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Electrochemical Real-Time Polymerase Chain Reaction

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We report the development of an electrochemistry-based real-time polymerase chain reaction (ERT-PCR) technique in this communication. Real-time PCR has established itself as the state-of-the-art technology for rapid and accurate specific nucleic acid sequence quantification.[1] The key feature is the capability of simultaneous target amplification and detection. Common to the three main detection chemistries, which include double-stranded deoxyribonucleic acid (ds-DNA) binding,[2] hydrolysis,[3] as well as hybridization probes,[4] the fluorescence signal of these probes is monitored at every PCR cycle and is proportional to the amount of amplicon produced.

To date, a number of real-time PCR thermal cyclers have been commercialized and widely used in clinical/research laboratories. Nevertheless, the footprint of these instruments is still too large for point-of-care applications due to the complex optics involved. Toward the goal of decentralized nucleic acid testing, the detection platform should be inherently simple, low-cost, and portable. One such candidate would be the electrochemical device, exemplified by the blood glucose meter for diabetic patients' daily checkup. Indeed, a great deal of work has been carried out for the electrochemical sequence-specific DNA detection over the past decade.[5] The majority was based on the hybridization of a target sequence to an oligonucleotide capture probe immobilized on an electrode surface, followed by signal transduction via a hybridization indicator. This post-amplificational detection strategy increases the overall assay time and risk of cross-contamination.

The most crucial issue of ERT-PCR lies in the compatibility of the electroactive hybridization indicator/detection platform with the PCR process. Among all the available indicators (groove binder/intercalator, covalently bound ferrocene probe, redox enzyme, and nanoparticle), the ferrocene (Fc) marker fulfills the requirements of thermal stability as well as negligible PCR inhibition arising from the nonspecific interaction between the indicator and PCR reagents (in particular the polymerase). In 2002, Wlassoff and King reported that the Fc marker could be enzymatically incorporated into PCR when some of the deoxythymine triphosphate (dTTP) was substituted by Fc-deoxyuridine triphosphate (dUTP).[6] With the Fc-labeled amplicon and Fc-dUTP both in solution phase, the detection was performed at the end of PCR using high-performance liquid chromatography−electrochemical detection method. Later, the same group demonstrated the incorporation of redox-active nucleotides into RNA and then electrochemical hybridization detection as well as the electrochemical detection of primer extension reactions on DNA self-assembled monolayers.[7]

To enable the real-time measurement of the Fc electrochemical signal associated with the specific amplicon, we take advantage of the solid-phase PCR technique for the progressive accumulation of the redox marker onto an electrode surface in response to the target amplicon generation, as schematically shown in Scheme 1. At the very beginning, the process behaves as the normal PCR to generate specific ds-DNA amplicon in the solution phase, except that it is labeled with ferrocene markers. It should be noted that $Vent_R$ (exo-) DNA polymerase is employed rather than the common Taq DNA polymerase because the former enzyme has a higher yield in the incorporation of modified deoxyribonucleotide triphosphates (dNTPs).[8] This ds-DNA is then heat denatured to its single-stranded (ss) form. At the annealing step, the ss-amplicon hybridizes to a complementary oligonucleotide extension probe immobilized on an indium tin oxide (ITO) electrode surface. Subsequent elongation of the probe by the polymerase during the PCR extension step results in a buildup of the Fc redox marker on the electrode surface. Eventually, all the probes are extended, and thus the solid-phase PCR process is completed.

Figure 1 shows the differential pulse voltammetric scans of the electrodes after PCR in the presence (sample) or absence (negative control: nonspecific or no template) of target sequence. The low level of redox signal from the negative control indicates a negligible Fc-dUTP adsorption/binding onto the electrode surface/extension probe as well as mis-priming/extension events. There are indeed a few important elements contributed to the successful solid-phase PCR. First, the linkage between the extension probe and electrode surface must be strong enough to survive through the temperature cycling during PCR (in particular the high denaturation temperature of 94 °C). Different immobilization chemistries were investigated, and we found that, with ITO as the electrode material, the attachment of an amino-modified oligonucleotide to an epoxysilane-functionalized surface achieved the required thermal stability (data not shown).[9] Second, the extension probe should be coupled to the transducer's surface at its 5′-end, allowing the addition of dNTPs as well as Fc-dUTP from its 3′-end by the $Vent_R$ (exo-) polymerase. Third, but equally important, the electrode and substrate surfaces have to be carefully passivated to avoid any unwanted adsorption of the PCR components that may inhibit the reaction efficiency and fidelity. In our system, ethanolamine was used to block the residual epoxide group after the probe immobilization step, while bovine serum albumin was included in the PCR reaction mixture to minimize the adsorption of the polymerase (dynamic passivation[10]).

