, demonstrating that PHP was folded into a hairpin structure and stained effectively by SYBR Green I (curve a). After PHP reacted with AMP, an obviously diminished fluorescence signal was obtained, which indicated the hairpin structure of a small amount of PHP was unfolded because of the binding of AMP and PHP (curve b). After PHP was incubated with AMP, phi29 and *Nt.AlwI*, we observed a significantly strong fluorescence signal, implying large amounts of amplification products were generated (curve c). In contrast, the fluorescence intensity remained nearly equal to that for PHP after incubation without AMP (curve d). This revealed the remarkably increased fluorescence intensity was caused by target-triggered quadratic recycling amplification. In addition, after PHP was incubated with phi29, *Nt.AlwI*, and penicillin in place of AMP, we found the fluorescence signal was consistent with that of PHP, which manifested the specificity of the proposed sensing system (curve e). These observations confirmed that the structure change of PHP and enzyme-assisted signal amplification were specifically attributed to the binding of AMP to PHP.

Optimization of Experimental Conditions. Typically, high concentration of MBP could gain high hybridization efficiency with ssDNA fragments released by nuclease-catalyzed isothermal amplification and the more significantly signal change as well. However, it often resulted in lower sensitivity and high background signal, thus was not conducive for the quantification of low-concentration of AMP. We examined the variance of the $(I_0 - I) / I_0$ value with the concentration of MBP, where I and I_0 were the DPV peak intensity at -0.25 V in the presence and absence of AMP, respectively. As shown in Fig. S2, the maximum value was obtained at the concentration of 10 μM.

So, we chose 10 μM as the optimal MBP concentration. Additionally, the formed amount of ssDNA fragments was greatly depended on the amplification reaction time. Fig. S3A showed the effect of different reaction time on the $(I_0 - I) / I_0$ value. It was found that a maximum value was acquired after 2 h. Longer reaction time did not obviously change the value. So, 2 h was used as the optimal reaction time in the subsequent research. According to the same procedure, 2 h was selected for the optimal conjugate time for free MBP to CP on electrode in the subsequent research (Fig. S3B).

Regeneration and Stability. The proposed biosensor can be regenerated 20 times with about 12% loss of the original signal by dipping the electrode in hot (> 80 ℃) ultrapure water for 10 min, followed by a rapid cooling in an ice bath for 10 min. The signal attenuation might be attributed to the loss of immobilized CP on electrodes. To evaluate the stability of this biosensor, five electrodes were fabricated independently under the same conditions and stored at 4 ℃ for 2 weeks. Then these electrodes were used to detect 10 nM AMP. The results showed about 94% of its initial response of the biosensor for AMP remained, which indicated this proposed biosensor had very desirable stability.

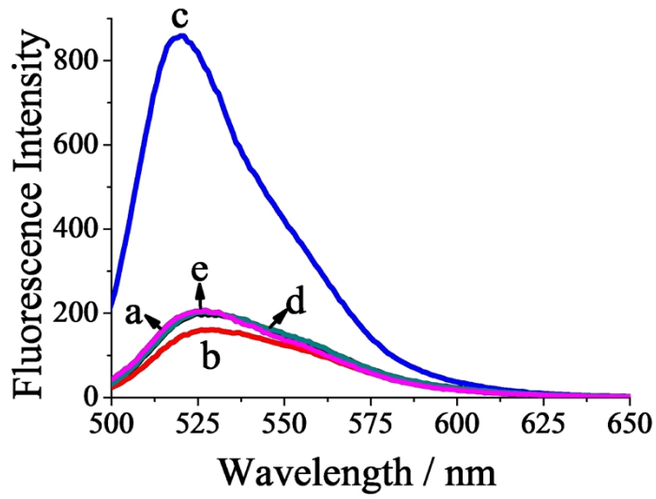

Fig. S1 Fluorescence spectra of target-triggered quadratic recycling amplification after the treatment of SYBR Green I with PHP (a), PHP in the presence of AMP (b), positive sample (c), blank sample (d), PHP in the presence of MBP, phi29, *Nt.AlwI* and penicillin (e).

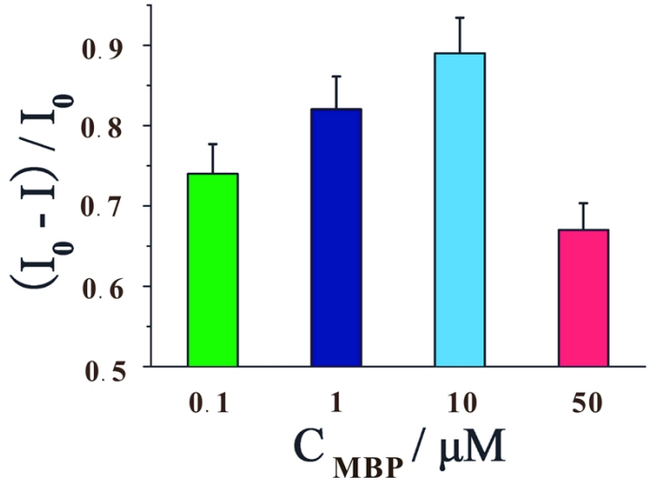

Fig. S2 Effect of the concentration of MBP on the electrochemical signal of biosensor.

