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Real-time gene analysis based on a portable electrochemical microfluidic system

Yichen Liu[a], Baiyang Lu[a], Yidan Tang[a], Yan Du[a,b,*], Bingling Li[a,b,*]

[a] State Key Lab of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Science, Changchun, Jilin 130022, China
[b] University of Science and Technology of China, Hefei, Anhui 230026, China

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ABSTRACT

Isothermal amplification, such as loop-mediated nucleic acid isothermal amplification (LAMP), is a highly promising technique that could revolutionize portable or point-of-care gene detection. However, the commonly used readouts of these isothermal reactions have been limited to a small number of options such as real-time fluorescence and colorimetric paper strips, which suffer from practical difficulties in further integration and quantitation, respectively. To enrich the readout library and provide more options suitable for a variety of detection requirements, we report a ready-to-use gene testing method based on the execution of isothermal nucleic acid amplification reactions on closed and portable PGE-LAMP electrochemical chips. Taking the HF183 gene of the fecal pollutant *Bacteroides* as a model target, this method allows both end-point and real-time transduction from genomic information to electrochemical signals with ultra-high sensitivity, specificity and a good signal-to-noise ratio, and with a detection limit as low as 80 copies. This approach is therefore highly promising for the development of point-of-care detection systems and accelerating the practical application of LAMP.

1. Introduction

Microorganisms such as pathogens, viruses, and parasites are a serious threat to human health and the living environment [1]. Therefore, portable, off-the-shelf, or even point-of-care (POC) detection is particularly important for rapid infection control and recovery [2–6]. Currently, the most universal, specific, and sensitive biomarkers for these microorganisms are their gene segments [7,8]. A classical gene analysis is usually executed on a real-time machine, during which the polymerase chain reaction (PCR) or a series of isothermal nucleic acid amplifications are required to enrich the concentration of targeted gene segments until the amplicons reach detectable amounts [9–18]. Fluorescence is the most commonly used readout method in real-time commercial kits and instruments. Even though this is effective and sufficiently fast, it is hard to transform these large-scale fluorescence instruments into portable devices. This is not only because of the relatively high costs, but also the high technological barriers to miniaturizing all the components (e.g. light sources, lasers, sensors, and cameras). Thus, when working towards a practical, portable or even POC gene detection system, readouts that are easy to integrate and have better digital signal modes, such as those produced by electronics and electrochemistry, are still urgently needed as alternatives to fluorescence.

To achieve the above aim, reliable and practical electrochemical gene detection should stringently require both convenient fabrication and portability of the sensing platform, together with accurate, sensitive and specific signals. More importantly, to overcome some general disadvantages of electrochemical sensors, such as the difficulty in obtaining a reproducible electrode surface and the problem of background signals, the sensing platform should be designed for the reaction conditions, even outputting signals as the amplification reaction progresses. This has been one of the biggest challenges hindering the development of real-world electrochemical gene sensors.

With the aim of providing a potential solution to the above challenge, here we innovatively construct a portable electrochemical microfluidic system that can achieve real-time isothermal nucleic acid amplification with results comparable to real-time fluorescence detection. As shown in Scheme 1, isothermal nucleic acid amplification (i.e. loop-mediated isothermal amplification, LAMP) is used to provide the ultra-sensitivity needed to detect extremely low amounts of gene targets, as required in practical applications [19–25]. To reduce the false positive signals that generally occur in most LAMP detection strategies, including electrochemical ones [19,26–29], a hemi-duplex oligonucleotide strand displacement (OSD) reporter is immobilized on the working electrode as a sequence-specific acceptor that only recognizes

* Corresponding authors.
E-mail addresses: duyan@ciac.ac.cn (Y. Du), binglingli@ciac.ac.cn (B. Li).

