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. 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. However, they 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 endonucleases or exonucleases. These enzymes allow selective hydrolysis of an oligonucleotide probe hybrized 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 proposed as well as clever probe designs. 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. 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. Since then, the Exo III-aided target recycling strategy has been adapted to diverse optical assay formats (homogeneous and heterogeneous) as well as different types of labeled oligonucleotide probes (stem-loop or linear molecular beacons, displacing probes or reporter nanomaterials). 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. 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. 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 chemical 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...
The catalytic reaction triggered by the addition of enzyme leads to a current response increase for an electrode immersed in an intercalated redox probe, the enzyme reaction should 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 replaced with 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 microtiter plate 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 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 U mL−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 ($S_t$) 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 μM 3′Exo III, 2 μM [Os(bpy)$_2$(dpdz)$_2$]³⁻ and 0.8 μM of P1–P2. (B) Plot of the signal recovery rate ($v_0$) as a function of target concentrations.

**Notes and references**


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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