Chapter 8
Piezoelectric Sensing for Sensitive Detection of DNA

Maria Minunni

Abstract Piezoelectric sensing has been widely applied for affinity sensing, and recently sensitive DNA detection has been reported in different matrices for different analytes (i.e. target sequences). In this chapter, the detection principle and the approaches used in DNA-based sensing with focus on detection of microsatellite DNA, present in high number of copy as well as target sequence detection of genes present in one or few copy number per haploid genome will be presented and discussed. Particular attention will be devoted to the pre-analytical steps which may influence the sensor response to the target analyte such as genomic DNA fragmentation and denaturation. Comparison between immobilization chemistries is also presented. In particular, finding in microsatellite detection with both biotinylated and thiolated probes is reported and discussed.

8.1 Introduction

The utilization of piezoelectric quartz crystal oscillators as microbalances (QCM) has been applied in Analytical Chemistry, both in gas and in liquid phases. In literature there is a wide number of papers based on commercially available or in house developed devices, using crystals ranging from 5, up to 10, 30 MHz, using in QCM in the fundamental frequency or in the relative overtones. Applications of QCM's as thin film monitors and controls; in surface science, plasma-assisted etching, analytical chemistry, and space system contamination and for aerosol mass measurements have been reported over the last 20 years. Details of their methodology are scattered widely throughout the literature.

M. Minunni (✉)
Dipartimento di Chimica "Ugo Schiff", Università di Firenze, Via della Lastruccia 3,
50019 Sesto F.no (FI) Florence, Italy
e-mail: maria.minunni@unifi.it

8.1.1 Piezoelectric Biosensors: Theory and Applications of Piezoelectric Effect

Piezoelectric, similarly to Surface Plasmon Resonance (SPR) transduction methods is widely used in biosensors development, mainly because it allows the detection of label-free targets and the kinetic measurement of molecular interactions in real-time. Moreover, piezoelectric crystals as SPR chips can be regenerated, allowing a multi-use of the sensor. Piezoelectric crystals are acoustic transducers generally utilized as microbalance or microviscosimeters. In particular, piezoelectric quartz crystals are the basic elements of quartz crystal microbalance (QCM) device. Quartz resonators are the most used crystals, a crystal variant of Silicon-dioxide (SiO_2).

However, independently from the material used, the term "piezoelectric" derived from the Greek word piezen meaning "to press". The first investigation on the piezoelectricity was performed in 1880 by Jacques and Pierre Curie [1], who observed that a mechanical stress applied to the surfaces of various kinds of crystal caused a corresponding electrical potential across the crystal, whose magnitude was proportional to the applied stress. The Curies also verified the converse piezoelectric effect, in which application of a voltage across these crystals caused a corresponding mechanical strain. This causes a vibrational, or oscillatory, motion in the crystal, resulting in the generation of acoustic standing waves at a characteristic resonant frequency. The wave is called bulk acoustic wave (BAW) or surface acoustic wave (SAW) in the case of propagation through the substrate or on the surface, respectively. A few naturally abundant crystals (i.e. quartz, tourmaline and Rochelle salt) are piezoelectric, but many other materials exhibit this effect, including quartz analogue crystals, such as berlinite ($AlPO_4$), ceramics with perovskite or tungsten-bronze structures ($BaTiO_3$, $KNbO_3$, $LiNbO_3$, $LiTaO_3$, $BiFeO_3$, $NaxWO_3$, $Ba_2NaNb_5O_5$, $Pb_2KNb_5O_{15}$). However, recently piezoelectric crystals based on gallium orthophosphate ($GaPO_4$) have been reported for different application, including biosensing [2, 3]. $GaPO_4$ production has been, first set up by AVL List GmbH, Graz Austria, for applications as pressure monitoring in motors combustion engines, since the thermal coefficient of this material is compatible with the high temperature reached in car engines. Characteristics of this material are, in particular, high sensitivity, stability up to 970 °C, no pyroelectricity, no stress induced twinning, high electric resistivity >1,015 Ωcm. For all these interesting properties different applications have been identified and investigated also within an European project entitled "Growth of Large $GaPO_4$ Single Crystals and their use for Special Sensor Applications", (contract n° G5RD-CT-2002-00709, coordinated by Prof. Dr. Peter Kremple at AVL), who had foreseen in particular some Bulk Wave Applications such as high shear $GaPO_4$ crystal rheometer, $GaPO_4$ crystal particulate microbalance (GCPM) and Nanobalance Affinity Sensor for DNA-based sensing (i.e. GMO) and drug detection. Within this project sensitive DNA detection was studied using conventional quartz crystals and eventually $GaPO_4$.

However, only quartz provides the unique combination of mechanical, electrical, chemical and thermal properties, which allowed its commercial success. Direct

and converse piezoelectricity has a wide range of applications. Piezoelectric quartz crystals are used in quartz watches, computers and in many high-performance devices (such as Atomic Force Microscopy) to apply tiny mechanical displacements on the scale of nanometers. Furthermore, such crystals are employed as quartz crystal microbalance for thickness monitoring.

The quartz crystal microbalance is a bulk acoustic wave device based on the converse piezoelectric effect, in which a quartz crystal is sandwiched between two electrodes. The resonant frequency of the quartz crystal depends on several parameters, such as size, density and cut. The most used devices employ AT-cut quartz crystals, sliced with an angle of $35°10'$ respect to the optical z-axis (Fig. 8.1a). AT-cut quartz crystals show a high frequency stability and a temperature coefficient close to zero between 0 and 50 °C [4]. AT-cut crystals oscillate in the thickness shear mode (TSM) [5].

The application of a voltage between the two electrodes causes a shear deformation of the crystal, which is maximized at the crystal faces, making the device sensitive to surface interactions. The resonant condition with the acoustic wave is satisfied by including the crystal into an oscillation circuit, where the frequency of the alternating potential difference applied to the electrodes matches the fundamental frequency of the crystal. The fundamental frequency depends upon the thickness of the wafer, its chemical structure, its shape and its mass [6]. Since the oscillation frequency depends on the crystal mass, deposition of thin films on the crystal surface increases the resonator thickness and decreases the frequency in proportion to the film mass. Measurements of the crystal frequency allow the detection of the film mass, therefore the device operates like a 'microbalance'. The first quantitative investigation of the piezoelectric effect was performed by Sauerbrey [7], who derived the relationship for the change in frequency ΔF (in Hz) caused by the added mass Δm (in g) in vacuum or in air:

$$\Delta F = -\frac{2F_0^2}{A\sqrt{\mu_Q \rho_Q}} \cdot \Delta m \tag{8.1}$$

where F_0 is the fundamental resonant frequency of unloaded quartz, μ_Q is the shear modulus of AT-cut quartz ($2.947 \times 1{,}011 \text{ g cm}^{-1} \text{ s}^{-2}$), ρ_Q is the density of the quartz (2.648 g cm^{-3}) and A is the surface area in cm^2. The Sauerbrey equation assumes a uniform distribution of mass on the entire electrode portion of an AT-cut quartz crystal. Mass sensitivity decreases monotonically with the radius, in a Gaussian manner becoming negligible at and beyond the electrode boundary [8]. Another assumption of this equation is that the mass added or lost at the crystal surface does not experience any deformation during the oscillation: this is true for thin, rigid layers in vacuum or in air. For thicker, less rigid layers, as it happens for quartz crystals operating in liquid, a more complex theory is necessary. Many factors such as density, viscosity, conductivity and dielectric constant of the liquid may influence the oscillating behavior. When a quartz crystal oscillates in contact with a liquid, a shear motion on the surface generates motion in the liquid near the interface.
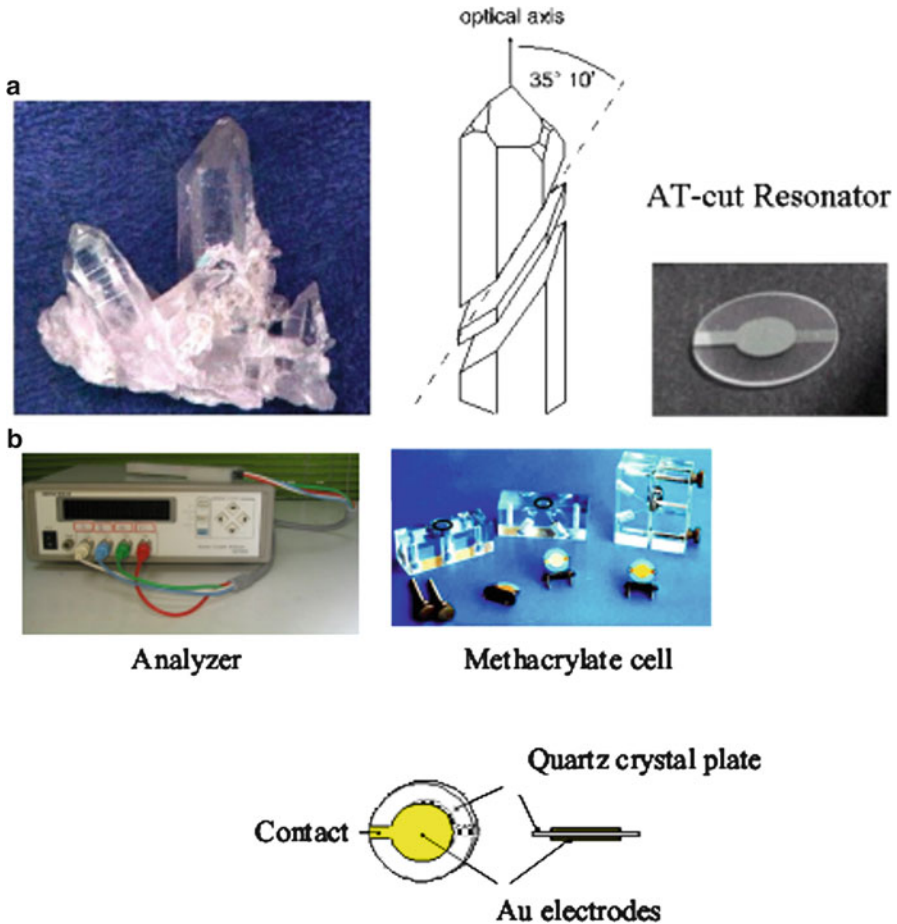
Fig. 8.1 (**a**) The cut-angle with respect to crystal orientation (so-called AT cut) determines the mode of induced mechanical vibration. AT-cut quartz crystals with a cut angle of 35° 10′ with respect to the optical z-axis perform shear displacements perpendicular to the resonator surface [Wegener website]; (**b**) instrumentation: Frequency meter with the oscillator; methacrylate cell where the crystal is housed and scheme of the sensor

