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Critical factors for the performance of chip array-based electrical detection of DNA for analysis of pathogenic bacteria

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Authors:

Yanling Liu[1], Bruno Elsholz[2], Sven-Olof Enfors[1] and Magdalena Gabig-Ciminska[1,3,*]

[1] *School of Biotechnology, Royal Institute of Technology (KTH), S-10691, Stockholm, Sweden*

[2] *Fraunhofer Institute for Silicon Technology, Itzehoe, Germany*

[3] *Laboratory of Molecular Biology (affiliated with the University of Gdansk), Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Kladki 24, PL-80822 Gdansk, Poland*

Running title: Factors for the performance of chip array-based electrical detection of DNA

Subject category: Enzymatic assays and analyses

*** Corresponding author:**

Magdalena Gabig-Ciminska, Department of Biotechnology, Royal Institute of Technology (KTH), S-10691, Stockholm, Sweden

Tel. +46-8-5537 8310; Fax: +46-8-5537 8323; E-mail: gabig@biotech.kth.se, gabig@biotech.ug.gda.pl

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ABSTRACT

Different factors influencing a chip array-based electrical detection of DNA for analysis of pathogenic bacteria were examined. Both, rehydration of capture probe layer of functionalized chip arrays, and also efficient hybridization of targets irrespectively of their length, resulted in signal enhancement when high ionic phosphate-buffered saline (i.e. 600 mM sodium chloride and 40 mM disodium hydrogen phosphate) was used. Similarly, placement of two adjacent capture and detection probes binding sites at a terminal part of the target strand resulted in significant signal increase. Moreover, 10 min ultrasonic fragmentation of targets amplified the signals up to two-fold for longer DNA strands (i.e. above 300 bp). No obvious effects on signals were visible for shorter than 400 bp PCR amplicons subjected to ultrasonication. For DNA strands of all sizes, over 10 min ultrasonication diminished the specific electrical responses. Our results demonstrate also that target analytes are detected with discrimination against mismatches, even for single nucleotide sequence alteration. The mismatch detection appeared in order of easiness of recognition as follows: triple random > quintuple middle > triple middle > single middle mismatch. Among the three variants of one-base mismatches, a sequence variation was most remarkable for adenine. On the other hand, no benefits in assay sensitivity were recognized by the use of longer capture probe linkers as the 6-C linker.

Keywords: Electrical chip arrays, Nucleic acid-based analysis, Toxin-encoding genes, Bacterial pathogenicity, *Bacillus cereus*

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INTRODUCTION

Routine tests used for the identification of pathogenic microorganisms are based on traditional classification with the use of microbiological and/or serological methods. However, in many cases only certain strains of a species are pathogenic and many toxins are produced by several species [1]. Therefore only genetic tests might provide unambiguous results when assessing the presence of pathogens in the tested material.

Consequently, nucleic acid-based sensing systems have become important tools for the detection of pathogenic microorganisms [2-8]. Various types of such platforms have been developed depending on signal transducer configuration [9,10]. However, some of the general properties of all these systems, such as sensitivity, specificity, reliability etc., represent crucial criteria in determining their sensing performance. Any improvement of these properties may increase the efficiency and broaden the scope of DNA chip-based analyses. This could, for example, reduce the cost and time required for nucleic acid examination, and thus assessing genetic predispositions to bacterial pathogenicity.

One of the sensing systems recently developed by our group is an electrochemical DNA chip array for confirmative analysis of presence of toxin-encoding genes in bacteria from primary enrichment cultures [11]. It is an automated detection system able to evaluate the presence of specific sequences coding for toxins in the applied sample by measuring changes of the electrochemical signal generated by redox recycling [11,12]. The platform is built on a silicon 16-position electrical chip array for simultaneous analysis of sequences of interest. In order to develop further this methodology and explore its potential, different parameters were analyzed and are reported in this work. Efforts made on optimization of various factors influencing the performance of the chip array-based DNA detection for determination of bacterial pathogenic capacity are summarized. Enteropathogenic *B. cereus* was used as a model, and its toxin-encoding genes were selected as targets for this study.

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First, a rehydration of surface-immobilized capture probe layer is investigated, followed by studies made on hybridization efficiency, all in respect to obtained signal. Additionally, employment of ultrasound with the purpose of fragmenting genomic DNAs for assay sensitivities, as well as different capture/detection probe-binding locations along DNA strands and their effects on signal generation is studied. At last, mismatch discrimination is explored.

