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Redox-Active Nucleic-Acid Replica for the Amplified Bioelectrocatalytic Detection of Viral DNA

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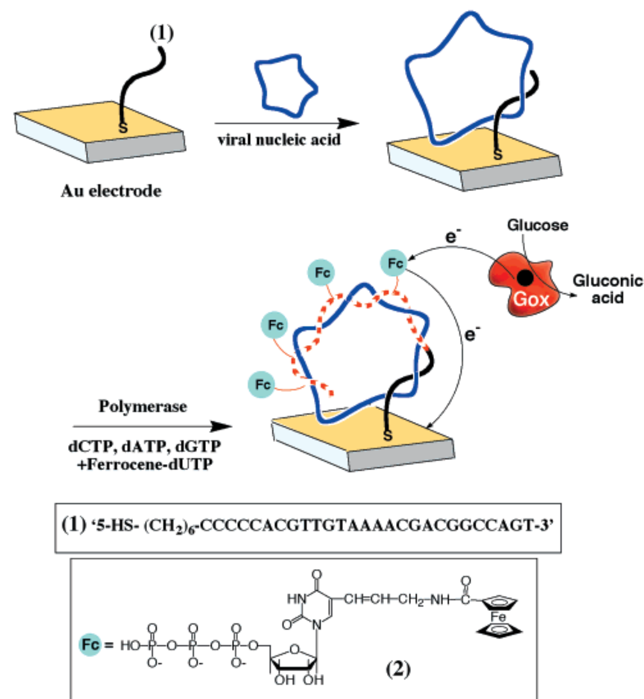
The detection of pathogens or autosomal recessive diseases is one of the future challenges in medicine and diagnostics.[1] Sensitive gene detection is accomplished by the amplification through the polymerase chain reaction (PCR).[2] Inherent limitations of PCR prohibit the application of the method for quantitative or parallel high-throughput analyses. Recent advances in DNA bioelectronics have addressed the amplified electronic transduction of nucleic acid recognition events. Dendritic, hyperbranched, oligonucleotides were employed to enhance the binding of DNA to electrodes.[3] Nucleic-acid-enzyme conjugates were employed as bioelectrocatalysts for the amperometric transduction of nucleic-acid recognition processes.[4] Biocatalytic conjugates that associate to nucleic-acid/DNA recognition pairs and stimulate the precipitation of an insoluble product on electrodes were used as an amplification system for DNA sensing.[5] Similarly, nucleic-acid-functionalized liposomes[6] or nanoparticles[7] were used as particulate labels for the amplification of the DNA sensing processes. Recently, the polymerase or reverse transcriptase-induced replication of biotin-labeled DNA/RNA, followed by the biocatalytic precipitation of an insoluble product on the transducer, was used for the amplified detection of viral genomes.[8] Faradaic impedance spectroscopy or microgravimetric quartz crystal microbalance measurements were used to transduce the detection processes. The amperometric detection of DNA was recently examined by using redox-labeled nucleic acids as probes, e.g. ferrocene-labeled nucleic acids.[9] Here we wish to report on the polymerase-induced generation of a redox-active DNA replica of the analyzed DNA. The redox-active replica is then coupled to the glucose-oxidase (GOx)-mediated oxidation of glucose that provides the bioelectrocatalytic amplification of the DNA detection process. The system provides a new facet in DNA bioelectronics as it demonstrates the ability to form redox-active DNA replicas that may activate bioelectrocatalytic cascades.

