Electric chips for rapid detection and quantification of nucleic acids

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Abstract

A silicon chip-based electric detector coupled to bead-based sandwich hybridization (BBSH) is presented as an approach to perform rapid analysis of specific nucleic acids. A microfluidic platform incorporating paramagnetic beads with immobilized capture probes is used for the biorecognition steps. The protocol involves simultaneous sandwich hybridization of a single-stranded nucleic acid target with the capture probe on the beads and with a detection probe in the reaction solution, followed by enzyme labeling of the detection probe, enzymatic reaction, and finally, potentiometric measurement of the enzyme product at the chip surface. Anti-DIG–alkaline phosphatase conjugate was used for the enzyme labeling of the DIG-labeled detection probe. *p*-Aminophenol phosphate (pAPP) was used as a substrate. The enzyme reaction product, *p*-aminophenol (pAP), is oxidized at the anode of the chip to quinoneimine that is reduced back to pAP at the cathode. The cycling oxidation and reduction of these compounds result in a current producing a characteristic signal that can be related to the concentration of the analyte. The performance of the different steps in the assay was characterized using *in vitro* synthesized RNA oligonucleotides and then the instrument was used for analysis of 16S rRNA in *Escherichia coli* extract. The assay time depends on the sensitivity required. Artificial RNA target and 16S rRNA, in amounts ranging from 10^{11} to 10^{10} molecules, were assayed within 25 min and 4 h, respectively.

Keywords: Electric biochips; Sandwich hybridization; Magnetic bead; Redox recycling; 16S rRNA

1. Introduction

Nucleic acid-based techniques, such as nucleic acid hybridization, polymerase chain reaction (Mullis et al., 1992), and chip-based analysis (Vo-Dinh, 1998; McGlennen, 2001), have made it possible to automate most of the processes required for gene expression analysis (Schena et al., 1995; Richmond et al., 1999; Wang et al., 1999; Oh and Liao, 2000) and identification of organisms through 16S rRNA or DNA analysis (Amann et al., 1995; Fuchs et al., 1998; Van de Peer et al., 1998). Nucleic acid hybridization is an essential component in many of the current standard molecular biology techniques including sample preparation, PCR, sequencing, genotyping, and nucleic acid detection with microarrays (Bej et al., 1991; Southern et al., 1999). Important factors in such applications are the stability of the duplex formed and the presence of secondary or tertiary structure in the target, capture or detection probe (Sohail et al., 1999).

Biosensors and DNA microarrays have been brought into focus due to their ability to provide the sequence-specific information in a fast and simple manner in comparison to the traditional hybridization assays (Watson et al., 2000). Usually they incorporate a biologically active layer as a recognition element and a transducer, which translates the

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biorecognition event into a signal proportional to the analyte concentration. In the case of the DNA microarrays, the surface is scanned or imaged after hybridization and washing to obtain the hybridization pattern (Taylor and Schultz, 1996; Wang, 2000).

Among other things, biosensor technology offers the possibility to monitor DNA and RNA hybridization kinetics. Furthermore, detection and quantification of specific sequences can be performed (Nilsson et al., 1995). The design of a DNA/RNA biosensor generally involves attachment of a capture oligonucleotide on a solid surface followed by detection of the target nucleic acid via hybridization to the capturing probe. Immobilization of oligonucleotides to solid supports has been developed recently (Steel et al., 2000; Kumar and Liang, 2001). By using small particles, like magnetic micro-beads, a large surface area is provided for nucleic acid attachment (Walsh et al., 2001). The magnetic properties of the beads make them also easy to move and fix in a liquid system.

The immobilization step should lead to a well-defined capture probe orientation and packing density, allowing the probe to be accessible for hybridization. Depending on the transducer, capture probes can be immobilized via thiol group linkage to gold electrodes (Herne and Tarlov, 1997), via biotin–streptavidin bonds (Buckle et al., 1996), by covalent coupling to functional groups (Bach et al., 1999), or by simple adsorption to carbon surfaces (Wang, 2000). Likewise, different methods of attachment to magnetic beads carrying different active groups on their surfaces have been described (Lund et al., 1988).

This paper presents the concept and the characterization of an electric chip-based biosensor for analysis of RNA and DNA. The performance is shown for analysis of synthesized mRNA fragments in buffer and for analysis of 16S rRNA in *E. coli* extract.

2. Experimental

2.1. The concept

The principle of the electric chip combined with bead-based sandwich hybridization (BBSH) is illustrated in Fig. 1. The detailed procedures of the different steps in the signal generation are described below. Magnetic beads with immobilized capturing probes are exposed to the sample containing the target nucleic acid that is permitted to hybridize to the capturing probe. Simultaneously a second hybridization is made with digoxigenin (DIG) labeled detection probe. The alkaline phosphatase (ALP) is then bound to the detection probe. Finally, the enzyme substrate (pAPP) is added and the enzyme product (pAP) concentration is measured with the chip. In this measurement the current is generated by the cyclic oxidation–reduction between pAP and quinoneimine (Niwa et al., 1993).

