Supporting Information

Label-Free Electrochemical CRISPR Platform Gated by Allosteric Transcription Factors for Ultrasensitive Small-Molecule Detection

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Experimental Section

Instruments

The CD spectra were performed on MOS-500 spectrophotometer (BioLogic, France) at room temperature. The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter. All electrochemical experiments were conducted on a CHI660A workstation using a conventional three-electrode system comprising a modified AuE working electrode, a saturated calomel electrode (SCE) reference electrode, and a platinum wire counter electrode. The fluorescence intensity tests were carried out on a 96-well reaction plate with Synergy™ Mx multimode microplate reader Receives (BioTek, USA). DNA double-strand hybridization was performed on an S1000™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA).

Nucleic Acid Preparation

DNA and RNA strands were dissolved in DEPC-treated water and quantified to 100 μM stock solution. Double-stranded DNAs (dsDNAs) and Hp were prepared by annealing equimolar amounts of promoters and templates in a 2.5 mM $MgCl_2$ solution, followed by heating at 95 °C and then slowly cooling to 4 °C.

Composition and assembly of the activated Cas12a complex

LbCas12a and crRNA were first mixed at 1 μM each in the RNP assembly mixture containing 1 mM $MgCl_2$ and incubated on ice for 30 min to form the Cas12a/crRNA ribonucleoprotein (RNP) complex. The preassembled Cas12a RNP was then combined with the corresponding dsDNA activator in 1× CRISPR cleavage buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 100 mM KCl, and 5% glycerol. In the final Cas12a activation mixture, the concentrations of Cas12a/crRNA RNP and dsDNA activator were 50 nM and 10 nM, respectively.

After activation, the Cas12a-containing reaction mixture was applied to the Hp-modified gold electrode and incubated at 37 °C for 30 min to allow trans-cleavage of the immobilized Hp probes. This cleavage step exposed accessible 3′-OH termini on the electrode-tethered Hp, which subsequently served as initiation sites for TdT-mediated elongation.

Circular Dichroism (CD) Spectroscopy for G-quadruplex Verification

To verify the formation of G-quadruplex (G4) structures, Hp cleavage products generated by activated Cas12a RNP were incubated with TdT and the dNTP mixture in buffer containing 50 mM K^+. Subsequently, the reaction mixture underwent phenol-chloroform extraction, and the resulting aqueous phase containing the purified nucleic acids was collected for subsequent experimental measurements. Then the reaction mixture was transferred to a quartz cuvette to

ensure high-fidelity signal acquisition. The CD signal was monitored by spectrophotometric scanning across a wavelength range of 220–280 nm using a CD spectrometer.

UV-vis Absorption Characterization of G4/Hemin Complex Formation

To directly verify the formation of G4/hemin complexes, UV-vis absorption spectroscopy was performed to monitor changes in the characteristic Soret absorption band of hemin. Reaction products were prepared under different conditions, including Hp, Hp/TdT, Hp/activated Cas12a, and Hp/activated Cas12a/TdT. Each reaction product was incubated with hemin in 20 mM HEPES buffer containing 50 mM KCl, 200 mM NaCl, and 1% DMSO at pH 8.0. Unless otherwise specified, the final concentration of hemin was 5 μM, and the reaction products were prepared from equivalent initial Hp concentrations. The mixtures were incubated at room temperature in the dark for 30 min to allow hemin binding to the generated G-rich/G4 structures. Subsequently, UV-vis absorption spectra were recorded from 320 to 640 nm using a quartz cuvette. The red shift and/or hyperchromic enhancement of the hemin Soret absorption band was used as spectroscopic evidence for G4/hemin complex formation.

Colorimetric Measurement of Peroxide-like activity

An equimolar mixture of each reaction product and hemin (1 μM, 50 μL total volume), incubated for 10 minutes at room temperature. Subsequently, 50 μL of single-component 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic substrate was added and thoroughly mixed. The experimental group's solution rapidly developed a characteristic blue coloration. The 100 μL reaction mixture was immediately transferred to a 96-well microplate, and absorbance was measured using a microplate reader. To minimize experimental variability, the measurement interval was maintained as brief as possible. Absorbance was determined by spectrophotometric scanning across a wavelength range of 500-800 nm.

