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Target label-free, reagentless electrochemical DNA biosensor based on sub-optimum displacement

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Abstract

One of the most time consuming and complex steps in the detection of DNA target with a biosensor is the previous labeling of the target. In this paper, a novel target label-free, reagentless and easy to use DNA biosensor is reported. Electrochemical transduction (cyclic voltammetry, differential pulse voltammetry and impedance spectroscopy) and optical red out by surface plasmon resonance were chosen for the platform optimization.

This target label-free DNA detection method is based on displacement of sub-optimum labeled oligonucleotide. This strategy requires the pre-hybridization of the capture probe immobilized on the electrode surface with a sub-optimum mutated oligonucleotide pre-labeled with an electrochemically active ferrocene moiety. Due to the higher affinity of the target that is fully complementary to the capture probe, the sub-optimum ferrocene-labeled sequence is displaced when the fully complementary target is introduced into the system. The decrease of the electrochemical signal from the ferrocene verifies the presence of the target, which is proportional to the target concentration. A variation of this strategy was employed to enhance the ferrocene signal. A diffusional mediator, ferrocyanide, was introduced in the system to help in this purpose. This platform attains a stable, specific and reproducible response (5–15%), with a detection limit in the range of μM.

This electrochemical sensor is the first example of this kind of sensor to detect cystic fibrosis, however, this configuration could be generically applied to any application where the detection of a DNA target is involved.

Keywords: Label-free electrochemical biosensor; e-SPR; Impedance; Cystic fibrosis gene

1. Introduction

DNA arrays have attracted the attention of medical diagnostic and analytical chemists. The broad range of applications that has been found for DNA arrays makes them an important analytical tool. DNA arrays are relevant for the diagnosis of genetic diseases [1], detection of infectious agents [2], study of genetic predisposition [3], development of personalized medicine, detection of differential genetic expression, forensic science [4], drug screening [5], food safety [6] and environmental monitoring [7].

Despite the great promise of DNA arrays in health care and their success in medical and biological research, the technology is still not employed in everyday use in the clinic and is even further from implementation in home-diagnosis [8]. The principal challenges to their more wide spread use include high cost and complexity. Expensive laboratory instrumentation and biological knowledge for the labeling of the DNA prior to the sample injection into the array are both required. Moreover, false positives from non-specific adsorption are more likely. Development of target label-free methods for DNA sensors can potentially address these challenges.

Some examples of electrochemical label-free DNA sensors have already been commercialized. The eSensor™ fabricated by Motorola Life Science Inc. [9], proceeds via a sandwich hybridization assay. Thiol-capture probe is self-assembled on gold electrode, where it is hybridized to the DNA target. A third ssDNA ferrocene labeled is required to readout the target hybridization. Other examples of electrochemical sandwich

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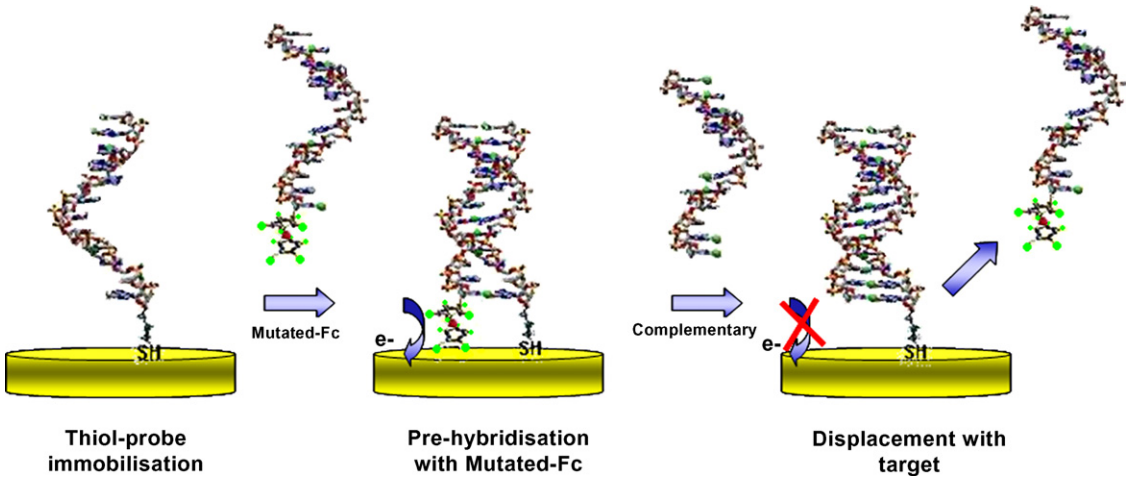


Scheme 1. Conceptual schematic of sub-optimum ferrocene-labeled mutated oligonucleotide displacement detection.

DNA sensors using dimethylcarbamyl ferrocene [10], or colloidal gold nanoparticles as labels [11] were reported.

Friz Biochem commercialized the EDDA™ (Electrically Detected Displacement Assay) for single nucleotide polymorphism (SNP) detection. The sensor was based on displacement of the target hybridized with the capture probe. The SNP that contains the target produces instability in the duplex with the capture probe, which allows the displacement of the target by a labeled oligonucleotide complementary to the capture probe [12]. A similar approach was reported by Mir and Katakis [13] but in this case the mismatched sequence was labeled and pre-hybridized with the capture probe. In this system is the target, which displaces the pre-hybridized labeled sequence. Thus, in this platform is not required a step after the addition of the target, which make it reagentless and more easy to used, increasing its applicability.

Toshiba developed the Genelyzer™. This DNA sensor is based on the electrochemical detection of the Hoechst 33258 molecule intercalated into the dsDNA [14]. When an intercalator such as Hoechst33258 or daunomycin [15] was inserted between the double-helix structures of the dsDNA which is facilitated by its planar aromatic ring, an enhancement in the intercalator's redox signal was observed. In contrast, some intercalators such as methylene blue have an affinity towards ssDNA, and a decrease of electrochemical signal was observed after hybridization [16]. Several metal complexes such as cobalt phenanthroline [17], cobalt bipyridine [18] ruthenium bipyridine [19], and anticancer agents such as echinomycin [20] and epirubicin [21], were used as labels for the detection of hybridization.