The resonant frequency change of a quartz crystal having one face in contact with liquid is described by the Kanazawa and Gordon equation [9]:

$$\Delta F = -F_0^{3/2} \left(\rho_L \eta_L / \pi \mu_Q \rho_L \right)^{1/2} \tag{8.2}$$

where ρ_L is the density of the liquid and η_L is the viscosity of the liquid. Piezoelectric crystals have been used as microbalances and as a microviscometer owing to their small size, high sensitivity, simplicity of construction and operation, low cost, lightweight and the low power required [10]. The quartz-crystal microbalance has

traditionally been used in many applications such as thin film deposition control, etching studies, aerosol mass measurements and space system contamination studies. Recently however, the interest in the application of piezoelectric devices in the field of analysis has increased, since it was realized that many opportunities for molecular sensing can be opened up once a suitable recognition layer or molecule is coated on the crystal [11]. Therefore, immobilizing an affinity ligand on the surface of a quartz crystal covered with gold or silver, an affinity mass sensor is realized. When the affinity reaction with the target in solution takes place, the binding ligand can be determined at nanogram levels from the piezoelectric frequency shift. Such piezoelectric biosensors have found a wide range of applications in food [12, 13], environmental [14] and clinical [15, 16] analysis. A typical instrumentation used is displayed in Fig. 8.1b.

8.1.2 Nucleic Acid–Based Piezoelectric Sensing

Among different Affinity Based Biosensors (ABBs), nucleic acid sensing has appeared to be one of the most explored. When developing nucleic acid based approaches, a capturing sequence (probe), responsible for the system selectivity has to be immobilized on the sensing surface. The sequence, complementary to the probe added in solution, binds the surface with consequent frequency decrease, due to surface hybridization reaction and complex (dsDNA) formation. In Fig. 8.2 a typical hybridization signal is displayed. The recorded analytical signal is the frequency shift between the frequency value before sample injection, taken in buffer (base line) and its value after sample injection followed by washing with buffer to remove the sample excess and unbound material, leaving on the surface just hybridized sequences. Ideally, the frequency shift should be different from 0 if the analyte, i.e. the target sequence, is present in the sample and 0 if not. For checking the system selectivity negative control (non complementary sequence) should be also injected on the surface and the recorded should be 0. Negative control should be always used to control eventually present unspecific binding i.e. adsorption, phenomena at the surface. This is extremely important for real analytical system application.

Generally, the complementary sequence to the target analyte (a gene, a fragment or a short oligonucleotide) is first considered during the choice of probe. The probe length is generally set ranging from 15 to 20 bases, with one end usually linked to a functional group to be exploited for the immobilization chemistry. For example, biotinylated probes are used in surface functionalization involving streptavidin; thiolated probes [17], are required for direct probe coupling to gold surfaces via Self Assembled Monolayer (SAM) formation [18]. In Fig. 8.3 is reported a scheme of the relative immobilization protocols.

Some immobilization approaches are summarized in Tombelli et al. [19, 20]. Another criterion taken into account in probe selection is the C-G base content (three hydrogen bonds vs. two with A-T pairing); preferred composition varies from at least 40 to 60 % to stabilize the hybrid on the surface [21]. Finally, to facilitate
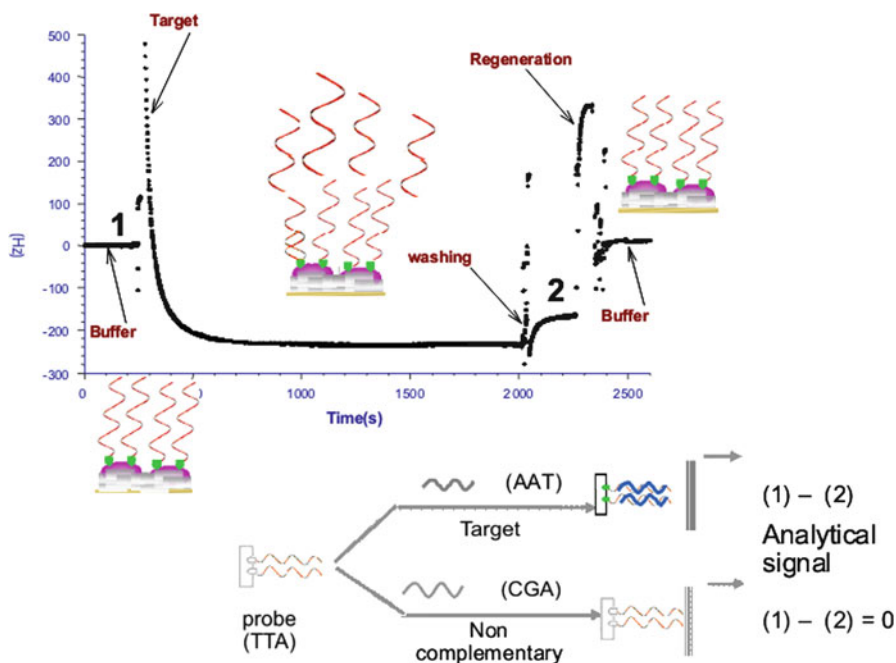
Fig. 8.2 Scheme of the signal recorded by piezolelectric sensing during a measurement cycle (see text). The hybridization reaction between the immobilized probe and the target sequence in solution is displayed

surface hybridization, it is important to avoid probe hybridization on regions that can assume conformations and may obscure the binding site of interest, i.e. by formation of secondary structures such as hairpins or loops.

Recently it has been eventually reported by our group, an optimized and reproducible strategy for probe design for nucleic acid-based sensing based on a free available software computational assisted approach. The *in silico* selection, was validated by experiments conducted using optical transduction for DNA-sensing development, in particular Surface Plasmon Resonance imaging (SPRi), demonstrating that "smart" probe design for DNA-sensing significantly improves the sensor's analytical performances for DNA-DNA hybridization measurements. This approach can be easily transferred to piezoelectric transduction [22].

Behind the probe selection, the immobilization chemistry is strategic for the success of the nucleic acid sensor development. Many immobilization chemistries, using different approaches, have been developed, depending from the sensor surface. Among the available methods, we mainly focused on thiols based approaches, using thiolated probes and dextran modified chip following the chemistry used in the gold chip of the SPR-based Biacore family instrumentation. During the sensor developments the system is first studied using synthetic oligonucleotides to evaluate the system's main analytical parameters such as sensitivity, selectivity, reproducibility expressed as coefficient of variation (CV%), detection limit (DL),
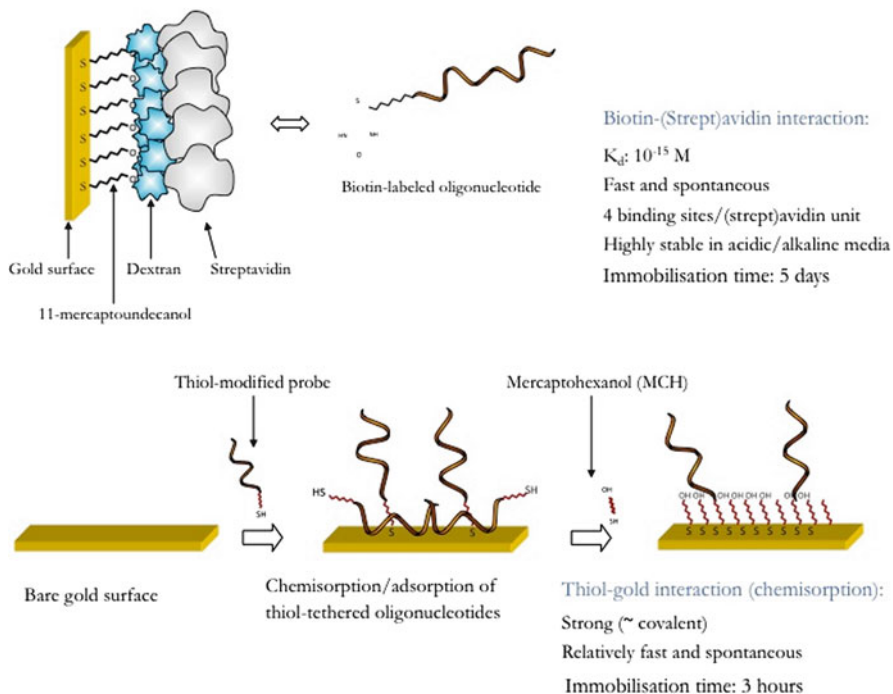
Fig. 8.3 Immobilization chemistry using (**a**) thiol/dextran/streptavidin modified surfaces for biotinylated probe binding; (**b**) direct coupling of thiolated probe with further surface passivation with short thiols

analysis time, etc. Once the assay conditions have been optimized one can move to real sample analysis. For real analysis thus matrix effects have to be evaluated and pre-analytical steps, i.e. sample-pre-treatment, should also be considered. In nucleic acid sensing, the sample pre-treatment is a key step. DNA can be detected in different cellular compartments (nucleic, mitochondrial, etc.) but it should be extracted from the cell. Double stranded DNA is the form in which the sample is obtained after its extraction.