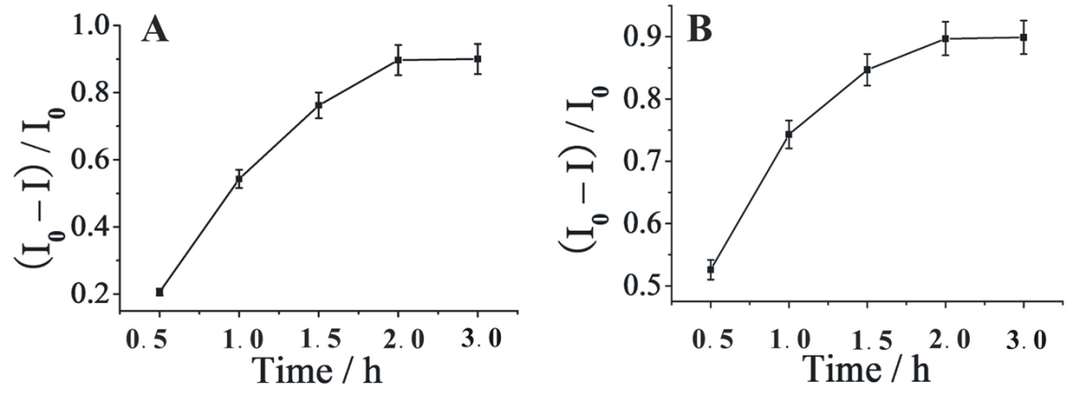

Fig. S3 (A) Effect of the isothermal amplification reaction time on the electrochemical signal of biosensor. (B) Effect of the conjugation time of the MBP with the capture probe on electrode on the electrochemical signal of biosensor.

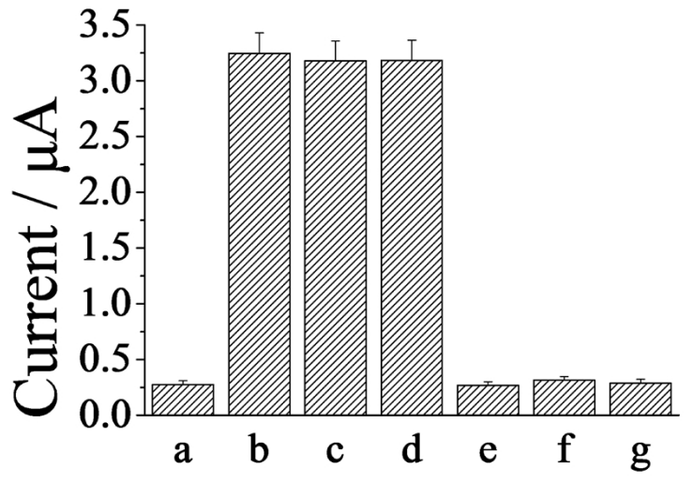

Fig. S4 DPV responses of the biosensor to (a) AMP (10 nM), (b) penicillin (1 μM), (c) amoxicillin (1 μM), (d) benzylpencillin (1 μM), (e) AMP (10 nM) and penicillin (1 μM), (f) AMP (10 nM) and amoxicillin (1 μM), (g) AMP (10 nM) and benzylpencillin (1 μM). Error bars are standard deviations across three repetitive experiments.

Detection method	Detection range	Detection limit	Detection time	reference
Fluorescence	2 μM - 70 μM	0.2 μM	~4 h	[9]
Electrochemical microfluidic	100 pM - 1 μM	100 pM	~6 h	[7a]
Colorimetry	21 nM - 285 nM	14 nM	~4 h	[7c]
Photoelectrochemistry	1 nM - 230 nM	0.2 nM	~6 h	[7d]
ELISA	0.428 nM - 100 nM	0.1 nM	~5 h	[5a]
Electrochemistry	5 pM - 10 nM	1.09 pM	~4.5 h	This work

Tab. 1 Comparison of different assay methods for AMP determination.

Spiked amount (nM)	Meased (nM)		Recocery (%) ± SD, n=5	
	aptasensor	ELISA	aptasensor	ELISA
1.000×10^{-2}	$(1.047 \pm 0.03)\times10^{-2}$	$(0.964 \pm 0.031)\times10^{-2}$	104.7 ± 3.4	96.4 ± 3.1
1.000×10^{-1}	$(0.934 \pm 0.041)\times10^{-1}$	$(1.039 \pm 0.017)\times10^{-1}$	93.4 ± 4.1	103.9 ± 1.7
1.000×10^{0}	$(1.028 \pm 0.057)\times10^{0}$	$(1.027 \pm 0.008)\times10^{0}$	102.8 ± 5.7	102.7 ± 0.8
1.000×10^{1}	$(0.983 \pm 0.049)\times10^{1}$	$(0.973 \pm 0.024)\times10^{1}$	98.3 ± 4.9	97.3 ± 2.4

Tab. 2 Application of the electrochemical aptasensor to detect AMP in milk and comparison with ELISA detection.

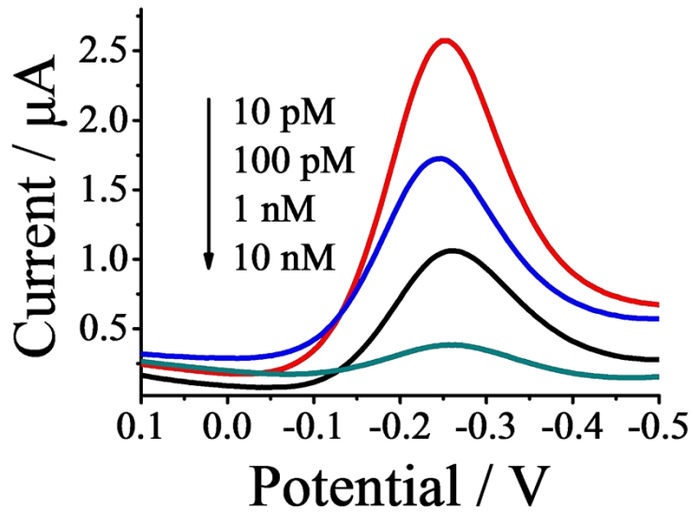

Fig. S5 Typical DPV responses of the biosensor to the different spiked milk samples.