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Electrochemical molecular beacon DNA biosensor for the detection and discrimination of the DF508 cystic fibrosis mutation

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ABSTRACT

Cystic fibrosis is one of the most common genetically inherited diseases in Northern Europe, consisting of a defect of chloride transport in the epithelium, with the DF508 mutation being the most common mutation associated with the disease. In this work the design and characterisation of a reagent-less electrochemical genosensor, based on the use of an electrochemical molecular beacon targeting the DF508 mutation is presented. Different aspects of the sensing platform including molecular beacon design and surface chemistry of the sensor surface were evaluated. Operational parameters such as assay buffer and assay time were also optimised. Using the optimised molecular beacon designs a clear differentiation between the targeted sequence (i.e. mutant) and potential interferent (i.e. wild type) was demonstrated, with a total required assay time of 20 min. The major advantage of the proposed reagent-less sensing platform is the fact that this only required, as intervention of the end-user, the addition of the sample.

1. Introduction

Cystic fibrosis (CF) is an inherited chronic disease resulting in lung and pancreatic damage, causing progressive disability, and, for most, early death [1,2]. Almost 1000 mutations have been identified in the CF gene, but a limited number of common mutations cause disease in most patients [3]; furthermore the severity of the disease is related with the nature of the mutation [1]. DF508 is the most common mutation, responsible for the deletion of the phenylalanine at position 508 in the amino acid sequence of the cystic fibrosis transmembrane conductance regulator (CFTR); this mutation is coded by a three bases deletion in the protein coding gene, located in chromosome 7. The DF508 frequency varies between ethnic groups but in general accounts for between 50% and 70% of CF patients. Currently cystic fibrosis diagnosis is performed using the so called "sweat test" as patients with cystic fibrosis present an abnormally high level of electrolytes in sweat [4] and/or on the screening of the immunoreactive trypsinogen in potentially affected young children, [5].

The outcomes of the Human Genome Project have resulted in an increasing demand for cost-effective tools for genetic analysis, [6–8], and the detection of cystic fibrosis associated mutation has been extensively used as model in the development of rapid genotyping approaches. Surface plasmon resonance imaging (SPRI) has been reported [9,10] for the simultaneous detection and discrimination of different cystic fibrosis mutations including DF508 and other single nucleotide polymorphisms (SNPs) located in the same region of the CFTR gene (Exon 10). Using SPRI Mannelli et al. reported on the label-less real time detection of several DNA targets (short oligonucleotides or a 377 bp PCR product) on a 196 spot DNA array [9]. The same author also reported the SPRI parallel detection of several cystic fibrosis associated mutations; DF508, DI507, M470V, Q493X, V520F and 1716 G > A [10].

Hahm and Lieber [11] reported on a conductivity DNA biosensor based on the use of silicon nitrate nanowire (SiNW) functionalised with a short chain peptide nucleic acid (PNA) probe specific for the DF508 wild type sequence; the authors, reported an increase in the system conductance as a result of the hybridisation with the target, due to the negative charge of the DNA target.

Millan et al. [12] reported on the combination of oligonucleotide modified carbon paste electrode and electroactive DNA intercalators, for the voltammetric detection of the DF508 mutation. More recently Marin and Mei [13,14] reported on the use of a nanoparticle based electrochemical sensor for the detection of CF, whilst Mir et al. reported on the use of an electrochemical displacement approach for the detection of cystic fibrosis DF508 mutation [15]. Recently, the Nasef et al. developed several approaches based on the use of methylene blue DNA interaction [16] on temperature modulated electrochemical impedance spectroscopy [17], and on electrochemical melting curves analysis [18] for the detection/discrimination of synthetic PCR analogues of the DF508 cystic fibrosis mutation and wild type sequences. All the reported approaches provided good differentiation with good linearity in the range

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between 10 and 100 nM, exhibiting a limit of detection (LOD) of 2.64 nM [16].

More recently Bonanni et al. [19] used electrochemical impedance spectroscopy, coupled with AuNPs label, to develop a very sensitive approach, with limit of detection of the order of 100 pM, for the detection of DF508 mutant amplicon.

Molecular beacons (MB) are short chains of ssDNA characterised by the typical stem loop structure [20,21] where the loop of the MB is designed to allow target recognition and the stem has the function of modulating the detection, with the termini of the oligonucleotide modified with fluorophore and quencher, respectively [22]. As a result of the stem structure, in the absence of target, the fluorophore and the quencher are forced to be in close proximity with no fluorescence occurring; when hybridisation with specific target is occurring the stem in the MB is forced to open resulting in an increase in fluorescence. In 2003 Fan and co-workers reported the first example of an electrochemical molecular beacon E-MB [23] where the quencher was replaced by a thiol group to allow self-assembling on Au electrodes, and the fluorophore was substituted by an electrochemical reporter (e.g. ferrocene, methylene blue) [24]. Upon interaction with the target molecule, the E-MB underwent a conformational change, distancing the electrochemical reporter from the electrode surface, resulting in a decrease in electrochemical signal. Lubin et al. [25] went on to exploit this architecture for the sensitive detection of DNA mismatches in several medium as urine, plasma, saliva and soil. Cash and co-workers [26] described an E-DNA sensing platform based on target-induced conformational changes in an electrode-bound DNA pseudoknot. In the absence of a target, the structure of the pseudoknot probe minimised collisions between the redox tag and the electrode, and as a result of target binding the pseudoknot structure was disrupted and a consequent quantitative increase in electrochemical response was observed. Recently Farjami et al. [27] presented the possibility of tuning the signal generation mechanism of MB by simply changing the length of the MB; when length was reduced from 33 to 20 nucleotides a change in signal generation mechanism, for on–off to off–on, was recorded. Ying et al. [28] reported on the use of electrochemical impedance spectroscopy-based MB, where the electroactive moiety was replaced by a CdTe quantum dot with a strong reduction in charge transfer resistance (Rct) recorded after hybridisation with specific target. Bonanni and Pumera [29] reported the use of MB like probes for the label-less detection of DNA sequences. In this specific work the authors took advantage of the different ability of ssDNA and dsDNA to adsorb onto graphene in order to differentiate between fully complementary and mismatched targets. Electrochemical MB like probes have also been used for the detection of metals [30] or proteins using specifically modified aptamers [31].

