Review

Electrochemical and piezoelectric DNA biosensors for hybridisation detection

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\textbf{A B S T R A C T}

DNA biosensors (or genosensors) are analytical devices that result from the integration of a sequence-specific probe and a signal transducer. Among other techniques, electrochemical and piezoelectric methods have recently emerged as the most attractive due to their simplicity, low instrumentation costs, possibility for real-time and label-free detection and generally high sensitivity.

Focusing on the most recent activity of worldwide researchers, the aim of the present review is to give the readers a critical overview of some important aspects that contribute in creating successful genosensing devices. Advantages and disadvantages of different sensing materials, probe immobilisation chemistries, hybridisation conditions, transducing principles and amplification strategies will be discussed in detail. Dedicated sections will also address the issues of probe design and real samples pre-treatment. Special emphasis will be finally given to those protocols that, being implemented into an array format, are already penetrating the molecular diagnostics market.

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\textbf{Contents}

1. Introduction .................................................................................................................. 140
2. Probe design .................................................................................................................. 141
   2.1. Linear oligonucleotide probes .................................................................................... 141
   2.2. Hairpin oligonucleotide probes .................................................................................. 141
   2.3. Peptide nucleic acids (PNAs) .................................................................................... 142
   2.4. Locked nucleic acids (LNAs) .................................................................................... 142
3. Immobilisation methods .................................................................................................. 142
   3.1. Carbon-based electrodes .......................................................................................... 143
       3.1.1. Direct physisorption ......................................................................................... 143
       3.1.2. Attachment of biotinylated probes to avidin-coated surfaces ..................................... 143
       3.1.3. Attachment to an electropolymerised layer .......................................................... 143
       3.1.4. Self-assembly .................................................................................................. 143

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1. Introduction

DNA biosensors (or genosensors) are analytical devices that result from the integration of a sequence-specific probe (usually a short synthetic oligonucleotide) and a signal transducer. The probe, immobilised onto the transducer surface, acts as the biorecognition molecule and recognises the target DNA, while the transducer is the component that converts the biorecognition event into a measurable signal. Assembly of numerous (up to a few thousand) DNA biosensors onto the same detection platform results in DNA microarrays (or DNA chips), devices which are increasingly used for large-scale transcriptional profiling and single-nucleotide polymorphisms (SNPs) discovery.

As clinical diagnostics and other applications (e.g., environmental screening) do not generally need the massive data accumulation typical of gene chips, alternative technologies are in development whose promise is to provide flexible and economical alternatives for applications that require relatively fewer measurements. Among other techniques, electrochemical and piezoelectric transductions are the most attractive due to their simplicity, low instrumentation costs, possibility for real-time and label-free detection and generally high sensitivity.

In the past few years, several excellent reviews have been published on both electrochemical and piezoelectric DNA sensing [1–7]. Focusing on the most recent activity of worldwide researchers, the aim of the present review is to give the readers a critical overview of those important aspects that contribute in creating successful genosensing devices. For the sake of clarity, this review will specifically focus on devices in which the biological recognition elements are surface-immobilised, and therefore in intimate contact with the transducer. Hence, the whole branch of literature recently
Advantages and disadvantages of different sensing materials, probe immobilisation chemistries, hybridisation conditions, transducing principles and amplification strategies will be discussed in detail. Dedicated sections will also address the issues of probe design and real samples pre-treatment. Special emphasis will be given to electrochemical protocols that, being implemented into an array format, are already penetrating the molecular diagnostics market.

2. Probe design

As the specificity of the hybridisation reaction is essentially dependent on the biorecognition properties of the capture oligonucleotide, design of the capture probe is undoubtedly the most important pre-analytical step. Thus, a number of probes, variable for chemical composition and conformational arrangement, have been used to assemble DNA biosensors.

Most commonly, the probes are linear oligonucleotides, either synthesised in situ or pre-synthesised and afterwards immobilised onto the sensor surface. However, structured (hairpin) oligonucleotides are being used with increasing frequency.