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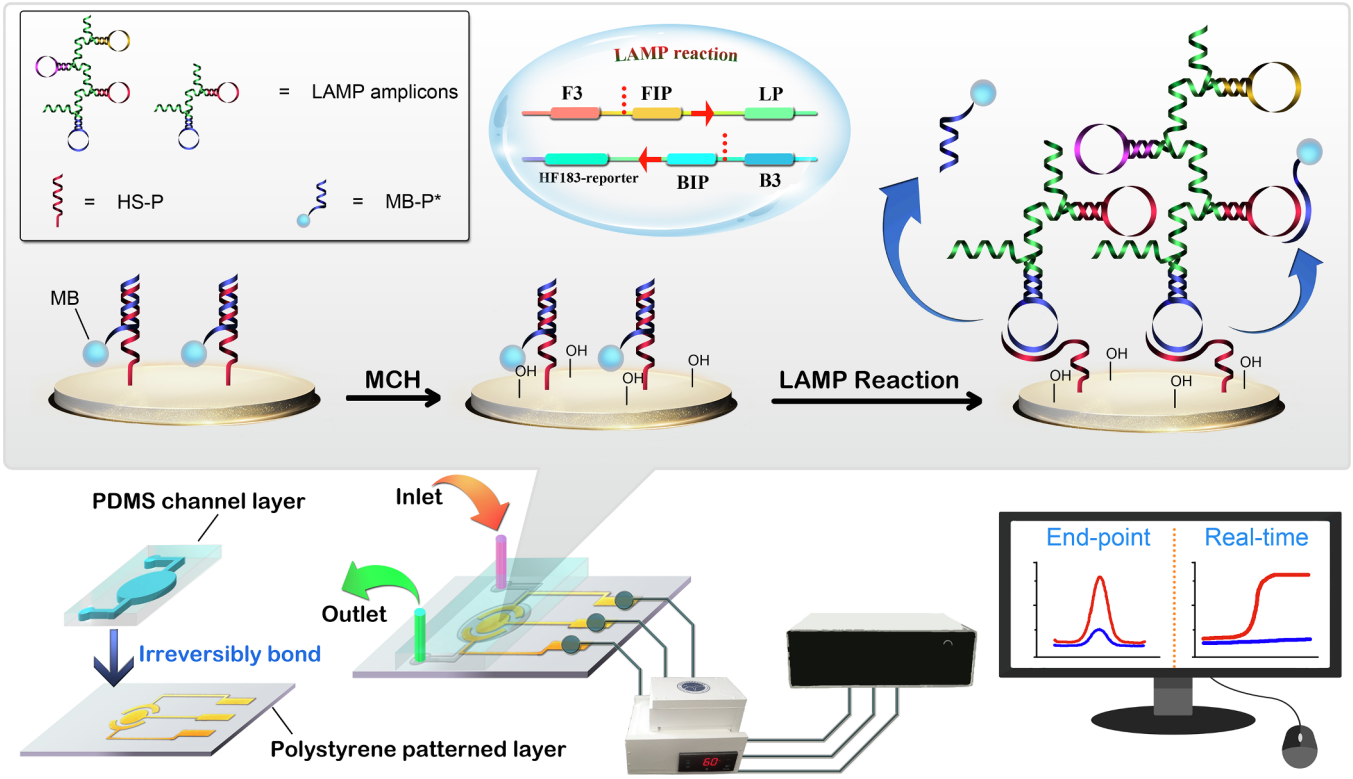

Scheme 1. Schematic illustration of LAMP reaction electrochemical detection on the PGE chip with microfluidic channel. Top: Electrode immobilization and the sensing strategy for detection of LAMP amplicons. Bottom: Principle of chip fabrication and electrochemical detection.

the correct amplicons. Here, the OSD reporter is constructed by hybridizing the complement of the LAMP amplicons (HS-P) with a short antisense strand conjugated to methylene blue (MB-P*). In the absence of amplicons, the MB is held in close proximity to the electrode surface, yielding effective electron transfer signals. After or during the LAMP reaction, the LAMP amplicons will displace or drag the MB-P* away from the electrode surface, leading to a sharp decrease in current. The whole sensing platform is constructed using disposable, plastic-gold electrodes (PGE) and sealed with a PDMS microfluidic system (Fig. 1). This PGE-LAMP chip is portable, cheap, thermo-stable, and may easily be patterned, meeting the requirement for portable integration of the whole detection process [30,31]. The whole sensing process can be monitored not only at the end-point, but also in a real-time mode as the LAMP reaction proceeds at 55–65 °C. A real-time curve can be drawn, such as that provided by a traditional fluorescence instrument. Because the whole detection process is carried out in a closed system, it successfully avoids contamination by aerosols [32]. For our model target, the HF183 gene (a gene of *Bacteroides*, an important marker for human fecal pollutants in water) [33,34], detection is demonstrated successfully both in a pure buffer and in real water sources from lake and tap water with a limit of detection (LOD) of 80 gene copies (5.3 amol·L^{-1}). This approach thus provides new opportunities to further improve the design of electrochemical genetic diagnostic systems.