Fan et al. [22] reported the realization of an electrochemical sensor involving a molecular beacon strategy. The molecular beacon was a ferrocene-labeled DNA stem-loop structure that self-assembles onto gold. Hybridization with the target sequence induced the opening of the stem-loop, which in turn significantly altered the electron-transfer tunneling distance between the electrode and the ferrocene label. The resulting change in electron transfer efficiency was readily measured by cyclic voltammetry.

The label-free detection of DNA hybridization was also achieved using a ferrocyanide solution. This negatively charged molecule is repelled by the negative charges of the ssDNA capture probe on the surface, but this repulsion is more evident when the negative charges were duplicated after hybridization with the oligonucleotide target, which changes the electron-transfer resistance that can be measured by impedance spectroscopy [23,24].

Here we report novel strategy to develop a target label-free easy to use electrochemical DNA sensor. This sensor is based on the displacement of a pre-labeled sub-optimum oligonucleotide by a label-free target (Scheme 1). The detection strategy requires the pre-hybridization of the capture probe immobilized on the electrode surface with a sub-optimum mutated oligonucleotide labeled with a ferrocene molecule. Due to the higher affinity of the target that is fully complementary to the capture probe, the sub-optimum label can be displaced when the complementary target is introduced in the system. The decrease of the signal from the redox label verifies the presence of the target that is proportional to its concentration.

An improvement to the system's sensitivity was introduced by the inclusion of a diffusional mediator to help in the transfer of electrons produced by the ferrocene moiety. Both strategies were tested with a variety of electrochemical detection techniques (cyclic voltamperometry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS)) in order to decide on the one that could confer the highest sensitivity and also surface plasmon resonance (SPR) to obtain more information about the surface modification. The cystic fibrosis gene is the target described here but the strategy could be generically applied to any DNA sequence.

2. Experimental

2.1. Reagents

The 15-mer oligonucleotide sequences specific for detection of the cystic fibrosis gene were purchased from VBC Biotech Service Gmbh (Austria) and Eurogentec (Belgium). The oligonucleotide sequences involved in cystic fibrosis gene detection by sub-optimum displacement are listed below:

- 15-mer amine-labeled mutated oligonucleotide, for its bioconjugation with ferrocene (ferrocene-sub-optimum): amine-C_6-5′-AATATCATTGGTGTT-3′.
- 15-mer thiol-labeled capture probe oligonucleotide (thiol-capture probe): 5′-ACACCAAAGATGATA- C_6-thiol-3′.
- 15-mer complementary oligonucleotide (target): 5′-TATC-ATCTTTGGTGT-3′.
- 22-mer non-complementary oligonucleotide (non-complementary) used for controls: 5′-GAGGCGATCACAC-CGCAGACGT-3′.

All stock oligonucleotide solutions were made at 1 mg mL^{-1} with autoclaved Milli-Q water and stored at −20 °C.

N, *N*′-Dicyclohexylcarbodiimide (EDC), sodium tetraborate, tris(hydroxymethyl)aminomethane-hydrochloride acid (Tris–HCl), polyethylene glycol sorbitan monolaurate (Tween), ethylenedinitril tetraacetic acid (EDTA), Denhard's solution, diammonium citrate, saline sodium citrate buffer (SSC), ferrocyanide, ferricyanide and 2-mercaptoethanol were purchased from Sigma. Sodium tetraborate, dimethylformamide, ferrocene acetic acid, dithiothreitol (DTT), and *N*-hydroxysuccinimide (NHS) were from Aldrich and 3-hydroxypicolinic acid (HPA) from Fluka. The Sephadex G-25 DNA grade resin was purchased from Amersham Pharmacia Biotech.

2.2. Instrumentation

For characterization of ferrocene-labeled oligonucleotide, matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) detection was carried out in a stainless steel plate with a Voyager DE-STR from Applied Biosystems.

The electrochemical surface plasmon resonance (e-SPR) measurements were performed with double channel Autolab ESPRIT™ equipment from Eco Chemie. The e-SPR electrochemical cell is a two-electrode system, with a 4.8 mm^2 gold layer sensor disk working electrode that is defined by a gasket when the cell is assembled. The 35 μL cell has platinum reference and counter electrode. The instrument can be programmed for automatic injection and washing steps. Electrochemical measurements were carried out in a CH Instruments Inc. potentiostat. The impedance curve fitting was performed with ZView 2 software.

2.3. Ferrocene DNA labeling

A freshly prepared solution of 125 nmol of NHS and 250 nmol of EDC in anhydrous dimethylformamide were added to a solution of 100 nmol of ferrocene acetic acid in the same solvent. The final volume was 300 μL. The reaction mixture was stirred at room temperature under argon until appearance of a precipitate. EDC forms an active ester functional group with carboxylate group using NHS. In this reaction a precipitate of *o*-acylurea is formed that was removed by centrifugation and the supernatant was added to 90 μL of 0.1 M sodium tetraborate buffer at pH 8.5 containing 10 nmol of the amino modified oligonucleotide and it was left to react for 6 h at room temperature. The active ester group in the presence of amine nucleophiles is attacked; the NHS group leaves as a precipitate, creating a stable amide linkage with the amine. The white precipitate was separated by centrifugation. The supernatant contained a ferrocene-labeled oligonucleotide. The modified oligonucleotide was purified with G-25 Sephadex column. UV spectrophotometry was used to collect the appropriate fraction from the column. The product was characterized by UV spectrometry, cyclic voltammetry and MALDI-TOF.

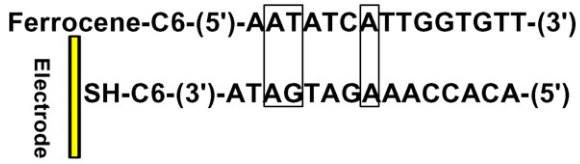
Cyclic voltammetry of ferrocene-aptamer was performed in 10 mM Tris–HCl buffer, 150 mM NaCl, pH 7.5 at 0.025 V s^{-1} with an 18 mm^2 gold working electrode and a reference and counter Ag/AgCl electrode. MALDI-TOF characterization was carried out using a HPA matrix. A 50 g L^{-1} solution of HPA in Milli-Q water and 50 g L^{-1} solution of diammonium citrate were prepared and both reagents were combined in a ratio of HPA:NH_4 citrate (9:1). The mixture was stirred for 1 min and centrifuged to separate the white precipitate. The same volume of supernatant was mixed with ferrocene-aptamer, in a final concentration of 12 pmol μL^{-1}. Two microliters of the mixture was allowed to air-dry on a stainless steel plate. Twenty thousand volts of accelerating voltage, 92% of grid voltage, 400 ns of extraction delay time, 1715 of laser intensity and 50 of shots were applied in a linear mode for the measurement.