The experimental variables affecting the hybridisation event at the transducer–solution interface are referred to as stringency. Such variables typically include hybridisation and post-hybridisation-washing buffers composition and reaction temperature. When dealing with more than a single probe at the time, the basic requirement for a functional system is the ability of all the different probes to hybridise their target sequences with high affinity and specificity under the same stringency conditions [8]. This aspect makes the design of complex sets of probes even more difficult.

The design of probes for the analysis of samples susceptible for degradation (such as RNA) requires additional attention if a sandwich hybridisation scheme is chosen. Selection of probes binding sites in close proximity to each other results particularly convenient [9], as it minimises the adverse effects of sample degradation on the success of the sandwich hybridisation reaction.

2.1. Linear oligonucleotide probes

Design of linear probes takes now great advantage of decades of experience, which has led to many commercially available software. A particular challenge is, however, still represented by design of full sets of arrayed probes for the screening identification of closely related and unrelated pathogens [10]. Upon retrieval of the genomic sequences from specific data banks and their assembly and alignment using dedicated software, PCR primers and reporter oligonucleotides are typically selected within highly conserved regions of each bacterial genome. According to the requirements of each specific application, capture oligonucleotides can be then designed within either hypervariable or highly conserved regions. Candidate sequences are finally tested for theoretical melting temperature ($T_m$), hairpins and dimers formation and for homologies using a Basic Local Alignment Search Tool (BLAST) search.

Use of capture probes in the order of 18–25 nucleotides usually confers higher levels of specificity to the hybridisation reaction. However, excessively longer capture oligonucleotides often exhibit particularly unfavourable hybridisation specificity and yields. While a single or a few mismatches are unlikely to significantly destabilise even a 30-mer probe–target duplex over a wide range of experimental conditions [11], the general hybridisation efficiency of similar or longer probes might be particularly low, due to intramolecular hydrogen bonding and consequent formation of non-reactive hairpin structures.

2.2. Hairpin oligonucleotide probes

Hairpin probes are structured oligonucleotides that combine a double-stranded stem region (due to intramolecular base pairing) and a single-stranded loop, which contains the capture sequence (e.g., Fig. 1A). Similarly to molecular beacons, the

![Fig. 1 – eBeacon (ferrocene-labelled hairpin probe): (A) before hybridisation (folded state); (B) after hybridisation (unfolded conformation).](image-url)
hairpin probes used for electrochemical sensing were functionalised at both 3' and 5'-end. One of these functionalities (e.g., an alkylthiol spacer arm) allowed for surface immobilisation of the biomolecule, while the other consisted of a redox active compound (e.g., a ferrocene derivative). In the absence of a target, the stem–loop configuration hold the redox moiety in proximity to the electrode. Hence, because of the short distance and easy electron transfer, the label generated large faradic currents. The conformational change which followed hybridisation displaced the label from the electrode surface, resulting in strong diminution of the electrochemical accessibility of the redox moiety. The extent of signal suppression thus reflected presence and concentration of the target sequence. The major advantage of using redox labelled hairpin probes was that hybridisation could be measured directly, obviating the need for labelling the target through additional and time-consuming steps and also avoiding use of further reagents.

Proper design of the hairpin probes is obviously crucial, as functionality, selectivity and sensitivity of such capture oligonucleotides are reported to strongly depend on the amplitude of the loop and the length of the stem region [12,13]. In particular, the hairpin probe used by Miranda-Castro et al. exhibited superior selectivity with respect to analogous linear oligonucleotides. Such an improved discrimination capability was attributed to the loop–stem structure of the probe, which stabilised the dissociated state of the probe–analyte duplex especially in the presence of mismatched base pairs [13]. However, other authors [12,14] showed different hairpin probes to yield non-specific hybridisation with a number of sequences which presented a certain degree of homology with their target. These results raised the question of whether the hairpins are truly advantageous in terms of selectivity. Also, most of the electrochemical studies were limited to the use of relatively short synthetic oligonucleotide targets (<52 bases), thus lacking the true challenge of real sample analysis.