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MATERIALS AND METHODS

Reagents

Bovine serum albumin 30% solution (BSA, protease-free), ethidium bromide solution (10 mg/ml), polyoxyethylensorbitan monolaurate (Tween 20), deoxynucleotide mix (each dNTP 10 mM), *Taq* DNA polymerase (5 units/µl) and PCR buffers were obtained from Sigma-Aldrich (Steinheim, Germany). 4-Aminophenyl β-D-galactopyranoside (pAPG) was also obtained from Sigma-Aldrich (Schnelldorf, Germany). Streptavidin-β-Galactosidase conjugate (Str-β-Gal) was obtained from Roche Diagnostics Corporation (Bromma, Sweden). All salts prepared for buffers were obtained from Merck KGaA (Darmstadt, Germany). Water used in all experiments was ultra-pure Milli-Q water (Millipore purification system). Phosphate-buffered saline (PBS) was prepared by dissolving 150 mM NaCl and 10 mM Na_2HPO_4 in water and adjusting to pH 7.4. TPBS buffer (pH 7.4) was prepared by adding 0.01% (v/v) Tween 20 to PBS. Chip rehydration buffer was prepared by mixing PBS solution with 0.01% (v/v) Tween 20 and 1% (v/v) BSA. DNA hybridization buffer was prepared by adding 4 mM EDTA to PBS solutions. Working buffer was prepared by dissolving 120 mM NaCl, 30 mM K_2HPO_4 and 1 mM $MgCl_2$ in water and adjusting to pH 7.2. Flushing buffer was prepared by adding 0.01% (v/v) Tween 20 to working buffer. Enzyme-conjugate dilution

buffer was prepared by mixing 1% (v/v) BSA to flushing buffer. All buffers were sterilized by autoclaving before use.

B. cereus ATCC14579 and F4810/72 cultures

Bacillus cereus strain ATCC14579 was obtained from the American Type Culture Collection, Manassas, USA. *B. cereus* F4810/72 was provided by Svensk Mjölk (Swedish Dairy Association), Lund, Sweden. Bacteria were grown aerobically in 10 ml nutrient broth (LB medium, Merck KGaA, Darmstadt, Germany), supplemented with 1% (w/v) glucose, with shaking (180 rpm) at 30 °C. 3.5 ml of the overnight culture was used to start a second culture that was grown to exponential phase (absorbance at 600 nm of 3). Cells were collected by centrifugation (5000 *g*, at 4 °C, 10 min). Pellets were washed once with PBS (pH 7.4) and then re-collected by the same centrifugation. Afterwards, cell pellets were resuspended in water to the concentration 3×10^9/ml for later ultrasound treatment.

Design of PCR primers, probes, oligo- and polynucleotides

Each upper/lower primer pair and capture/detection probe pair (Table 1) were designed by OLIGO Primer Analysis Software, Version 6.88 (MedProbe, Oslo, Norway), according to the sequences from eight toxin-related genes, *hblA, hblC, hblD, nheA, nheB, nheC, cytK*-2 and the toxin cereulide synthetase-encoding gene *cesA*. The corresponding sequence accession numbers are AE017008 (*hblA, hblC, hblD*), AE017003 (*nheA, nheB, nheC*), AE017001 (*cytK*-2), and BD402606 (*cesA*).

The upper and lower primers were single-stranded (ss) oligonucleotides with 25 nucleotides complementary to the selected sequences within each toxin-related genes. Capture and detection probes were designed with the same principle as primers and chosen to bind to target sequences adjacently. An additional four nucleotides-spacer (non-

complementary to selected targeting sequences) was added to both the 5' end of capture probes (HBLA_Cp, HBLC_Cp, HBLD_Cp, NHEA_Cp, NHEB_Cp, NHEC_Cp, CYTK-2_Cp and CESA_Cp) and the 3' end of detection probes (HBLA_Dp, HBLC_Dp, HBLD_Dp, NHEA_Dp, NHEB_Dp, NHEC_Dp, CYTK-2_Dp and CESA_Dp). The spacer was labeled with a thiol group via a 6-carbon linker in capture probes and with a biotin in detection probes. NCBI BLAST function was applied to evaluate the sequence specificity of all selected capture and detection probes by aligning sequence similarities with all bacterial genes available from NCBI, EMBL, DDBJ and PDB databases. There were no significant sequence similarities obtained with any other kinds of genes for all capture and detection probes designed here.

Moreover, one negative control probe, which was non-biotinylated and non-relevant to any of the selected eight toxin representative sequences, and one positive control probe with the same sequence as negative control probe, but being mixed with 1% biotinylated probes in non-biotinylated ones, were designed. The accession number of these two control sequences is Z99108.

Different mismatched (MM) *cytK*-2 capture probes (Table 1), i.e. three types of single middle-mismatched (M1MM) probes: M1aMM_Cp, M1cMM_Cp, M1gMM_Cp; M3MM_Cp and M5MM_Cp, were designed by replacing one, three and five contiguous matched nucleotides with mismatched nucleotides in the middle region of *cytK*-2 capture probe, respectively. The random 3MM probe (R3MM_Cp) was designed by allocating three mismatched nucleotides distributed along the *cytK*-2 capture probe strand. Single-stranded (ss) 51-mer HBLC polynucleotide was designed to complement with both HBLC_Cp and HBLC_Dp, respectively. Ss 25-mer CYTK-2 oligonucleotide was designed to complement to CYTK-2_Cp and labeled with biotin via four nucleotides-spacer at the 5'-end. CYTK-2_Cp9,

CYTK-2_Cp12 and CYTK-2_Cp18 were designed by linking 9, 12 and 18 carbons between CYTK-2_Cp nucleotides and the thiol-group label, respectively.