Figures

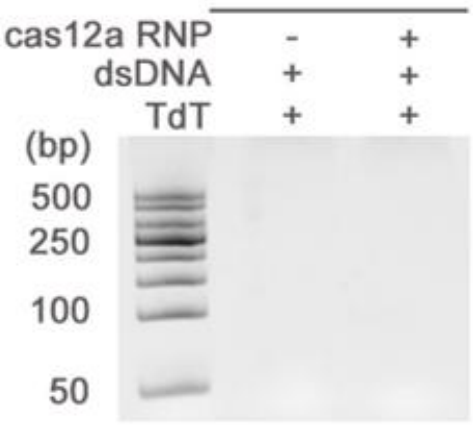


Figure S1. Characterization of TdT-mediated DNA extension. Native PAGE (12.5%) analysis of the reaction products: lane 1, 500 bp DNA ladder; lane 2, dsDNA (10 nM) with TdT; lane 3, dsDNA (10 nM) with activated Cas12a RNP and TdT.

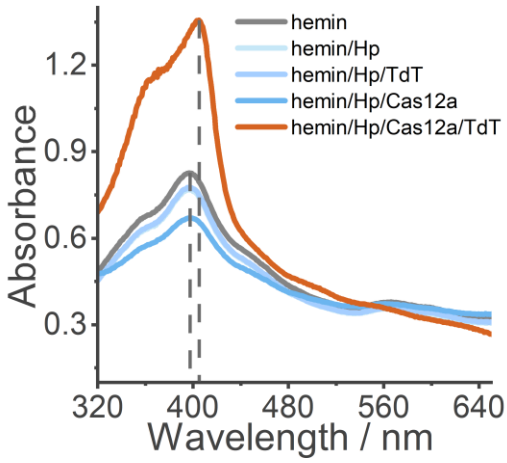


Figure S2. UV-vis absorption characterization of G4/hemin complex formation. UV-vis absorption spectra of hemin incubated with Hp, Hp/TdT, Hp/activated Cas12a, and Hp/activated Cas12a/TdT respectively.

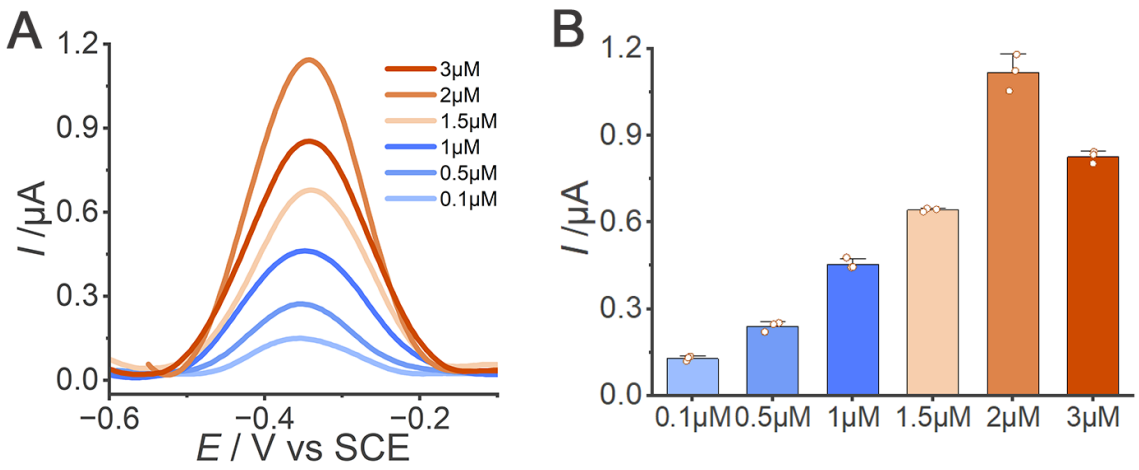


Figure S3. Optimization of the immobilized Hp concentration. (A) Differential pulse voltammetry (DPV) curves and (B) corresponding peak current responses at various concentrations of hairpin DNA (0.1, 0.5, 1, 1.5, 2, and 3 μM). Error bars represent the standard deviation of three independent measurements.