2. Experimental section

2.1. Material and reagents

All the DNA sequences were purchased from Sangon Biotech (Shanghai, China) and stored in 1 × TE (pH 7.5) at −20 °C; the sequences are provided in Tables S1 and S2. All of the DNA was purified by high-pressure liquid chromatography (HPLC) and quantified with a DeNovix DS-11 + FX spectrophotometer (DeNovix Inc., Wilmington, DE, USA). All reagents used in the LAMP reaction and chip fabrication were of analytical grade unless otherwise stated. All solutions were prepared with ultra-pure water (resistance > 18.25 MΩ·cm) from a Milli-Q purification system. Details of the materials and buffer used in the experiment are given in the Supplementary data.

2.2. Fabrication of the PGE-LAMP chips

The PGE-LAMP chips consist of a PDMS channel layer and a polystyrene (PS) patterned layer [30,31] (Fig. S1, see more detailed information in the Supplementary data). Briefly, the 10-mm thick PDMS-based microfluidic channel frame was molded against master molds by standard photolithography using a SU-8 photoresist [35]. The PS patterned layer was irreversibly bonded with the PDMS channel layer by air plasma cleaning at room temperature [36]. The size of the complete PGE-LAMP chip is 15 mm × 11 mm and the volume of the microfluidic chamber is approximately 5 μL (Fig. 1B–D).

2.3. Fabrication of HS-P: MB-P* sensing surface on a gold working electrode

30 μL of 1 μM HS-P:MB-P* reporter duplex solution was prepared and pumped into the PDMS channel for immobilization. The assembly was kept at room temperature for 4 h and thoroughly rinsed with 1 × TNaK buffer, after which 1 mM of MCH (used as a blocker) was pumped into the channel and kept at room temperature for 2 h.

2.4. HF183 LAMP reaction

The standard procedures of the LAMP reaction were optimized for end-point and real-time electrochemical detection with the specific OSD probe immobilized on the electrode surface. For end-point detection, different concentrations of HF183 template and LAMP primers (at final concentrations of 1.6 μM FIP, 1.6 μM BIP, 0.4 μM F3, 0.4 μM B3, 0.8 μM LP) were added to the 1 × Iso buffer with 0.4 mM dNTPs and