8.2   Development of Piezoelectric Sensing for DNA-Based Sensitive Detection

The extracted DNA can be then purified by precipitation and different widely used protocols are available [23]. Re-suspension of DNA can be achieved in buffer or distilled water. In any case the extracted DNA is a double helix, with the target sequence hybridizing the probe immobilized on the sensor surface, hidden in the double helix. Thus it is clear that the dsDNA should be opened to allow surface hybridization of the target sequence.

8.2.1 Sample Pre-treatment for Target Sequence Analysis

8.2.1.1 DNA Fragmentation

Fragmentation of DNA is achieved using ultrasound generating random fragments or by enzymatic digestion by nucleases that breaks the helix at specific sites, different for the various nucleases, able to originate fragments eventually containing the intact target sequence. In literature related to genomic detection of sequences by DNA-based sensing using ultrasound has been successfully reported [24, 25]. Alternatively fragmentation can be achieved by restriction enzymes, proteins that recognize specific, short oligonucleotides sequences (the "restriction enzyme cutting sites") and cut DNA at those sites and nowhere else [26]. Bacteria contain over 400 such enzymes that cut over 100 different DNA sequences. The restriction enzymes cleave DNA in a very specific fashion. Type II restriction enzymes, most commonly used for DNA analysis and genetic engineering, have a unique nucleotide sequence at which they cut a DNA molecule. The recognition sequence is often a six base pair palindromic sequence (the top DNA strand from 5′ to 3′ is the same as the bottom DNA strand from 5′ to 3′), but some recognize four or even eight base pair sequence. Some sites occur frequently in DNA (e.g. every several hundred of base pairs), other much less frequently (rare-cutter; e.g., every 10,000 base pairs).

There are two kinds of restriction enzymes: restriction "endonuclease" and restriction "exonuclease". Nuclease is the general term for enzymes that catalyse the hydrolysis of nucleic acids by clearing chains of nucleotides into smaller units. The endonuclease is a nuclease that claves nucleic acid at interior bonds and so produces fragments of various size; while exonuclease is a nuclease that releases one nucleotide at a time (serially) starting from a nucleic acid termination. Other restriction enzymes can be used to further characterize a particular DNA molecule. The location of these restriction enzyme cleavage sites on the DNA molecule can be compiled to create a "restriction enzyme map". These maps are very useful for identifying and characterizing a particular DNA plasmid or region. Restriction fragment length polymorphism map (RFLP) are widely employed in clinical diagnostic for point mutation detection as well as in food analysis (see below). The selection of the enzyme to be applied in genomic DNA digestion is performed by the analysis with free available software.

8.2.1.2 Denaturation of dsDNA

In this section we will discuss about the importance of the sample pre-treatment in terms of dissociating the two strands of the DNA double helix (dsDNA) using different strategies. We strongly believe that for sensitive DNA detection, such as direct sequence analysis in unamplified genomic DNA, the dissociation step is of key importance. For this reason we spent quite a lot of time in studying and evaluating different approaches, starting from the most popular denaturing

thermal treatment up to the addition of suitable chemicals or oligonucleotides to the sample to prevent strand re-annealing. Thermal treatment is very simple and for this reason is widely employed, consisting in heating up the dsDNA to 90 °C for 5 min with further cooling and injection into the instrumentation. Many examples demonstrated the success of this procedure, which is particularly suited with amplified DNA material, i.e. by PCR. This procedure, on the contrary, ends in very low reproducible results, not applicable analytically, when target sequence sensitive detection has to be achieved, as in the case of single copy sequences per haploid genomic DNA in systems without any amplification [25]. Thermal denaturation can be eventually applied to satellite DNA, significantly (i.e. in many copies) present in haploid genome [27], as we will discuss later on in this chapter. We had this feeling already with optical detection using Biacore family instrumentation (BIAcore X™) where we demonstrated that in this system re-annealing of the injected sequence occurs with important reflection on the system analytical performances [28]. To be clearer, in the case of this instrumentation or eventually in flow mode systems, we think re-annealing of the strands can occur, preventing hybridization from the analyte (target sequence) with the immobilized probe. As consequence reduced amount of ssDNA analyte are available for hybridization. This reduced amount may became significant and critic in case of genomic DNA, where only few copies of target are available for probe hybridization. If significant target depletion by re-annealing occurs, no significant analytical signal may be registered, unless when signal amplification systems i.e. by gold nanoparticles are used [25].On the base of our initial findings with sample thermal denaturation, we then focused on dedicated sample pre-treatments optimization for improving sensitive DNA sensing performances, eventually starting with amplified DNA to go further into genomic DNA target sequence detection. We initially studied different approaches both at the PCR and post-PCR level aiming to improve sensor signals and reproducibility for further application to sensitive DNA sensing. The developed approaches have a general validity, independently of the transduction principle used. We focused our approaches in particular on two sequences dealing with detection of transgenosis markers contained in the promoter region (P35S) of the cauliflower mosaic virus (CAMV) ribosomal RNA and TNOS terminator, indicated in the official protocols as the target analyte for genetically modified organisms (GMO) detection [29]. The rational we used in these approaches was to evaluate the possibility either to isolate single stranded target DNA or to prolong the half-life of the ssDNA in the denaturation step, by preventing re-annealing. ssDNA can be obtained using magnetic particles for capturing and precipitation or by enzymatic digestion [13]. The approaches used to obtain selective extraction of the single strand, containing the target sequence, are based on the use of proper primers, modified by biotinylation or phosphorylation. In particular streptavidin modified particles were used to bound biotinylated DNA, while one phosphorylated strand was obtained in the amplified DNA, using phosphorylated primers. The modified ssDNA was selectively recognized and digested by a lambda exonuclease. Other researchers, as well as our group, have employed asymmetric PCR with optical sensing to obtain significant amount ssDNA target in solution as with asymmetric

amplification (i.e. SPR, Biacore family instrumentation), by suitable ration of reverse and forward primers, an excess of ssDNA containing the target sequence can be synthetized, preventing re-annealing with the other strand of the dsDNA, now present in much lower amount [30–32]. We will not further comment these just mentioned approaches because they can be applied only to amplified DNA samples, since primers have to be employed. However the full understanding of encountered problems and the developed solutions, stresses the importance of the denaturing step in improving DNA-based sensing performances. Thus further strategies have to be introduced for sensitive sequence detection in genomic DNA. To this aim, behind this PCR-based mentioned approaches, we evaluated other denaturation methods assisted by the use of chemical reagents added to the DNA sample. In particular denaturation treatments can be performed by the combination of a strong alkaline environment and a formamide treatment at 42 °C. Denaturation of dsDNA in strong alkaline conditions is well established. At a pH 13 the charge of the DNA bases changes, thus preventing H-bond formation [33]. On the other hand, the influence of organic compounds such as formamide, urea and formaldehyde on the thermal DNA denaturation process has been well documented [23]. Formamide is a helix destabiliser that replaces the native DNA bases for inter-strand hydrogen bonds, thus inducing the denaturation of dsDNA [34]. Results were obtained again with amplified and genomic DNA [32], using different concentration of chemical, but still the applied treatment was found to be insufficient to generate reproducible and significant SPR sensor signals. These treatments however were eventually applied to piezoelectric DNA to evaluate the ability of sensitive genomic DNA detection [35]. Finally an innovative approach was developed, based on the use of small selective oligonucleotides added to the denaturing mixture, for hybridizing, at a selected annealing temperature, the two ssDNA of the helix. The oligos attachment site can be designed and should not overlap the ssDNA region involved in the surface probe binding. The hybridization of these small oligos prevents re-annealing of the two strands, leaving the target sequences to hybridize the immobilized probe, thus prolongating the single stranded state of the target sequence. For its re-annealing blocking behaviour, the approach was named "denaturation by blocking oligos".

In Fig. 8.4 is displayed a scheme of the developed approach and the differences with the conventional thermal denaturation. Initially Biacore X™ instrumentation was used [36] in the protocol optimization as proof of principle. Once demonstrated the suitability of the "blocking oligonucleotides approach" for improving sensitive DNA detection of three different sequences in genomic DNA, the latter developed denaturing approaches based on the addition of formamide at different percentages or of blocking oligos were applied to sensitive detection of target DNA present in single or few copies per haploid DNA by piezoelectric sensing [37].