2.4. Immobilization and hybridization of thiol-capture probe

Twelve micrograms per milliliter of thiol-capture probe in 1 M KH_2PO_4 was immobilized for 1 h at room temperature on the gold e-SPR chips. One millimolar of mercaptoethanol was used for 1 h at room temperature to block the remaining gold surface non-occupied by the capture probe. Pre-hybridization of ferrocene-modified oligonucleotide was performed by incubating 48 μg mL^{-1} of this oligonucleotide in 10 mM Tris–HCl, 1 mM EDTA, 0.3× SSC and 2× Denhard's solution at pH 7.5 (hybridization buffer) for 60 min at room temperature. After pre-hybridization CV, DPV and EIS were carried out in buffer and in ferrocyanide/ferricyanide. Afterwards 30 μg mL^{-1} of target in hybridization buffer was incubated for 30 min at room temperature. After displacement CV, DPV and EIS were recorded in the same manner. After each step the cell was washed with a solution of 100 mM Tris–HCl pH 7.5, 150 mM NaCl and 1% Tween.

2.5. Electrochemical monitoring of direct displacement

The e-SPR cell was used to detect the pre-hybridization of the ferrocene-modified oligonucleotide and the subsequent displacement by CV, DPV and EIS. The conditions used for the electrochemical measurements were; for CV a scan rate of 0.1 V s^{-1}, between −0.5 and 0.5 V was applied, in the case of DPV scanning between −0.2 and 0.2 V, with an increment of potential of 0.005 V, amplitude of 0.05 V, pulse width of 0.05 s and a pulse period of 0.5 s was used and for EIS initial potential of 0.005 V, frequencies from 0.1 MHz to 0.1 Hz and amplitude of 0.005 V was applied. All experiments were carried out in 35 μL of 10 mM PBS, 150 mM NaCl pH 7.5.



Scheme 2. Representations of capture probe sequence and mutated oligonucleotide ferrocene labeled. The three mutations that contain the sub-optimum oligonucleotide are inside the rectangle.

2.6. Electrochemical monitoring of mediated displacement

The same measurements CV, DPV and EIS were carried out with an equimolar mixture of ferrocyanide and ferricyanide at a concentration of 1 mM in 10 mM PBS, 150 mM NaCl pH 7.5. Afterwards the cell was cleaned with wash buffer and cleanliness was verified by CV. The same CV shape as before the detection of ferrocyanide/ferricyanide should be obtained to assure the cleanliness of the surface.

3. Results and discussion

The sequence of the ferrocene-labeled sub-optimum oligonucleotide used in the displacement assay is offset from the capture probe by 1 bp and contains three mutations. The 1 bp offset might enhance the unzipping and zipping by the complementary oligonucleotide (target) contributing to easier displacement. Two of the mutations in the sub-optimum are separated from the first base by one base to facilitate the displacement. Thus, initiation only requires breaking 1 bp to start the sub-optimum displacement by the target (Scheme 2).

The following four parameters that impact both detections and surface architecture were examined with the goal of optimizing sensor performance.

- The position of the ferrocene label position for impacting hybridization as well as in the electrochemical detection.
- The concentration of the capture probe in solution during electrode modification for controlling the surface coverage.
- The concentration of ferrocene-sub-optimum for reducing the change in the zero current and increasing the efficiency of displacement.
- The NaCl concentration in the hybridization buffer for improving the pre-hybridization stability of the duplex.

The criteria that was used for optimization was the efficiency of displacement as percentage of the initial signal (pre-hybridization of labeled sub-optimum oligonucleotide) after 30 min of displacement when incubated with 30 $\mu g\,mL^{-1}$ concentration of target. At a second level the inferred (as explained below) non-specific or "zero" displacement by buffer was used as a criterion (such "zero" displacement was minimized).

Optical and electrochemical techniques were used to investigate this system. SPR provided information on surface processes without the need for labeling. SPR detection can monitor in real time the immobilization of the thiol-capture probe on gold chips, the pre-hybridization of ferrocene-sub-optimum oligonucleotide, and displacement of this pre-hybridized by the complementary target. Electrochemical methods were primarily used to record the difference in electrochemical response before and after displacement. Various electrochemical techniques (CV, DPV and EIS) were used to record the system response. Since each technique is based on different principles, advantages of sensitivity signal/ratio etc. can be discovered by a survey of techniques.

Two different strategies were followed to detect ferrocene-sub-optimum displacement by the target. The first was the direct detection of the ferrocene label, and the second was the indirect detection of this label through the mediation of a ferrocyanide solution.

3.1. SPR detection

The non-specific or "zero" displacement due to the presence of buffer can be obtained from SPR. A general rule of thumb used for the calculation of surface coverage in Autolab SPR is that 120 m° of change corresponds to 1 $ng\,mm^{-2}$. The coverage of a monolayer ($3.7 \times 10^{-13}\,mol\,mm^{-2}$, [25]) of target ($MW = 4564\,g\,mol^{-1}$) should yield about 200 m° of response. Loss of the base structure in buffer occurs and it can be readily detected. However, when the ferrocene-sub-optimum oligonucleotide ($MW = 5003\,g\,mol^{-1}$) is displaced by another oligonucleotide with similar molecular weight, a very small difference in refractive index results. The typical response for the hybridization of the sub-optimum is in the range of 220 m°. We consider any SPR results that show an angle change much higher than 20 m° after displacement are the result of non-specific displacement by buffer. Obviously, the higher this delta, the higher the non-specific displacement is, and the less reliable the architecture. Thus, an additional criterion (displacement value over 20 m°) was used for the qualitative exclusion of configurations if other criteria were similar.