In the work reported herein, we present a reagent less electrochemical genosensor exploiting molecular beacons for the detection and discrimination of the cystic fibrosis associated DF508 mutation, which was chosen because of its prevalence. Different approaches for the MB immobilisation, all based on the chemisorption of the E-MB on the sensor surface, were investigated; furthermore optimisation of the detection buffer and detection time was performed. Discrimination between the mutant and wild type was demonstrated, exhibiting the suitability of the proposed sensing platform the rapid and specific detection of the DF508 mutation.

2. Materials and methods

2.1. Chemicals and DNA

All chemicals and reagents were of analytical grade and used without further purification. Trizma base, Potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$), sulphuric acid (H_2SO_4), phosphate buffer saline pH 7.4 (PBS), saline sodium citrate buffer (SSC) and phosphate buffer saline pH 7.4 with Tween-20, (PBS-T) were purchased from Sigma (Germany); hydrochloric acid (HCl 6 M), potassium dihydrogen phosphate (KH_2PO_4) and sodium chloride (NaCl) were purchased from Scharlau (Spain); sodium hydroxide pellets (NaOH) were supplied by Panreac; 6-mercaptohexanol (MCH) and potassium hexacyanoferrate (II) ($K_4Fe(CN)_6$) were purchased from Fluka (Germany) and HEPES buffer was supplied by Acros Organics (USA). Alumina powder (1, 0.3, 0.05 micron) used for the mechanical cleaning of the Au electrodes was supplied by CHI Instruments (USA). All solutions were prepared in ultra pure water (18 MΩ cm) obtained using a "Simplicity Water Purification System" from Millipore (France). The oligonucleotides, used in this study are listed in Table 1 and were provided by Biomers.net (Germany).

The wild type and mutant target oligonucleotides used in this work are analogues to the PCR products obtained using an amplification protocol, based on a "Multiplex Amplifiable probe Hybridisation (MAPH)" strategy [32] and reported previously [17].

2.2. Instrumentation and measurements

The electrochemical studies were carried out using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System (GPES) software (Eco Chemie B.V., The Netherlands). A classical three-electrode cell was used. A set of commercially available polycrystalline Au disc (2 mm diameter) electrodes (CHI Instruments) were used as working electrode, a Ag/AgCl (KCl 3 M) was used as reference electrode (CHI Instruments) and a Pt wire (Sigma) used as counter electrode. The working electrodes were mechanically cleaned, to a mirror finish, with alumina (using three different sizes: 1, 0.3, 0.05 μm) aqueous slurry. After rinsing with ultra pure water the electrodes were electrochemically cleaned by cycling them (scan rate 100 mV s^{-1}) in 0.5 M H_2SO_4 between −0.2 and 1.6 V. The number of cycles, ca. 40, was not fixed: the Au electrodes were considered clean when, in a cyclic voltammetric experiment with a ferro/ferri equimolar solution, a peak to peak separation of ca. 80 mV was recorded.

Fluorescence measurements were performed using a Cary Eclipse Spectrofluorimeter equipped with Cary temperature controller (Varian, Spain). All measurements were performed at 25 °C. Black quartz cuvettes with 1 cm path length and with a total volume of 250 μl (Hellma) were used. Excitation and emission

Table 1
Summary of the oligonucleotides sequences used in this work.

Mut_{DF508} DF508 mutant amplicon	5′ GCC GCG AAT TCA CTA GTG TGG CAC CAT TAA AGA AAA TAT CAT TGG TGT TTC CTA TGA TGA ATA TAA TCG AAT TCC CGC GGC C 3′
Wt_{DF508} DF508 wild type target amplicon	5′ GCC GCG AAT TCA CTA GTG TGG CAC CAT TAA AGA AAA TAT CAT CTT TGG TGT TTC CTA TGA TGA ATA TAA TCG AAT TCC CGC GGC C 3′
F-MB_{DF508} Fluorescence MB specific for DF508 mutant sequence	5′ FAM–GCG AGA AAG AAA CAC CAA TGA TAT TTA GCC TCG C – BHQ 3′
E-MB_{DF508} Electrochemical MB specific for DF508 mutant sequence	5′ MB–GCG AGA AAG AAA CAC CAA TGA TAT TTA GCC TCG C – SH 3′