2.2. Electrode fabrication

Thin film gold microelectrode arrays consisting of four pairs of interdigitated electrodes plus two auxiliary electrodes were manufactured using silicon technology (Fig. 2) (Paeschke et al., 1995; Hintsche et al., 1997). The chips were fabricated on 6 in. thermally oxidized silicon wafers. Gold electrodes with titanium for the adhesion layer were manufactured by deposition on wafer and photolithography. The chips were structured using the lift-off technique. Each

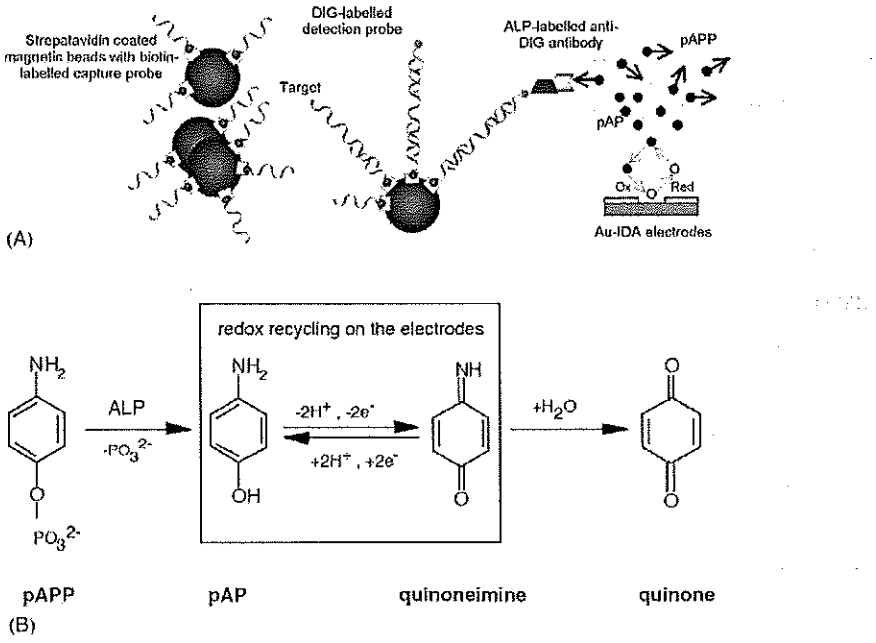


Fig. 1. Principle of the electric chip-based nucleic acids assay (panel A) and the redox recycling scheme (panel B).

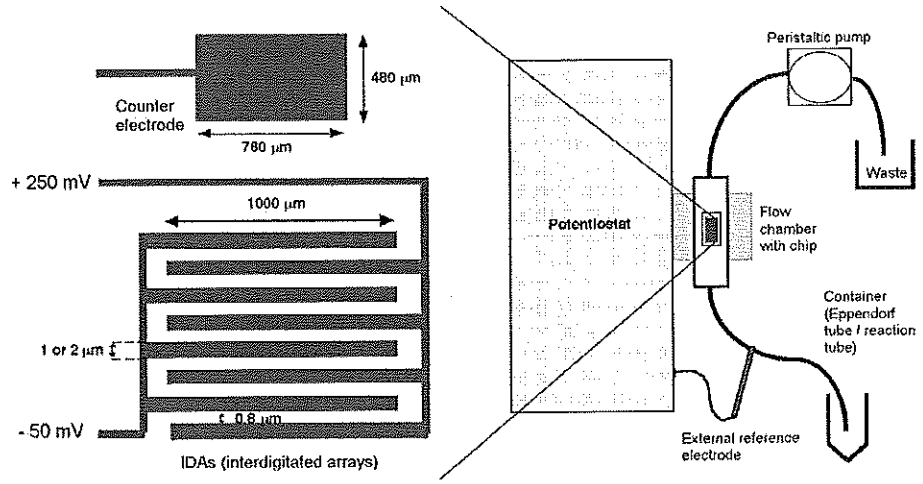


Fig. 2. Top view of detailed interdigitated electrodes on the chip (left). The schematic diagram of the integrated fluidic system (right).

chip (6.4 mm × 5 mm) was encapsulated by 500 nm thick silicon oxinitride using chemical vapor deposition, excluding the electrode and connecting areas. The electrode fingers of the interdigitated structure are 1000 µm long and 1 µm or 2 µm wide. The gaps are 0.8 µm and the number of electrode fingers per band is 70. The auxiliary/counter electrode on the chip is a thin-film gold pad of 0.5 mm^2. The layout of the interdigitated electrodes on the chip and the schematic diagram of the integrated fluidic system are shown in Fig. 2.

2.3. Measurement device

A microprocessor-controlled portable potentiostat developed by the Fraunhofer Institut für Siliziumtechnologie (ISIT, Itzehoe, Germany) and manufactured by eBiochip Systems GmbH (Itzehoe, Germany) was used for the measurement with the chips. This device allows amperometric measurements at two independent channels in a current range of 2.5 pA to 100 nA. The chips are mounted on printed circuit boards and connected to the potentiostat by a special socket in the front of the device. The chip is covered by a flow cell (volume of 2.5 µl), which allows to pump liquids to the surface of the chip. An external Ag/AgCl reference electrode is included in the fluidic flow to allow a three electrode measurement together with the working electrode pairs and the counter electrode on the chip (Fig. 2).