2.3. Peptide nucleic acids (PNAs)

PNAs are DNA mimics in which the nucleobases are attached to a neutral N-(2-aminoethyl)-glycine pseudopeptide backbone (Fig. 2B). Following the Watson–Crick rules, PNA probes form hybrids stabilised by hydrogen bonding and base stacking. However, because of the neutral backbone of PNA, PNA/DNA hybrids display melting temperatures higher than the corresponding DNA/DNA duplexes (generally +1 °C/nt), stability against nucleases and proteases, relative insensitivity to ionic strength and usually higher selectivity against single-base mismatches [15].

If compared to the conventional oligonucleotide probes, PNAs have appeared particularly interesting for the development of electrochemical genosensing concepts, the main reason being the drastically different electrical characteristics of their molecular backbone.

The supporters of PNAs claim that these probes are able to bind their complementary sequence with comparably higher affinity and specificity than usual oligonucleotide probes, while discriminating mismatched targets to a larger extent. However, despite careful control of the hybridisation conditions, different authors observed relatively strong non-specific signals for a number of SNP-containing targets [16,17]. An example of how the selectivity of PNA probes can be further improved is offered by the work of Marchelli’s group, which exploited the effect exerted by the introduction of chiral aminoacids into the PNA backbone [18]. A group of three adjacent d-lysine chiral monomers (referred to as “chiral box”) was introduced in the middle of a 15-mer PNA specific for the cystic fibrosis W1282X point mutation. When compared to analogous achiral PNAs or oligonucleotides, such a modified probe exhibited enhanced mismatch recognition capability, allowing easy differentiation of healthy, heterozygous and homozygous mutant patients.

2.4. Locked nucleic acids (LNAs)

Locked nucleic acids (LNA™, www.exiqon.com) are a class of nucleic acid analogues in which the ribose ring is “locked” by a methylene bridge connecting the 2'-O atom with the 4'-C atom (Fig. 2C). DNA oligos incorporating LNA nucleosides show increased thermal stability (+2 to 6 °C/included monomer) and further improved discriminative power with respect to single-base mismatched targets (ΔTm up to 8 °C). To these authors’ knowledge, LNA probes have not, yet, been used for electrochemical or piezoelectric sensing of DNA. However, the outstanding properties of such DNA mimics are likely to find massive utilisation for the future development of SNPs typing assays.

3. Immobilisation methods

The ability to immobilise the probe in a predictable manner while maintaining its inherent affinity for the target DNA is
crucial to the overall device performance. Hence, independently on the molecular identity and conformation of the probe, some general aspects must be taken into account when considering its surface attachment. Being obvious that the choice of the most appropriate immobilisation protocol is strictly dependent on the characteristics of the transducing material, robust immobilisation chemistries are usually preferred, in order to prevent desorption of the probes from the sensing layer. As it will be illustrated below, retention in a polymeric matrix, covalent attachment on a functionalised support, affinity immobilisation and self-assembling are, to date, the most successful approaches. This is mainly because each of these immobilisation strategies can lead to ordered sets of end-point attached and properly oriented probes. Moreover, such chemistries also allow to control the conformational freedom of the probes and the corresponding interchain space through the modulation of the surface coverage. Hybridisation efficiencies as high as 100% have even been reported in the most favourable cases, thus demonstrating that the resulting bio-architectures are fully accessible for interaction with the solution-phase target, while offering only minimal steric impediments.

While the electrodes applied onto piezoelectric crystals are typically made of gold (with platinum being a rare exception), the availability of a range of conducting materials have made the family of electrochemical genosensors and related immobilisation chemistries much broader. Besides classical bulky electrodes, detection platforms that are suitable for mass production, miniaturisation and possibly allow for simultaneous multi-site immobilisation and detection are the most attractive. In fact, such devices combine the capability to fully characterise a given sample in a single assay (e.g., when screening for several single-nucleotide polymorphisms) with use of minimal sample volumes and short analysis times.

Functionalisation of the sensing interface with the capture probe must be done following two basic criteria. As a general rule, the probe has to be immobilised in such a way that its biorecognition capability is preserved as much as possible. Additionally, as a particular need of electrochemical detection schemes, the attached bio-layer does not have to behave as a total insulator, thus allowing for electrochemical interrogation of the surface. The most interesting immobilisation approaches, specific for each electrode material, are described below.

