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Short communication

Direct electrochemical genosensing for multiple point mutation detection of *Mycobacterium tuberculosis* during the development of rifampin resistance

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ABSTRACT

We present a robust and simple method for the direct detection of multiple point mutations in the *Mycobacterium tuberculosis* rpoB gene during the development of rifampin (RIF) resistance using an electrochemical genosensor. The device contained five different capture probes which are designed to hybridize with several sequence segments within the bacterial rpoB gene hotspot region. Point mutations were detected by monitoring the guanine oxidation with differential pulse voltammetry after hybridization between PCR amplicons and inosine modified capture probes at graphite surface. Changes in the peak voltage corresponding to guanine oxidation provide an electrochemical signal for hybridization that can be used to determine the presence of point mutations conferring rifampin resistance. The analytical parameters (sensitivity, selectivity and reproducibility) were evaluated. High selective discrimination against point mutation of bacteria at hot-spot region was observed. Several mutations were detected at several parts of the amplicon from 21 positive samples.

1. Introduction

Mycobacterium tuberculosis infection remains one of the most significant causes of death worldwide and leads to millions of deaths annually. A rise in the incidence of drug-resistant strains of *M. tuberculosis* has accompanied the increased incidence of tuberculosis (Telenti et al., 1993; Mani et al., 2001). In the Aegean region 8.2% of *M. tuberculosis* strains isolates were found to be resistant to rifampin (RIF). Many DNA sequencing studies have demonstrated that more than 95% of RIF-resistant bacteria strains possess mutations within the 81-bp hotspot region between the 507th and 533rd codons of the rpoB gene (Cavusoglu et al., 2002, 2006).

Recent advances in biosensors based on nucleic acid hybridization recognition have led to the development of genosensor technology for DNA sequence analysis (Millan and Mikkelsen, 1993; Palecek, 1996; Mikkelsen, 1996; Wang et al., 1997, 1998; Erdem et al., 2000). Specifically, electrochemical hybridization biosensors demonstrate great promise for pathogen identification, mutation detection, and genomic sequencing (Thorp, 1998; Sawata et al., 1999; Mascini et al., 2001; Pividori and Allegret, 2003; Kara et al., 2004; Wong et al., 2005).

Herein, we have demonstrated a genosensor for the direct electrochemical detection of multiple point mutations in the *M. tuberculosis* genome during the development of RIF resistance. This analytical method relied on graphite electrode arrays, modified with five different guanine-free aminohexyl-tethered oligonucleotide probes. Samples of genetic material for genotyping the bacteria of interest were obtained from cell culture and subsequent PCR amplification of the corresponding rpoB gene. These amplicons were captured via hybridization to five different capture probes, which recognized several different parts of the amplicon, at five different electrode interfaces. Differential pulse voltammetry (DPV) was employed to detect hybridization and to discriminate between point mutations via guanine oxidation signals. The results of this study demonstrate great promise for practical applications in the development of clinical assay techniques and the design of oligonucleotide chips.

2. Experimental

2.1. Chemicals and Apparatus

2.1.1. Chemicals

For surface preparation, covalent attachment buffer containing 5 mmol/L EDC and 8 mmol/L NHS was used. Binding buffer containing 0.5 M acetic acid, 20 mM NaCl (pH 4.8), and capture probes was used during immobilization of oligonucleotides on electrode sur-

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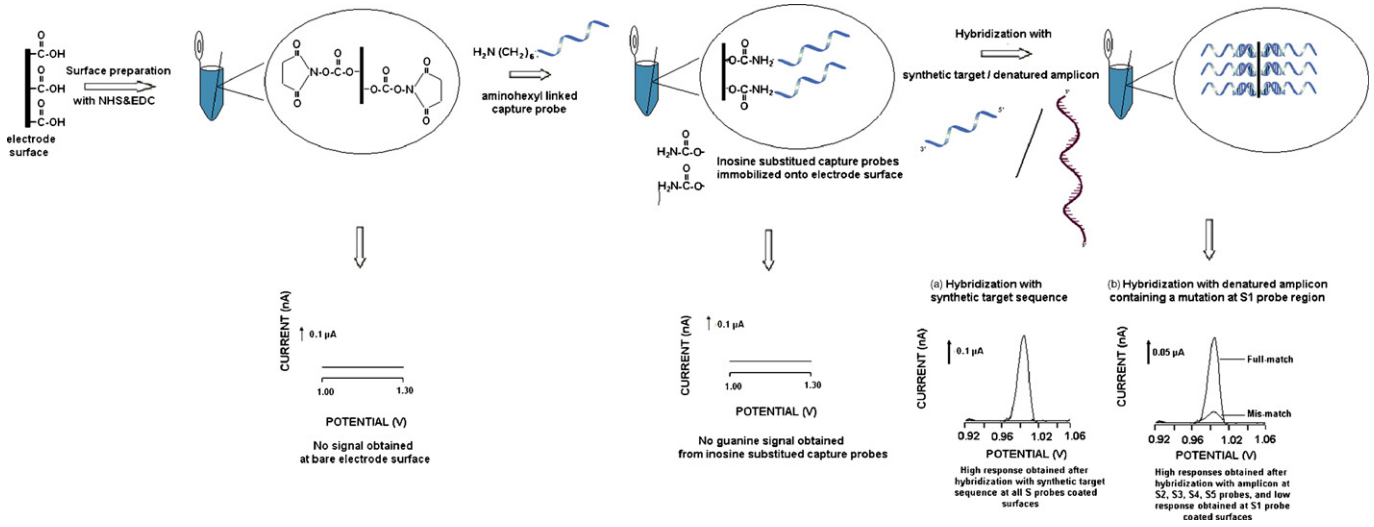
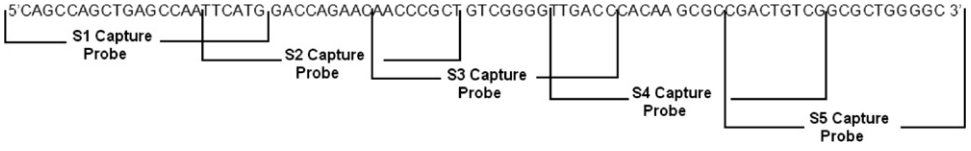