All PCR primers, probes, oligo- and polynucleotides were obtained from Thermo Electron GmbH (Ulm, Germany). These sequences are listed in Table 1.

PCR and amplicon preparation

Genomic DNAs from *B. cereus* strains, i.e. ATCC14579 and F4810/72, were used as templates for amplification of selected sequences of *hblA, hblC, hblD, nheA, nheB, nheC*, *cytK*-2 and *cesA* genes, respectively. One bacterial colony was treated by ultrasonication for 5 min. After a heat treatment (95 °C, 10 min) and removal of solid particles by centrifugation (5000 *g*, 4 °C, 10 min), cell lysates were ready for use as genomic DNA templates in PCR. All PCRs were performed in a DNA Thermal Cycler (PTC-200, MJ Research, USA). Reaction components in 50 μl volumes contained bacterial genomic DNA (10^7 cells), 0.5 μM primers, 200 μM each of four kinds of dNTPs, 2.5 units of *Taq* polymerase in reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3). The PCR was performed according to the designed program: step 1, pre–denaturation (95 °C, 4 min); step 2, denaturation (95 °C, 45 s); step 3, primers annealing (56 °C, 1 min); step 4, elongation (72 °C, 2 min); step 5, repeat from step 2 to step 4 for 34 cycles; step 6, final extension (72 °C, 10 min). After the completion of reactions, resultant PCR products were analyzed by agarose gel electrophoresis.

PCR amplicons were purified from PCR mixtures with the use of PCR centrifugal filter devices (Millipore, USA) according to the manufacture's instruction, for later use. The purified PCR amplicons were quantified by UV absorbance at 260 nm using a spectrophotometer.

Ultrasound treatment for PCR amplicon fragmentation

Ultrasound treatment of bacterial cells or PCR amplicons was carried out by an ultrasound disruptor UP100H (Dr. Hielscher GmbH, Stuttgard, Germany) equipped with a microtip 1 mm in diameter. The operating frequency was 30 kHz and effective output power was 100 W. During treatment, samples were cooled in ice-water bath.

Ultrasound was applied to the 100 µl of PCR amplicons, i.e. 874-bp *hblA*, 187-bp *hblC*, 989-bp *hblD*, 274-bp *cesA*, 310-bp *nheA* and 413-bp *nheC*, 1 nM each. After ultrasonication, 0.2 nM of each fragmented PCR amplicon was applied for chip assays.

Chip array elements and analyzer system

Chip arrays and the corresponding instrument 'eMicroLISA' were obtained from Fraunhofer Institute for Silicon Technology and AJ eBiochip GmbH (Itzehoe, Germany), respectively. 16 circular interdigitated array electrode positions, contact pads and their connections were embedded at chip surfaces. All these electrode positions can be functionalized by DNA probes via thiol-gold interaction. After that, chip arrays were placed into a reaction chamber. Details of the chip array elements and instrument were described previously [13].

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Chip array functionalization

Prior to immobilization, thiol-labeled capture probes with a concentration of 100 µM were dissolved in Na_2HPO_4 solution (25 mM, pH 7.0). These probes were spotted onto 16 circular interdigitated array electrode positions by using a piezo electric nanodispenser NP 2.0 (GeSiM, Groβerkmannsdorf, Germany). After incubation in a humid chamber at RT for about 2 h, the functionalized chips were streamed by water to remove unbound probes and dried for later analyses. For detailed information see [13].

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Each chosen capture probe was spotted on random triplicate electrode positions of the chip array. Furthermore, one negative control probe position (i.e. non- biotinylated and non-relevant to any of selected eight toxin representative sequences) and one positive control probe position (i.e. the same sequence as negative control probe, but being mixed with 1% biotinylated probes in non-biotinylated ones) were placed at the front and back site of the chip array, respectively, in order to validate detection specificity and assay performance.

Target DNA analyte preparation and assay program

Target DNA analytes were mixed with detection probes (1 μM working conc. for each) in hybridization buffer. The mixture (200 μl total volume) was incubated at 95 °C for 5 min, then immediately cooled on ice for 1 min. After that, it was directly transferred in the chip reaction chamber for hybridization. The assay program was designed with steps in a sequence (Table 2). An internal iridium oxide reference electrode (+100 mV anode / -400 mV cathode) was used for all measurements.

Chip array storage and probe layer rehydration

After functionalization, chip arrays were stored in the dry condition under the protection of nitrogen gas at RT. Before assay, single chip array was applied to the reaction chamber and flushed with 4×PBS at 50 °C for 5 min.

Strategy of chip array-based electrochemical DNA detection system

Figure 1 shows a scheme of the target DNA binding to capture probe layers and the following signaling process. Firstly, a mixture of target DNA analyte and detection probes was applied to the chip surface, which was previously functionalized with specific capture probes spotted on the different electrode positions, to form and bind the sandwich hybrid. After introducing

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the Streptavidin-β-Galactosidase conjugate (Str-β-Gal), it interacts specifically to the bound biotin labeled to the detection probe. Following the flushing of pAPG, the attached enzyme conjugate catalyzes the pAPG to 4-Aminophenol (pAP). Hydrolysis of pAP and its oxidative form quinoneimine (QI) between interdigital electrode fingers results in an increasing electron redox-recycling and finally generate amperometric curves from all 16 electrode positions simultaneously after the stop flow of pAPG. After completion of the reaction the electric read out (nA/min) was made in an 8-s period in "stop flow mode" with a time offset of 2 sec.

