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A DNA-based piezoelectric biosensor: Strategies for coupling nucleic acids to piezoelectric devices

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Abstract

A DNA-based piezoelectric biosensor has been here studied in terms of probe immobilisation and DNA sample pre-treatment. The biosensor is specific for the detection of the mecA gene of methicillin-resistant *Staphylococcus aureus* (MRSA).

Methicillin-resistant *S. aureus* is responsible of several infections in humans, like pneumonia, meningitis and endocarditic. MRSA is also a major cause of hospital-acquired infections worldwide. The antibiotics resistance is conferred by the gene mecA, codifying for an anomalous protein.

Two different immobilisation procedures of the probe specific for mecA gene are reported: immobilisation via streptavidin-biotin interaction and direct immobilisation of thiolated probes.

After the study with synthetic oligonucleotides, the system has been applied to the analysis of bacterial DNA from MRSA, amplified by polymerase chain reaction. These samples were pre-treated with two different denaturation procedures and the performances of the sensor in the two cases were compared.

The two immobilisation methods and denaturation protocols were here used to study the influences of these parameters on the performances of the sensor, applied here to the detection of the mecA gene. Better results in terms of sensitivity and reproducibility were obtained when using the biotinylated probe and the PCR-amplified samples treated by a denaturation procedures involving the use of high temperature and blocking oligonucleotides.

Keywords: Piezoelectric biosensor; *Staphylococcus aureus*; Denaturation; DNA-based sensing; Probe immobilisation

1. Introduction

Recently, there has been an increasing interest in biosensor technology for fast hybridisation detection using easy and rapid procedures. Building of such devices involves the immobilisation of single-stranded oligonucleotides (probe) on the surface of the transducing element and recording the variations of the transducer signal caused by the hybridisation between the probe and the complementary strand in solution (target). Several types of nucleic acid-based biosensors have been developed over recent years [1–3]. In particular, piezoelectric biosensors offer the possibility of monitoring the hybridisation reaction in real time, without the use of any label [4–8] and they have been presented as alternatives to gel electrophoresis and to traditional methods where labelled probes are often required.

In this work, the development of a piezoelectric biosensor for the detection of the mecA gene, present in methicillin-resistant *Staphylococcus aureus* (MRSA) strain, is described. Methicillin-resistant *S. aureus* is the causative agent of a wide variety of infections and toxin-mediated diseases [9–11]. MRSA is also a major cause of hospital- and community-acquired infections worldwide. Methicillin-resistant *S. aureus* strains account for 60% of *S. aureus* clinical isolates in Japan, 50% in Italy and 34 % in the United States [12,13].

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One of the characteristics of this strain is its resistance to β-lactamic antibiotics. β-Lactamic antibiotics, such as penicillin and methicillin, are substrate analogs of penicillin-binding proteins (PBPs) [14]. Methicillin resistance in MRSA strains is due to acquisition of the mecA gene [12] which encodes for PBP2a, a new protein with a low affinity for the β-lactamic antibiotics [15].

Historically, isolates were distinguished by phenotypic methods, including antibiotic susceptibility testing and bacteriophage typing. Both methods have limitations, as genetically unrelated isolates commonly have the same antibiogram, and many *S. aureus* isolates are non-typeable by phage typing [16]. With the advent of molecular biology, strain typing focused on DNA-based methods, included Southern blot hybridisation using gene-specific probes, polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (PFGE) [17–19]. These methods require subjective interpretation and comparison of patterns and fingerprint images and they remain difficult to standardize between laboratories, and the image-based information is difficult to organize for rapid search and retrieval by computer [20].

In DNA-based piezoelectric biosensors hybridisation detection is performed following the frequency changes resulting from the interaction between a specific probe immobilised on the gold electrode of a quartz crystal and the complementary strand in solution.