The thiolated 27-base nucleic acid (**1**) was assembled on an Au-electrode. The surface coverage of **1** was determined by microgravimetric quartz crystal microbalance experiments[10] to be 2×10^{-11} mol·cm^{-2}. The thiolated monolayer of **1** is complementary to the cyclic viral gene of M13ϕ DNA that includes 7229 bases. Accordingly, the monolayer-functionalized electrode was hybridized[11] with different concentrations of the M13ϕ DNA. Microgravimetric quartz crystal microbalance experiments indicate that in the presence of a bulk concentration of M13ϕ DNA corresponding to 1×10^{-9} M, the surface coverage of the hybridized cyclic DNA is 1.5% of the primer **1** coverage. The scheme for the generation of the redox-active DNA replicas and the amperometric amplified bioelectrocatalytic analysis of the gene is outlined in Scheme 1. The double-stranded assembly is interacted with the nucleotide mixture dNTP that includes the synthetic ferrocene-tethered dUTP (**2**) as a redox-labeled nucleotide,[12] in the presence of polymerase, Klenow fragment I. Replication of the target DNA results in a ferrocene-labeled, redox-active, DNA replica that can be analyzed electrochemically. Furthermore, as the ferrocene units act as electron-transfer mediators that contact redox-enzymes, e.g. glucose oxidase, with electrodes,[13,14] the bioelectrocatalytic oxidation of glucose provides an amplification route for the primary generation of the redox-active replica. Figure 1A shows the differential pulse voltammograms (DPV) corresponding to the ferrocene-functionalized replica formed upon the polymerase-induced replication of the double-stranded assembly formed between **1** and the M13ϕ DNA, 1×10^{-9} M, at different time intervals of polymerization. As polymerization proceeds, the electrical response of the redox-replica increases and it tends to reach saturation after ca. 60 min. Figure 1A, inset I, shows the cyclic voltammogram of the replicated redox-active DNA formed after 60 min of polymerization. These results clearly indicate that the ferrocene-labeled-dUTP is incorporated in the replicated DNA. Coulometric analysis of the redox wave of the ferrocene units after 60 min of replication, Figure 1A, inset I, indicates that ca. 3.6×10^{-11} mol·cm^{-2} of ferrocene are electrically contacted with the electrode. A parallel replication process with an Au-quartz crystal as transducer reveals a frequency change of $\Delta f = -67$ Hz upon polymerization for 60 min, indicating a replication efficiency of ca. 59%. Knowing the

Scheme 1. Amplified Detection of Viral DNA by the Generation of a Redox-Active Replica and the Bioelectrocatalyzed Oxidation of Glucose



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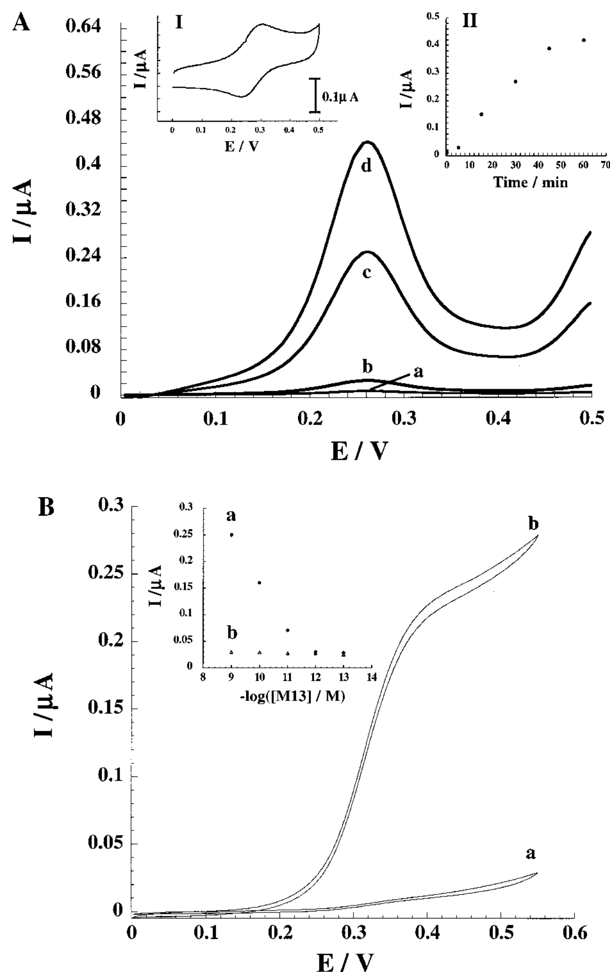