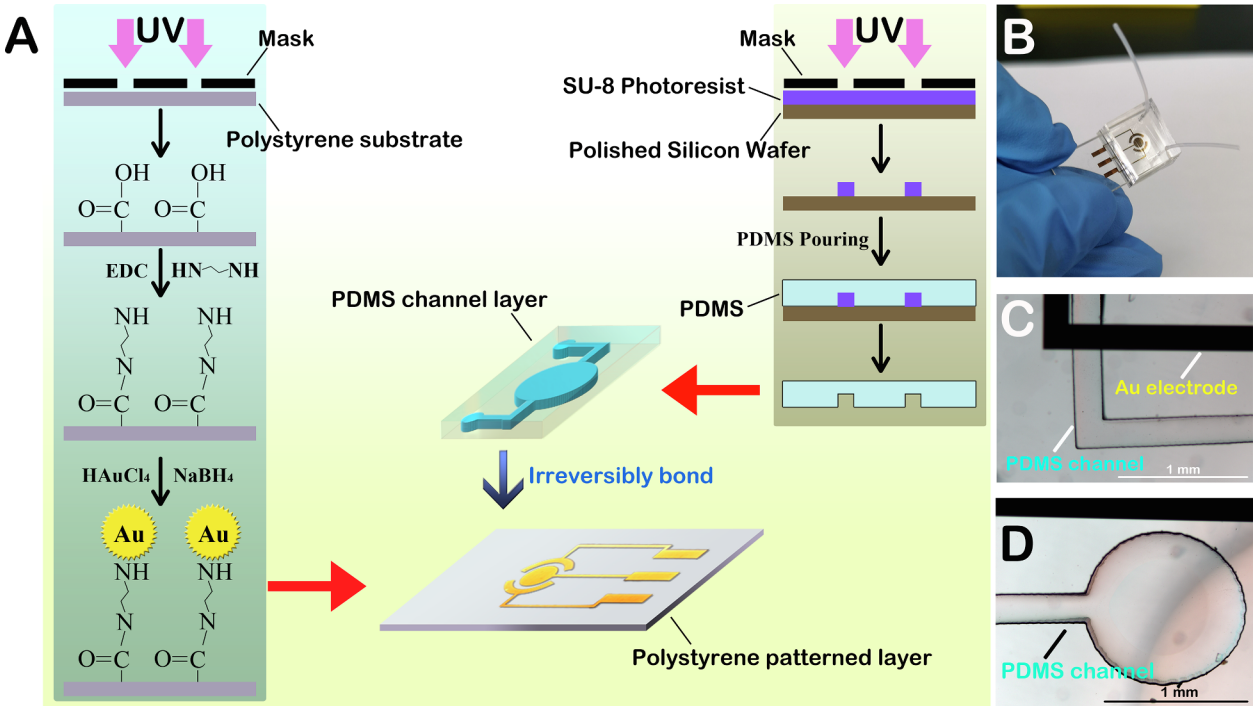

Fig. 1. (A) Fabrication of PGE-LAMP chips. Detailed PGE-LAMP chip fabrication process for the polystyrene patterned layer and the PDMS channel layer. (B) A PGE-LAMP chip with a microfluidic channel. (C, D) Drawings of the microfluidic channel on the PGE-LAMP chip.

2 mM Mg^{2+}. Then, 1.5 μL of 8000 units/mL Bst 2.0 DNA polymerase as a LAMP reaction initiator were added to the reaction with a total 25 μL reaction at 60 °C for 1.5 h. The resulting LAMP product is suitable for on-chip OSD reaction and electrochemical end-point detection. For real-time detection, LAMP reactions in different concentration of HF183 templates were performed using a similar protocol to the end-point detection. Here, 25 μL of LAMP reaction mixture was injected into the PDMS channel for isothermal amplification and real-time electrochemical detection was performed for 100 min at 60 °C using a home-made auxiliary device. In order to determine the normal output of LAMP isothermal amplification, we also prepared a fluorescence OSD reaction containing 100 nM OSD-reporter F and 200 nM OSD-reporter Q for different concentrations of HF183 templates. The fluorescence signals were recorded with a LightCycler® 96 Instrument (Roche Diagnostics GmbH, Germany).

3. Results and discussion

3.1. Characterization and chemical modification of the gold working electrode

The successful fabrication of the gold working electrode was verified by cyclic voltammetry using electrochemical cleaning in 100 mM H_2SO_4. (Fig. S2). After a brief rinse with deionised water, the freshly prepared working electrodes were used to assemble OSD reporters as a sensing interface. Briefly, 1 μM HS-P: MB-P* solution and 1 mM MCH were pumped in succession onto the gold electrode via the PDMS channel [30]. The thiol group tagged on the HS-P was used for Au-S bond attachment, while MCH was used as a blocker to decrease non-specific absorption. Square wave voltammetry (SWV) was used to validate the fabrication of the sensing interface and the detection of the gene target (Fig. 2A). The sensing platform showed a clear and sharp oxidized peak attributed to MB (Fig. 2A, inset). In the presence of the mimic target (shortened as Mimic, designed as the loop sequence within the LAMP amplicons), the Mimic was fully complementary to HS-P and induced a toehold-mediated strand displacement reaction that drove the MB-P* away from the electrode surface. Thus, a sharp reduction in the MB peak could be observed.