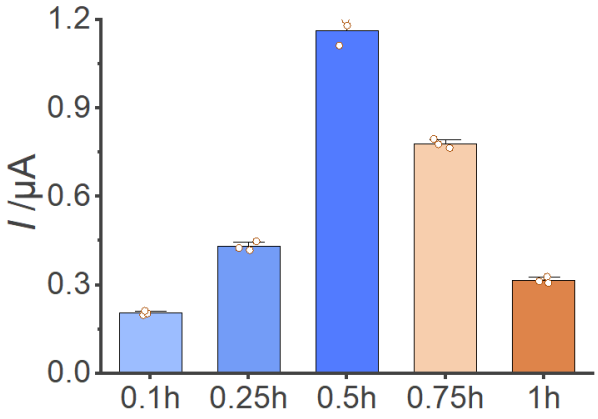


Figure S4. Optimization of the Cas12a cleavage time. The effect of incubation time for the CRISPR/Cas12a-mediated trans-cleavage reaction on DPV signal response (0.1 h to 1 h). Error bars represent the standard deviation of three independent measurements.

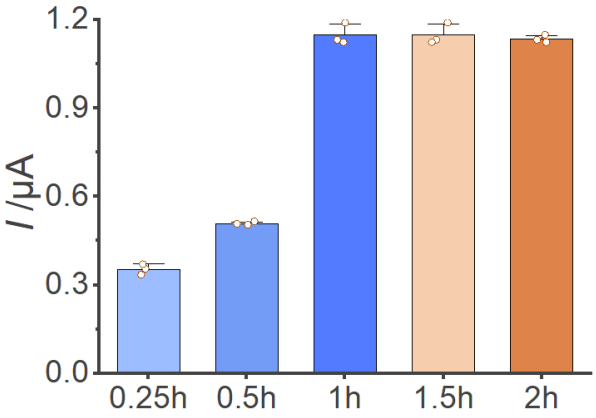


Figure S5. Optimization of the TdT-mediated DNA polymerization time. Effect of the TdT reaction duration (from 0.25 h to 2 h) on the DPV signal response. Error bars represent the standard deviation of three independent measurements.

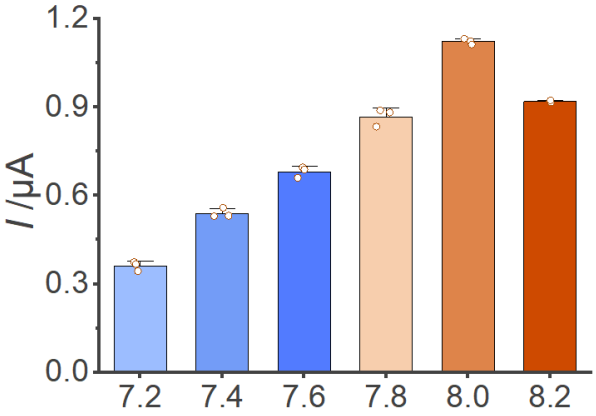


Figure S6. Optimization of the reaction pH. The influence of buffer pH (ranging from 7.2 to 8.2) on the DPV signal response. Error bars represent the standard deviation of three independent measurements.

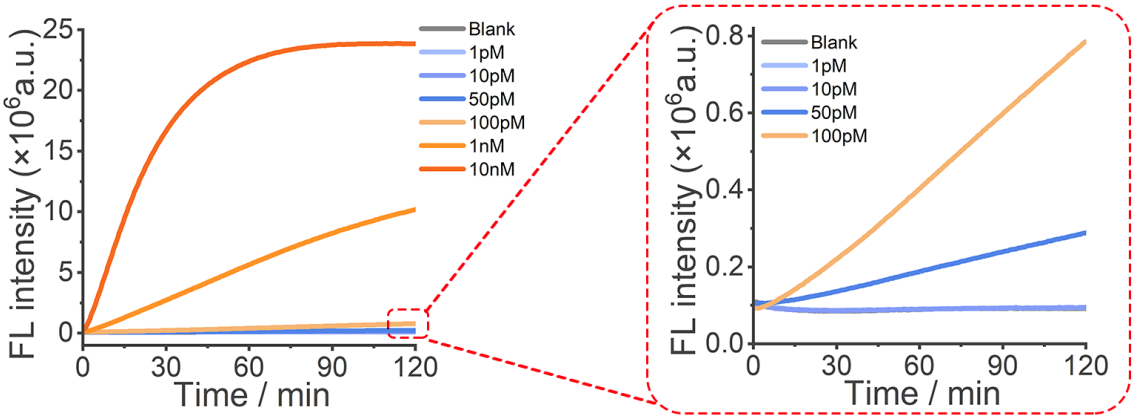


Figure S7. Fluorescence-based detection of dsDNA at varying concentrations. Time-dependent fluorescence intensity curves for different concentrations of dsDNA (ranging from 1 pM to 10 nM) to validate the Cas12a activation efficiency.