To illustrate the usefulness of this methodology for electrochemically monitoring the PCR in real-time, rather than a single end-cycle PCR measurement, it is essential to follow the process at different cycles. Figure 2 presents a plot of the current signal (at −0.46 V vs Pt pseudo-reference electrode) as a function of the cycle number (0, 1, 3, 5, 15, and 25 cycles). It is clear that the current signal of the sample increases significantly with the cycle number, whereas that of the negative control remains more or less the same. One interesting point about this electrochemical scheme is that the onset (the point at which the sample signal is distinguishable from the negative control one) occurs much earlier

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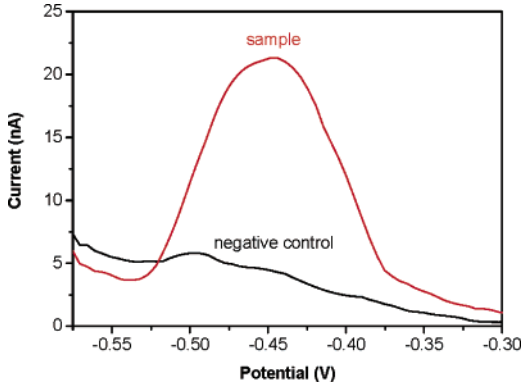
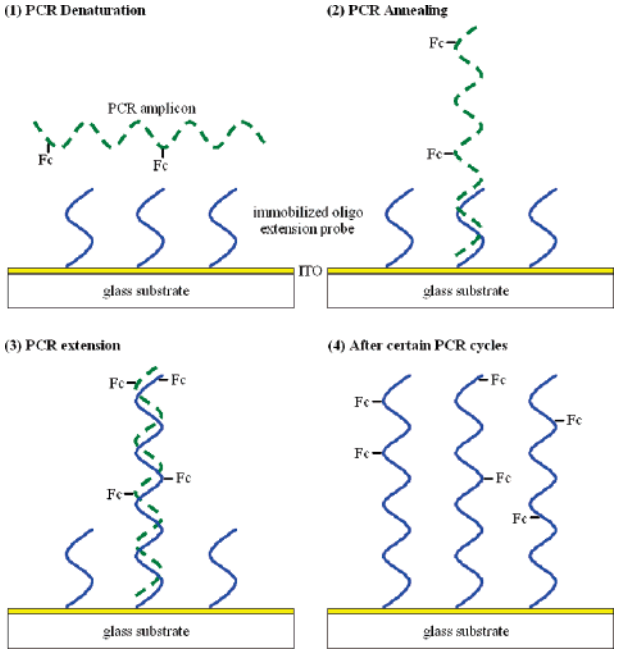


Figure 1. Differential pulse voltammetric scans of the indium tin oxide electrodes after 25-cycle PCR with (sample) or without (negative control) the target template. The electrochemical measurements were carried out using a pulse amplitude of 100 mV/s and a scan rate of 25 mV/s. Platinum wires were used as the counter and pseudo-reference electrodes. The experimental procedure is detailed in the Supporting Information.

Scheme 1. A Schematic Representation Showing the Working Principle of the Electrochemical Real-Time PCR Technique Based on the Solid-Phase Extension of Oligonucleotide Capture Probe with Ferrocene (Fc) Redox Marker



than that for the optical counterpart (usually 15−20 cycles, under a similar starting template amount used in this experiment). This suggests that this method could either be used for the real-time low-cycle PCR amplification or for the quantification of trace amounts of DNA.

The proof-of-concept experiments described here were conducted with an ITO-coated glass chip modified by the extension probe in a conventional thermal cycler setting. A series of samples were run in parallel, and the electrochemical measurements were made at different PCR cycles. To demonstrate this technique to be effective for point-of-care applications, the future studies include the implementation of this new strategy to a previously developed microfabricated PCR electrochemical device,[11] where the simultaneous PCR product amplification and electrochemical sequence−sequence detection could be achieved utilizing the on-chip component of thin film heaters and sensors for the PCR thermal cycling and the patterned three-electrodes system for the electrochemical signal sensing. Arrayed electrodes to enhance the multiplexing capability that is usually very limited in the conventional optical real-time PCR[12] will also be explored.

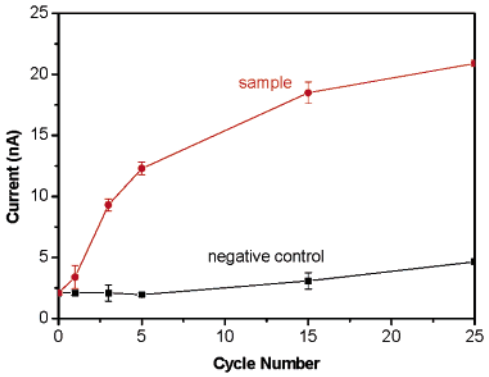


Figure 2. Plots of current signal against PCR cycle number for the extension probe-immobilized indium tin oxide electrode subjected to PCR in the presence (sample) or absence (negative control) of the target template.

In conclusion, we have demonstrated the utilization of solid-phase extension of the oligonucleotide capture probe with redox-active Fc-dUTP marker for electrochemical real-time monitoring of the PCR process. This new method can be used for the electrochemistry-based real-time monitoring of nucleic acids in decentralized applications.

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Supporting Information Available: Instrumentation and experimental procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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