We will here report about the finding obtained initially by our group and more recently appeared in literature relative to sequence detection in unamplified genomic DNA. We will first discuss the detection of specific micro satellite DNA detection with application to food analysis and further detection of sequences present in few copies per haploid genome, identified as marker of transgenosis.
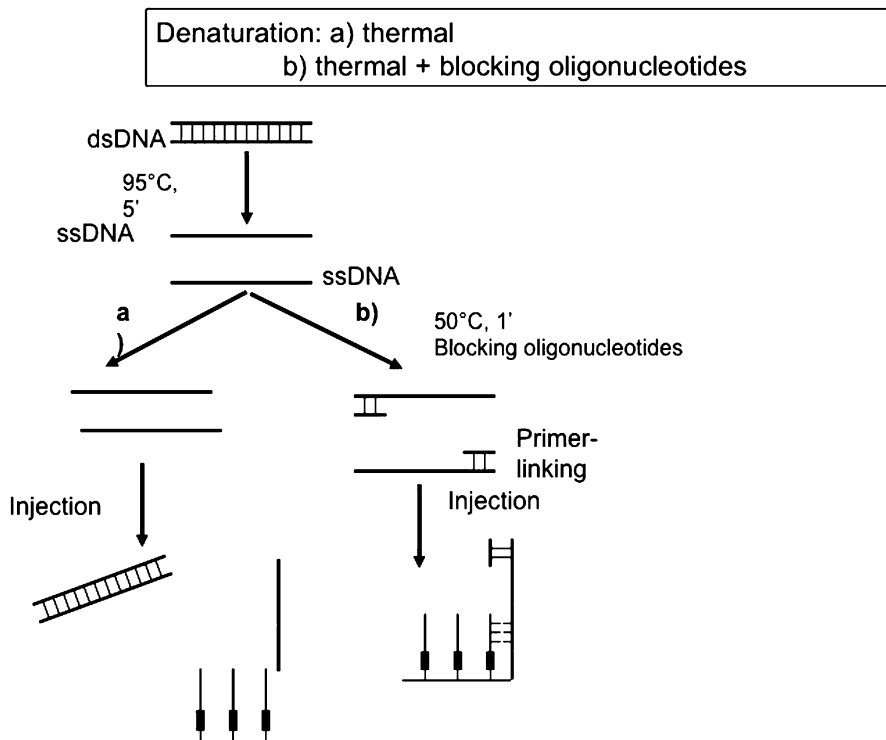
Fig. 8.4 Denaturation of dsDNA (**a**) thermal denaturation; (**b**) thermal with blocking oligonu-
cleotides

8.3   Detection of Microsatellite Sequences in Bovine
Genomic DNA

First the detection of microsatellite sequences was achieved to demonstrate the
ability of piezoelectric sensing to operate directly in genomic DNA. To approach
the problem of direct sequence detection in unamplified genomic DNA, we first
focused on target sequences highly repeated in genomes, which are species specific
and, in some cases, could represent a significant part of the genome. In particular,
detection of the highly repeated sequence called satellite 13, present in *Bos taurus*
genome was achieved in genomic DNA, for the first time by piezoelectric sensing.
In the bovine genome, eight highly repetitive and several minor repetitive sequences
have been detected comprising a total of 27% of the DNA [38]. The target
sequence chosen in this case is situated in highly repeated DNA and is therefore
present in the genome in a high number of copies, increasing the amount of target
sequence available for the hybridization with the probe [39] immobilized on the
sensing element. The developed quartz crystal microbalance-based sensor has been
applied to the identification of animal species in meat samples, which represents

Table 8.1 Oligonucleotide sequences of immobilized probe, modified with biotin or SH in 5′ end, complementary and non complementary sequences (negative control) used in the optimization assays and blocking oligonucleotide used in the denaturation protocol

Biotinylated and thiolated probe:	Biotin or SH-5′ TCACGCAGCTCAGCAGGCCCT 3′
Complementary target:	5′ AGGGCCTGCTGAGCTGCGTGA 3′
Non-complementary sequence:	5′ GGCAGAGGCATCTTCAACGATGGCC 3′
Blocking oligonucleotide 1:	5′ GTCTGCTCATCTGCTTGACAATTTC 3′
Blocking oligonucleotide 2:	5′ TAATCAAGTAGATGAGCAGGCAG 3

a relevant problem in food analysis for economical, religious or public health concerning reasons. Numerous analytical methods, currently available for species differentiation, rely on protein analysis, such as electrophoresis techniques [40, 41], liquid chromatography [42, 43], and immunoassays [44].

Modern methods for meat identification are instead based on DNA analysis. They allow species-specific DNA sequences identification, which has some advantages over protein analysis [45]. Molecular biology methods allow the determination of DNA also in heat-treated nourishment and are, therefore, suitable for the identification of species-specific DNA in meat and bone meal and concentrate mixtures [46]. Furthermore, they allow the discrimination between related species, such as sheep and goat or chicken and turkey [47].

Earlier DNA sequence analysis was performed using genomic DNA as species-specific probe, which was hybridized to DNA extracted from meat samples [48–51]. Later, probes derived from highly repetitive (satellite) DNA sequences were developed [52].

The analysis of restriction fragment length polymorphism (RFLP) of PCR fragments is an alternative DNA detection system and it has already been successfully applied to species differentiation [53–56]. PCR-RFLP allows the amplification of a conserved region of DNA sequence using PCR, and the detection of the genetic variation between species by digestion of the amplified fragment with restriction enzymes. This technique was used for speciation by exploiting DNA sequence variations within the mitochondrial D-loop region [57], cytochromeB (cytB) gene [52] and satellite DNA sequences [58].

More recently, real-time PCR for meat species identification has also been reported [59].

DNA-based piezoelectric sensing was tested as alternative method in the identification of species-specific DNA sequences for meat analysis, based on determination of a highly repetitive and species-specific DNA sequence present in bovine (*B. taurus*) genomic (non-amplified). The sensor was developed by immobilizing the specific probe on the surface using thiol chemistry with thiolated probes, and surface saturation with short thiols, following the protocol previously developed [27]. Behind thiolated probes, dextran modified surfaces using biotinylated probes were also used. The base sequences of the 5-thiol functionalized probe (21-mer), complementary (21-mer) and non-complementary (25-mer) oligonucleotides and blocking oligonucleotides (25-mer and 23-mer) are reported in Table 8.1. The analytical parameters of the sensor are evaluated first by synthetic oligonucleotides,
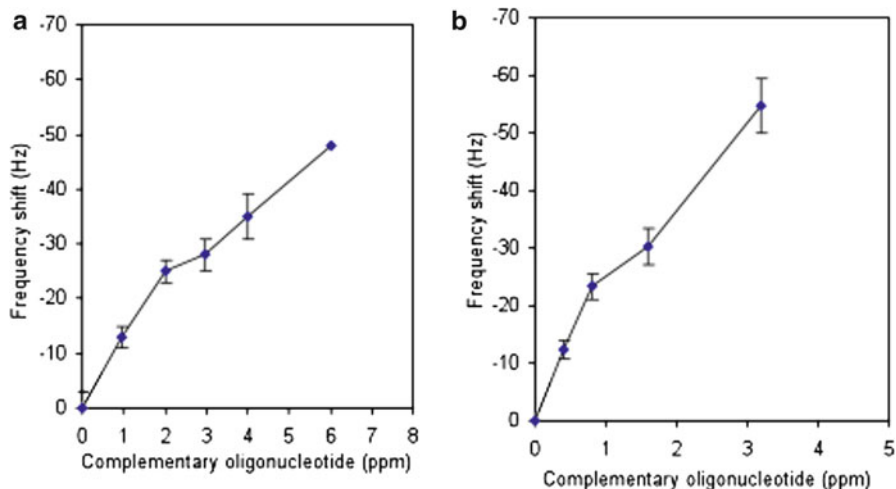
Fig. 8.5 (**a**) Biotinylated oligonucleotide probe. Hybridisation with synthetic oligonucleotide target. (**b**) Thiolated oligonucleotide probe. Hybridisation with synthetic oligonucleotide target

complementary and non-complementary to the 21-mer immobilized probe; once assessed good analytical behavior in terms of selectivity, sensitivity and repro-ducibility, the system is then applied to real samples.

8.3.1 Sensor Optimization: Biotinylated and Thiolated Probes

A synthetic 21-mer biotinylated and thiolated probes, complementary to a sequence, which is present inside the *Bos taurus* satellite n.13 (247 bp), were used. The selectivity of the systems was tested with the non-complementary sequence. Comparison between the two immobilization chemistries was achieved to select the best performing sensor with standard solutions for further application to sequence detection in genomic, unamplified DNA.

