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Homogeneous electrochemical monitoring of exonuclease III activity and its application to nucleic acid testing by target recycling†

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A simple and fast electrochemical nucleic acid assay based on a target recycling strategy and the release of a double-stranded DNA intercalating redox probe upon digestion of a specific duplex by exonuclease III is demonstrated.

The development of portable, easy-to-use, fast, and inexpensive sensitive nucleic acid assays for point-of-care analysis of pathogenic agents is an important step forward in public health, forensic analysis, food industry or environmental monitoring.¹ Among the different strategies proposed for the development of such nucleic acid assays, isothermal nucleic acid amplification-based technologies, including LAMP, EXPAR, RPA, HDA, and MDA, were the most widely considered for the reason that they are very sensitive and specific.² They however require precise temperature control as well as complex sets of primers, enzymes and handling procedures which restrict their scope of applications. Methods based on signal amplification instead of target amplification are an attractive and much simpler alternative, in particular those based on DNA target recycling wherein a nucleic acid sequence takes part in multiple hybridization events to achieve a greatly enhanced sensitivity. The target recycling reaction can be achieved both with nicking endonucleases³ or exonucleases.⁴ These enzymes allow selective hydrolysis of an oligonucleotide probe hybridized to the target nucleic acid sequence, which in turn releases the intact target template for further rounds. In the case of nicking endonucleases, the oligonucleotide probe is cleaved at a specific nucleotide position, restricting thus possibilities in terms of probe design and choice of DNA target. In an attempt to overcome this limitation, new restriction endonucleases have been proposed⁵ as well as clever probe designs.⁶ However, a relatively high level of temperature control is still required for the selective melt of cleaved probes. The use of an exonuclease III (Exo III) for DNA target recycling appears to be a more versatile approach.⁷ This enzyme has the particularity to specifically catalyse the stepwise removal of

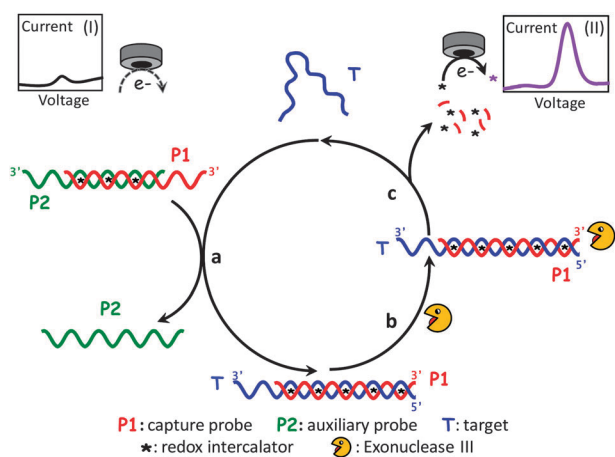
mononucleotides from blunt or recessed 3'-hydroxyl termini of double-stranded DNA (dsDNA), irrespective of the sequence present at the 3' end. Since Exo III works efficiently at low temperature, target recycling can be simply done at room temperature.

Okano and Kambara were the first to demonstrate the principle of DNA target recycling by Exo III in combination with a fluorescence detection method.^{4a} Since then, the Exo III-aided target recycling strategy has been adapted to diverse optical assay formats (homogeneous⁸ and heterogeneous^{4b}) as well as different types of labeled oligonucleotide probes (stem-loop^{4c} or linear⁹ molecular beacons, displacing probes¹⁰) or reporter nanomaterials.¹¹ Although fluorescent-based optical detection methods were the most widely used, they are not so easily amenable to the development of cost-effective and low-power handheld readout devices. By comparison, electrochemical detection methods can overcome these limitations since they are inherently more robust, simpler, less expensive and easier to miniaturize than optical ones, with the further advantages of being able to work with cloudy and/or colored samples. Until now, very few efforts have been made to combine the advantages of an electrochemical readout with a DNA target recycling amplification, and most of them were based on a heterogeneous assay wherein the target recycling process occurs directly on the electrode surface.¹² The problem with heterogeneous formats is their higher complexity and demand in terms of electrode preparation, as well as their slower DNA hybridization and enzyme kinetics compared with homogeneous assays. The development of faster and easier-to-use electrochemical detection strategies such as those taking advantage of homogeneous DNA hybridization remains thus highly desirable. Very recently, a homogeneous electrochemical assay based on an Exo III-aided target recycling strategy has been developed by Hsing and colleagues.¹³ Their approach is based on the detection of a redox label that is released by Exo III upon hydrolysis of a redox-labeled oligonucleotide probe. Although interesting, the method needs the nucleic coupling of a redox label to an oligonucleotide probe.