The first parameter investigated was the position of the label. Ferrocene labeled at the 5′-end of the mutated oligonucleotide (close to the surface) should offer higher electrochemical signal due to its proximity to the electrode surface. However, steric impediment of the label might cause problems in the hybridization event. The pre-hybridization of the ferrocene-sub-optimum with the immobilized capture probe was tested with two different ferrocene-sub-optimum oligonucleotides, one labeled in the 5′ and another labeled in the 3′-end. The SPR result shows that the labeling at 3′ produced slightly more hybridization (79 m°), than the 5′ labeled (70 m°), indicating some impact to hybridization due to the position of the label.

However, a large difference was observed for the electrochemical detection of the ferrocene. CV in buffer carried out after the hybridization of ferrocene-sub-optimum with the capture probe showed ferrocene peaks in the case of ferrocene label at 5′. No peaks were detected in the case of ferrocene label at 3′. All subsequent experiments were performed with the ferrocene label at the 5′ end.

Using the previous approach procedure the concentration of capture probe immobilized on the gold surface was optimized.

The optimal density of capture probe on the surface is a balance between maintaining sufficient distance between strands to allow for high hybridization efficiencies while at the same time maintaining the highest density of capture probes to provide the greatest number of hybridization events. The trade-off is that maximum hybridization signal and maximum hybridization efficiency are not obtained at the same probe density [26]. DNA probes that are too closely packed cannot participate in the hybridization reaction due to steric hindrance or electrostatic interactions [27,28].

As shown in Fig. 1, the increase of capture probe concentration in solution increases the amount of immobilized probe on the surface and results in a greater amount of hybridization with ferrocene-sub-optimum oligonucleotide. At a concentration of 24 $\mu g\,mL^{-1}$ the capture probe was close to the hybridization saturation. However, at the 24 $\mu g\,mL^{-1}$ concentration a higher non-specific or "zero" displacement was also observed. Probably higher repulsion from the closer neighboring strands is obtained in a more packed capture probe configuration, destabilizing the duplex. We subsequently selected 12 $\mu g\,mL^{-1}$ of capture probe for further experimentation.

The next variable optimized was the concentration of ferrocene-sub-optimum oligonucleotide that was used for pre-hybridization with the capture probe. An increase of the pre-hybridization of the ferrocene-sub-optimum contributes to a system with higher signal, being easier to detect the displacement at lower target concentrations.

It appears that with a concentration of 48 $\mu g\,mL^{-1}$ ferrocene-sub-optimum system arrives to the hybridization saturation and a higher non-specific displacement was observed at lower concentrations of ferrocene-sub-optimum, therefore 48 $\mu g\,mL^{-1}$ of ferrocene-sub-optimum was chosen as the optimum concentration by this technique.

The last variable to be optimized was the concentration of sodium chloride in the hybridization buffer. An increase of cations in the hybridization environment might stabilize the duplex because both strands in the duplex are negatively charged and suffer mutual repulsion.

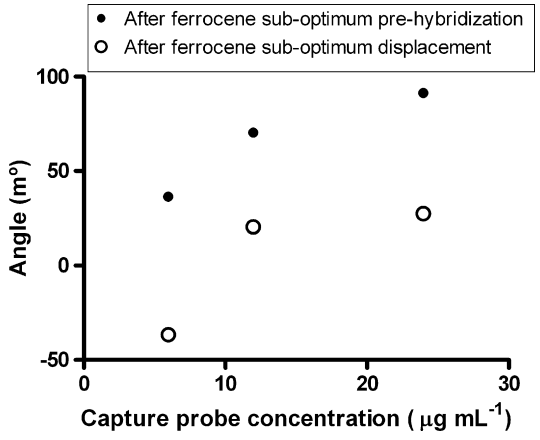


Fig. 1. Results obtained by SPR from the pre-hybridization of 12 $\mu g\,mL^{-1}$ ferrocene-sub-optimum oligonucleotide and the subsequent displacement with 30 $\mu g\,mL^{-1}$ complementary target oligonucleotide at different concentrations of capture probe (6, 12 and 24 $\mu g\,mL^{-1}$) in 1 M KH_2PO_4.

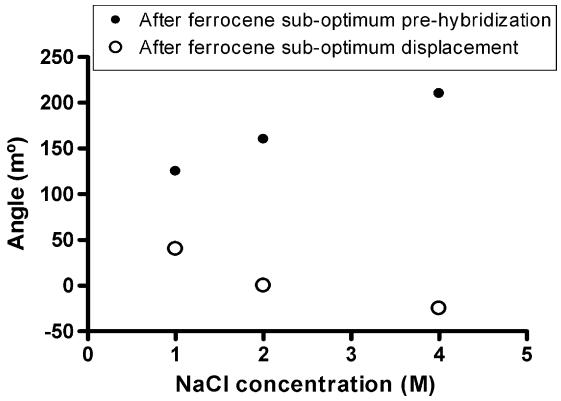


Fig. 2. Results obtained by SPR from the pre-hybridization of ferrocene-sub-optimum oligonucleotide and the subsequent displacement with complementary target oligonucleotide at different concentrations of NaCl (1, 2 and 4 M) in hybridization buffer during the pre-hybridization of ferrocene-sub-optimum oligonucleotide. Electrodes modified with 12 $\mu g\,mL^{-1}$ of capture probe and 48 $\mu g\,mL^{-1}$ of ferrocene-sub-optimum.

Fig. 2 shows a high enhancement of hybridization of ferrocene-sub-optimum oligonucleotide with the capture probe when the concentration of NaCl in hybridization buffer increased. So it seems correct the hypothesis that higher concentrations of salt during hybridization stabilize the duplex, however, at the same time, it appears that extreme non-specific displacement also is enhanced when the target appear in 1 M NaCl hybridization buffer, because higher destabilization of the duplex is obtained when more drastic change of the ionic strength is applied.

For this reason, 1 M of NaCl in pre-hybridization and displacement was chosen as optimum.

3.2. Direct ferrocene-sub-optimum displacement electrochemical detection

Ferrocene label is direct detected in this strategy after and before displacement in buffer 10 mM PBS, 150 mM NaCl pH 7.5.