The potentiostat is connected to a PC through a serial interface (RS232). Custom-made software controls the potentiostat, collects the measurement data and transfers data to the commercial data analysis and visualization software Origin™ from OriginLab Corporation (Northampton, MA, USA).

2.4. Reagents

Anti-digoxigenin-alkaline phosphatase (anti-DIG–ALP) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Streptavidin coated paramagnetic beads with 2.8 µm diameter (Dynabeads® M-280 Streptavidin) are products of Dynal A.S. (Oslo, Norway). *p*-Aminophenol phosphate (pAPP) was from ICN Biomedicals Inc. (Aurora, OH, USA). *p*-Aminophenol (pAP), bovine serum albumin (BSA), and diethyl pyrocarbonate (DEPC) were obtained from Sigma–Aldrich (Steinheim, Germany). Binding and washing buffer (B & W buffer) contained 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 2 M NaCl. Trisbuffered saline (TBS) was prepared by dissolving 30 mM tris(hydroxymethyl)aminomethane and 100 mM sodium chloride in water and adjusting to pH 8.0 by adding hydrochloric acid. Phosphate-buffered saline (PBS) buffer at pH 7.4 contained 10 mM sodium phosphate and 150 mM sodium chloride.

2.5. Bacterial strain and culture conditions

The *E. coli* K-12 strain W3110 [F^-, IN(*rrnD-rrnE*)1, λ^-] (Genetic Stock Center, Yale University, New Haven, CT) was grown aerobically with shaking in LB broth at 37 °C.

2.6. Preparation of cell samples for RNA sandwich hybridization

Culture samples were analyzed during exponential growth at an optical density (600 nm) of 0.4. Cells were pipetted and quickly transferred to cold acidified phenol, pH 4.5 at the ratio 1:1, quickly frozen in liquid nitrogen and stored at −70 °C.

2.7. RNA extraction

Lysis of *E. coli* cells for RNA isolation was performed as described above. Lysed cells were thawed at 0 °C and extracted with phenol–chloroform–isoamyl alcohol (25:24:1) at 4 °C and then with chloroform–isoamylalcohol (95:5) at the same temperature. Total RNA was precipitated with two

Table 1
Sequences of oligonucleotide probes used in this work

Probe[a]	Sequence[b] (5′–3′)
aroGA probe (44-mer)	GGCUUAGAGUGGCAGUCAGAAAUAAUGUGGCCAGUUUUGUCAUU
aroGC probe (20-mer)	TGACTGCCACTCTAAGCCACX
aroGD probe (20-mer)	YACAATGACAAAACTGGCCAC
rrsAC probe (28-mer)	AGGTAAGGTTCTTCGCGTTGCATCTTAAX
rrsAD probe (29-mer)	AGTGTGGCTGGTCATCCTCTCAGAGTCGAY

[a] The probe names, aroGA probe, aroGC probe, aroGD probe, rrsAC probe, and rrsAD probe, are abbreviated from aroG artificial, aroG capture, aroG detection, rrsA capture, and rrsA detection probe, respectively.
[b] X and Y stand for biotin and digoxigenin, respectively.

volumes of ethanol in 0.3 M sodium acetate (pH 5.2). The precipitates were washed with ethanol (70%), dissolved in water, and stored at −70 °C. The RNA concentration was determined by absorption at 260 nm. All equipment was treated with diethyl pyrocarbonate (DEPC) to inactivate RNase. Solutions were made in DEPC-treated water. DEPC, 0.1% (v/v), was added and incubated at 37 °C for 1 h followed by autoclaving at 121 °C for 15 min.

2.8. Probe design

All oligonucleotide probes (with or without 5′- or 3′-biotin or digoxigenin modification) were obtained from Thermo Hybaid GmbH (Ulm, Germany). The sequences are listed in Table 1. The biotin or digoxigenin was linked to the probes with a spacer sequence of a few bases in length, each of which was selected to be non-complementary to the target strand. The capture and detection probes were designed to be complementary at different parts of the target RNA, after searching the *aroG* and *rrsA* gene sequences of *E. coli* from GeneBank (www.ncbi.nlm.nih.gov). All probes except aroGA that was used as analyte for the characterization work were DNA probes.

2.9. Capture probe immobilization

Conjugation of the biotinylated single-stranded capture probe to M-280 streptavidin beads was performed using the following protocol. Ten microliters of stock suspension of beads (6.7×10^6 beads) were first washed twice with 10 μl of B & W buffer, and then diluted in 20 μl of this buffer. To immobilize, an equal volume of the biotinylated probes was added to the beads and the resulting mixture was placed on a rotator to allow conjugation. After conjugation, the beads were washed and diluted to desired concentration in TBS, pH 8.0 buffer. The amount of immobilized capture probe was determined by measuring the amount of oligonucleotide left in solution spectrophotometrically at 260 nm. The efficiency of the immobilization schemes is expressed as percentage immobilized probes.

The number of captured probes bound to the surface of a single bead, N, can be calculated with the equation:

$$N = \frac{\text{conc.} \times E \times N_A}{25 \times N_{\text{beads}}},$$

where E is the immobilization efficiency, N_A the Avogadro constant (6.022×10^{23} molecules/mol) and N_{beads} is the number of beads in the reaction (6.7×10^6 for the 40 μl reaction scale). The geometric bead surface is declared to 24.63 μm^2.