For probe immobilisation, two different chemistries are here presented. The first one is based on the probe coupling via biotin–streptavidin binding, on previously modified surfaces with a thiol-dextran layer. The second one is based on the direct coupling of thiol-derivatised probes (probe-C6-SH) to bare gold sensor surfaces. The interaction of the immobilised probe with a complementary and a non-complementary synthetic oligonucleotides was investigated to test the sensor specificity. The analytical parameters of the sensor were also studied.

Moreover, the developed sensor was applied to the analysis of DNA samples amplified by polymerase chain reaction, extracted from bacterial DNA. The DNA was from MRSA and *Staphylococcus lugdunensis*, sensitive to methicillin, used as negative control [17]. A recent denaturation procedure [21,22] of PCR-amplified DNA is here also used and further optimised for this specific application.

2. Experimental

2.1. Apparatus, reagents and samples

10 MHz AT cut quartz crystals (14 mm) with gold evaporated (42.6 mm^2 area) on both sides were obtained from Mistral (Latina, Italy). For the measurements, the quartz crystal was housed inside a methacrylate cell where only one side of the crystal was in contact with the solution. The frequency variations were continuously recorded using a quartz crystal analyser, Model QCA917 (Seiko EG&G, Chiba, Japan). The frequency shifts reported in the paper are the differences between two stable frequency values (±0.5 Hz).

11-Mercaptoundecanol, diglyme (bis-2-methoxyethyl ether) and ethanolamine hydrochloride were purchased from Sigma (Milan, Italy); Dextran T500 was purchased from Amersham (Uppsala, Sweden), epichlorohydrin and *N*-hydroxysuccinimide (NHS) from Fluka (Milan, Italy). Ethanol and all the reagents for the buffers were purchased from Merck (Darmstadt, Germany).

2.2. Samples

Biotinylated synthetic oligonucleotides and PCR primers were purchased from MWG (Germany), synthetic oligonucleotides having a group C6-SH at the 5′ termination were purchased from Sigma-Genosys (Cambridge, UK).

Bacterial DNA for PCR amplification was purchased from LGC Promochem (London, UK). Genomic DNA from the methicillin-resistant strain *S. aureus* subsp. *aureus* Rosenbach (ATCC 700699) was used as specific sample whereas DNA from *S. lugdunensis* (ATCC 43809D) was used as negative control since this strain is known to be methicillin sensible.

The immobilised probe was designed to recognise a region of the mecA gene. The sequence of probe is included in the fragment amplified by PCR (Fig. 1a).

The base sequence of the synthetic oligonucleotides is reported below:

Biotinylated probe: 5′ biotin-TTCCAGGAATGCAGAAA-GACCAAAGCA-3′ (27-mer)
Thiolated probe: 5′ HS-(CH2)6-TTCCAGGAATGCAGA-AAGACCAAAGCA-3′ (27-mer)
Target: 5′-TGCTTTGGTCTTTCTGCATTCCTGGAA-3′ (27-mer)
Non-complementary strand: 5′-CAAGGGAGGGAGAGA-CAGAGAGGCC-3′ (25-mer)

The oligonucleotides used in the denaturation procedure (thermal + blocking oligonucleotides) were as follows:

MecA1 (P1): 5′-TAATAGTTGTAGTTGTCGGGTTTG-3′
MecA2 (P2): 5′-GGTTTTAAAGTGGAACGAAGGTAT-3′
MecA3 (P3): 5′-GTTAGATTGGGATCATAGCGTCATT-3′
MecA4 (P4): 5′-AATTCCACATTGTTTCGGTCTAAAAT-3′