3.2. End-point electrochemical detection with the PGE-LAMP chip

To evaluate the demand for more accurate and reliable detection of the gene amplicons, the LAMP reaction and five primer sets for HF183 previously designed by the Ellington group were directly borrowed here due to their excellent efficiency [20]. It thus remained as a single strand and allowed for further hybridization with HS-P. The sensitivity and selectivity of the LAMP reaction were also verified using standard fluorescence readouts (Figs. S3 and S4). The sensitivity is 80 copies, consistent with previously reported results.

Before carrying out real-time detection, the electrochemical sensing platform was first demonstrated in end-point mode. After the LAMP reactions had been carried out for 1.5 h at 60 °C, 25 μL of the reaction products were pumped into the microchannel and incubated with working electrodes modified with HS-P:MB-P* and with pre-SWV background scanning. After 1.5 h incubation at 37 °C, the electrodes were washed with 1 × TNaK buffer and then SWV scanning was carried out. Clearly, the HF183 positive samples produced a significant reduction in the MB peak current, while the currents for pure buffer control or non-specific amplicons were almost unchanged (Figs. 2B and S5). This suggests the successful detection of HF183 LAMP amplicons only. As shown in Fig. 2C, the 8000 to 8,000,000 copy samples all blocked at least 90% of the normalized MB peak current. The 80 and 800 copy samples only produced a partial reduction in the MB current. The detection signal was therefore sufficiently sensitive, selective and significant. It should be noted here that there is no dose dependence with a 1.5 h LAMP reaction. All primers would be consumed for most concentrations of the templates (Fig. S3). This is because no matter how much template was present, all the primers would be consumed and thus the same amount of LAMP amplicons should be generated. When other water sources, such as local lake water, pond water, and tap water, were used (see detailed information in Supplementary data), very similar and satisfactory results were achieved (Figs. 2D and S6). By contrast, LAMP amplicons of randomly-picked control genes, including OMPA (plasmid of *Enterobacter sakazakii*), MALB (plasmid of