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Positive controls generated signals similarly as described above, but only from enzyme conjugate Str-β-Gal binding, following substrate pAPG hydrolysis, and the resulting pAP redox recycling at the chip electrodes, and with excluded hybridization step.

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Functionalized chip arrays were placed into the reaction chamber and subjected to assays by applying sample analytes onto the chip array surface. After the first hybridization, the electric signals were generated and the whole assay was completed. Next, the electrodes on the same chip array were stripped by flushing 75 °C hot water for 4 min to remove all captured molecules and make the capture probe layers available for the 2nd hybridization and subsequent detection. The same procedure was applied to all repeated assays.

RESULTS

Rehydration of surface immobilized probe layer

The chip arrays are usually stored under nitrogen gas after their functionalization with capture probes [13]. Therefore, effects of chip array surface rehydration conditions on signal level were studied. Before applying sample, PBS solutions with different ionic strengths (see MATERIALS AND METHODS, in Reagents) were flushed onto a surface of a functionalized chip array to rehydrate the immobilized capture probe layer for 15 min. Different temperatures of incubation were also tested. The rehydration of the capture probe layer was examined by measuring the signals obtained from chip positive control (PC) positions, on which the biotinylated capture probes can react to and capture the Str-β-Gal conjugates without previous hybridization, followed by the electrical detection. Rehydration performed with buffers of ionic strength only 150 and 300 mM NaCl, in 10 and 20 mM Na_2HPO_4, respectively (i.e. 1× or 2×PBS consequently), and at RT or 40 °C gave a similar signal level of around 60 nA/min (data not shown). In contrast, rehydration by the 4×PBS resulted in a much higher signal (176 nA/min), which was three times of that from rehydration buffers of lower ionic strengths. While rehydration completed with the use of 5×PBS decreased the PC signal level to about 110 nA/min. Additionally, when the rehydration time was reduced from 15 to only 5 min, no remarkable changes in the signal patterns were observed (data not shown). Different temperatures, 40, 50 or 60 °C, did not affect the PC signal levels visibly.

As a consequence of these observations, all chip arrays were rehydrated by treatment with 4×PBS at 50 °C for 5 min prior to their utilization in subsequent analyses.

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Effect of the hybridization buffer ionic strength on signal generation

To examine the effect of buffer ionic strength on hybridization carried out at the chip arrays and to acquire the optimal buffered condition for the improved signal level, hybridization solutions of different concentrations of PBS (i.e. 1×, 2×, 4× and 5×) were studied. Measurements with PCR amplicons, 274-bp of *cesA,* 310-bp of *nheA,* 317-bp of *nheB*, 413-bp of *nheC,* 811-bp of *cytK*-2 and 989-bp of *hblD*, correspondingly, were carried out. For all PCR products the signals amplified with increasing buffer ionic strengths until the 4×PBS, while 5×PBS decreased the signal level obviously (Figure 2). It was concluded that the 4×PBS used for hybridization provided the highest signal level, no matter what sequences and sizes of target DNA was provided as an analyte.

Influence of linker length of capture probe on signal generation

Probes with different linker lengths were designed in order to study the influence of steric hindrance of capture probes on the target hybridization and resulting signal level. Carbon-linkers (C-linkers) tether the capture probes to the sensing Au electrodes via the reaction of its end-labeled thiol group to the gold. Commonly used linker backbone consists of six carbons. In this study, identical in sequence capture probes of the *cytK-2* gene, but varying in linker length, i.e. CYTK-2_Cp: 6-C-linker, CYTK-2_Cp9: 9-C-linker, CYTK-2_Cp12: 12-C-linker, and CYTK-2_Cp18: 18-C-linker, were used for functionalization of the chip arrays, respectively. Two different *cytK*-2 target formats, i.e. 25-mer CYTK-2 oligonucleotides and 811-bp *cytK*-2 PCR amplicons, were applied in order to validate the possible effect of linker length. Figure 3 revealed that similar signals were generated from the 6-, 9- and 12-C linked capture probe positions, when both targets analytes were utilized. In case of use of capture probe with 6-C-linker signals of 51.6 nA/min for 0.05 nM 25-mer CYTK-2 oligonucleotides, and 0.2 nM 51 nA/min for 811-bp *cytK*-2 PCR amplicons were obtained. However, a 1.6-fold

increase in signal level was obtained from the 18-C linked probe position. To test the detection specificity, a non-target DNA analyte (i.e. F4810/72 genomic DNA) was exposed to the array surface. The lowest background signal (2 nA/min) was generated from the 6-C linked probe positions. 9- and 12-C linked probe positions produced a bit higher background signal with the average of 3.5 nA/min. 18-C linked positions caused the highest background signal, around 9 nA/min (data not shown). In conclusion, since no profit in assay sensitivity from the use of longer capture probe linkers was visible, the 6-C linker of capture probe was utilized in the following work.