The hybridization between the probe and 21-mer complementary oligonucleotide solutions were tested and the results are shown in Fig. 8.5. In case of sensor modified with biotinylated probe, the CV% has been calculated for all the concentrations and the average is 10 %. For the thiolated probe, the sensor performances are similar to the biotinylated one. The average CV% calculated is 10 % for both. The selectivity of the crystals surfaces just towards the target was confirmed by the absence of frequency shift with non-complementary sequence 1.8 ppm in both modified surfaces. Both sensor were regenerable. The surface was regenerated by treatment with HCl (10 mM, 30 s), which allowed to perform up to 20 hybridization cycles on a single surface, without affecting the sensor surface.
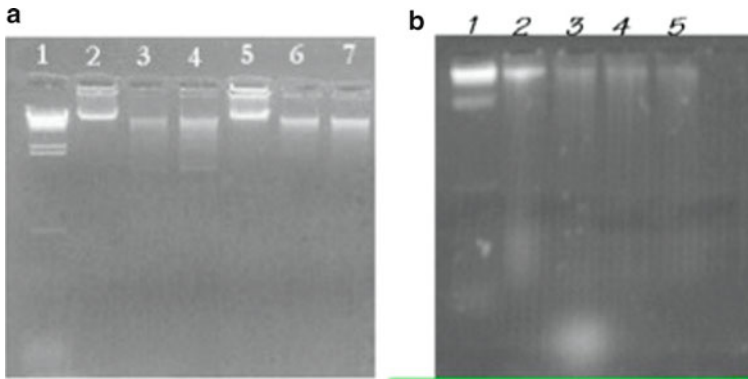
Fig. 8.6 Electrophoretical analysis of digestion reaction. (**a**) Commercially available genomic DNA (Novagen). Lanes: *1* marker l-DNA-HindIII, *2* genomic bovine DNA, *3–4* digested genomic bovine DNA, *5* genomic porcine DNA, *6–7* digested genomic porcine DNA. (**b**) Real sample genomic DNA. Lanes: *1* marker l-DNA-HindIII, *2* genomic bovine DNA, *3-4-5* digested genomic bovine DNA

In this case of *Bos taurus* micro satellite detection was achieved first in Genomic bovine (*B. taurus*); Porcine DNA (*Sus scrofa*) was used as negative control. DNA commercially available and finally the sensor were applied to real samples consisting in genomic bovine DNA extracted from animal muscle.

8.3.2   *Sample Pre-treatment*

8.3.2.1   DNA Fragmentation

To allow detection of target sequence in genomic DNA, fragmentation of the sample is necessary, achieved by enzymatic digestion. Enzymatic digestion was applied here to keep the target sequence in one piece, although this procedure is much longer then fragmentation by ultrasounds. The genomic DNA was digested using the restriction enzyme EcoRI (overnight), to obtain DNA fragments of around 400–500 bp containing the target sequence (available on GenBank accession number no. V00122).

It was verified that the consensus sequence recognized by the enzyme was not present inside the *B. taurus* satellite no. 13 (247 bp) to maintain integer the target sequence thus allowing the maximum amount of target sequence in solution hybridizing the probe. Checking the restriction site of the enzyme respect the target sequence is important to ensure that the fragmentation does not affect the ability of the target sequence to hybridize to the immobilized probe. The enzymatic fragmentation can be then confirmed by electrophoretical analysis, on agarose gel (1 % in TAE electrophoresis buffer) (Fig. 8.6).
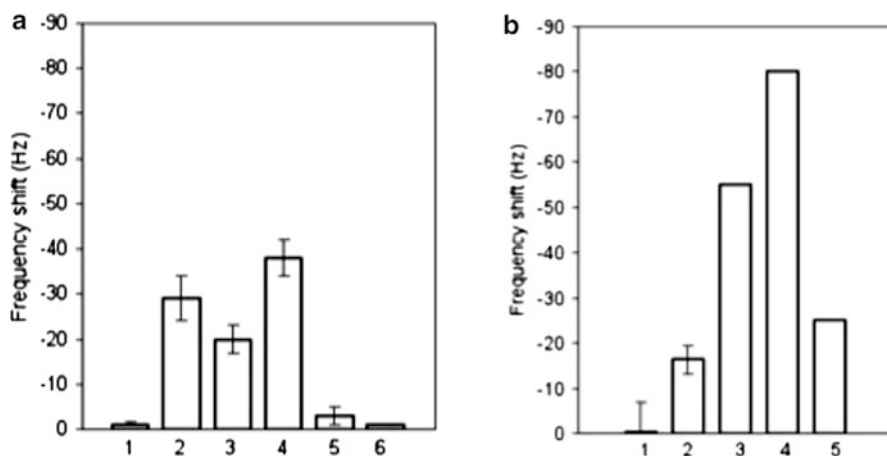
Fig. 8.7 Genomic bovine DNA: thermal denaturation. (**a**) Biotinylated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 2 ppm (n = 3), *3* Bovine genomic DNA 10 ppm (n = 3), *4* Bovine genomic DNA 20 ppm (n = 3), *5* Porcine genomic DNA 10 ppm (n = 3), *6* Blank solution (n = 1). (**b**) Thiolated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 1 ppm (n = 3), *3* Bovine genomic DNA 25 ppm (n = 1), *4* Bovine genomic DNA 10 ppm (n = 1), *5* Porcine genomic DNA 10 ppm (n = 1)

8.3.2.2 DNA Denaturation: Thermal Versus Thermal and Blocking Oligos

The genomic fragmented DNA, after precipitation in ethanol and re-suspension, underwent denaturation. Both simple thermal and thermal with blocking oligos were applied to the samples and results compared. In the case of thermal blocking oligo, after the addition of the two oligonucleotides (1 μM), the sample was incubated at 95 °C for 5 min and then 1 min at 50 °C. This second temperature is the appropriate temperature for the annealing of the oligonucleotides to the complementary DNA sequences. The choice of the oligonucleotides depends on the length of the digested fragment and on the position of the target inside the fragment.

8.3.3 Analysis of Genomic Bovine DNA

8.3.3.1 Thermal Denaturation: Biotinylated Versus Thiolated Probe

Hybridization signals obtained after the interaction between both biotinylated and thiolated probes, and the commercially available genomic DNA samples previously treated with the restriction enzyme (EcoRI) and thermally denatured are next discussed.

Figure 8.7 shows the signals relative to the hybridisation of the probes with: non-complementary and complementary oligonucleotide; bovine genomic DNA; porcine

genomic DNA as negative control and blank solution, obtained by applying the digestion treatment and the thermal denaturation to a solution containing all the reagents but DNA [60].

Both sensors are capable to distinguish between the complementary sequence (bovine DNA) and non-complementary solutions (porcine DNA and blank solution). Hybridisation with a double concentration of bovine DNA reports a frequency shift nearly double. In the case of biotinylated surface, the regeneration was fully accomplished with HCl 1 mM.

On the contrary, in the case of thiolated probes, although it was possible to recognize the bovine from the porcine DNA, maybe there is aspecific adsorption that causes a significant signal of the non-complementary sequence. Furthermore, because of this adsorption, the regeneration solution (HCl 1 mM) optimized for oligonucleotides was not sufficient to regenerate the sensor surface and so improved regeneration approach, has to be tested. A different regeneration treatment was also employed. This treatment consists of a first addition of alkaline solution (15 s with NaOH 100 mM), which dissociated the two strands, followed by 30 s with regeneration solution (200 mmol/l Tris·Cl, pH 7, 0.1×SSC, 0.1 % (w/v) SDS). The alkaline pH separates the double strand DNA and then the surfactant, contained in the regeneration solution, removes the target sequence. Several surface washing steps with hybridization buffer were necessary to remove all the surfactant in the regeneration solution.

The surface performances were controlled, using standard solutions of synthetic oligonucleotides, before and after the hybridisation-regeneration cycles. However, after many regeneration cycles, only a partial surface regeneration was achieved, consequently the surface activity results lower. In the case of thiolated probe results were not as satisfying as the ones obtained with the biotinylated one, based on dextran-streptavidin-biotin protocol.

8.3.3.2 Thermal with "Blocking Oligos" Denaturation: Biotinylated Versus Thiolated Probe

The frequency shifts in Fig. 8.8 were obtained after the interaction between the probe and commercially available genomic DNA samples previously treated with the restriction enzyme (EcoRI) and then thermally denatured with blocking oligonucleotides.

Hybridization signals relative to the hybridisation of the probe with: non-complementary and complementary oligonucleotide; bovine genomic DNA; porcine genomic DNA as negative control and blank solution, are shown in Fig. 8.8.

The results are similar to the ones found with thermal denaturation with biotinylated probe (Sect. 8.3.3.1) with regard to the values of frequency shifts and the easiness of regeneration with HCl.

Considering that the results obtained with thermal denaturation (Sect. 8.3.3.1, thiolated probe) do not meet the requirements for analytical applications, the
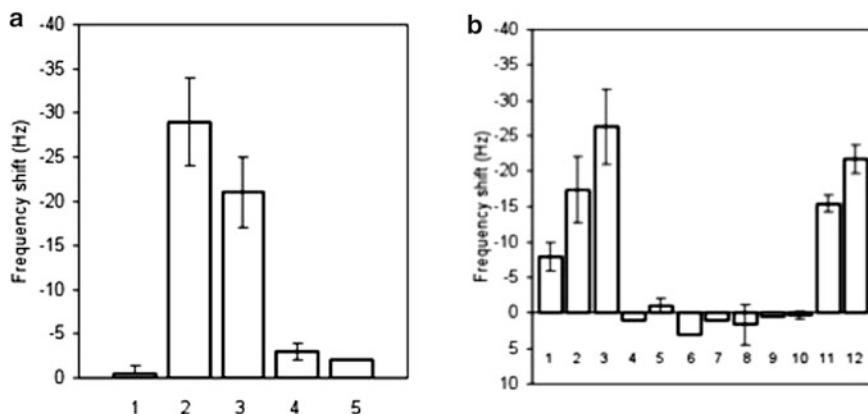
Fig. 8.8 Genomic bovine DNA: thermal with blocking oligonucleotides denaturation. (**a**) Biotinylated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 2 ppm (n = 3), *3* Bovine genomic DNA 10 ppm (n = 3), *4* Porcine genomic DNA 10 ppm (n = 3), *5* Blank solution (n = 1). (**b**) Thiolated oligonucleotide probe. Signals: *1* Bovine genomic DNA 5 ppm (n = 4), *2* Bovine genomic DNA 10 ppm (n = 6), *3* Bovine genomic DNA 20 ppm (n = 4), *4* Porcine genomic DNA 5 ppm (n = 1), *5* Porcine genomic DNA 10 ppm (n = 3), *6* Porcine genomic DNA 20 ppm (n = 1), *7* Blank solution for 5 ppm (n = 2), *8* Blank solution for 10 ppm (n = 3), *9* Blank solution for 20 ppm (n = 2), *10* Non-complementary oligonucleotide 8 ppm (n = 3), *11* Complementary oligonucleotide 1 ppm (n = 3), *12* Complementary oligonucleotide 2 ppm (n = 3)

denaturation treatment based on the use of "blocking oligo" was combined to the improved regeneration procedure.