2.10. Bead-based sandwich hybridization

One hybridization was used to attach a target nucleic acid to the capturing probe on the magnetic beads, and another (simultaneous) hybridization was used to bind the DIG-labeled detection probe to the target. Initially, the fluidic system interconnected by Silicon tubing (1.0 mm i.d., Reichelt Chemietechnik GmbH, Heidelberg, Germany) was flushed with 0.1N NaOH followed by washing with Millipore water and TBS buffer, pH 8.0. The buffer was pumped through the tubes to flush out air bubbles. After flushing, the system was ready to use. Prior to use in the BBSH assay, all Eppendorf tubes were washed with 3% BSA in TBS buffer, pH 8.0 to minimize non-specific binding of the conjugates to the reaction tubes. RNase-mediated degradation of the target RNA was inhibited by preparing buffer solutions in DEPC–H_2O. The hybridization was carried out in an Eppendorf tube. In a typical assay, first capture oligonucleotide-polystyrene beads were washed in TBS buffer, pH 8.0, next suspended in PBS buffer, pH 7.4 and then introduced into an Eppendorf tube by pipetting. Afterward, the magnetic beads were exposed to the sample and DIG-labeled detection probe and subjected to simultaneous hybridization of the target with the capturing probe and the detection probe. Upon completion of the hybridization, the magnetic beads were separated from the solution by means of an external magnet. The beads were washed carefully using a pipette with TBS buffer, pH 8.0 containing 3% BSA. Then, they were exposed to an anti-DIG–ALP conjugate (diluted to the appropriate concentration in TBS buffer, pH 8.0) that labels the captured molecules with enzyme.

2.11. Electrochemical detection of the BBSH complex

To measure the bound ALP activity, a 4 mM solution of pAPP in TBS pH 8.0 was added to the Eppendorf tubes and incubated for 10 min or longer, at 30 °C or 40 °C. The ALP complex catalyzed the hydrolysis of pAPP, yielding electroactive pAP, which was detected amperometrically

after transfer to the chip. The potential of one finger set of the interdigitated electrodes was fixed at +250 mV and that of another finger set at −50 mV. The redox recycling of pAP/quinoneimine was used for amplification of the amperometric current response. The difference between the anode and cathode's response was determined as signal. The change in this signal with time was measured and a peak current (EL signal, nA) was usually registered within about 1–2 min. Fig. 1B illustrates the measuring principle for pAP/quinoneimine.

3. Results

3.1. Immobilization of the capture probe on the magnetic beads

Different immobilization strategies can be used to couple nucleic acids onto surfaces (Ramsay, 1998). In this work, immobilization of biotinylated oligonucleotides to streptavidin-coated magnetic beads was used. Streptavidin is a protein with four identical subunits, each containing a high affinity binding-site for biotin (Freitag et al., 1997). The influence of the concentration of the probe during the immobilization on the beads was investigated. Unbound capture probes after the different coupling reactions were measured photometrically at 260 nm, and the immobilization efficiency was calculated afterward as described in Section 2. Fig. 3 shows that the probe density on the beads increased with increasing probe concentration in the reaction solution up to about 1.25 μm aroGC. The maximum probe density was about about 2 μg/mg bead. Thus, at a probe concentration of 1.25 μM a maximum surface density of 1.2 fg/μm^2 bead surface was achieved. This corresponds to 30 fg capture probe per bead or 2.4×10^6 probe molecules per bead. Higher probe concentration did not increase the probe density further on the bead. The immobilization yield, i.e. the percentage of the probes that were immobilized, was relatively constant at 80–95% up to a probe concentration of 0.5 μm but then declined linearly with increasing probe concentration in the reaction. The 0.5 μm reaction solution was used for further experiments. Data in Fig. 3 refer to single stranded DNA capture probes, but comparative values were obtained for RNA probes (data not shown).

The time dependence of the immobilization of the single-stranded aroGC DNA probe to streptavidin-coated beads was also investigated. Samples were analyzed using biotin-labeled capture probes at 0.5 μm probe concentration with a bead concentration of 2.5 μg/μl and immobilization times between 0 and 20 min. The efficiency of attaching the capture probes to the beads was around 90% after 5 min of incubation, and it reached 100% at longer reaction times (data not shown). Based on the results an immobilization time of 10 min seems to be optimal. Exactly the same data were obtained in the case of using RNA capture probe in the experiment (data not shown).