Ultrasound-assisted analyte material preparation for signal amplification

In order to study the signal level as a function of target analyte fragmentation ultrasound was applied for 1, 2.5, 5, 10 and 14 min, respectively, to several PCR amplicons being products of different gene sequences and representing different lengths, i.e. 187-bp of *hblC*, 274-bp of *cesA*, 310-bp of *nheA*, 413-bp of *nheC*, 874-bp of *hblA*, and 989-bp of *hblD*. Fragmentation via ultrasonication of short DNA strands (i.e. 187-bp of *hblC*, 274-bp of *cesA* and 310-bp of *nheA*) did not enhance signals when compared to the non-sonicated analytes (Figure 4). Additionally, electrical response obtained from the 187-bp PCR amplicon of *hblC* was dramatically reduced after 10 min treatment. However, clear improvement in signal levels was observed once longer DNA strands (413-bp of *nheC*, 874-bp of *hblA* and 989-bp of *hblD*) after 10 min ultrasonication were subjected for the assays, correspondingly, while further extended sonication caused decrease in the electrical signals.

Placement of capture and detection probes along the target sequence

It was previously shown that the placement of the capture and detection probes in close proximity to each other along the target strand yielded in signal amplification [14]. Also,

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positioning of enzyme conjugates (Str-β-Gal) in close vicinity of chip array electrodes resulted in improved signals [15]. In the present work, different capture/detection probe-binding locations along DNA strands and their effects on signal generation were studied (Figure 5A). Two additional PCR amplicons of *nheB*, i.e. 332- and 321-bp, contained the same probe-binding sequences and similar strand sizes as the one used in earlier studies (described above as 317-bp of *nheB*), but different probe-binding locations along the target sequence. Probe-binding sites were located at the 5'-end, 3'-end or in the middle region of the targeted *nheB* strands, respectively. The same principle was applied to three *cytK*-2 PCR amplicons, i.e. 815-, 811- and 847-bp. It was evident that the highest signals for both, *nheB* and *cytK*-2 PCR amplicons, were obtained when a placement of the two adjacent capture and detection probes was located at 5'-end of the target strand (Figure 5A(2), 5B(2)). A large drop in signals appeared when probe-binding area at 3'-end of the DNA strand for both *nheB* and *cytK*-2 PCR amplicons was occupied for hybridization (Figure 5A(1), 5B(1)). At last, the lowest signals were achieved for the use of probe-binding location in the middle region of the target strand, again for both *nheB* and *cytK*-2 PCR amplicons (Figure 5A(3), 5B(3)). Worthy of note is the fact that a shift of probe-binding sites from 5'-end to middle region at the target strand drastically reduced the signal about 10-fold for the longer PCR amplicon of *cytK*-2, while for the shorter PCR strand of *nheB* the signal was reduced only from 96 to 50 nA/min by the same shift of probe-binding sites.

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Mismatch recognition

In this experiment, *cytK*-2 capture probes with different mismatch sequences were designed and spotted on chip arrays. An 811-bp PCR amplicon of *cytK*-2 was chosen to test the assay specificity by comparing signal levels from array positions functionalized with perfect matched CYTK-2_Cp and different mismatched (MM) capture probes (Figure 6A). Four

mismatched capture probes, M1aMM_Cp, M3MM_Cp, M5MM_Cp and R3MM_Cp (i.e. CYTK-2_M1aMM_Cp, CYTK-2_M3MM_Cp, CYTK-2_M5MM_Cp and CYTK-2_R3MM_Cp, correspondingly), carrying respectively single middle, triple middle, quintuple middle and triple random mutations, were investigated. Assay conditions, i.e. hybridization temperature, hybridization buffer ionic strength and stringent washing after hybridization, affecting mismatch recognition were studied. Neither altering hybridization temperature nor buffer ionic strength allowed sensible discriminating electrical signals. Only implementation of a stringent washing, that is at 55 °C, after hybridization gave improvement and good signal differentiations between perfect matched and different MM capture probes. It was visible that the mismatch recognition was most remarkable for triple random variant, while less for quintuple middle, triple middle and single middle mismatch. Under this washing operation, additional decrease of buffer ionic strength (from the 4× to 2×PBS) did not further progress the specificity of the assays.

To investigate further the assay specificity, and especially specificity for one-base mismatch, when electric chip arrays are employed for detection, supplementary two types of single middle mutations in capture probe (M1gMM_Cp and M1cMM_Cp) were designed and validated. In addition, in order to obtain more representative data, three kinds of PCR amplicons, i.e. 811-bp and 847-bp of *cytK-2*, and 317-bp of *nheB* were used. Hybridization was performed at 55 °C in the 4×PBS, and followed by 55 °C stringent washing with flushing buffer. The extent of mismatch discrimination was evaluated as above by comparing the electrical signal levels of the array positions functionalized with probes carrying a perfect match sequence and various single middle, triple middle, quintuple middle and triple random mismatched probes (Figure 6B). In fact, for all three types of targeted PCR amplicons, the results indicate that the recognition effect of single base mismatches is prominent with adenine, i.e. for M1aMM_Cp (i.e. CYTK-2_ M1aMM_Cp or NHEB_ M1aMM_Cp). The