Figure 8.8b shows the frequency shifts after the hybridisation reaction between the probe and: non-complementary and complementary oligonucleotides; bovine genomic DNA, porcine genomic DNA and different volumes of blank solution.

The sensor was able to distinguish between complementary and non-complementary sequences both in synthetic oligonucleotidic samples and in digested non-amplified genomic DNA, both with biotinylated and eventually with thiolated probes immobilized on the sensor surfaces.

8.3.3.3   Analysis of Real Genomic Bovine DNA Samples, Extracted from Muscle by "Blocking Oligos" Denaturation; Biotinylated vs Thiolated Probes

Real samples were analysed, with biotinylated probe and with thiolated probe. Both samples were bovine genomic DNA extracted from animal muscle; and they were denatured with the thermal and blocking oligonucleotide treatment since it yielded the best results with both modified surfaces.
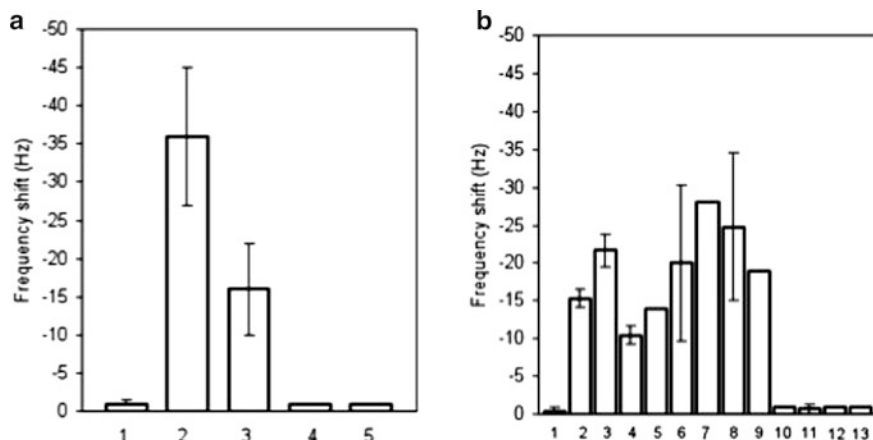
Fig. 8.9 Real genomic bovine DNA sample, extracted from muscle (thermal denaturation + blocking oligonucleotides). (**a**) Biotinylated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 2 ppm (n = 3), *3* Real sample (bovine genomic DNA) 10 ppm (n = 3), *4* Porcine genomic DNA 10 ppm (n = 1), *5* Blank solution (n = 1). (**b**) Thiolated oligonucleotide probe. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 1 ppm (n = 3), *3* Complementary oligonucleotide 2 ppm (n = 3), *4* Real sample (bovine genomic DNA) 5 ppm, 20 min (n = 4), *5* Real sample (bovine genomic DNA) 5 ppm, 35 min (n = 1), *6* Real sample (bovine genomic DNA) 10 ppm, 20 min (n = 5), *7* Real sample (bovine genomic DNA) 10 ppm, 35 min (n = 1), *8* Real sample (bovine genomic DNA) 20 ppm, 20 min (n = 5), *9* Real sample (bovine genomic DNA) 20 ppm, 35 min (n = 1), *10* Porcine genomic 5 ppm, 20 min (n = 2), *11* Porcine genomic DNA 10 ppm, 20 min (n = 1), *12* Porcine genomic DNA 10 ppm, 35 min (n = 1), *13* Blank solution (n = 2)

Figure 8.9 shows the signals relative to the hybridisation of the probe with: non-complementary and complementary oligonucleotide; the real sample (bovine genomic DNA); porcine genomic DNA as the negative control and blank solution.

The frequency shifts obtained with biotinylated probe (Fig. 8.10a) with the real sample are well comparable with the ones found with commercially available DNA (Sect. 8.3.3.1), with regard to the values of frequency shifts and the easiness of regeneration with HCl.

Figure 8.9b shows the signals relative to the hybridisation of the probe with: non-complementary oligonucleotide, complementary oligonucleotide, real sample (bovine genomic DNA); porcine genomic DNA as negative control and blank solution.

As in the previous case the frequency shifts are comparable with the ones found with commercially available DNA. The signals 4, 6 and 8 have been recorded employing 20 min as contact time; while the 5, 7 and 9 are relative to 35 min contact time. There are no significant differences between the two data series. This is in contrast with the results obtained with the commercially available bovine DNA (see signals 4 and 5 in Fig. 8.9b). A reason of this can rely on the degradation of the
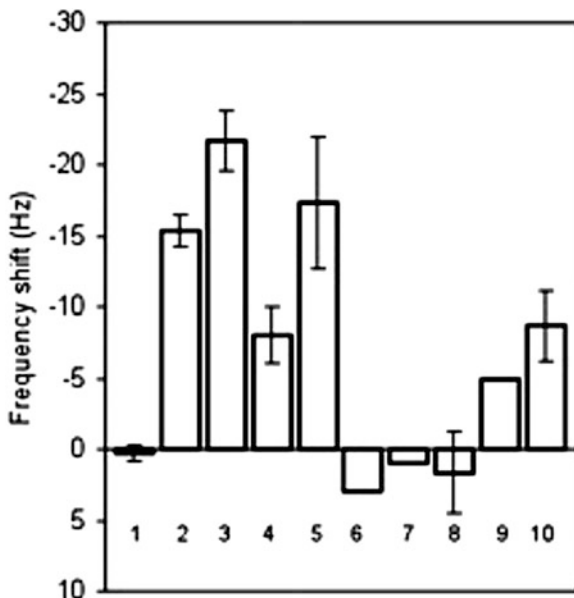
Fig. 8.10 Thiolated oligonucleotide probe. Porcine 20 ppm. Signals: *1* Non-complementary oligonucleotide 8 ppm (n = 3), *2* Complementary oligonucleotide 1 ppm (n = 3), *3* Complementary oligonucleotide 2 ppm (n = 3), *4* Bovine genomic DNA 5 ppm, 20 min (n = 4), *5* Bovine genomic DNA 10 ppm, 20 min (n = 6), *6* Porcine genomic DNA 20 ppm, 20 min (n = 1), *7* Blank solution for 5 ppm, 20 min (n = 2), *8* Blank solution for 10 ppm, 20 min (n = 3), *9* Porcine 20 ppm + Bovine 5 ppm genomic DNA, 20 min (n = 1), *10* Porcine 20 ppm + Bovine 10 ppm genomic DNA, 20 min (n = 3)

real sample, observed in the electrophoresis analysis, that causes a smaller number of non damaged copies. The aspecific adsorbtion was absent, as confirmed by the signals 10, 11, 12 and 13 relative to porcine genomic DNA samples.

8.3.3.4   Analysis of Mixture of Bovine and Porcine Genomic DNA Samples, by "Blocking Oligos" Denaturation

In order to investigate the applicability of this sensor to mixtures composed by DNA of different species, some mixtures with bovine and porcine DNA in different proportion have been prepared and analysed. The bovine and porcine DNA have been first separately digested by restriction enzymes, then mixed together and denatured with the new thermal and blocking oligonucleotides procedure. Considering that both modified surfaces showed similar behaviour, the experiments were performed employing the thiolated probe because of its easiest and quickest preparation.

In Fig. 8.10 results obtained with genomic DNA mixtures, previously digested with the restriction enzyme (EcoRI), and denatured are shown. The samples were prepared adding various bovine DNA concentrations to a fixed porcine DNA concentration.

Comparing the signals of the bovine DNA samples and the ones of the mixtures of bovine and porcine DNA it is evident that the signals obtained with mixtures are specific and comparable to the ones observed with the solution containing just the bovine DNA. No matrix effect has been found, confirming the applicability of this system to sequence detection even in mixed samples, containing DNA from different species. More in detail, the comparison must be made between the signals 13 and 6, 11 and 4, 12 and 5. The contact time for the hybridization was increased from 20 to 35 min, in order to obtain a higher signal from the samples because of their complexity. In fact, the presence, in solution, of many non-complementary sequences hinders the target sequence that can't reach easily the surface. The employment of a longer contact time allowed an increase in the frequency shift while the aspecific adsorbtion did not rise. It is confirmed by the signal 8, obtained with 10 ppm of porcine DNA and a contact time of 35 min. From the comparison of the two denaturing methods we can conclude that although with simple thermal denaturation specific signal is recorded, unspecific bindings occurs and regeneration of the surface is not achieved by simple 1 mM HCl treatment. The sample pretreatment, in term of denaturation step, was here strategic for the sensor re-use and reproducibility. This already in case of highly frequent sequences. We will see how the same step is crucial in case of single of few sequences per haploid genome directly in genomic DNA. In conclusion, the sensor is able to distinguish between complementary and non-complementary sequences both in synthetic oligonucleotide samples and in digested genomic DNA. It is also able to recognize the presence of bovine DNA extracted from muscle (real sample) and also in mixed samples. The only required sample pre-treatment is the enzymatic digestion to fragment the DNA. The system is specific, sensitive, and reusable, with fast analysis time. On the base of our findings, the sensor could represent an alternative method, to the traditional biomolecular techniques, for the identification of species-specific DNA sequences, directly in enzymatically digested DNA, bypassing the PCR step.