3.2. Stability of the enzyme substrate and product

The current of the electric chip is a function of the concentration of the enzyme product pAP that reaches the electric chip. pAPP of 4 mM was earlier reported to be suitable as substrate concentration for ALP and was used in all experiments (Niwa et al., 1993). However, the commercial substrate pAPP that was used, was contaminated by the product pAP. The stability of the enzyme substrate, pAPP, was determined in a 4 mM pAPP solution (TBS, pH 8.0) at 30 °C. Fig. 4 shows that the chip signal gradually dropped

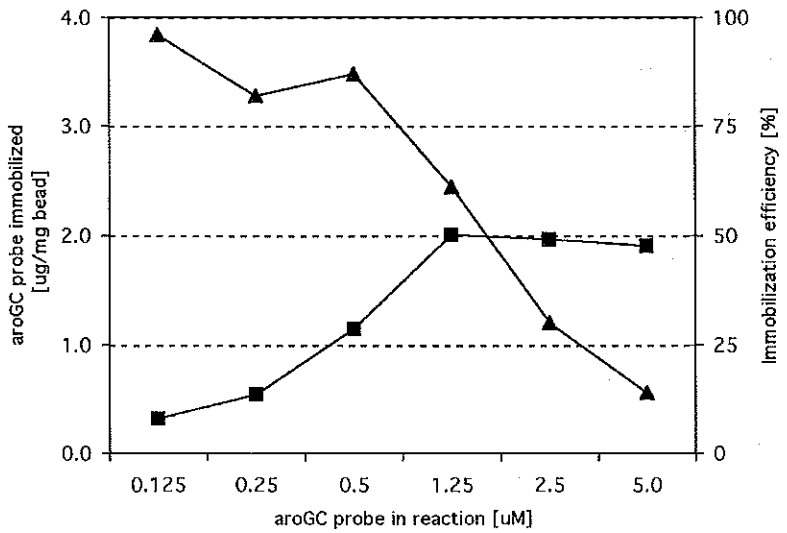


Fig. 3. Relationship between the amount of single stranded DNA capture probe (aroGC) in the immobilization reaction and the immobilized capture probe density on the beads (μg/mg) (■). The immobilization efficiency (▲) at each capture probe concentration was determined. Total concentration of beads was 2.5 mg/ml. The capture probe was immobilized during 5 min to magnetic beads via streptavidin–biotin interactions.

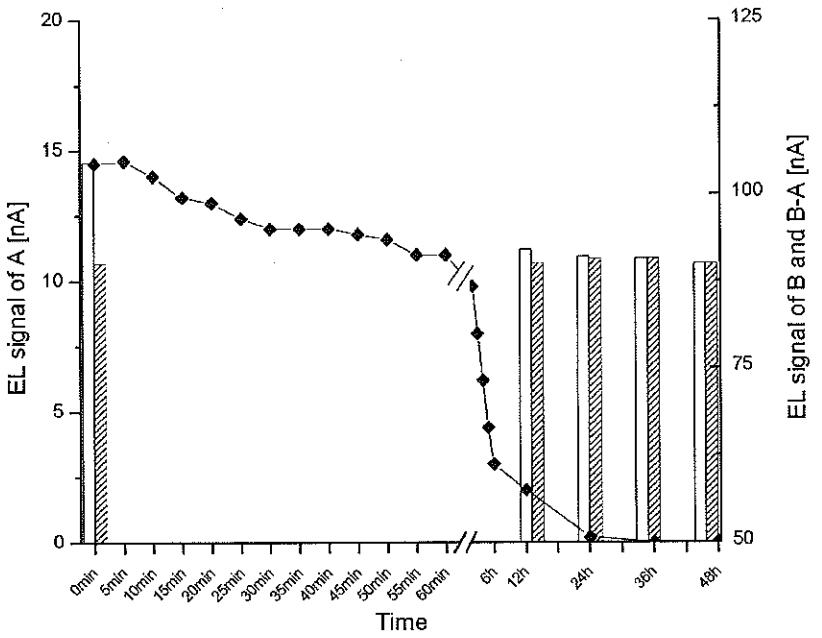


Fig. 4. Influence of the pAP impurity background signal in the substrate (pAPP) solution. Signal from the pAP contamination in the solution (A, ◆). Signal from enzymatic hydrolysis of pAPP containing pAP (B, transparent bar). The striped bars (B–A) show the difference between those two signals which is constant.

from 100 nA and stabilized at 90 nA after about 24 h. This was due to the presence of an impurity of pAP in the pAPP solid. The initial background signal slowly decayed and was zero after 24 h. Thus, when the pAP was eliminated, a stable signal was obtained, indicating that non-enzymatic hydrolysis of pAPP did not occur. Moreover, the enzymatic hydrolysis of this pAPP solution was measured at different times (0 min, 12, 24, 36, 48 h in Fig. 4). The amount of pAP was approximately the same (corresponding to 90 nA) in all measurements. Thus, to reduce measurement errors, at least 3 days old substrate solution in TBS buffer, pH 8.0 is recommended for the assay.

The pAP is known to be sensitive to oxidation to quinone (manufactures information). Therefore, the stability of the product, pAP, was investigated in an O_2^- saturated and in an oxygen-depleted (N_2-saturated) TBS buffer, at pH 8.0. An aliquot of the originally 5 μm pAP solution was injected to the electric chip at different times after preparation and the resulting peaks of current were plotted versus time (Fig. 5). The decomposition of pAP caused by its oxidation