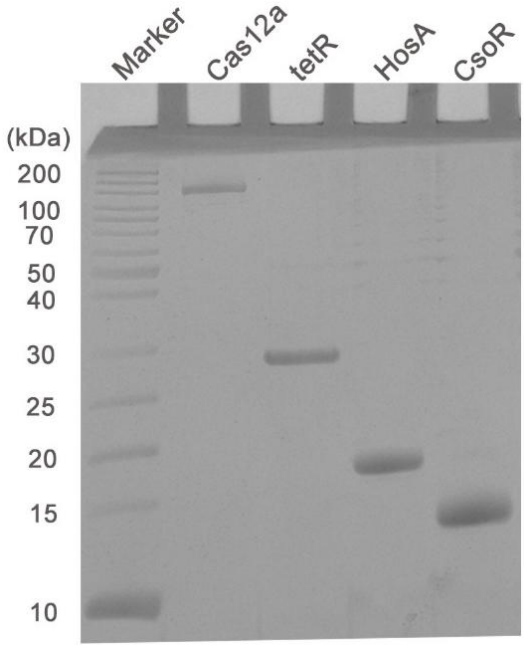


Figure S8. SDS-PAGE (10%) gel electrophoresis analysis of purified proteins. Characterization of the molecular weight and purity of LbCas12a, TetR, HosA, and CsoR proteins used in this study.

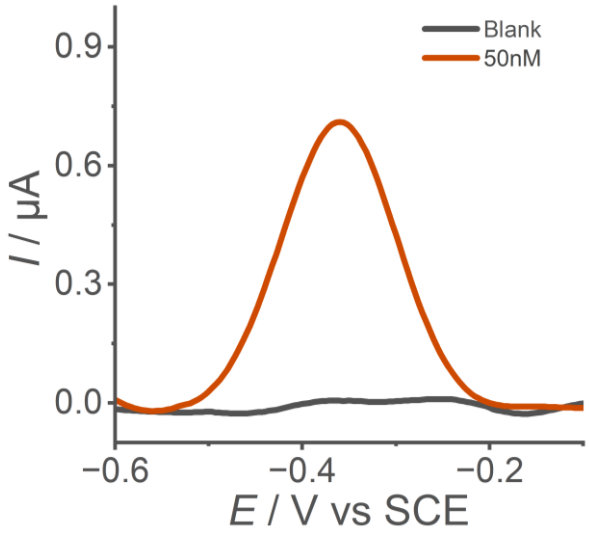


Figure S9. Feasibility of the electrochemical biosensor. Comparison of DPV responses between the blank (control) and the sample containing 50 nM of the target analyte, demonstrating the "turn-on" sensing capability.

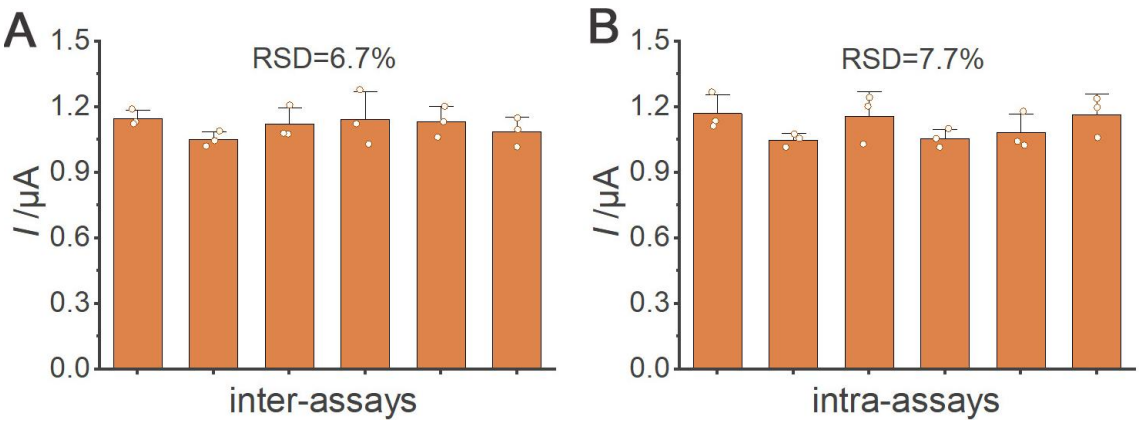


Figure S10. Reproducibility and precision of the biosensor. (A) Inter-assay and (B) intra-assay stability tests, with Relative Standard Deviation (RSD) values of 6.7% and 7.7%, respectively. Error bars represent the standard deviation of three independent measurements.