3. Sensor development

3.1. Immobilisation of the probe

3.1.1. Biotinylated probe

The gold electrode was cleaned with a boiling solution consisting of H_2O_2 (30%), NH_3 (30%) and milliQ water in a 1:1:5 ratio. The crystals were dipped in this solution

taatagttgtagttgtcgggtttgGTATATATTTTTATGCTTCAAAAGATAAAGAAATTAATAATACTATTG
ATGCAATTGAAGATAAAAATTTCAAACAAGTTTATAAAGATAGCAGTTATATTTCTAAAAGC
GATAATGGTGAAGTAGAAATGACTGAACGTCCGATAAAAATATATAATAGTTTAGGCGTTAA
AGAATAAACATTCAGGATCGTAAAATAAAAAAAGTATCTAAAAATAAAAAACGAGTAGATG
CTCAATATAAAATTAAAACAAACTACGGTAACATTGATCGCAACGTTAAATTTAATTTTGTTA
AAGAAGATGGTATGTGGAA*gttagattgggatcatagcgtcatt*ATTCCAGGAATGCAGAAAGACCAAAGC
ATACATATTGAAAATTTAAAATCAGAACGTGGTAA*aattttagaccgaaacaatgtggaatt*GGCCAATACA
GGAACACATATGAGATTAGGCATCGTTCCAAAGAATGTATCTAAAAAAGATTTAAAGCAATC
GCTAAAGAACTAAGTATTTCTGAAGACTATATCAACAACAAATGGATCAAAATGGGTACAAG
ATG**atacctttcgttccactttaaaacc**

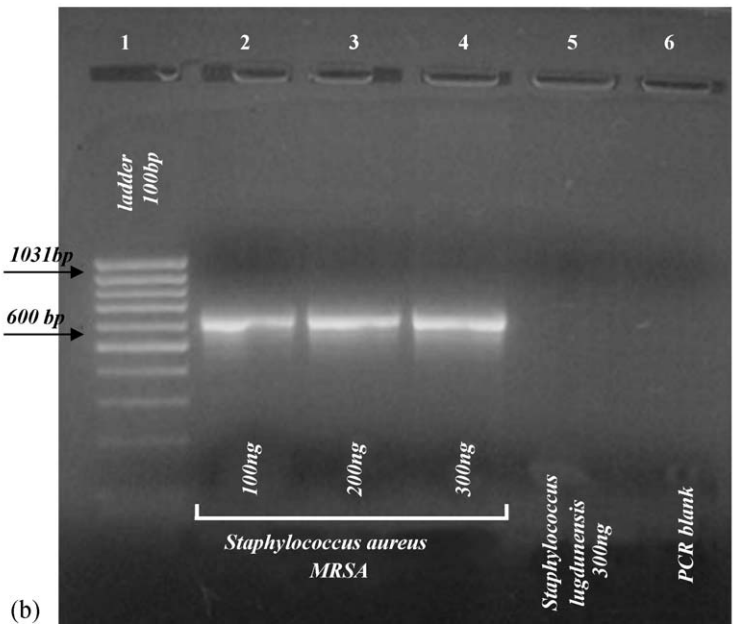

Fig. 1. (a) Sequence of bases of the fragment amplified from the mecA gene. The primers (P1 and P2) used for the amplification are reported in bold. The blocking oligonucleotides (P3 and P4) are indicated in italic and the immobilised probe is underlined. (b) Gel electrophoresis of the PCR amplification conducted as reported in Section 3.2.

for 10 min. They were then thoroughly washed with distilled water and immediately used.

The gold electrode was further modified as reported in Lofas et al. [23] for the immobilisation of streptavidin. The biotinylated probe was then bound to the streptavidin layer. The whole immobilisation protocol is reported in Mannelli et al. [24].

3.1.2. Thiol-derivatised probe

The clean crystal surface (as previously described) was treated with 200 μl of a solution of the thiolated probe (1 μM) in immobilisation solution (KH_2PO_4 1 M, pH 3.8) for 2 h. After washing, 200 μl of a blocking thiol (MCH, 1 mM) were added to the cell and the reaction was allowed to proceed for 1 h. After washing with water, the final step involved the substitution of water with 100 μl of hybridisation buffer to equilibrate the system.