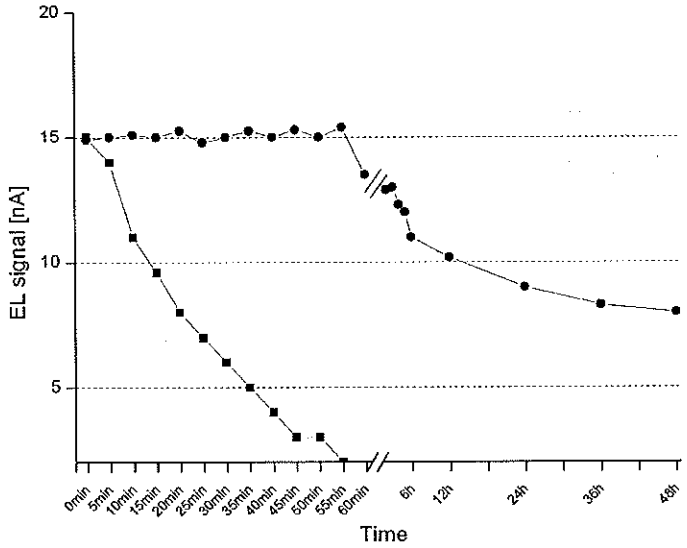


Fig. 5. Stability of pAP under different oxygen concentrations. Electric signal from 5 μM pAP in air-saturated (■) and in oxygen-depleted (●) TBS buffer, pH 8.0.

into quinone by dissolved oxygen is evident after 5 min in oxygen-saturated buffer. In the case of oxygen-depleted buffer, pAP was stable for more than 1 h.

3.3. The temperature effect on the ALP-complex activity

The signal depends not only on the reaction time between the addition of the substrate and the analysis, but also on the amount of enzyme that has been bound to the beads, and on its activity, i.e. the incubation temperature and pH. For highest sensitivity of the assay, the assay should be performed when the enzyme activity is at highest. This may lead to the conclusion that optimal temperature for the enzyme activity should be used. However, extending the reaction time is also a way to increase the sensitivity and then also the enzyme stability becomes important.

The optimum temperature for the enzymatic hydrolysis of pAPP to pAP was determined. Therefore, the anti-DIG–ALP enzyme complex was incubated for 10 min at temperatures ranging from 4 to 60 °C. The temperature optimum was found to be about 40 °C (results not shown). Higher temperatures led to a significant inactivation of the enzyme complex. In order to evaluate long-term effects of elevated temperatures, the enzyme was incubated for extended times at 30 and 40 °C. The enzyme activity was measured as an average activity of the last 10 min of the reaction time and the average activity from 0 to 10 min was defined as 100%. The amount of total product generated during the first hour was slightly higher at 40 °C than at 30 °C, but then the activity of the enzymatic reaction was lower at 40 °C compared to 30 °C resulting in lower amount of total generated product (data not shown). These results are of particular importance, if long enzymatic reaction times are used to enhance the sensitivity of the assay. In such a case, 30 °C incubation temperature should be chosen.

3.4. Non-specific binding

There are many potentially disturbing factors associated with the analysis in crude cell extract, and therefore the assay was first characterized using a 44-mer target RNA synthesized *in vitro* as analyte. This analyte was assayed in buffer. The signal was measured in experiments with adding neither target in the sample nor detection probe to evaluate possible unspecific adsorption of the anti-DIG–ALP complex in the assay. It was reported previously that a critical requirement in the hybridization assay using magnetic beads is the inclusion of SDS in the hybridization buffer, as this reagent was effective in reducing non-specific binding of the conjugate probe. In addition, pre-siliconized Eppendorf tubes were used for those assays (Lund et al., 1988). Here, we found that a crucial step in the BBSH was the addition of bovine serum albumin (BSA) to the washing buffer. All Eppendorf tubes were washed with TBS buffer containing 3% BSA to minimize non-specific binding of the conjugates to the reaction tubes. A signal was obtained only when both the target and the detection probe were present in the assay (data not shown). In this case, exclusion of BSA from the reaction buffer resulted in non-specific binding of the conjugates to the reaction tube and bead surface. Removal of BSA resulted also in a marked increase in the signal generated in the hybridization reaction with a negative control, i.e. the assay without a target or detection probe. Moreover, a non-specific binding of the analyte, the detection probe, ALP-conjugate, and pAPP to the beads washed with BSA was investigated to further characterize the behavior of the conjugate-based BBSH system. In these experiments, no capture probes were fixed to the beads as usual. None of the combinations resulted in a signal after exposure to the beads and washing, indicating that no unspecific binding occurred when BSA was present in the buffer (data not shown).