Figure 1. (A) Differential pulse voltammograms (DPV) of the ferrocene-tethered replicated DNA at different time intervals of replication: (a) before replication; (b) after 5 min; (c) after 30 min. and (d) after 60 min. Insets: (I) The cyclic voltammogram of the ferrocene-tethered DNA after 60 min of replication, scan-rate 100 $mV{\cdot}s^{-1}$. (II) Peak−current of the DPV curves for the ferrocene-tethered DNA at different replication time intervals. In all experiments the sensing interface was hybridized with M13ϕ DNA, 1×10^{-9} M. Replication was performed in Tris buffer, 20 mM, pH 7.5, that included 10 mM $MgCl_2$ and 60 mM KCl, in the presence of polymerase, Klenow fragment, 20 $U{\cdot}mL^{-1}$, and dCTP, dATP, dGTP, and ferrocene-dUTP, (**2**), each 1×10^{-3} M. (B) Cyclic voltammograms corresponding to (a) the ferrocene-tethered DNA generated upon analysis of M13ϕ DNA, 1×10^{-9} M, after replication for 60 minutes, in the presence of GOx, 2 $mg{\cdot}mL^{-1}$. (b) After addition of glucose, 5×10^{-2} M to the system described in (a). Data were recorded in phosphate buffer, 0.1 M, pH 7.4, under argon, scan-rate 2 $mV{\cdot}s^{-1}$. Inset: (Curve a) Peak−currents of bioelectrocatalytic anodic waves observed upon the analysis of different concentrations of M13ϕ DNA through the generation of the respective redox-active replica and the mediated bioelectrocatalyzed oxidation of glucose by GOx, 2 $mg{\cdot}mL^{-1}$, glucose, 5×10^{-2} M. (Curve b) The peak−currents of the anodic redox waves upon the analysis of different concentrations of M13ϕ DNA, through the generation of the respective ferrocene tethered replica, but in the absence of the GOx/glucose amplification system. All cyclic voltammograms were recorded in phosphate buffer, 0.1 M, pH 7.4, under Ar, scan-rate 2 $mV{\cdot}s^{-1}$.

surface coverage of the redox-label associated with the replicated DNA, we estimate that the average loading of a DNA replica with the ferrocene units corresponds to ca. 350 ferrocene units per replica (∼9% of all added bases). It should be noted that at a 59% yield of replication, and taking into account the number of A-bases, only ca. 40% of the ferrocene units that are incorporated in the DNA replica communicate with the electrode. This is probably due to the dimensions of the M13ϕ DNA that blocks the electrical communication of remote ferrocene units with the electrode. Figure

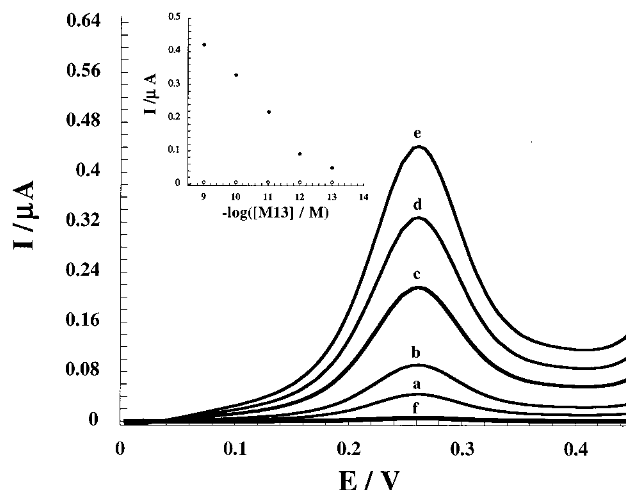

Figure 2. Differential pulse voltammograms (DVP) of the ferrocene-tethered replica formed upon the analysis of different concentrations (M) of M13ϕ DNA: (a) 1×10^{-13}, (b) 1×10^{-12}, (c) 1×10^{-11}, (d) 1×10^{-10}, and (e) 1×10^{-9}. (f) A control experiment where denatured calf thymus DNA, 5×10^{-7} M, is analyzed according to Scheme 1. In all of the experiments a fixed replication time corresponding to 60 min was used. Inset: Peak−currents of DPV of the ferrocene replica formed upon analysis of different concentrations of M13ϕ DNA.