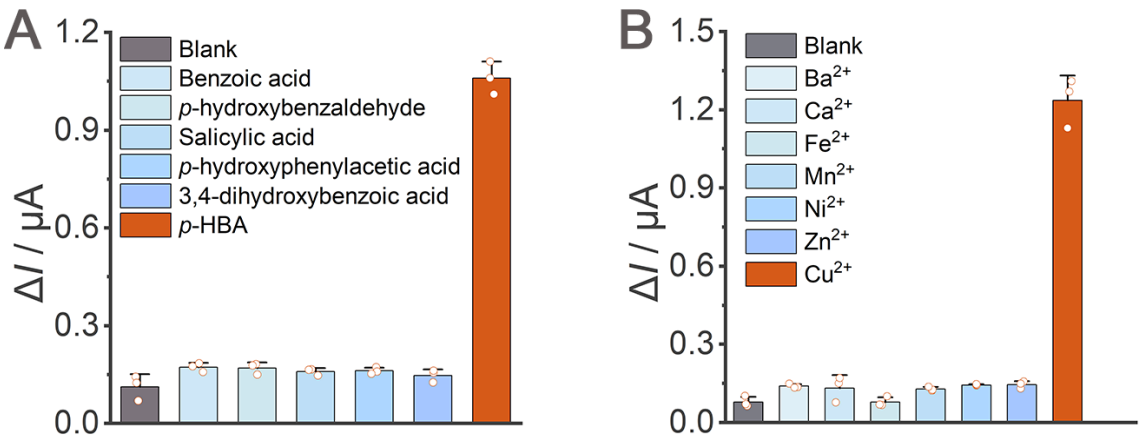


Figure S11. Specificity of (A) HosA-regulated p-HBA detection system toward p-HBA (100 nM) and structurally related aromatic interferents(each at 1 μM) (B) CsoR-regulated Cu^{2+} detection system toward Cu^{2+} (100 nM) and representative competing metal ions(each at 1 μM). Error bars represent the standard deviation of three independent measurements.

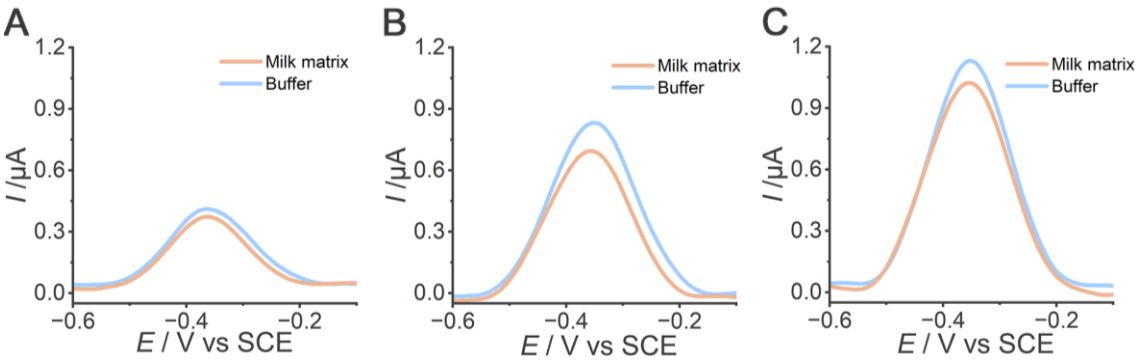


Figure S12. Comparison of the DPV peak current changes (△*I*) for Tc in buffer and pre-treated milk matrix. (A) 1 nM, (B) 50 nM and (C) 1 μM.