In the present work, we propose a homogeneous label-free electrochemical detection strategy which relies on a DNA target recycling amplification through the specific Exo III-digestion of a non-labeled oligonucleotide probe and an electrochemical readout which takes advantage of the difference in diffusion rates between a free and a bound dsDNA-intercalating

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Scheme 1 Principle of the Exo III-aided target recycling strategy and its associated homogeneous electrochemical detection.

redox probe. The method is exemplified for the detection of a DNA sequence specific to the *Salmonella typhimurium* bacterium, one of the main organisms causing the outbreaks of foodborne illnesses.

The principle of the method is depicted in Scheme 1. It consists in starting from a short dsDNA reservoir (**P1–P2**) in which an intercalating redox probe is bound. After adding (a) the target sequence **T** and forming a thermodynamically more stable **P1–T** duplex (having a 3′-blunt end at **P1** and a protective 3′-protruding end at **T**), (b) the Exo III can selectively digest the **P1** probe from the blunt 3′ end of the complementary **P1–T** duplex, thereby (c) releasing **T** for a new catalytic cycle. The catalytic reaction triggered by the addition of **T** thus leads to consumption of the **P1–P2** reservoir which in turns allows continuous release of the intercalating redox probe into the bulk solution. Assuming that the free intercalating redox probe is more easily electrochemically detected than the intercalated one, the enzyme reaction should thus lead to a current response increase for an electrode immersed in the reaction mixture. As a **P1–P2** duplex, a 15-bp dsDNA protected against Exo III activity by 3′-protruding ends at both strands was selected and designed for selectively hybridizing to a 37-mer target sequence **T** specific to the *S. typhimurium* bacterium (DNA sequences are listed in Table S1 in ESI†). As an intercalating redox probe, we have used the $\text{Os}[(\text{bpy})_2(\text{dppz})]^{2+}$ complex (where bpy = 2,2′-bipyridine and dppz = dipyrido-[3,2-*a*:2′,3′-*c*]phenazine), the latter being known to strongly intercalate in dsDNA and being sensitively detected by square wave voltametry (SWV) down to a few nanomolar.¹⁴

As shown in Fig. 1A, in the presence of 0.8 μM **P1–P2** duplex, the SWV response of 2 μM $\text{Os}[(\text{bpy})_2(\text{dppz})]^{2+}$ (a, green curve) is drastically diminished (b, black curve) because of intercalation of the osmium complex into the dsDNA. The signal decrease reflects thus the lower accessibility of the dsDNA-bound redox probe as well as its lower rate of diffusion to the electrode. Addition of 2 $\text{U } \mu\text{L}^{-1}$ of Exo III to the mixture does not substantially change the SWV signal (c, red curve), indicating that the duplex reservoir protected at its 3′-end is not significantly hydrolyzed by Exo III. This is in contrast to the large SWV peak current increase obtained after the addition of 100 nM **T** (d, blue curve).

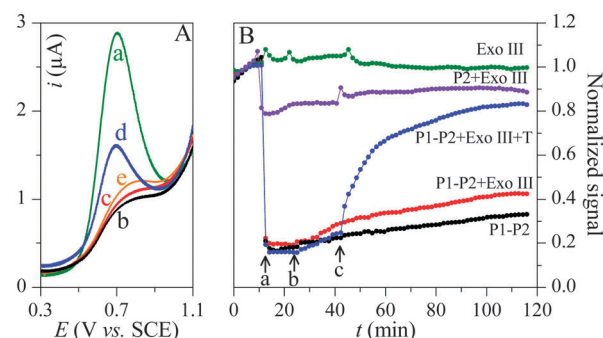


Fig. 1 (A) SWV curves recorded after successive additions of (a, green) 2 μM $[\text{Os}(\text{bpy})_2(\text{dppz})]^{2+}$, (b, black) 0.8 μM **P1–P2**, (c, red) 2 $\text{U } \mu\text{L}^{-1}$ Exo III, and (d, blue and e, orange) 100 nM **T** or **R**, respectively. (B) Real-time monitoring of the integrated SWV peak currents of 2 μM $[\text{Os}(\text{bpy})_2(\text{dppz})]^{2+}$ as a function of time and successive additions of (blue curve) (a) **P1–P2** duplex, (b) Exo III, and (c) **T**. The other plots are control experiments where some of the components were omitted (for details see Table S2 in ESI†).

This increase indicates that the triggering of catalytic reaction through the specific displacement of **P2** by **T** with concomitant formation of the 27-bp **P1–T** duplex leads to the release of intercalated redox probes by selective digestion of **P1**. Since several intercalating redox probes can be released per each duplex reservoir during a single hybridization–hydrolysis cycle, a more highly sensitive electrochemical detection can thus be expected compared with a strategy involving a single-labeled oligonucleotide probe. Such a catalytic reaction finally leads to a first order rate amplification of the electrochemical response as a function of time as soon as the duplex reservoir is not too much depleted. The specificity of the reaction was confirmed by the absence of signal increase when the target is substituted by a random non-complementary DNA sequence **R** (e, orange curve). A further control experiment was done with the Exo III beforehand deactivated at 70 °C for 20 min. In this case no signal change could be observed (data not shown).