1B, curve b, shows the cyclic voltammogram of the ferrocene-functionalized replica/M13ϕ DNA double-stranded assembly in the presence of glucose oxidase, GOx, 1 $mg{\cdot}mL^{-1}$ and glucose, 100 mM. An electrocatalytic anodic current is observed at the ferrocene-units oxidation potential. Control experiments reveal that no electrocatalytic current is observed in the absence of GOx or glucose. Also, replication of the target M13ϕ DNA with the nucleotide mixture dNTP/polymerase without the incorporation of **2** does not yield any electrocatalytic current in the presence of GOx/glucose. Thus, the tethered ferrocene components mediate the GOx oxidation of glucose.

As the surface coverage of the sensing interface by the M13ϕ DNA is controlled by its bulk concentration, the electrical response of the replicated ferrocene units and the electrocatalytic anodic currents resulting from the interaction with GOx/glucose are controlled by the bulk concentration of the M13ϕ DNA. Figure 2 shows the differential pulse voltammograms of the ferrocene-DNA replicas generated upon the polymerization of the double stranded assemblies formed between the **1** interface and different concentrations of M13ϕ DNA for a fixed time interval corresponding to 60 min. As the bulk concentration of M13ϕ increases, the DPV response of the system is enhanced. Figure 2, curve f, shows the DPV observed upon an attempt to analyze by the **1**-functionalized electrode the foreign denatured calf thymus DNA followed by an attempt to perform the polymerase-induced replication in the presence of **2**, dCTP, dATP, and dGTP. The lack of any amperometric signal indicates that no nonspecific adsorption of **2** onto the electrode takes place, and that the generation of the redox response of the ferrocene-tethered replica is a result of the specific formation of a double-stranded assembly between **1** and M13ϕ DNA. The resulting redox-tethered, double-stranded interfaces, formed in the presence of different concentrations of the analyte DNA, were then interacted with GOx/glucose as a bioelectrocatalytic amplification system. Figure 1B, inset, curve a, shows the calibration curve corresponding to the amperometric responses of the ferrocene-tethered DNA replica in the presence of GOx/glucose as the biocatalytic amplification system, at different concentrations of M13ϕ DNA. For comparison, the amperometric responses of the

ferrocene-tethered-replica without GOx and glucose (recorded at a scan rate of 2 $mV \cdot s^{-1}$) are presented by Figure 1B, inset, curve b.

In conclusion, we have introduced a new concept in DNA bioelectronics involving the generation of redox-active nucleic-acid replica and their use as electron-transfer relays for the activation of bioelectrocatalytic transformations. This method may be further broadened to electrode arrays (DNA-chips) for the amplified electrochemical detection of viral genes.

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(11) Hybridization of M13ϕ DNA with the **1**-functionalized electrode was performed in a phosphate buffer solution, 0.1 M, pH 7.5, that included NaCl, 0.2 M. Time of hybridization was 4 h.

(12) The *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid was prepared according to the following: Adamczyk, M.; Fishpaugh, J. R.; Heuser, K. J. *Bioconj. Chem.* **1997**, *8*, 253−255. The NHS-ferrocene ester, 5 mg, was added to a stirred solution of the amino-dUTP in a mixture of DMF (100 μL) and TEA (10 μL). The reaction was kept at room temperature for 4 h and then loaded onto a DEAE-cellulose column (1 × 25 cm). A linear gradient from 0.05 to 0.35 M TEAB (pH 7.4) was used. Fractions containing the ferrocene-labeled dUTP were collected, partially desalted, and lyophilized.

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