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A "four-ferrocene" modified stem-loop structure as a probe for sensitive detection and single-base mismatch discrimination of DNA

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ABSTRACT

We report the use of a four-ferrocene modified oligonucleotide as a probe for DNA detection with a gold electrode microsystem. This oligonucleotide is synthesized by automated solid-phase synthesis with four successive ferrocene moieties at the 5′-end and a C6-thiol modifier group at the 3′-end. The grafting of this 4Fc-DNA probe on a gold electrode microsystem results in the appearance of the ferrocene redox couple in cyclic voltammetry. The probe sequence is a stem-loop structure that folds efficiently on the electrode, thus optimizing electron transfer. Such architecture serves as sensor for DNA detection which is based on hybridization. The resulting disposable voltammetric sensor allowed direct, reagentless DNA detection in a small volume (20 μL). Electrochemical response upon hybridization with complementary short sequence (30-base length) and long sequence (50-base length) strands was observed by differential pulse voltammetry. Current variations were compared. The longer the sequence, the greater the decrease in current. The system's detection limit was estimated at 3.5 pM (0.07 fmol in 20 μL) with the 50-base length target and provided a dynamic detection range between 3.5 pM and 5 nM. Single mismatch detection showed a good level of sensitivity. The system was regenerated twice with no significant loss of Fc signal. Finally, 1 pM sensitivity was reached with a long chain analog of DNA PCR products of Influenza virus.

1. Introduction

The detection of nucleic acid sequences at the point-of-care is useful for a wide range of applications including clinical and research diagnostics [1], food safety control [2], forensics [3] and environmental monitoring [4]. In these fields, many research projects are currently devoted to the elaboration of miniaturized, integrated systems allowing efficient detection of biomolecules in very low volumes of biological fluids [5]. For this purpose, various optical, piezoelectric and electronic transduction techniques are employed [6]. Among them, electrochemical DNA sensors are widely recognized as being a promising solution for point-of-care diagnostics because electrochemical detectors are simple, portable and inexpensive [7,8]. Furthermore, this detection method can be easily adapted to a multiplexed array format.

The direct detection of nucleic acid sequences without enzymatic amplification is difficult, because the amount of nucleic acid available from samples is typically below 20 fM [9,10]. The standard sandwich-type strategy commonly employed for *in vitro* assays is far from achieving such levels of sensitivity. It is important to investigate systems able to detect very low concentrations of DNA (close to or below 1 pM), with efficient discrimination of single mismatches in DNA transcript sequences. Systems achieving such sensitivity should enable the reduction of the number of enzymatic amplification cycles before detection. Indeed, a more sensitive detection system means that the enzymatic amplification step required prior to detection is more cost-effective and less time-consuming [1]. This reduction of enzymatic amplification cycles is also a parameter required by the lab-on-chip technology aimed at integrating both amplification and DNA detection in a common microfluidic device [11].

Some groups working on high performance electrochemical sensors have described a very high level of sensitivity using enzymes for signal amplification. Liu et al. reported fM sensitivity by using an enzyme-based signal-ON E-DNA sensor that employed stem-loop structured probes which could be specifically conjugated with HRP enzymes upon DNA target hybridization [12]. Zhang et al. amplified the positive response resulting from the DNA target hybridization by using bilirubin oxidase (BOD) for the electrocatalytic reduction of ambient oxygen to water. They claimed the detection of 3000 copies of *Shigella* DNA (fM sensitivity) without enzymatic amplification [13]. Another work also achieved good sensitivity (1 pM) with a ligase-mediated electrochemical DNA sensor, particularly suitable for mismatch detection [14]. Nevertheless, the use of enzymes is expensive and systems require a rather complex architecture involving cumbersome steps, often resulting in

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a strong background signal. Alternatively, gold nanoparticles and quantum dots were used for signal amplification, in conjunction with stripping voltammetry. These methods achieved good detection limits for DNA (between 0.5 fM and 5 pM) [15–17]. Wang et al. reported the use of Fc-capped gold nanoparticle/streptavidin conjugates to amplify the electrochemical response of sandwich-type assays, enabling a detection limit of 2 pM [18]. In this case, the amplification of the voltammetric signals was attributed to the attachment of a large number of redox (Fc) markers to each DNA duplex formed. The same amplification was applied to biotinylated DNA target samples and a concentration level as low as 0.25 pM was detected [19]. More recently, Xia et al. confirmed the advantage of using a multiple label strategy to improve the detection limit. A sandwich-type construction with multiple methylene blue moieties proved to amplify the faradic current recorded on the test run with the complementary DNA target [20]. A subpicomolar detection limit was achieved with this multiple label strategy. Nevertheless, all these very sensitive methods are based on sandwich-type assays. They are all indirect methods which require a series of steps for target detection (hybridization and washing before signal measurement).

In the past decade, many researchers have focused on developing original strategies for DNA detection applications requiring direct, sensitive and economical analysis techniques. These requirements can be met by electrochemistry. In electrochemistry, the main strategies are based on the use of a redox-tagged single-stranded oligonucleotide as a probe for immobilization on electrochemical sensors [21–26]. Heeger [24] and Immoos et al. [23] pioneered the use of electrochemical stem-loop DNA as a capture probe for sensors. Hybridization of the target with the loop region induces a large conformational change in the surface-confined DNA and thus significantly affects the electron transfer rate between the redox moiety and the electrode. The detection limit of these systems is generally between 10 pM and 10 nM, as recorded by Liu et al. [12].