3.2. Bacterial DNA amplification

The amplification protocol (forward primer: MecA1 5′-TAATAGTTGTAGTTGTCGGGTTTG-3′; reverse primer: MecA2 5′-GGTTTTAAAGTGGAACGAAGGTAT-3′) was adapted from the procedure reported in Jenison et al. [25]. The 100 μl of reaction mixture contained 100–300 ng of bacterial DNA, 4 units of Taq polymerase (Amersham, Uppsala, Sweden), 400 nM of forward and reverse primer and 200 μM of each dNTP (Amersham, Uppsala, Sweden). PCR conditions were as follows: 95 °C for 4 min, 53 °C for 1 min and 72 °C for 1 min (30 cycles), 72 °C for 4 min.

The PCR amplification was conducted by using a MJ Research MiniCycler (M-Medical, Milan, Italy).

Screening of the PCR products (617 bp, Fig. 1a) was performed by gel electrophoresis and visualised through a UV transilluminator (Fig. 1b). The control solution (blank) contained all the PCR reagents except the DNA template. The negative control, DNA from the methicillin-susceptible *S. lugdunensis*, was processed by the same PCR protocol.

The DNA concentration of the amplicons was determined by fluorescence measurements (Fluorometer TD-700 from Turner Biosystems).

3.3. Hybridisation with synthetic oligonucleotides

The hybridisation with synthetic oligonucleotides was performed by adding 100 μl of the oligonucleotide solution

in hybridisation buffer (NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4) to the cell well. The reaction was monitored for 10 min and then the crystal washed with the hybridisation buffer to remove the unbound oligonucleotides. We report the frequency shift as the difference between this final value and the value displayed before the hybridisation reaction.

In all the experiments, the single-stranded probe was regenerated by 30 s treatment with 1 mM HCl (for two times). All the experiments were performed at room temperature.

3.4. Hybridisation with amplified PCR samples

The PCR fragments were diluted with hybridisation buffer to obtain a final volume of 100 μl. To obtain ssDNA, the sample was then denatured by using two different denaturation procedures. After the denaturation treatment, the sample was added in the cell and the hybridisation reaction was allowed to proceed for 20 min and evaluated as reported for the synthetic oligonucleotides.

The sensor surface was then regenerated by two subsequent treatments (30 s) with 1 mM HCl.

3.5. Denaturation treatments

The amplicons obtained by PCR have a double helix structure and the two strands should be separated (denatured) to allow the hybridisation with the probe immobilised on the sensor surface. To obtain a single strand from the amplicons, two approaches were followed: thermal denaturation and thermal denaturation with oligonucleotides.

3.5.1. Thermal denaturation

This denaturation method involves a 5 min incubation step at 95 °C followed by 1 min in ice. This procedure is well documented in many previous studies [4,5], and will be here compared with the following approach.

3.5.2. Thermal denaturation with oligonucleotides

This denaturation method was found to be a simple and useful way to obtain DNA available for hybridisation after PCR amplification [21,22]. The principle of this method relies on the use of small (10–30 bases) oligonucleotides (which can correspond to the PCR primers) added to the denaturation mixture. These oligonucleotides are complementary to some sequences on the strand which hybridises to the immobilised probe, but are positioned laterally and do not overlap the portion forming the complex with the probe. By the interaction between the thermally separated DNA strands and these oligonucleotides, re-association between DNA strands of PCR amplicons is prevented, and surface hybridisation can occur. The whole denaturation procedure consisted in 5 min incubation at 95 °C and 1 min at a specific temperature, which is the suitable temperature for primers annealing used in the PCR procedure [21].

Different combinations of primers were used: P1–P2, P3–P4, P2–P3 and P2–P4. They map in different regions of the 617 bp PCR fragment of mecA gene (Fig. 1a).

The primers (1 μM) were added to the PCR fragment solutions before the first thermal treatment at 95 °C 5 min, then the temperature was decreased to 50 °C for oligonucleotides annealing.

The sample was then injected in the sensor cell for hybridisation reaction.

4. Results and discussion

4.1. Hybridisation with synthetic oligonucleotides

The sensor was first optimised with synthetic oligonucleotides and the main analytical parameters were studied, e.g. specificity, sensitivity, reproducibility.