To have a real-time analysis, the enzyme kinetic reaction was monitored in a custom designed electrochemical micro-titer plate^{14b} with 48 independent microwells (working volume of 50 μL), all being able to be addressed quasi-simultaneously by SWV. A series of kinetic plots showing the integrated SWV peak current as a function of time (recorded here every 94 seconds) is given in Fig. 1B. The blue curve shows the progression of a typical Exo III-aided target recycling amplification. Starting from 2 μM osmium complex in the digestion buffer, successive injections of (a) 0.8 μM **P1–P2**, (b) 2 $\text{U } \mu\text{L}^{-1}$ Exo III, and (c) 100 nM **T** were done. In parallel, different control experiments were conducted to determine the possible influence of each assay component on the electrochemical response. In the presence of **T**, a concentration-dependent signal increase was systematically observed, with a tendency to asymptotically reach a value similar to the one obtained when only **P2** is in solution (violet curve), a situation corresponding thus to the total digestion of **P1**. In that experiment, the signal value slightly decreased after the injection of **P2**, suggesting an interaction of the osmium complex with the ssDNA (probably by electrostatic binding). The remaining SWV response finally represents the maximal recoverable signal. Two different

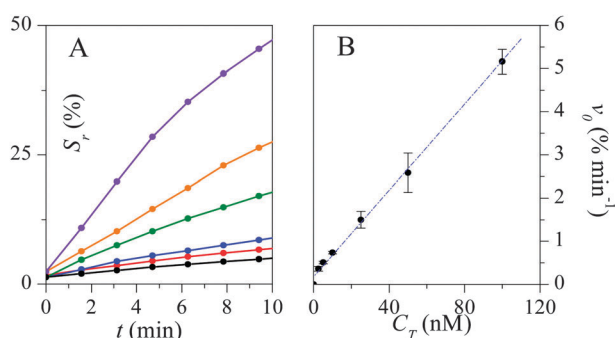


Fig. 2 (A) Time-dependent signal recovery (S_r) for different target concentrations (from bottom to top: 2.5, 5, 10, 25, 50, and 100 nM T). Experiments were performed in the presence of 1 U μL^{-1} Exo III, 2 μM [Os(bpy)₂(dppz)]²⁺ and 0.8 μM of P1–P2. (B) Plot of the signal recovery rate (v_0) as a function of target concentrations.

kinetic phases were observed for the enzymatic reaction (blue curve in Fig. 1B), *i.e.* an initial burst of hydrolysis (50% of the signal is recovered in less than 10 min) followed by a slower DNA digestion (an additional 1 h period is required to achieve the level-off). Assuming that the total recovery corresponds to the complete digestion of 0.8 μM P1, the kinetics of nucleotide hydrolysis for the initial phase is around 50 pmol nucleotide per minute, a value in line with former kinetic measurements.¹⁵

The kinetic plots were repeated for different target DNA concentrations ranging from 2.5 nM to 100 nM, and the percentage of signal recovery (S_r) with time is reported in Fig. 2A. The initial recovery rates (v_0) obtained from the slopes of the kinetic plots vary linearly with the target DNA concentration within the range 0–100 nM [$v_0/\% \text{ min}^{-1} = (5.0 \pm 0.1) \times 10^{-3} C_T/\text{nM} + (0.18 \pm 0.05)$; $r = 0.998$; $n = 7$] (Fig. 2B). The detection limit for the sensing system is 2.5 nM, and the assay reproducibility 15%. A key factor affecting the detection limit is the possible slow nonspecific digestion of the P1–P2 duplex. Different Exo III-resistant 3'-protruding ends were thus designed and tested, considering not only the influence of the tail length but also its base sequence (Fig. S2, ESI†). Some of the selected sequences were observed better for reducing the nonspecific hydrolysis. The assay selectivity was finally evaluated from comparative study between the perfectly matched T sequence and a double (M_2) or triple (M_3) mismatched sequence. Under the same experimental conditions, the signal recovery rate for M_2 was found to be 40% lower than the one obtained for T, meanwhile no signal recovery was observed for M_3 (see Fig. S3, ESI†).

In summary, we have demonstrated the feasibility of a homogeneous, label-free, single-step, electrochemical assay for the specific detection of a nucleic acid sequence at room temperature by target recycling amplification. This has been illustrated with the quantitative assay of a *S. typhimurium* specific DNA sequence down to nanomolar concentration (*i.e.*, ~ 0.1 pmol in 50 μL) in less than 10 min, which is competitive with optical DNA assays that use linear amplification by target recycling. On account of the sequence-independent Exo III activity this methodology might be extended to the detection of a wide range of nucleic acid targets, as well as small molecules or proteins able to specifically interact with structured nucleic acids (*e.g.*, aptamers).

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