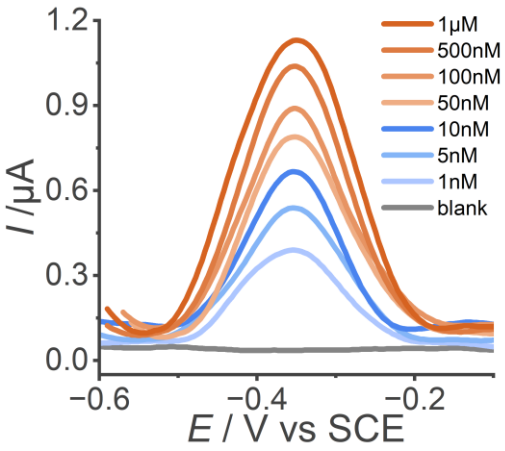


Figure S13. DPV signal responses of this developed biosensor for Tc in milk matrix (blank and ranging from 1 nM to 1 μM).

Tables

Table S1. Sequences of the dsDNA used in this work.

Name	Sequences (5'-3')
dsDNA	5'- GACGACAAAACTTTAGATCGTTACGCTAACTATGA -3'
	3'- CTGCTGTTTTGAAATCTAGCAATGCGATTGATACT -5'
$cDNA_I$	5'-TCCCTATCAGTGATAGAGATTTATCCCTATCAGTGATAGAGAG-3'
(tetR)	3'-AGGGATAGTCACTATCTCTAAATAGGGATAGTCACTATCTCTC-5'
$cDNA_{II}$	5'-GTTCGTATACGAACTTTAGTTCGTATACGAACAACTAT-3'
(HosA)	3'-CAAGCATATGCTTGAAATCAAGCATATGCTTGTTGATA-5'
$cDNA_{III}$	5'-GTAGCCCACCCCCAGTGGGGTGGGATACTTTAGATCGTTACGCTAACTATG
(CsoR)	TAGCCCACCCCCAGTGGGGTGGGATAC-3'
	3'-CATCGGGTGGGGGTCACCCCACCCTATGAAATCTAGCAATGCGATTGATAC
	ATCGGGTGGGGGTCACCCCACCCTATG-5'

The tetO sequence is depicted in green, the PAM sequence is depicted in red, the HosA binding sequence is depicted in blue, the CsoR binding sequence is depicted in yellow, an underline represents the complementary sequence recognized by the crRNA.

Table S2. Sequences of the RNA used in this work.

Name	Sequences (5'-3')
crRNA for dsDNA	UAAUUUCUACUAAGUGUAGAUGAUCGUUACGCUAACUAUGA
crRNA for $cDNA_I$ (tetR)	UAAUUUCUACUAAGUGUAGAUUCCCUAUCAGUGAUAGAGAG
crRNA for $cDNA_{II}$ (HosA)	UAAUUUCUACUAAGUGUAGAUGUUCGUAUACGAACAACUAU
crRNA for $cDNA_{III}$ (CsoR)	UAAUUUCUACUAAGUGUAGAUGAUCGUUACGCUAACUAUGA

The 5'-handle and 3'-spacer regions of crRNA are shown in blue and pink, respectively.

Table S3. Comparison of the analytical performance of the proposed biosensor with other reported methods for tetracycline (Tc) detection

Method	Linear range	LOD	Ref.
Fluorescence	0.1 – 5μM	13 nM	1
SERS[a]	1nM – 10μM	1 nM	2
Colorimetry	0.1 – 20μM	25 nM	3
Fluorescence	1.125 –18μM	60.1 nM	4
Electrochemistry	100pM – 1μM	36 pM	This work

[a] Surface-Enhanced Raman Scattering (SERS)

Reference

(1) Wang, K.; Liu, S.; Zhou, S.; Qileng, A.; Wang, D.; Liu, Y.; Chen, C.; Lei, C.; Ni e, Z. Ligand-Responsive Artificial Protein–Protein Communication for Field-Deployabl e Cell-Free Biosensing. *Angew. Chem. Int. Ed.* **2025**, *64* (4), e20-2416671. https://d oi.org/10.1002/anie.202416671.

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(3) Cui, Y.; Zhao, N.; Wang, S.; Yan, H.; Han, D. Colorimetric Detection of Tetracyc line by Enhancing Peroxidase-like Activity of Polymerized MnO_2@ZIF-67 Nanocomp osites. *Biosens. Bioelectron.* **2025**, 267, 117726. https://doi.org/10.1016/j.bios.2025.11 7726.

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