The results observed with the three electrochemical techniques for detection of direct displacement event is described. First CV in buffer was used, after pre-hybridization of ferrocene-sub-optimum this technique detected the redox peak of the ferrocene molecule at −0.26 and 0.06 V versus Pt. When complementary oligonucleotide displaced ferrocene-sub-optimum oligonucleotide, the height of the peak decreases depending on the amount of ferrocene molecules that are removed close to the electrode

Since CV results in small changes, DPV in buffer was used as an alternative. As in CV, the height of the peak is directly related with the amount of ferrocene molecules immobilized, so after displacement the peak decreases in comparison to the peak before displacement. Current DPV allows for a very effective correction of the charging background current, which should allow better sensitivity. Fig. 3, shows a typical DPV.

Finally impedance spectroscopy in buffer was also used to try to discern differences of the spectrum before and after

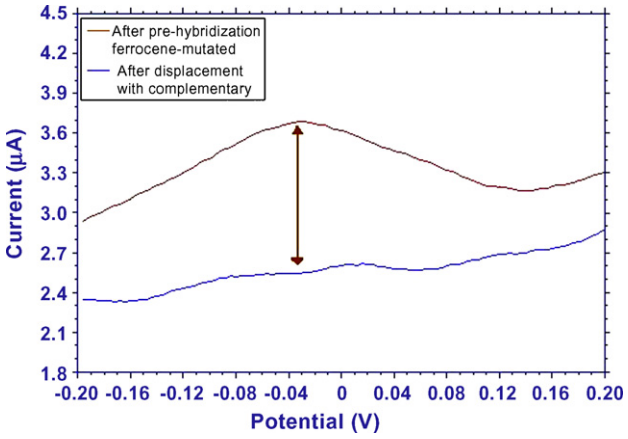


Fig. 3. Typical DPV before and after displacement of ferrocene-sub-optimum oligonucleotide. DPV was performed between −0.2 and 0.2 V, with an increment of potential of 0.005 V, an amplitude of 0.05 V, a pulse width of 0.05 s and a pulse period of 0.5 s in the presence of 10 mM PBS, 150 mM NaCl, pH 7.5. The highlighted signal difference is indicative of displacement.

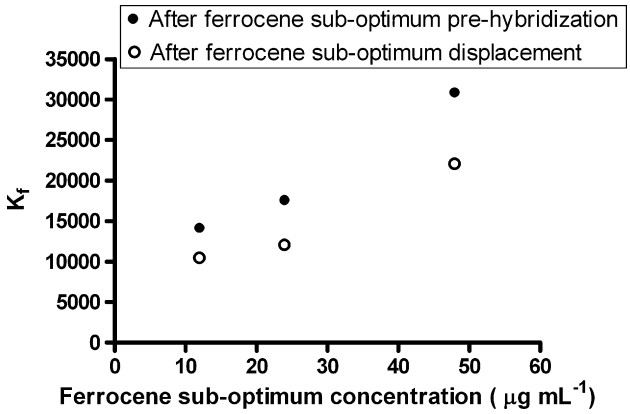


Fig. 5. Results obtained by EIS from the immobilization of 24 μg mL^{-1} capture probe oligonucleotide and displacement with 30 μg mL^{-1} complementary target oligonucleotide at different concentrations of ferrocene-sub-optimum (12, 24 and 48 μg mL^{-1}).

displacement. The change of ferrocene concentration close to the electrode surface affects mostly the electron-transfer resistance.

Laviron [29] related the parameters of electron-transfer resistance (R_{et}) and capacitance of the immobilized layer (C_{ads}) with the heterogeneous electron-transfer rate constant (k_f) through the following equation:

$$k_f = (2R_{et}C_{ads})^{-1}$$

When the amount of ferrocene molecule increases enhancement of electron transfer between the label and the electrode was observed ($k_f = 30{,}800$ for a pre-hybridization of 48 μg mL^{-1} sub-optimum oligonucleotide). After displacement the amount of ferrocene molecules decreases with the subsequent decrease of k_f ($k_f = 22{,}000$ for a displacement with 30 μg mL^{-1} complementary target oligonucleotide).

Impedance data was analyzed by fitting it to an equivalent electrical circuit model, which is required to obtain the values of R_{et} and $C_{ads.}$ Impedance values detected in displacement of ferrocene-sub-optimum system in buffer were fitted to the next circuit (Fig. 4).

In this circuit, R_s is the solution resistance, C_{dl} is the double-layer capacitance, R_{et} is the electron-transfer resistance and CPE_{ads} is a constant phase element of immobilized molecules. CPE is capacitor that does not behave ideally, due to surface roughness and/or different ion adsorption/desorption kinetics.

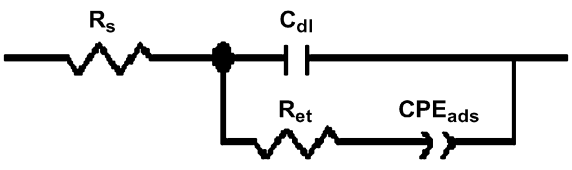


Fig. 4. Equivalent circuit for displacement of ferrocene-sub-optimum oligonucleotide system in buffer.

Additionally optimization of all the variables were made as mentioned by means of the different electrochemistry techniques.

The results obtained by CV in buffer showed an increase of peak height at higher concentrations of capture probe, which was also corroborated with SPR results. It is assumed that this increase corresponds to higher amount of ferrocene-sub-optimum hybridized. After displacement of ferrocene-sub-optimum by the target a decrease of peak height was obtained, which demonstrate the functionality of the system. Best signal displacement was obtained with 24 μg mL^{-1} of capture probe. Similar results were obtained with DPV and in impedance.

In ferrocene-sub-optimum oligonucleotide concentration optimization similar trends but higher signals were obtained as with the capture probe concentration optimization. In this case also an increase of concentration of ferrocene-sub-optimum oligonucleotide showed an increase of electrochemical signal and a decrease of signal after displacement As with SPR better displacement was obtained with 48 μg mL^{-1} of ferrocene-sub-optimum (Fig. 5).