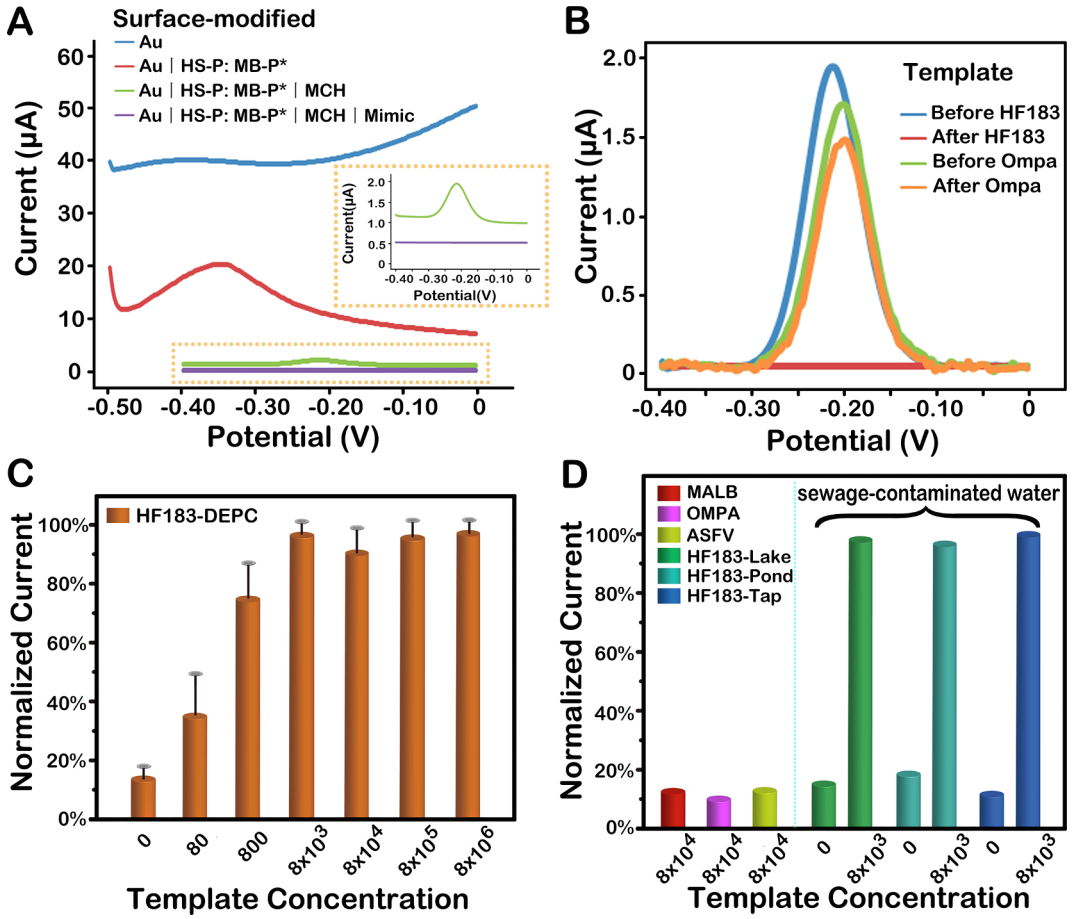

Fig. 2. End-point electrochemical monitoring of PEG-based LAMP detection. (A) SWVs of surface modified electrodes. (B) SWV curves before and after detecting positive HF183 and negative OMPA samples. (C) Normalized end-point current traces for different numbers of copies of HF183 plasmid target. (D) Normalized end-point current for negative samples (80,000 copies of MALB, OMPA and ASFV), and 0 and 8000 copies of HF183 LAMP products in lake water, pond water and tap water, respectively.

enterohemorrhagic *Escherichia coli*) and ASFV (plasmid of African swine fever virus), have no non-specific signals (Fig. 2D). Meanwhile, the detection is also applicable and effective when using HF183 genes isolated from lab-grown recombinant *E. coli* HF183 (bacterial colony counts were measured by plating a series of 10^8–10^9-fold dilutions on Luria Bertani agar plates containing 100 mg/ml ampicillin (Fig. S8). Therefore, this approach to detection could provide the ultra-sensitive YES/NO answer required for a practical pathogen assay.

3.3. Real-time electrochemical data acquisition by normalized current

As mentioned above, a major advantage of this system should be its suitability for real-time detection. To achieve this, the electrochemical PGE-LAMP chip was further connected to a new, self-manufactured auxiliary device. The device consists of two important parts, a digital temperature control module and a chip-connecting module. The temperature control has a theoretical controllable temperature range of 0–200 °C, which allowed the signal to be collected while the LAMP reaction was taking place (Fig. 3A and B). Use of further smart designs could reduce the temperature fluctuation of the heating zone and further stabilize the heating temperature during real-time detection. The chip-connecting module could be connected to the PGE-LAMP chip and the electrochemical workstation for real-time detection during heating.

To monitor real-time gene amplification and its specific detection, after the working electrode of the PGE chip had been modified with HS-P:MB-P* duplex, 5 μL LAMP reaction reagents with or without the HF183 gene plasmid were pumped into the sealed PDMS chamber. The PGE chip was then placed into the home-made device maintained at 60 °C, followed by SWV scanning every 4 min (Figs. 3C, D and S7). Finally, time-dependent curves for real-time electrochemical detection were generated for different concentrations of template (Fig. 3E). These were of similar shapes to those obtained from fluorescence reporters, and thus allowed potential quasi-quantitation according to the cycle threshold (ct) value. Because the template and LAMP reagents were completely sealed in a closed environment, the amplicons had very limited ways to diffuse out into the air. Therefore, the contamination induced by the aerosol could be effectively reduced, which has been one of the biggest challenges delaying the practical application of most electrochemical gene sensors.

4. Conclusions

In conclusion, we report a very practical electrochemical PGE-LAMP chip for gene plasmid detection. With this chip, ultra-sensitivity, specificity, and portability are achieved simultaneously. Through integration of the patterned electrodes and a sealed PDMS channel, both end-point and real-time detection can be carried out. For real-time detection in particular, the kinetic curves are very similar to those obtained from a standard real-time fluorescence reading. Because electrochemical techniques have particular advantages, being fast, sensitive, and easy to integrate and miniaturization, this method is well suited for use in an integrated portable device, showing great promise for real-world point-of-care detection. In addition, the approach could be extended to multiplex analysis by fabricating an electrode array with comparable soft- or hardware capable of reporting multiple channels.