presence of the other two kinds of examined one-base mismatches in M1gMM_Cp (i.e. in CYTK-2_ M1gMM_Cp or NHEB_ M1gMM_Cp) and M1cMM_Cp (i.e. CYTK-2_ M1cMM_Cp or NHEB_ M1cMM_Cp) resulted in comparable signal as obtained for perfect matched CYTK-2_Cp. Besides, triple middle M3MM_Cp (i.e. CYTK-2_ M3MM_Cp or NHEB_ M3MM_Cp), quintuple middle M5MM_Cp (i.e. CYTK-2_ M5MM_Cp or NHEB_ M5MM_Cp), and triple random mismatched R3MM_Cp (i.e. CYTK-2_ R3MM_Cp or NHEB_ R3MM_Cp) exhibited exactly the same trend on discrimination level when compared to perfect matched probe as it was observed before (at Figure 6A).

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A test for chip arrays reusability was performed. Individually, four target sequences, i.e. 51-mer HBLC polynucleotide, PCR amplicons of *cesA*, *nheA*, *hblC*, and *hblA* genes, were applied for hybridization with chip arrays functionalized with CESA_Cp, NHEA_Cp, HBLC_Cp, and HBLA_Cp. After an initial hybridization with target analytes, chip arrays were stripped by flushing hot water (75 °C), and then re-hybridized with target and detection probe mix two times (Figure 7). In general, rinsing resulted in regeneration of the capture probes for sequential experiments and caused chips available for re-use. However, regeneration of signals from one chip array gradually decreased with each following application. In the second use of the chip arrays above 80% of the original signal was obtained. Once the chip arrays were regenerated after the first hybridization, the resultant background signals were within 0.5-2 nA/min, as it was in the first assay. Here, background signals indicate the unspecific signals generated from non-target components due to cross-reaction with the sensing probe layer on chip array surfaces. The third use of chip arrays resulted in even lower signals, about 60% of the original signals. Meanwhile, the background signals increased to around 4 nA/min. Most chips were excluded from the use for the fourth assay due to high background signals.

DISCUSSION

Limitations of most current electrical detecting technologies include complex procedures which may result in poor reliability and sensitivity of the recognition. Only continued progress in nucleic acid detection technologies will make the electro-bio-testing a reality. In this respect, efforts were made to advance the technique and the results are being reported in this paper. The study was performed with the use of electrical chip arrays conjugated to automated analysis system 'eMicroLISA'.

This work seeks to give fundamental insight into issues that impact the structure and behavior of surface-immobilized capture probe layer influencing the detection sensitivity. The present investigation compares signal levels obtained from assays performed with rehydrated capture probe layer of chip arrays with that of responses from analyses with non-rehydrated surfaces. PBS solutions of varying ionic strength were individually employed for rehydration of the functionalized chip arrays. The results indicate that the amplified buffer ionic strength up to 4×PBS enhances the signal by nearly 300%. Further increase of buffer ionic strength, i.e. with the use of 5×PBS, led to a decrease in signal. The same tendency was observed when assays (i.e. with hybridization) were made for detection of target *cesA* PCR amplicon at the chip arrays (data not shown). It is assumed that the 5×PBS may stimulate the formation of secondary structures intra- or inter-probe strands, which results in steric hindrance causing a decrease of the probe layer accessibility. From previous studies it is known that the single stranded capture probes attached to the gold surface through a sulfur-gold linkage, typically form a compact layer with their multiple contacts to the surface and with potential cross-talk between neighboring strands [16-18]. Rehydration with high ionic phosphate-buffered saline causes most probably a repulsion of the immobilized probes from solid surface making them swelled and extended further into the solution. The conversion of the probes from the coiled conformation towards and extended, more upright conformation

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provides layer of better sterical accessibility to the reactive compounds applied for assays performed at chip arrays.

It is well known from a previous systematic work that ionic strength has a major influence on hybrid duplex formation and stability [19]. Thus, the influence of hybridization buffer ionic strength on the electric DNA chip signal as a matter of hybridization efficiency was studied by testing different ionic strengths of the hybridization solution (Figure 2). In course of these investigations, the 4×PBS was realized as being the optimal buffer for hybridization at the electrical DNA chip arrays irrespectively of the length of target analyte applied. The increase in ionic strength, resulting in neutralizing counterions such as metal mono- and multivalent ions (e.g. Na^{+}, Mg^{2+}) in the hybridization environment, reduces the electrostatic repulsion among the negatively charged DNA strands, which promotes capture probes to hybridize to complementary targets, and also strongly stabilizes the duplexes once they are formed. This effect was visible for this work when using buffer up to the 4×PBS. Further increases of buffer ionic strength diminished the generated signal, which may be due to a change in structure of capture probes.