The experiments were performed with different concentrations of the target oligonucleotide (0.06–0.75 μM), complementary to the probe immobilised on the crystal. Hundred microliters were added to the crystal surface modified with biotinylated or thiolated probes (Fig. 2).

A higher response (26% as average for all the tested concentrations) was obtained with the crystal modified by using the biotinylated probe. The two curves show a similar profile with a plateau for concentrations higher than 0.5 μM.

The specificity of the interaction was tested by using the 25-mer non-complementary oligonucleotide (1 μM). The signal resulting from this interaction when using the biotinylated or the thiolated probe was negligible (<2 Hz), evidencing the specificity of the system in both cases.

The reproducibility of the measurements has been also estimated. In Table 1 the results obtained after the hybridisation reaction with a concentration of the target oligonucleotide, are reported. In particular, the reproducibility of the signal was estimated for the results obtained on the same

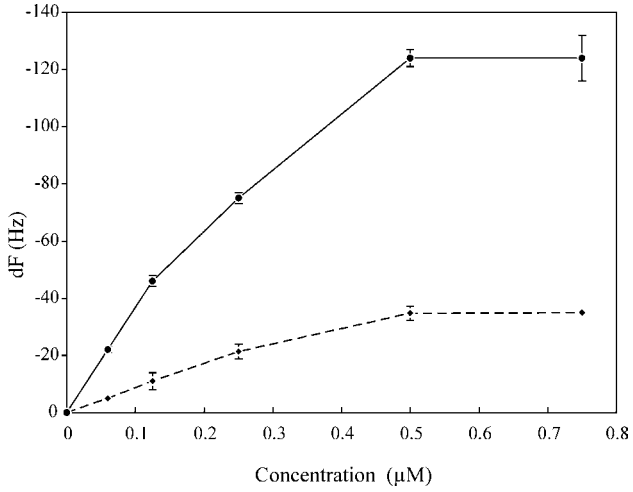

Fig. 2. Calibration curves obtained with synthetic oligonucleotides on the crystal modified with the biotinylated (—) or the thiolated probe (- - -).

Table 1
(a) Results recorded with the same concentration (0.125 μM) of the complementary oligonucleotide ($N = 3$) on the same crystal (intracrystal) and on different crystals ($N = 13$) (intercrystals), when the biotinylated probe was immobilised; (b) results recorded with the same concentration (0.250 μM) of the complementary oligonucleotide ($N = 3$) on the same crystal (intracrystal) and on different crystals ($N = 7$) (intercrystals), when the thiolated probe was immobilised

	$\Delta F_{average}$ (Hz)	S.D. (Hz)	CV%
(a) Biotinylated probe			
Intracrystal ($N = 3$)	−46	2	4
Intercrystal ($N = 13$)	−42	7	7
(b) Thiolated probe			
Intracrystal ($N = 3$)	−21	2	9
Intercrystal ($N = 7$)	−24	7	11

crystal (reproducibility intracrystal) and on different crystals (reproducibility intercrystals). Due to the different sensitivity of the system modified with the two immobilisation methods, for the surface modified with the biotinylated probe, the tested concentration was 0.125 μM while a 0.250 μM target solution was used with the thiolated probe modified surfaces.

The reproducibility was good for both the sensors. It must be considered that the procedure for the modification of the crystal is non-automated and involves several coating steps, which were performed in the laboratory starting from the gold surface. Moreover, the reproducibility intracrystal estimated as CV% at one concentration corresponds to average reproducibility calculated for all the concentrations used in the calibration curve (4 and 8% for biotinylated and thiolated probe, respectively).

4.2. Hybridisation with PCR amplified samples

Bacterial DNA was amplified by PCR following an adapted protocol from the one reported by Jenison et al. [25]. The amplified DNA fragment, internal to the mecA gene, was 617 bp in length and contained the complementary sequence to the immobilised probe.

Different template concentrations were tested (100, 200 and 300 ng DNA) for the amplification, as well as a negative control (DNA from *S. lugdunensis*).