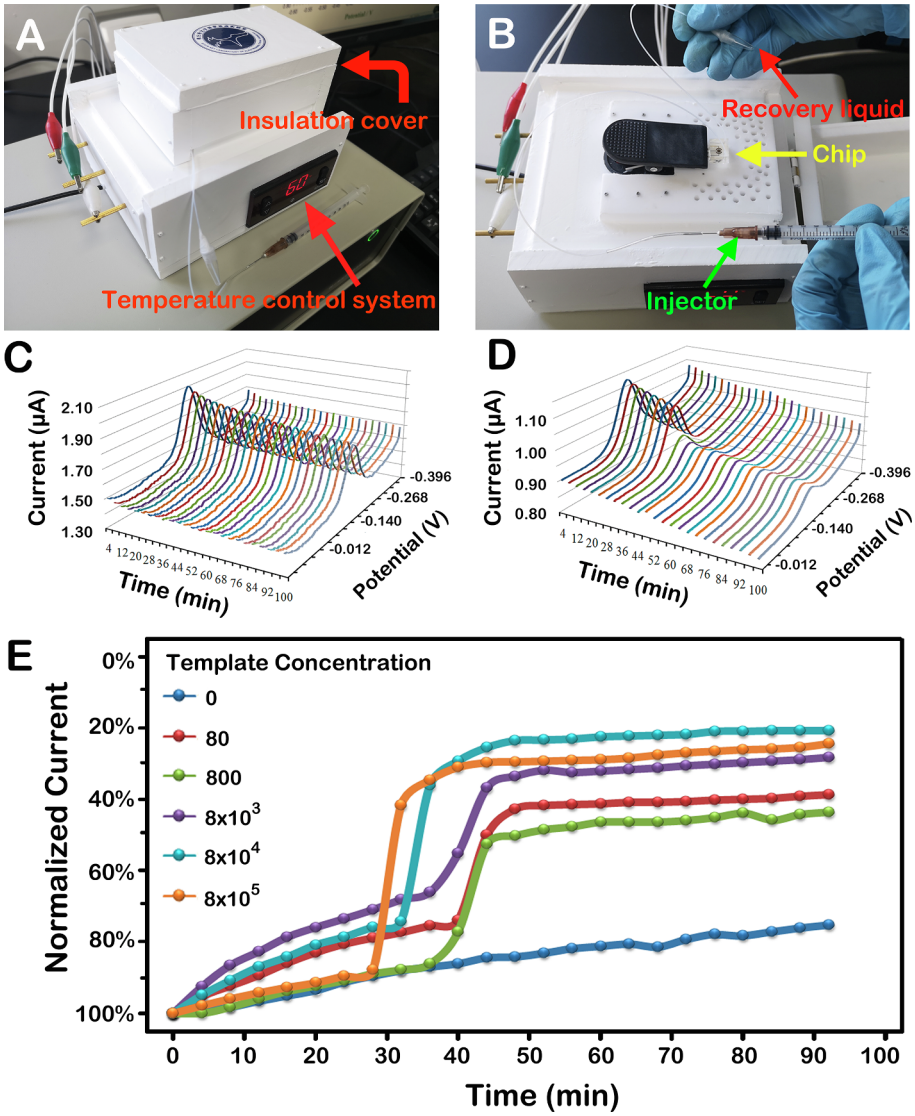

Fig. 3. Real-time electrochemical detection using the home-made electrochemical detection auxiliary device. (A, B) The structure and function of the electrochemical detection auxiliary device. (C) Real-time SWV curves of 0 copies of LAMP reaction at different times. (D) Real-time SWV curves of 80,000 copies of LAMP reaction at different times. (E) Normalized currents for the real-time LAMP-OSD reaction with different concentrations of HF183 templates.

CRediT authorship contribution statement

Yichen Liu: Conceptualization, Investigation, Methodology, Software, Data curation, Formal analysis, Writing - original draft. **Baiyang Lu:** Methodology, Validation. **Yidan Tang:** Methodology, Validation. **Yan Du:** Supervision, Validation, Funding acquisition, Writing - review & editing. **Bingling Li:** Conceptualization, Supervision, Formal analysis, Funding acquisition, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.elecom.2020.106665.

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