A few years ago, we focused on developing multiferrocenyl probes which, after grafting on a gold electrode microsystem, were expected to improve the detection limit significantly and offer high sensitivity for mismatch discrimination [27–29].

Unlike fluorescence, whose quenching processes do not permit the accumulation of numerous dyes in order to increase signal intensity, the incorporation of numerous ferrocenes close together in the oligonucleotide sequence enhances their electrochemical response exponentially, as observed by cyclic voltammetry (CV) [27].

In this paper, four ferrocene moieties were introduced at the 5′ end of a stem-loop oligonucleotide by automated synthesis, in the hope of obtaining a better electrochemical response from the system upon hybridization. After purification and characterization, this 4Fc-DNA was immobilized on the surface of a gold electrode microsystem *via* standard thiol chemistry. This paper deals with the electrochemical characterization of the 4Fc-DNA functionalized microsystem and presents our results in terms of sensitivity for DNA detection, single-mismatch discrimination and regeneration of the genosensor. The sensitivity of our system has been validated with a long synthetic DNA target of 50-base length and an analog of DNA PCR (polymerase chain reaction) products from Influenza virus RNA in complex medium.

2. Materials and methods

2.1. Chemicals

Ferrocene bis propanol was purchased from EZUS (Villeurbanne, France). DNA synthesis reagents and solvents were purchased from Glen Research (Sterling, Virginia). The other chemicals, such as electrochemical salts and solvents, were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

2.2. Fc phosphoramidite synthon synthesis

The 1-[3-*O*-dimethoxytritylpropyl]-1′-[3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidityl) propyl] ferrocene was synthesized in two steps from the ferrocene bis propanol according to the protocol described in a previous paper [30]. After synthesis, the synthon was characterized by ^{31}P NMR and ^{1}H NMR and dried overnight in a vacuum immediately before use. A solution of the Fc phosphoramidite synthon (0.12 M) in dry acetonitrile was used to incorporate ferrocene moieties in the oligonucleotide sequences during automated synthesis.

2.3. Design of the DNA model for influenza detection

The stem-loop sequence described in this article was designed by bioMérieux for Influenza B virus RT-PCR product analysis. Previous thermodynamic studies have demonstrated that the folded structure is not destabilized by incorporating numerous Fc molecules inside or at the extremities of the hairpin [28]. Ferrocenes bound to oligonucleotide probes act as electrochemical markers, affording a sharp electrochemical response which is highly sensitive to ionic and steric surrounding media. In the literature, Fc modified stem-loop structures have already been described as efficient probes for electrochemical DNA detection after anchoring on electrode surfaces [8,24,31]. In comparison to the papers already published in this field, the main advantage of our work lies in the incorporation of numerous ferrocenes in the probes to enhance the system's electrochemical response.

2.4. Oligonucleotide synthesis

The 4Fc-DNA probe is a 37 mer hairpin oligonucleotide bearing 4 successive Fc-phosphates at the 5′-end and a mercaptohexyl arm at the 3′-end in the form of a bis-hexanol disulfide. The 4Fc-DNA sequence is: 5′Fc-Fc-Fc-Fc-CGATCGGGAGAAGACGTCCAAAAACTCGATCGT-*O*-C_6H_{12}-S-S-C_6H_{12}-OH 3′. It was synthesized using standard phosphoramidite chemistry on an Applied Biosystems DNA synthesizer 394 (Foster City, USA) according to a method described in a previous paper [30]. The thiol modifier was incorporated using a homemade thiol derivatized CPG easily obtained by reacting bare CPG with bis-hexanol disulfide [32,33]. CPG loading with the thiol-modifier was 16 μmol g^{-1}. The bifunctional ferrocene phosphoramidite (1-[3-*O*-Dimethoxytritylpropyl]-1′-[3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidityl)propyl] ferrocene) was used in the DNA synthesizer to incorporate Fc directly into the oligonucleotide sequence during solid-phase synthesis. The coupling yield per cycle of the nucleotide phosphoramidites was calculated by dimethoxytrityl (DMT) quantification. The A, T, C and G synthons typically reacted with an average coupling yield of 98%. The average Fc coupling yield obtained from HPLC analyses of crude synthesis was 97%. The purity of the expected 4Fc-DNA in the crude synthesis was estimated at around 60% by HPLC (see Supplementary Information). The 4Fc-DNA was purified by HPLC using a DeltaPak C18 15 μm 300 Å with an acetonitrile gradient from 0 to 50% in 0.05 M triethylammonium acetate buffer (TEAAc), pH 7. Oligonucleotide purity was controlled by HPLC using an X-terra MS C18 2.5 μm column from Waters (Versailles, France). Analyses were carried out with an acetonitrile gradient from 5 to 50% in 0.05 M TEAAc, pH 7, in 65 min at 60 °C. Then, the oligonucleotide was characterized by MALDI-TOF Mass Spectrometry on an Applied Biosystems Voyager DE-PRO (Foster city, USA), using