Fig. 1. Analytical procedure for the detection of multiple mutations in the *M. tuberculosis* rpoB gene.

faces. 2× SSC solution (30 mM sodium citrate, 0.3 M NaCl, pH 7.4) containing the target sequence served as the hybridization buffer, and 1× SSC + 0.1% SDS solution (0.15 M NaCl, 15 mM sodium citrate, 0.1% SDS) was used as washing buffer for all experiments.

Capture probes were designed to target several parts of the rpoB gene's hotspot region in which mutations frequently develop.



HPLC-purified synthetic capture probes and 81-mer target sequences were obtained in lyophilized powder form from TIB MOLBIOL (Germany). The sequences of the oligonucleotides, synthetic targets, and amplicons are as follows:

Oligonucleotide	Sequence
S1 capture probe	5′-NH_2-$(CH_2)_6$-CAT IAA TTI ICT CAI CTI ICTI-3′
S2 capture probe	5′-NH_2-$(CH_2)_6$-AI CII ITT ITT CTI ITC CAT IAA-3′
S3 capture probe	5′-NH_2-$(CH_2)_6$-IIT CAA CCC CIA CAI CII ITT-3′
S4 capture probe	5′-NH_2-$(CH_2)_6$-CIA CAI TCI ICI CTT ITI IIT CAA-3′
S5 capture probe	5′-NH_2-$(CH_2)_6$-I CCC CAI CGC CIA CAI TCI-3′
Synthetic target sequence (represents the 81-mer hot-spot region)	5′-CAGCCAGCTGAGCCAATTCATGGACCAG AACAACCCGCTGTCGGGGTTGACCCACAAGCGCCG-ACTGTCGGCGCTGGGGC-3′
PCR amplicon related to *M. Tuberculosis* isolates	5′-CCACCCAGGACGTGGAGGCGATCACACCGCAGAC-GTTGATCAACATCCGGCCGGTGGTCGCCGCGATCAA-GGAGTTCTTCGGCAC**CAGCCAGCTGAGCCAATTCATG-GACCAGAACAACCCGCTTCGGGGTTGACCCACAAGC-GCCGACTGTCGGCGCTGGGGC**CCGGCGGTCTGTCAC-GTGAGCACCCGTCGCACTACGGCCGGATGTGCCCGA-TCGAAACCCCTGAGGGGCCCAACATCGGTCTGTCGG-CTCGCTGTCGGTGTACGCGCGGGTCAACCCGTTCGG-GTTCATCGAAACG-3′
Synthetic non-complementary target	5′-TTG AGG ATC CTG GAA TTA-3′
Non-complementary PCR amplicon	5′-ATC AAC TTC GAC TGG CCC TTC TTG CTG GCC AAG CTG ACG GAC ATT TAC AAG GTC CCC CTG GAC GGG TAC GGC CGC ATG AAC GGC CGG GGC GTG TTT CGC GTG TGG GAC ATA GGC CAG AGC CAC TTC CAG AAG CGC AGC AAG ATA AAG GTG AAC GGC ATG GTG AGC ATC GAC ATG TAC GG-3′

I = inosine. The underlined bold part represents the hot-spot region.

2.1.2. Electrochemical apparatus

Differential pulse voltammetry experiments were performed using an AUTOLAB PGSTAT-30 electrochemical analysis system (Eco Chemie, The Netherlands). The three-electrode system consisted of a disposable graphite electrode, an Ag/AgCl reference electrode, and a platinum wire auxiliary electrode.

2.2. Methods

2.2.1. PCR amplification from M. tuberculosis isolates

PCR amplicons were prepared f rom several *M. tuberculosis* isolates by C. Cavusoglu at the Mycobacteriology Laboratory of Ege University (Cavusoglu et al., 2006).

2.2.2. Electrochemical assay

The detection procedure was broken down into the following steps: electrode biomodification, hybridization and washing, and surface voltammetric transduction (Fig. 1).

2.2.2.1. Electrode biomodification. Surface was chemically modified by exposure to freshly prepared covalent attachment buffer for probe immobilization. Capture probes were then immobilized onto the different modified electrode surfaces by placing the electrodes into binding buffer containing 10 μg/mL oligonucleotide for 1 h.

2.2.2.2. Hybridization. Hybridization experiments were performed with both synthetic oligonucleotides and PCR-amplified target sequences by placing the electrodes into target solutions for 30 min.

Synthetic or PCR-amplified target sequences were diluted to the desired concentration (20 μg/mL) with hybridization buffer. The analyte double-stranded DNA from *M. tuberculosis* PCR amplicons was thermally denatured in a water bath at 95 °C for 8 min. The sample was then cooled in an ice water bath for 5 min to slow the rate of amplicon strand annealing. The PCR blank, the non-complementary