An influence of steric hindrance on DNA hybridization by solid surface-anchored capture probes has been investigated by other researchers [20-23]. This interference is derived from the proximity of capture probes to an anchoring support. A close distance provides to the probes limited freedom of movement for target to contact and hybridize alike in the solution-phase. Thus, it is believed that the longer linker lengths enhance DNA hybridization and consequently improve the chip array performance with respect to the detection sensitivity, also to signal-to-noise ratio [21]. In order to evaluate this observation, four different C-linker lengths of capture probe were employed for the use of electrical chip arrays presented in this work (Figure 3). The results illustrated that capture probe C-linker lengths ranging from 6 to 12 carbons did not influence neither specific responses, nor the

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unspecific background signals, no matter what target type (i.e. varied in sizes, being single- or double-strands) was applied. While the utilization of capture probe C-linker length of 18 carbons yielded a specific signal amplification, the higher unspecific background responses were observed as well. Overall, as no benefits in assay sensitivity from the use of longer capture probe linkers were demonstrated, the 6-C linker of capture probe was used in further work.

Employment of ultrasound first to lyse bacterial cells, and secondly with the purpose of fragmenting genomic DNAs for assay sensitivities was studied previously [11,12,24]. However, further studies were needed for better understanding the relationship between fragmentation of target analytes and hybridization efficiency, being directly associated with method sensitivity. Thus, in this investigation PCR amplicons of different sequences and sizes were introduced. Results showed that ultrasonic fragmentation enhances specific signals up to two-fold for longer DNA strands (i.e. above 300 bp) by 10 min treatment (Figure 4). No obvious effects on generated signals were visible for shorter than 400 bp PCR amplicons subjected to ultrasonic treatment. For DNA strands of all sizes, over 10 min ultrasonication diminished the specific signals, presumably due to the loss of hybridization sites on the targets caused by extensive DNA fragmentation. The current observations and also other group's work confirm that the efficiency of hybridization process on solid surfaces is strongly correlated with the DNA fragment size [12,24,25].

Earlier studies revealed that the placement of the capture and detection probes in close proximity to each other along the target strand resulted in signal intensification [14]. Furthermore, it was also reported that the placement of enzyme conjugates (Str-β-Gal) nearby the chip array electrodes resulted in increased signals [15]. In this work, different capture/detection probe-binding locations along DNA strands and their effects on signal generation were studied. The results proved how important these factors are in respect to

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hybridization efficiency and enzyme-catalyzed redox signal generation (Figure 5A, 5B).

Various contributions to the observed effects have been identified. First of all, straightforward access of target strands to surface bound capture probes as well as enzyme conjugates to resulting hybrids is seen when the probe binding site is a terminal part of the target strand. Furthermore, a placement of a detection probe complementary to region adjacent to the binding site of the surface-immobilized capture probe results in a most favorable distance between the enzyme that is bound on the top of a hybrid and the electrode surface, in respect to effective contact of redox active molecules with the transducer [15]. At the same time, the use of adjacently positioned capture and detection probes along the targeted sequence is believed to be attributed at least partly to a helper effect for efficient nucleation process as has been seen as mentioned above in our previous investigations [14].

Invention of biosensing systems is often related to the detection of mismatches, and in particular to the discrimination of perfectly matched hybrid from mismatched one. In this work, various conditions (i.e. temperature and buffer ionic strength upon hybridization and following washing) favoring the formation of perfectly matches over mismatched duplexes were studied. *cytK*-2 capture probes with different mismatches were used for capturing of *cytK*-2 PCR amplicon. Figure 6A shows well visible mismatch discrimination, even for single nucleotide sequence alteration. To obtain more comprehensive information for the properties of mismatch recognition, examination of three kinds of PCR amplicons, for different variants of mismatched capture probes was performed (Figure 6B). Supplementary two types of single middle mutations in capture probe (M1gMM_Cp and M1cMM_Cp) were validated here. The results indicate that the mismatches are ordered by easiness of recognition as follows: triple random > quintuple middle > triple middle > single middle mismatch. Among the three variants of one-base mismatches, a sequence alteration was most remarkable for adenine. In general, the results exhibit a general trend in mutation recognition

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by the electrical chip arrays, and indicate that the target analyte could be detected with good discrimination against mismatches.

This paper covers a number of issues related to the challenges facing electrical chip arrays-based detection of DNA. It identifies and evaluates various critical factors that must be considered and overcome for reliable and sensitive biosensing. The knowledge obtained from this work is undoubtedly useful for many other detection systems.

ACKNOWLEDGMENTS

This work was supported by the European Commission (EC project LSHB-CT-2004-512009 eBIOSENSE) and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS).

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Figure 1:

Scheme of the assay performance on the chip array. The application of target and detection probe mix to the chip reaction chamber results in the formation of a sandwich hybrid-complex: capture probe/target DNA/detection probe. After the following enzyme conjugate binding and the substrate pAPG hydrolysis, an electrical signal is generated.

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Figure 2:

Influence of the hybridization buffer ionic strength on signal. 0.1 nM of each PCR amplicon, i.e. 274-bp of *cesA*, 310-bp of *nheA*, 317-bp of *nheB*, 413-bp of *nheC*, 811-bp of *cytK*-2 and 989-bp of *hblD*, respectively, was applied for hybridization performed at PBS representing a range of ionic strength (i.e. 1×, 2×, 4× and 5×). Normalized signals were calculated by referring to the highest signals for each individual amplicon. Error bars represent the standard deviations on at least three independent determinations per column.