NaCl concentration in hybridization buffer was also optimized. By SPR an increasing hybridization of ferrocene-sub-optimum was obtained with increasing concentration of NaCl, whereas by CV, DPV (Fig. 6) and impedance in buffer a decrease of electrochemical signal was detected at increasing concentration of salt. A similar behavior was already reported by Liu et al. [30] and Hartwich et al. [31]. They assume that at low ionic strength the duplex are oriented perpendiculars to the surface, because of the electrostatic repulsion between strands, facilitating the electron transfer. In contrast, high ionic strength produces a shield of the DNA charge, resulting in a collapsed configuration of the duplex, forming an obstacle for the electron transfer. Another reason for this behavior might be the formation of chloride film on the electrode surface due to the high concentration of NaCl during the pre-hybridization incubation, which might affect electron transfer

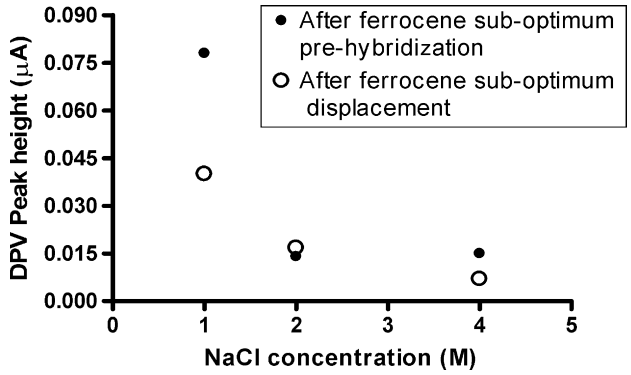


Fig. 6. Results obtained by DPV from the pre-hybridization of ferrocene-sub-optimum oligonucleotide and the subsequent displacement with complementary target oligonucleotide at different concentrations of NaCl (1, 2 and 4 M) in hybridization buffer during the pre-hybridization of ferrocene-sub-optimum oligonucleotide. Electrodes modified with 12 μg mL^{-1} of capture probe and 48 μg mL^{-1} of ferrocene-sub-optimum.

Thus as also demonstrated SPR 1 M NaCl is the optimum concentration for pre-hybridization of the sub-optimum oligonucleotide with the capture probe.

3.3. Mediated ferrocene-sub-optimum displacement electrochemical detection

In order to improve the sensitivity of the ferrocene-sub-optimum detection a mediator was added in solution. Ferrocyanide/ferricyanide can help to transfer the electrons produced in the redox reaction of ferrocene label to the electrode surface enhancing the signal. As well the ferrocyanide/ferricyanide solution has a catalytic effect that improves the ferrocene detection. However, this modification renders to a system no longer reagentless.

Steel et al. [32] and Choi et al. [33] used ferricyanide in solution and CV to detect changes in the surface charge of gold electrodes and the consequent electrochemical interaction between $Fe(CN)_6^{3-}$ and the electrode when a dsDNA was hybridized on the electrode surface. Because when hybridization was done the reduction/oxidation of negative charges on the sugar-phosphate backbone of dsDNA cover the electrode surface, which prevents $Fe(CN)_6^{3-}$ from approaching the surface, decreasing current signal. So a similar decrease of current might be obtained in the displacement system. However, this system can not be directly compared with the Steel and Choi experiments because a ferrocene label was attached to the 5′-end of the hybridized species. The positively charged ferrocene molecule linked to the negatively charged oligonucleotide, produced an opposite effect of $Fe(CN)_6^{4-}$/$Fe(CN)_6^{3-}$ attraction, hence increasing the current signal.

The effect of the attraction of ferricyanide to a positively charged molecule attached to DNA was already reported by Park and Hahn [34]. A dsDNA-modified electrode was treated with an intercalating agent, proflavine, then the negative charges on the duplex strands were compensated with the positive charges of intercalator molecules, which provides a surface-charge condition that facilitates the approach of $Fe(CN)_6^{3-}$ to the electrode surface, detecting an increase of signal when proflavine was intercalated in the dsDNA.

Furthermore an effect of enhancement of ferricyanide signal with a ferrocene label was reported by Heeger et al. [35]. Their approach worked by the addition of an electrochemical mediator, ferricyanide, that was reduced by the ferrocene label, thus in the presence of ferricyanide the electrode repeatedly reduced each ferrocene increasing the peak currents. A similar system was reported by Ostatna et al. [36] and Boon et al. [37], in this case the reduction of methylene blue was considerably improved in the presence of an electron acceptor, $Fe(CN)_6^{3-}$.

So a double effect of attraction and enhancement of the signal is produced by the ferrocene label to ferrocyanide/ferricyanide, which allows more sensitive detection of the pre-hybridization of ferrocene-sub-optimum and the subsequent displacement.

The redox reaction produced between ferrocene label and ferricyanide is shown Scheme 3.

As the scheme shows, this system is based on transfer of the electrons from the reduced form of ferrocene to $Fe(CN)_6^{3-}$ resulting in the conversion of $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$ and the oxidation of ferrocene, which is reduced on the electrode surface. So in the presence of ferricyanide the electrode repeatedly reduces each ferrocene molecules immobilized on the electrode surface increasing the current signal.

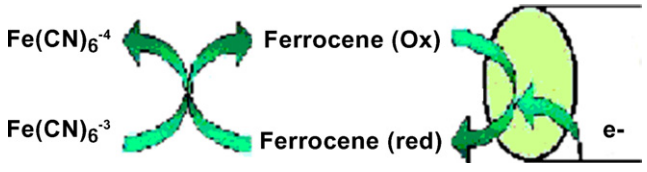
To demonstrate this hypothesis chronoamperometry of a modified electrode with complementary oligonucleotide hybridized with capture probe immobilized on the surface and other electrodes with ferrocene-sub-optimum oligonucleotide hybridized with immobilized capture probe was carried out.

Each electrode was immersed in 15 μL of 10 mM PBS, 150 mM NaCl pH 7.5 in a two-electrode cell set up with platinum as reference and counter electrode. A potential of −0.2 V was applied to record reduction of ferrocene. When the current arrived at the base line (after 100 s) 5 μL 4 mM of $Fe(CN)_6^{-3}$ in 10 mM PBS, 150 mM NaCl pH 7.5 was injected into the cell to record the effect of ferrocyanide in the reduction of ferrocene.