In order to obtain a hybridisation signal from the sensor, the amplified samples have to be denatured to dissociate the dsDNA into ssDNA, ready for the binding with the probe. The two denaturation procedure described in Section 3.5.

4.2.1. Biotinylated probe

PCR blanks, negative controls and the mecA amplified product (0.03 μM) were initially tested after the thermal treatment and a measurable signal (-27 ± 5 Hz) could be observed only for the specific sample.

The denaturation procedure involving oligonucleotides was further used on these samples (0.03 μM): different combination of the oligonucleotides, hybridising in different regions of the amplified fragment, were tested in order to study the effect of the position of these sequences on the hybridisation reaction (Fig. 3). The thermal reaction is also reported in the figure together with the results obtained with PCR blanks and negative controls.

All the combinations of oligonucleotides allowed a successful hybridisation of the sample with the immobilised probe. Moreover, the signals obtained with the second denaturation approach are higher than the one recorded when only thermal denaturation is applied. In particular, the lowest signal (-27 ± 4 Hz) was observed for oligonucleotides P3–P4 mapping very close to the target sequence recognised by the probe. This could be due to a steric hindrance of the two oligonucleotides P3 and P4 in the target-probe binding.

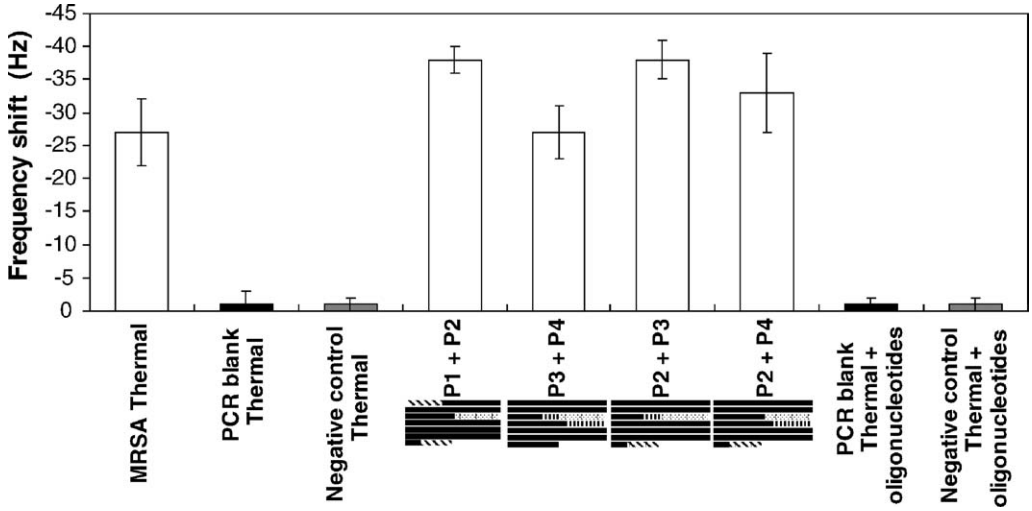

Fig. 3. Results obtained on the biotinylated probe with PCR samples denatured by the thermal + oligonucleotides procedure using different combinations of the oligonucleotides. All the PCR samples were tested at a concentration of 0.03 μM. The blocking oligonucleotides varied in the position within the amplified DNA, P1–P2 (▨) are positioned at the end of the amplified fragment, while P3–P4 (▥) are in the middle, flanking the complementary target sequence (▤).

Table 2
Summary of the results obtained with the two immobilisation procedures and the two denaturation methods when testing PCR-amplified samples (0.03 μM)

	PCR mecA (Hz)	PCR negative control (Hz)	PCR blank (Hz)
Thiolated probe Thermal denaturation	−22 ± 2	−4 ± 2	−2 ± 1
Thiolated probe Thermal + blocking oligonucleotides denaturation	−27 ± 5	−3 ± 3	−1 ± 1
Biotinylated probe Thermal denaturation	−31 ± 4	−1 ± 1	−2 ± 1
Biotinylated probe Thermal + blocking oligonucleotides denaturation	−38 ± 3	−1 ± 1	−1 ± 1

The best results, in terms of signal and reproducibility, were found for the couples P1–P2 (−38 ± 3 Hz, CV% = 5%) and P2–P3 (−38 ± 2 Hz, CV% = 8%). The specificity of the sensor was evidenced by the very low signals (−1 Hz) obtained with PCR blanks and negative controls for all the tested denaturation procedures.