8.4 Detection of Target Sequences Present in Single Copy Per Haploid Genome in Plant Genomic DNA

Once assessed the ability of the sensor to detect highly present sequences per haploid DNA we moved further to the detection of target DNA present in single or in few copies in haploid genomes. For this reason we focused on markers of transgenosis sequences, in particular we developed a system for detecting the P35 promoter in Genetically Modified Organisms (GMOs). Here we used a genetically

modified (GM) tobacco plant, *Nicotiana glauca*, an ornamental plant, transformed with pBI121, which contained a gene cassette carrying the P35 form cauliflower virus. A gene cassette consists of a promoter (P), a coding region and a terminator (T). Officially established method on European scale for GMOs control are based on the search of Promoter region (P35S) of the CAMV (cauliflower mosaic virus) ribosomal RNA and on NOS terminator (TNOS) of the nopalin synthase gene from the soil bacterium *Agrobacterium Tumefaciens* [29]. This promoter is used in approved GM soya and maize. Analytical methods for GMOs are based on real-time PCR (required for labelling) or end-point PCR [61]. Our group has been quite active in the development of GMOs DNA-based sensor using different transduction principles with application to GMO Reference Certified Material or real-samples i.e. optical (SPR), piezoelectric and electrochemical. We thus selected the P35S as target analyte for the study of the piezoelectric based detection of genomic DNA. The GM plant *Nicotiana glauca* (GR4), was not commercially available, but was transformed at Laboratorio di Genetica, Università di Firenze, Italy. The not transformed plant (wild type) was taken as negative control in all the experiments.

Similarly to the bovine DNA, extraction of the plant DNA (from leaves) was achieved using available protocols [23].

8.4.1 Sample Pre-treatment

8.4.1.1 DNA Fragmentation

Fragmentation was conducted by enzymatic digestion with DNA digestion enzymes Bam HI e Hind III, which ended into a 872 bp fragment containing the P35S target sequence.

The DNA immobilized probe mapped within this fragment. The enzyme selection was achieved by free available software (DNA club). Purification of fragmented DNA by precipitation in ethanol and re-suspension is necessary prior testing on the sensor.

8.4.1.2 DNA Denaturation

Here three different denaturation protocols were tested and compared. In particular we compared chemical denaturation with formamide used in our previous work with optical sensing (SPR) [32] and blocking oligos" denaturation.

The sensor surface was modified by thiol/dextran chemistry and biotinylated probes used for the immobilization via streptavidin anchoring [35].

The oligonucleotides used are reported in Table 8.2.

The P35S sensor has been already optimized and tested on PCR amplified DNA in different real matrices such as soft drinks, crackers, as well as in PCR amplified DNA from *Nicotiana glauca*, thus we will directly report the results on

Table 8.2 Olignucleotide sequences of immobilized probe, modified with biotin in 5′ end, complementary and non complementary sequences (negative control) used in the optimization assays and blocking oligonucleotide used in the denaturation protocol

Biotinylated probe (35S, 25 mer):	5′ Biotin-GGCCATCGTTGAAGA TGC CTC TGC C-3′
Complementary target (35S, 25 mer):	5′-GGC AGA GGC ATC TTC AAC GAT GGC C-3′
Non complementary (Tnos, 25 mer):	5′-GAT TAG AGT CCC GCA ATT AAT CAT T-3′
Blocking oligonucleotide 1 (35S1, 21 mer):	5′-GCT CCT ACA AAT GCC ATC ATT-3′
Blocking oligonucleotide 2 (35S2, 18 mer):	5′-CTC CAA ATG AAA TGA ACT-3′

the optimized system, using this time genomic DNA samples extracted from GM plant, fragmented by enzymatic reaction, and denatured differently to compare, also in this case, the different sample pre-treatments with the aim to stress the key importance of denaturation steps. In parallel, as control oligonucleotides and PCR sample undergoing to same treatments were used as controls, to study any possible interference with the sensor.

8.4.1.3 Analysis of PCR DNA Samples, by Chemical and "Blocking Oligos" Denaturation

Three approaches were tested: thermal (95 °C for 5 min, cooling in ice for 1 min), chemical (20 % formamide, 0.3 M NaOH at 42 °C for 30 min), and thermal combined with blocking oligonucleotides.

The complementary 35S oligonucleotide (25-mer, 1 ppm), a non complementary oligonucleotide (25-mer, 8 ppm), the 35S PCR fragment (243 bp), a PCR negative control (180 bp), and PCR blanks were analyzed after the three denaturation procedures (Fig. 8.11a, b).

The effect of the chemical denaturation on oligonucleotides resulted in an increase (46 %) of the signal if compared with the one obtained with the thermal treatment. This increase could be due to a non specific effect since it is also observed with the non complementary oligonucleotide. On the contrary, the thermal and blocking oligonucleotides' treatment did not affect the hybridization signal. The same findings were obtained with the same treatments applied to PCR fragments and PCR negative controls.

The same hybridization signal was obtained after treating the 35S samples with the three denaturation methods, but the reproducibility (expressed as coefficient of variation, CV%) (n = 3) was quite different (9 % for the thermal one, 12 % for the thermal and blocking oligonucleotides one, and 25 % for the chemical one). The same denaturation treatments applied to the negative controls gave less homogeneous results since a high signal was observed in the case of the chemical treatment, while negligible results were found in the cases of thermal plus blocking and thermal treatments. The sensor behaviour is shown in Fig. 8.11a.
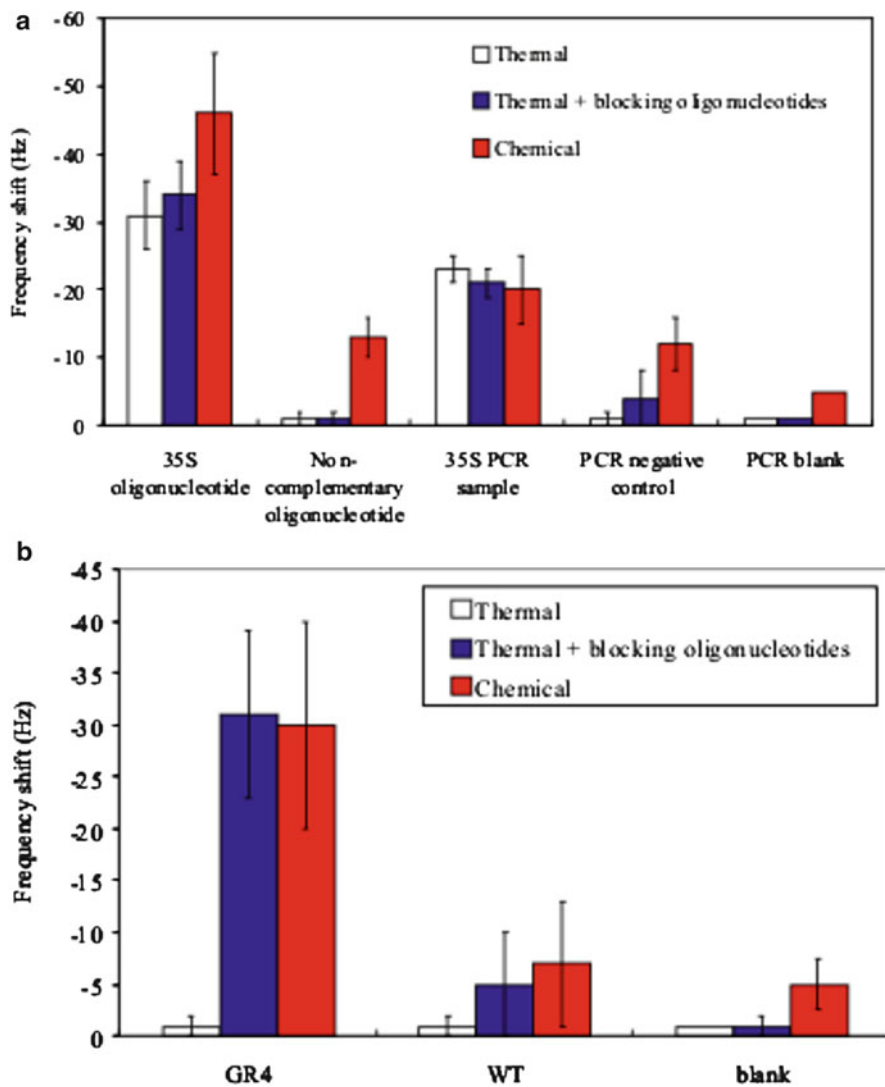
Fig. 8.11 (**a**) Effect of denaturation treatment on oligo and on PCR samples. Samples analyzed: oligonucleotide complementary p35S, oligonucleotide non complementary, fragment PCR 35S (54 ppm), PCR negative control, (35 ppm) and blank (n = 3); (**b**) Analysis of target P35S sequence in fragmented genomic transgenic DNA (10 ppm) GR4 (n = 6), non transgenic WT DNA (n = 6) and blank (n = 3). DNA denatured by three approaches (thermal, thermal with blocking oligonucleotides, and chemical). (Reproduced with permission from Minunni et al. [35]. Copyright (2005) American Chemical Society)

After the initial evaluation of the sample pre-treatments on the sensor behavior, these were finally applied to more complex, unamplified genomic DNA.