Figure 3:

Effect of linker length of capture probe on signal. The electrode positions were functionalized with CYTK-2_Cp, which contained C-linkers with different lengths (6, 9, 12 and 18 carbons, respectively). 0.1 nM of biotinylated 25-mer CYTK-2 oligonucleotide (light grey columns) and 0.2 nM of 811-bp *cytK-2* PCR amplicon (black columns) were applied for assays, individually. Normalized signals were calculated by referring to the signal from the 6-C linked probe positions. Each column is an average of three independent determinations and the error bars represent the standard deviations.

Figure 4:

Signal level as a function of target analyte fragmentation. Ultrasound was applied for 1, 2.5, 5, 10 and 14 min, respectively, to PCR amplicons representing different lengths, i.e. 187-bp of *hblC*, 274-bp of *cesA*, 310-bp of *nheA*, 413-bp of *nheC*, 874-bp of *hblA*, and 989-bp of *hblD*; afterwards 0.2 nM of each analyte was individually applied for chip assay. Normalized signals were calculated by referring to the signal correspondingly obtained from assays with non-sonicated target. Error bars represent the standard deviations of the mean of three repeated determinations per column.

Figure 5:

A. Different probe-binding locations along *nheB* and *cytK*-2 PCR amplicon strand. Probe-binding sites were located at the 3'-end (1), 5'-end (2) or in the middle region (3) of the targeted strands, respectively. Grey bars attached to gold surface represent capture probes, dark bars labeled with B represent biotinylated detection probes, and long single-strands represent the hybridized strands from PCR amplicon targets.

B. Influence of different probe-binding locations along *nheB* and *cytK*-2 PCR amplicon strand on signal. Three *nheB* PCR amplicons (317-, 332- and 321-bp) and three *cytK*-2 PCR amplicons (815-, 811- and 847-bp) comprised the same probe-binding sequences and similar strand sizes, however were captured at the 3'-end (1), 5'-end (2) or in the middle region (3). 0.1 nM of each type of *nheB* and *cytK*-2 PCR amplicons was applied for chip assays, correspondingly.

The averages of data obtained from at least three independent determinations are plotted. Error bars were generated from the standard deviations of the replicates.

Figure 6:

A. Mismatch recognition in respect to different assay conditions. Chip arrays were functionalized with the CYTK-2_Cp, CYTK-2_M1aMM_Cp, CYTK-2_M3MM_Cp, CYTK-2_M5MM_Cp, and CYTK-2_R3MM_Cp (described on graph as _Cp, M1aMM_Cp, M3MM_Cp, M5MM_Cp and R3MM_Cp, respectively). 0.2 nM of 811-bp *cytK*-2 PCR amplicon was applied to study assay conditions (i.e. H=hybridization temperature; hybridization buffer ionic strength; and W=washing temperature) affecting mismatch recognition. Normalized signals were calculated by referring to CYTK-2_Cp signals.

B. Detection specificity as a function of mismatch recognition. Chip arrays were functionalized with CYTK-2_Cp or NHEB_Cp, CYTK-2_M1aMM_Cp or NHEB_M1aMM_Cp, CYTK-2_M1gMM_Cp or NHEB_M1gMM_Cp, CYTK-2_M1cMM_Cp or NHEB_M1cMM_Cp, CYTK-2_M3MM_Cp or NHEB_M3MM_Cp, CYTK-2_M5MM_Cp or NHEB_M5MM_Cp, and CYTK-2_R3MM_Cp or NHEB_R3MM_Cp (described on graph as _Cp, M1aMM_Cp, M1gMM_Cp, M1cMM_Cp, M3MM_Cp, M5MM_Cp and R3MM_Cp, respectively), mismatched capture probes. 1 nM 847-bp *cytK*-2, 0.2 nM of 811-bp *cytK*-2 and 317-bp *nheB* PCR amplicons were applied to study the detection specificity among various mismatched sequences. Normalized signals were calculated by referring to _Cp signals.

Error bars represent the standard deviations on at least three independent determinations per column.

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Deleted: Figure 7:
Chip array reusability test. Chip arrays were functionalized with NHEA_Cp, HBLA_Cp, HBLC_Cp and CESA_Cp and applied for hybridization with 0.1 nM of each target, i.e. 51-mer HBLC polynucleotide, PCR amplicons of *nheA*, *hblA*, *hblC* and *cesA* genes in the 4×PBS, respectively. After the first analysis (white columns) the hybridized targets were stripped by flushing hot water (75 °C for 4 min). Then, the chip arrays were re-hybridized with target and detection probes, and the signals are represented by grey bars. Repeated stripping and the third hybridization resulted in signals represented by dark bars. Normalized signals were calculated by referring to responses obtained in the first assay. Error bars represent the standard deviations on at least three independent determinations per column.

Table 1:

Sequences and locations of PCR primers, probes, oligo- and polynucleotides.

Table 2:

Program for DNA detection at ‘eMicroLISA’.