Further experiments were conducted with the PCR specific samples at different concentrations (0.015, 0.03 and 0.06 μM). PCR blanks and the negative control at the same concentrations were also tested using the thermal + oligonucleotides treatment (P2–P3) (Fig. 4). The signal obtained with the specific sample increased with the concentration up to a value of −64 ± 1 Hz for the concentration 0.06 μM. This dependence of the signal from the concentration was not found for the negative control and for PCR blanks since the measurable signals obtained (−7 for the negative control and −10 for the blank) were probably due to non-specific adsorption of some component of the PCR mixture on the sensor surface.

4.2.2. Thiolated probe

The same experiments using the two denaturation procedures, with the crystal modified with the thiolated probe, are summarised in the first two rows of Table 2. For the thermal with blocking oligonucleotides denaturation, oligonucleotides P2 and P3 were used. All the samples were tested at a concentration of 0.03 μM.

Also with this probe immobilisation procedure, a slightly higher signal was obtained for the thermal with oligonucleotides denaturation protocol. The sensor resulted very specific with both the denaturation methods as evidenced by the very low signals obtained with PCR blanks and negative controls.

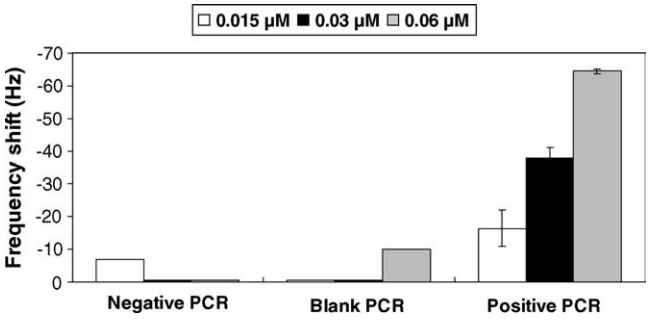

Fig. 4. Different concentrations of the PCR samples were tested after denaturing the samples with the thermal + oligonucleotides method, using the P2 + P3 combination.

The results previously described for the biotinylated probe are summarised in the last two rows of Table 2, for a direct comparison between the two probe immobilisation methods. Higher signals can be observed for the biotinylated probe when using both the two different denaturation procedures. The sensors developed with the two probes showed similar reproducibility in terms of CV% also when using PCR samples. The specificity was good for both the sensors, but lower signals were obtained with the negative control samples on the biotinylated probe.

5. Conclusions

A study on the performances of a DNA-based piezoelectric biosensors has been reported focusing on the probe immobilisation method and on the samples pre-treatment. The biosensor has been applied to the analysis of the mecA gene of the methicillin-resistant *S. aureus*.

A specific probe for this gene has been immobilised onto the gold electrode of quartz crystals by using two different immobilisation procedures. The hybridisation reaction has been studied using both synthetic oligonucleotides and PCR-amplified samples.

Two denaturation protocols were used for amplicons strand separation and the one involving the use of blocking oligonucleotides has been further optimised for this particular application.

The analytical characteristics of the biosensor has been evaluated with a comparison between the different methodologies used and the best results were obtained when a biotinylated probe was immobilised and the PCR samples were treated with a thermal denaturation involving blocking oligonucleotides.

Since, the samples used were PCR-amplified DNA from bacterial DNA commercially available, a more general application to PCR-amplified DNA from real clinical samples can be foreseen for the proposed biosensor, once the PCR conditions have been optimised for these more problematic

samples. In this way a method for quick identification of MRSA, supporting a prompt prophylaxis intervention, could be developed.

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