Fig. 7 shows the results obtained in the chronoamperometry. Red and blue lines show a sustained reduction current of about 0.47 μA due to the cycle between ferrocene and ferrocyanide shown in Scheme 3. This cycle was not produced in the absence of ferrocyanide (black line) or of ferrocene (green line)

These experiments demonstrate an electrocatalytic cycle that results in signal amplification when ferrocyanide was present in the system with a ferrocene label.

The CVs and DPV scans of the capture probe ferrocene-sub-optimum duplex showed an enhancement in signal in the presence of ferrocyanide/ferricyanide in solution.



Scheme 3. Redox catalytic reaction produced between ferrocene label and ferricyanide.

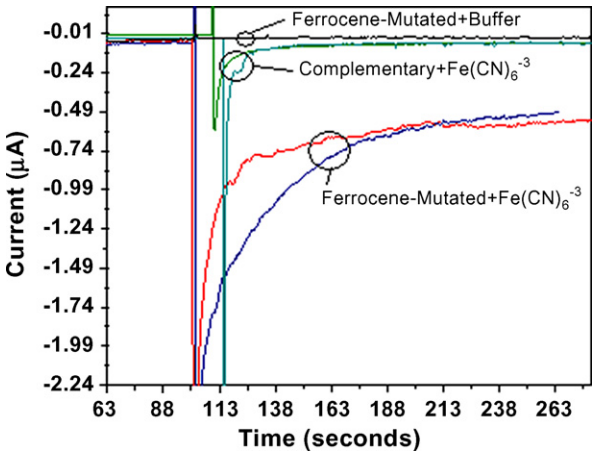


Fig. 7. Chronoamperometry of ferrocene-sub-optimum oligonucleotide hybridized with immobilized capture probe (red and blue line) and complementary oligonucleotide hybridized with immobilized capture probe (sky-blue and green line) with 1 mM ferrocyanide in solution. Ferrocene-sub-optimum hybridized electrodes were also detected in buffer without ferrocyanide as control (black line).

Pre-hybridization of ferrocene-sub-optimum oligonucleotide resulted to a higher reduction current comparing with the signal after displacement or with the bare gold electrode. It is believed that the ferrocene electron transfer is catalyzed by ferrocyanide/ferricyanide increasing the current and after displacement this effect is reduced due to the relative absence of ferrocene molecules. It also should be noted that this effect is more pronounced for the reduction reaction of ferrocene as explained before.

The use of ferrocyanide/ferricyanide in impedance spectroscopy has been widely used for the detection of biomolecules interactions. The change on the electrode surface due to the hybridization event reveals an increase in the electron-transfer resistance at the electrode surface upon the construction of the double-stranded assembly. This is attributed to the electrostatic repulsion of ferrocyanide upon formation of the negatively charged double-stranded superstructure [38]. However, in this case the ferrocene molecule immobilized on the surface has to be taken into account in the system. Thus, when ferrocene-sub-optimum was hybridized on the surface, $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ ions suffered two electrostatic effects, the repulsion by the negative charged phosphate backbone of the DNA and an attraction by the positive charged ferrocene molecule, to this effect was added the electron transfer produced by the ferrocene molecules on the electrode surface. Whereas after displacement only the repulsion produced by the double-stranded DNA to the $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ ions increased the electron-transfer resistance value.

The results obtained with ferrocene-sub-optimum displacement with ferrocyanide/ferricyanide in solution were fitted to the next circuit (Fig. 8).

R_s is the solution resistance, CPE_{dl} is the double-layer pseudo-capacitance, R_{et} is the electron-transfer resistance and W is the Warburg element, which is related with the diffusion of the $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$ ions.

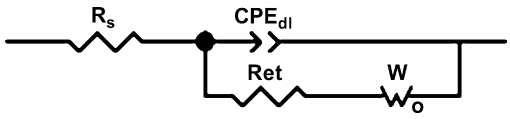


Fig. 8. Equivalent circuit for displacement of ferrocene-sub-optimum oligonucleotide system in ferrocyanide/ferricyanide.

The previous variables were also optimized with this strategy using the same electrochemical techniques.

CV and DPV in the presence of ferrocyanide/ferricyanide showed in general also an increase of signal with an increase of capture probe concentration. Impedance in ferrocyanide/ferricyanide detected a decrease of R_{et} when the concentration of ferrocene on the surface increases, so after displacement an increase of R_{et} was detected as expected. In this case, the most pronounced displacement effect was detected at 24 μg mL^{-1} of capture probe. Although most of the cases 24 μg mL^{-1} appeared to be the optimum concentration, taking into account the possible non-specific adsorption detected by SPR, 12 μg mL^{-1} of capture probe was chosen as optimum concentration to enhance specific displacement.

In the optimization of ferrocene-sub-optimum concentration CV and DPV in ferrocyanide/ferricyanide showed an increase of peak height at higher concentrations of the oligonucleotide (Fig. 9), due to the higher amount of ferrocene immobilized on the surface. For the same reason a decrease of R_{et} response was obtained by impedance when the ferrocene-sub-optimum concentration increased and an increase after displacement. As demonstrate previous techniques, the optimum concentration of ferrocene-sub-optimum concentration was 48 μg mL^{-1}.