8.4.1.4   Analysis of Plant Genomic DNA Samples, by Chemical and "Blocking Oligos" Denaturation

Genomic DNA, previously fragmented by enzymatic digestion by restriction enzymes (BamH), previously checked to leave intact the target sequence. The length of the fragments containing the target sequence, complementary to the immobilized probe, was 872 bp. All of the denaturation procedures were applied to 10 ppm of sample. The results are reported in Fig. 8.11b.

The thermal treatment did not results in a measurable signal. On the contrary the thermal plus blocking oligonucleotides and chemical denaturations allow significant hybridization of the samples. Furthermore, thermal plus oligo oligonucleotide denaturation, results in a better reproducibility then the one obtained by the chemical one. Also the treatment may influence the sensor lifetime, since little regeneration was achieved with chemical denaturation, while with thermal with blocking the surface could be regenerated more then ten times, before losing sensitivity and specificity, thus more then doubling the sensor life-time.

This results, stresses again the importance of selecting the best sample-denaturation.

The piezoelectric sensor detection was very selective, the target sequence was detected in the transgenic sample (GR4) and the signal were significantly different from the negative control (wild type, WT), except when the thermal treatment alone was applied. Moreover, the best results were found when the thermal plus blocking oligonucleotides' treatment was applied, as demonstrated by an evaluation of the reproducibility and the lower nonspecific effect when testing the negative controls, as confirmed by the findings observed with ssDNA and PCR-amplified DNA. This is in line with our previous finding with microsatellite DNA detection in bovine genomic DNA.

This work demonstrated that it is possible to detect the target sequence directly in unamplified genomic DNA, even considering the low concentration of the target in the sample (4×10^5 copies in 10 ppm of sample). PCR-amplified DNA (243 bp) represents an enriched sample where the target sequence is present in a very high number of copies (4×10^{11} copies). To explain the detection of such a low number of copies of target DNA, additional contributions (i.e., viscoelastic effects) to the biosensor signal other than mass loading may be taken into account. Moreover, it must be considered that the signals due to oligonucleotides, to the PCR samples, and to the genomic digested DNA on the surface cannot be compared due to the very different matrix complexity of all these samples and to the different secondary structure once the DNA is hybridized to the probe.

The real-time and label-free DNA sequence detection in non amplified DNA, as reported here, represents an important improvement in DNA analysis. Since the specificity of the system relies on the probe immobilized on the surface, the

applicability of direct genomic sensing is wide, from environmental to food and clinical analysis. Recently some work on sensitive DNA in genomic DNA has been reported by some authors and will be here summarized and discussed.

8.5   State of Art

Recently some groups have reported about sensitive DNA detection by piezoelectric sensing. In Table 8.3, are summarized the approaches. Crystals with different fundamental frequencies, from 5 up to 12 MHz, are used. Different frequency means also different sensitivities as can be deduced by Sauerbrey model, (Eq. 8.1) [7]. Also different operating temperatures (from room temperature up to 55 °C) and flow modes are used, instead, except the case of "Dip and Dry" approach, were the crystal is exposed to the target solution, followed by rinsing to remove the unbound material and then dried. The frequency shift is taken before and after target exposure, when the crystal is dried.

Gene detection directly in genomic DNA is achieved in microorganisms, i.e., *Mycobacterium tuberculosis,* is detected in human specimens (sputum), and the approach used followed our first report, in other words the sample is digested by enzymatic reaction and sample denaturation is achieved by thermal with blocking oligonucleotides [62]. Signal requires amplification and the detection approach is by "dip and dry" method, not directly in liquid. This procedure may be cumbersome and may have important reflections on system reproducibility, at least to our experience The QCM, for its sensitivity to humidity is also used as hygrometer, and in the DNA measurement this effect may be present and difficult to be controlled. However the application reported encourages further development for real clinical application of these devices.

Hepatitis B virus (HBV) genomic DNA is genotyped by hybridization of target sequence to biotinylated Peptide nucleic acids (PNA) probes, which have been proved to be suitable probes especially in mismatch detection [63]. The analysis of the one and two base mismatch is performed without any fragmentation of the DNA molecule and simply by thermal sample denaturation. The hybridization temperature is set at 55 °C. To the dsDNA complex is further bond Rec protein to improve system sensitivity reported to be 8 pg/L in standard solution. Detection of the target sequences with DNA samples extracted from animal feed containing 30 % RR soybean amplified by the PCR and unamplified DNA. The detection limit for genomic DNA was in the range of 4.7·105 numbers of genomic copies contained EPSPS gene in the QCM cell. Mismatch analysis was achieved in DNA extracted from human blood cells. Good correlation (r = 0.9613) was found with RT-PCR method, indicating potential real application of the device to detect HBV in clinical diagnostics.

Two papers deal with genetically modified material. In particular detection in Soybean Roundup Ready 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, which is an active component of an insert integrated into RR soybean genome,

Table 8.3 Literature reporting about DNA genomic detection by piezoelectric sensing

Target analyte	T °C	Probe mer	Digestion	Denaturation	Sample	Signal amplification	Ref.
Mycobacterium tuberculosis Gene IS6110	Room	25	Enzymatic BstDSI	Thermal with blocking	Sputum	Streptavidin	[62]
Hepatitis, point mutation in L50 (2562–2613)	55	29 PNA with linkers	No	Thermal	Blood	Rec -protein	[63]
OGM EPSPS gene	Room	21	No	Thermal	R-Ready soybean 30 %	No	[64]
OGM P35S GM tobacco plants	Room	25	EnzymaticBamHI/HindIII vs ultrasounds	Thermal	GM tobacco plants	No	[65]

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is reported [64]. EPSPS gene renders plants' resistance against herbicide glyphosate (Roundup Ready). Glyphosate is toxic for plants because it prevents the production of aromatic aminoacids (tryptophan, tyrosine, phenylalanine). A 21-mer single stranded biotinylated oligonucleotide (probes) is immobilized on sensor (8 MHz quartz crystal) surface via avidin binding, previously covalently attached to gold. No sample digestion has been performed and thermal denaturation applied. Very low reproducibility is reported: CV% 20 %, may be due to pre-analytical steps i.e. sample pre-treatment. The sensor proposed in this study was tested for the detection of EPSPS sequence in PCR non-amplified DNA samples extracted from animal feed containing 30 % of the genetically modified soybean Roundup Ready. The sensor was able to distinguish transgene sequence between modified and unmodified soybean DNA (extracted from animal feed) at the following level: 3.6, 4.6 and 5.4 μg of genomic DNA in 200 μl of QCM cell.

The influence of different used probe on the sensor behavior is also reported, confirming the importance of probe optimization in the development of nucleic acid-based sensing as underlined before [22]. The negative control, genomic DNA extracted from unmodified soybean, generated small frequency shift, which might be attributed to the direct adsorption of DNA on gold electrode surface without hybridization to the probe or weak, non-specific interactions between the probe and partially complementary sequences present in very long genomic DNA. The detection limit was in the range of 4.7×10^5 numbers of genome copies with EPSPS gene in the QCM cell with 200 μl of investigated samples.

Finally, detection of P35S has also been reported in pflp (ferredoxin like protein)-gene inserted in *Nicotiana tabacum* plants [65]. Wild type tobacco DNA was used as a control. The extraction protocol applied was the same applied in our laboratory, i.e. CTAB, and BamH/Hind III restriction enzymes were used in the fragmentation by enzymatic digestion, while we used only BamH. Sonication in parallel was used to compare the fragmentation efficacy of the two approaches. It is interesting the comparison between the two fragmentation approaches performed in this work. By the comparison can be observed the same frequency decreases were observed at the same concentrations of both digested and sonicated samples but with different standard deviations, having the sonicated samples the highest. This result was assumed since fragmentation of the DNAs was non-selective during sonication process. However, the sonication even if nonselective is much faster to performed (minutes compared to overnight digestion used in enzymatic fragmentation).

Very sensitive target sequence detection directly in unamplified DNA can be achieved by label free and real-time detection using piezoelectric sensing. The sensor development as well as the preanalytical steps are strategic for the success of the detection.

First the probe immobilization chemistry should allow selecting binding by preventing any unspecific adsorption from the bulk to the surface. For this reason we have selected and compared in the presented application both biotinylated and thiolated probes. Biotinylated probes are bound to the previously modified surface by thiol/dextran/strepatvidin, while thiolated probes are directly bound to the surface. At the same time the probe selection is also a key step. In fact the presence

of loops or hairpins in the probe structure could affect the hybridization with the target sequence, thus careful selection, eventually assisted by *in silico* selection, could be employed. The presence of secondary structures can be also prevented by tuning the denaturation protocols to prevent non only re-annealing but also intra strand bonding.

Fragmentation of genomic DNA is also important, but enzymatic digestion may be too long for use in biosensing. In this sense, once assessed the sensitive DNA detection one could move on to ultrasound fast fragmentation.

The application of these systems to a wide panorama of clinical, food and environmental analysis is the next challenge. In this sense, multi-array approaches based on piezoelectric sensing are welcome, allowing parallel analysis of target sequences. For that different sensor designs can be foreseen. Over years interesting papers dealing with the development of automated instrumentation have been presented.

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