3.5. Influence of concentrations of capturing probe and detection probe on the sensitivity of the assay

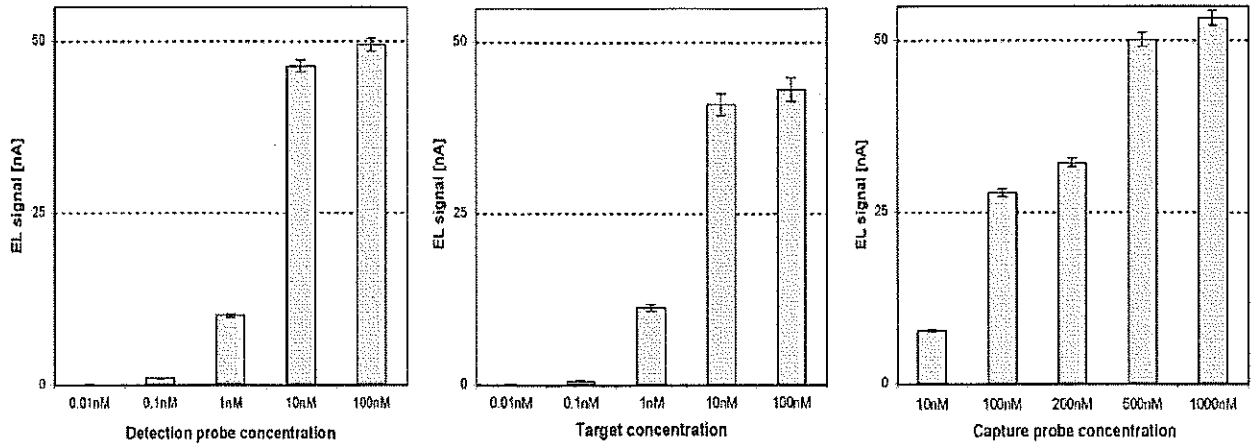
The signal generated from the reaction should depend on the concentration of the analyte, but it also depends on the concentration of capture probe, i.e. the beads, and the detection probe. To investigate the relevant concentration ranges for these parameters, the signal was recorded under conditions where one of these parameters was varied while the others were constant. The results are summarized in Fig. 6.

At a constant bead concentration of 5 μg/μl with 1.4 μg (200 pmol) of capture probe per 1 mg beads, the detection level for 10 nM of the analyte oligonucleotide was reached at about 0.1 nM of detection probe. In the range of 1–10 nM detection probe the signal was strongly dependent on the detection probe concentration, but at higher concentration the signal seemed to be saturated (Fig. 6, left panel). This indicates that saturation is reached at a detection probe to analyte ratio of about one. Similar results were obtained when the analyte concentration was varied over the same concentration range with a detection probe concentration fixed to 10 nM. The detection limit was about 0.1 nM analyte and the signal increased in the range of 1–10 nM analyte, but no further increase of the signal was detected when the analyte to detection probe ratio exceeded 1 (Fig. 6, mid panel).

The sensitivity should also be related to the concentration of the capturing probe, which was varied by the concentration of the beads while keeping the concentrations of both the detection probe and the analyte at 10 nM. The right hand panel of Fig. 6 shows that the signal is proportional to the capturing probe (bead) concentration in a range between 10 and 500 nM with a tendency to become saturated at bead concentration exceeding 5 μg/μl that corresponds to a capture probe concentration of 1000 nM.

3.6. Assay of 16S rRNA in E. coli extract

The results from the previous experiments were applied to set up an electric chip-based procedure for analysis



Capture probe conc. Capturing bead conc.	1000 nM 5 μg/μl	1000 nM 5 μg/μl	10 – 1000 nM 0.05 – 5 μg/μl
Target conc.	10 nM	0.01 - 100 nM	10 nM
Detection probe conc.	0.01 - 100 nM	10 nM	10 nM

Fig. 6. Influence of concentration of detection probe (left panel), target (mid panel), and capturing beads (right panel) on the signal. Total procedural time was around 25 min (2 min hybridization; 10 min enzyme binding; 10 min enzymatic reaction). The reactions for each concentration point were carried out in duplicate.

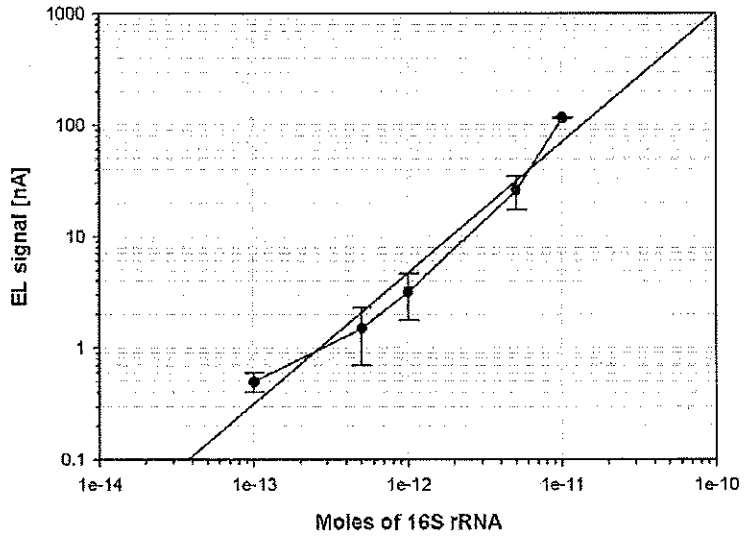


Fig. 7. The electrical signal generated in the assay using 16S rRNA in *E. coli* extract as a target. The hybridizations were carried out at 60 °C. The assay was completed in 4 h (1 h hybridization; 30 min enzyme binding; 2 h enzymatic reaction).

of 16S rRNA of *E. coli*. Samples from an *E. coli* culture were subjected to total RNA extraction. The BBSH method was used for hybridization and the electric chip for the signal generation. Reproducible detection signal was accomplished for the samples in the range 10^{-13} to 10^{-11} mol as shown in Fig. 7. However, it should be noted that electric signal obtained here was observed when the time of the procedure was increased up to approximately 4 h.