Opposite results comparing with the direct electrochemical detection was obtained in the optimization of NaCl concentration. CV and DPV in ferrocyanide/ferricyanide show the same trend as SPR, probably because mixture of ferrocyanide/ferricyanide competed with the metal-chloride in the surface compensating the effect that this film produced in the electrochemically active surface area. It is

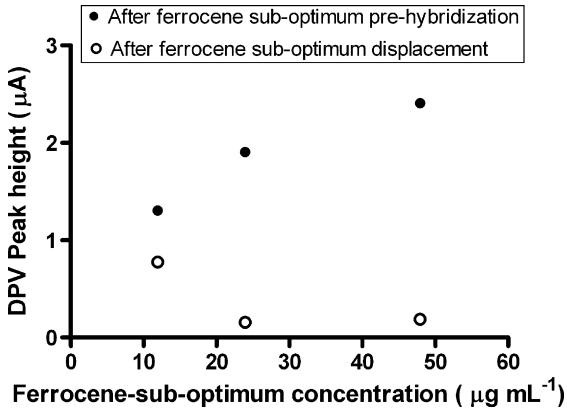


Fig. 9. Results obtained by DPV from the pre-hybridization of ferrocene-mutated oligonucleotide and the subsequent displacement with complementary oligonucleotide at different concentrations of ferrocene-sub-optimum (12, 24 and 48 μg mL^{-1}) in the presence of 1 mM of ferrocyanide/ferricyanide in 10 mM PBS, 150 mM NaCl, pH 7.5. 12 μg mL^{-1} of capture probe and 30 μg mL^{-1} of target was used in these experiments.

also significant the low values of R_{et} were obtained in impedance with ferrocyanide/ferricyanide in solution compared to previous optimizations. At higher concentrations of NaCl, high concentrations of ions were free in the solution, so the increase in the concentration of electrolyte enhances the electron transfer, decreasing the electron-transfer resistance.

After optimization, the best conditions for pre-hybridization of ferrocene-sub-optimum oligonucleotide and subsequent displacement with complementary target oligonucleotide were: incubation of 12 $\mu g\,mL^{-1}$ capture probe, pre-hybridization with of 48 $\mu g\,mL^{-1}$ ferrocene-sub-optimum and 1 M of NaCl in hybridization buffer. DPV was the electrochemical technique that provides maximum advantages for detection because offered higher sensitivity and shorter detection times. The addition of the ferrocyanide mediator in the system offered higher signals currents, however, the use of a mediator leads to a sensor that is no longer reagentless.

3.4. Reproducibility, background and specificity evaluation

The reproducibility of the detection of ferrocene-sub-optimum displacement was evaluated in a home made 20 μL two electrode thin layer cell with a 18 mm^2 square gold sheet as a working electrode opposite to a solid state platinum reference and counter electrode by DPV with ferrocyanide/ferricyanide in solution. Peak height of DPV was measured before and after displacement obtaining $9.9 \pm 0.5\,\mu A$ ($n = 5$) after the pre-hybridization of ferrocene-sub-optimum and $4.5 \pm 0.7\,\mu A$ ($n = 5$) after displacement with complementary target.

Non-specific displacement signal was show in Fig. 10. Displacement of pre-hybridized ferrocene-sub-optimum oligonucleotide was detected by DPV in ferrocyanide/ferricyanide. Displacement was carried out with a complementary oligonucleotide as target, with buffer as control and another control was performed with a 22-mer non-complementary oligonucleotide.

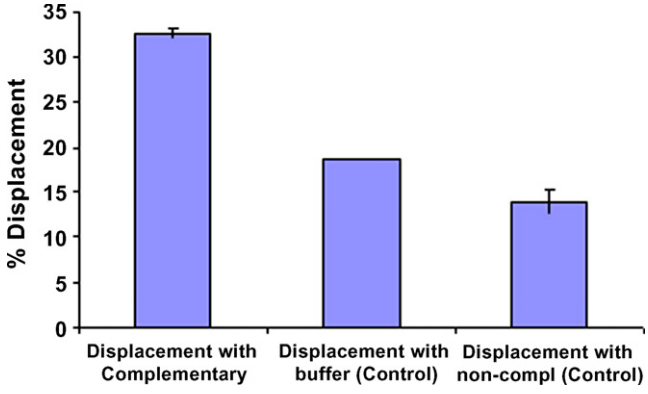


Fig. 10. Percentage of electrochemical displacement detection of pre-hybridized ferrocene-sub-optimum by complementary oligonucleotide target. The controls were carried out displacing with buffer or with a non-complementary oligonucleotide instead of complementary. These results were obtained with DPV in ferrocyanide/ferricyanide. The electrode was modified with 12 $\mu g\,mL^{-1}$ of capture probe, 48 $\mu g\,mL^{-1}$ of ferrocene-sub-optimum and the signal was displaced with 16 $\mu g\,mL^{-1}$ of complementary target.

32.6% of ferrocene-sub-optimum displacement with complementary oligonucleotide was achieved while 18.7% was obtained with buffer and 13.9% with a non-complementary oligonucleotide. Thus the specific displacement was equal to 14% (Fig. 10). More studies about stringency of pre-hybridization and displacement of ferrocene-sub-optimum should be carried out to decrease the background due to the non-specific displacement, which could improve the sensitivity of the system.

Proportionality of signal displaced with the concentration of the target was tested. Higher percentage of displaced signal was detected at higher concentrations of target, a fact that demonstrates the functionality of the system to detect label-free oligonucleotide hybridization. However, below 1.2 μM of target the displacement cannot be differentiated from the non-specific displacement by the buffer.

4. Conclusions

Ferrocene-sub-optimum displacement assay was demonstrated to be a useful platform towards a label-free reagentless and easy to use electrochemical DNA biosensors.

Different techniques were used to demonstrate this system (SPR, CV, DPV and EIS), obtaining a faster and higher response with DPV.

Two different strategies were followed to perform displacement assay. First the direct detection of the ferrocene label and then to increase the signal response an indirect detection of ferrocene labeled was performed by means of a ferrocyanide solution. This mediator helps in the electron transfer and produces an electrocatalytic cycle that results in signal amplification. However, the addition of this mediator solution leads this approach not longer reagentless.

33% of the signal produced by the pre-hybridization of the ferrocene-sub-optimum oligonucleotide was displaced by the target and this percentage was demonstrated to be proportional to the target concentration. The specificity of the system was tested with a non-complementary oligonucleotide that non-specifically displaced 14% of the signal. A good reproducibility was obtained with this approach that was 5–15% of the signal.

However, this approach is still far from a commercial biosensor. Further studies should be carried out to improve the sensitivity of the system. A decrease of the non-specific displacement by the buffer or other molecules should be carried out. With this aim, stringency conditions of pre-hybridization and displacement of ferrocene-sub-optimum need to be tested and optimized. Moreover stability of the biomolecules on the surface and large quantities fabrication reproducibility should be tested.

This label-free electrochemical sensor was designed to detect cystic fibrosis, however, this system can be applied in any application where the detection of a DNA targets are involved.

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