4. Discussion

Biosensors based on the combination of base-pair recognition of nucleic acid probes and electrochemical transducers currently receive large attention (de Lumley-Woodyear et al., 1999; Marrazza et al., 1999; Mascini et al., 2001; Lucarelli et al., 2002; Zhang et al., 2002). This paper describes an instrument based on BBSH and enzymatic labeling as bio-recognizing system and potentiometric de-

tection of the enzyme product by means of a silicon-based electric chip. The concept is illustrated in Fig. 1. This approach offers a large flexibility and can be seen as a step towards the design of an integrated silicon chip in which also the bio-recognition system is integrated. By means of using magnetic beads for the bio-recognition several of the chemical steps included before the signal generation could be studied without the demand of including the probe immobilization already in the chip manufacturing process, which must be done with advanced and specialized laboratories and equipment.

Alternative methods for the immobilization of the capturing probes are available (Ramsay, 1998; Steel et al., 1998). The use of streptavidin–biotin was chosen because of the simplicity and the fast reaction that completed the immobilization in 5–10 min. In an application situation, the beads are stock material for the assay and more elaborate methods for immobilization may be preferred.

Protocols for the extraction of the target nucleic acid have not been extensively investigated here. The optimal protocol will certainly depend much on the properties of the target and the sample. The total RNA extraction used in the analysis of 16S rRNA was, however, simple and required about 5 h, once the cells were lysed.

Early studies of molecular interactions provided valuable basic information and led to the provision of general rules for the design of capture and detection probes (Southern et al., 1999). In the analysis of 16S rRNA of *E. coli*, the choice of about 30-mer sized probes worked well even if we have no information about the fraction of the target molecules that was hybridized. When working with chromosomal DNA the number of target molecules will be known when the cell number is known. However, such calibrations are complicated in RNA analyses. Furthermore, in analysis of mRNA, the formation of secondary structures can hamper the hybridization to the probe (Mir and Southern, 1999). The effect of secondary structures is minimized in filter-based assays, since the target is immobilized in denatured form. It is essential that the probe is located at a site where secondary structure formation is less likely. Several software tools are available for the simulation of secondary structure formation, but there is still a lack of confidence with respect to the applicability of these simulations to realistic sample conditions (Van de Peer et al., 1998; Fuchs et al., 1998; Fuchs et al., 2001).

The investigation on the influence of the concentrations of detection probe during the hybridization showed that the ratio of detection probe to target could be as low as one. Higher concentration of detection probe did not increase the sensitivity (Fig. 6), although the excess of detection probe to the target is always recommended to drive hybridization to completion. Our results indicate that the hybridization efficiency of the two probes was about the same in this case. However, it is not possible to estimate the capture probe hybridization efficiency from the present data. The experiments also showed that the capture probe concentration, measured as concentration of beads, must be optimized for an assay (Fig. 6).

In the analysis of the synthesized mRNA, 200 fmol (corresponding to 10^{11} molecules) was detected in less than 25 min (Fig. 6). To reach maximum assay sensitivity the probe concentration parameters must be considered (Fig. 6), but sensitivity can also be increased by extending the enzyme reaction time before the electrochemical measurement. By this way and some other improvements, detection limits down to 10^9–10^6 molecules of target should be readily achievable. However, when extending the incubation time, the stability of the enzyme must be taken into account. The investigation shows that a lower temperature, e.g. 30 °C instead of 40 °C, may give an improved sensitivity even if the enzyme activity optimum is closer to 40 °C, due to the better enzyme stability at lower temperature.

To reach good sensitivity also the background noise must be eliminated. The use of BSA to prevent unspecific binding in the system was shown to be essential and very efficient. Another potential source of background signal is the impurity of pAP in the substrate pAPP. In freshly prepared pAPP solution, the pAP concentration contributes to about 20% of the signal under the conditions applied in Fig. 4. Fortunately, this background could be completely eliminating by storing the substrate in equilibrium with air for 36 h before use, during which time the pAP was oxidized to the electrochemically inert quinone.

All electrochemical measurements were made in pH 8 Tris buffer solution. This potential of hydrogen was found to be advantageous for the electrochemical reversibility of pAP because oxidized pAP (quinoneimine) reacts with hydrogen ions below neutral pH, which breaks the electrochemical recycling (Niwa et al., 1993). Furthermore, this pH value was relatively effective for the enzymatic reaction carried out by ALP.

In conclusion, in this paper we report on the characterization of several aspects of the bio-recognition in the electric biochip, and for most of this work a synthesized fragment of mRNA was chosen. The feasibility of this biochip principle was then tested in the quantification of 16S rRNA in an *E. coli* RNA extract. The sensitivity is high for a system working without prior amplification via PCR: already at this early stage of development, RNA target in the range of 10^{11}–10^{10} molecules can be assayed, which in the case of chromosomal DNA analysis often means the same number of cells. This concept is now available for development of many types of analyses where quantification of specific nucleic acids is the goal, as quantification of pathogenic microorganisms via measurement of specific DNA sequences related to the pathogenicity, quantification of selected gene expression via measurement of specific mRNAs or group classification of bacteria via 16S rRNA analysis.

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