3.1. Carbon-based electrodes

During the past decade, carbon electrodes have experienced great popularity, mainly because of favourable characteristics such as suitability for mass production (via screen-printing), low cost, wide electrochemically accessible potential window, etc. Such a popularity has been renewed and reinforced by the advent of carbon nanotubes [19]. However, carbon-based transducers are unlikely to play, at least in the near future, an important role in DNA diagnostic devices. This is because on bare carbon surfaces immobilisation of the probe usually proceeds randomly, via multiple interactions between the transducer surface and both the phosphate backbone and the hydrophobic nitrogenous bases of the oligonucleotides. Random adsorption probably accounts for a significant fraction of the surface-confined strands also when attempting carbodiimide/N-hydroxy-succinimide (EDC/NHS) covalent coupling of derivatised oligos to activated surfaces. Besides being non-oriented, physisorbed molecules are limited in their mobility, which makes the hybridisation event sterically inhibited. Efficient blockage of the surface (e.g., to prevent the non-specific adsorption of DNA sequences, redox labels, etc.) can additionally be non-trivial.

Along with an example of immobilisation by simple adsorption, the most relevant strategies developed to circumvent the drawbacks of carbon surfaces are described below. The one exploited by Gorodetsky and Barton [20] appears, between the others, of particular interest.

3.1.1. Direct physisorption

Kerman et al. [15] choose physisorption as a rapid and simple method to modify the surface of an electrochemically pre-treated glassy carbon electrode (GCE) with PNA probes. However, confirming the validity of the above-mentioned concerns, the non-specific signal from the Co(NH3)63+ label was difficult to be minimised. Significant signals were also obtained only for micromolar concentrations of the target, which reflected the low hybridisation efficiency of the physisorbed PNA molecules.

3.1.2. Attachment of biotinylated probes to avidin-coated surfaces

Metfes et al. [9] used a layer of neutravidin adsorbed onto a carbon paste electrode as the platform for immobilising biotin-labelled probes. Even prior to attach such capture oligonucleotide, the electrode surface was blocked using casein, which effectively minimised the non-specific adsorption of the components of the bioassay.

Similarly, Hernandez-Santos et al. modified the surface of disposable screen-printed carbon electrodes with streptavidin prior to immobilisation of the biotinylated capture sequence. Blocking of the surface with BSA was also found important in order to reduce non-specific interactions [21].

3.1.3. Attachment to an electropolymerised layer

Tan and co-workers [22] used a monolayer of 4-aminobenzoic acid (4-ABA) electropolymerised on the surface of a glassy carbon electrode for the covalent immobilisation of an aminomodified capture oligonucleotide. The capture probe was covalently attached to the carboxylic functionalities of the 4-ABA monolayer via EDC/NHS cross-linking reaction. Voltammetric transduction of the hybridisation events (accomplished in the presence of methylene blue as the redox label) revealed, however, modest performances for such a biorecognition layer.

3.1.4. Self-assembly

Gorodetsky and Barton recently demonstrated that pyrene-functionalised oligonucleotides can be conveniently self-assembled onto highly oriented pyrolytic graphite electrodes (Fig. 3) [20]. Characterisation of these films evidenced the features previously observed for thiol-tethered oligos on gold surfaces: closely packed oligonucleotides with helices oriented in a nearly upright conformation. This technique might, therefore, provide the oligonucleotides immobilised on carbon sur-
faces of unprecedented biorecognition efficiencies, thus giving a renewed impulse to the use of carbon-based transducers.

3.2. ITO electrodes

Tin-doped indium oxide (ITO) is the material of choice for fabrication of a number of optoelectronic devices. However, ITO electrodes have also found application for DNA sequence-specific detection due to their particular amenability for electro-catalytic oxidation of guanine residues [23].

3.2.1. Covalent attachment to a self-assembled monolayer

While earlier papers described the use of randomly adsorbed oligo- or poly-nucleotides sequences, ordered and oriented immobilisation of specific probes was subsequently demonstrated for these metal-oxide surfaces. According to the method of Popovich et al., the oligonucleotide probes were bound to an ITO electrode by firstly forming a self-assembled monolayer (SAM) of 12-phosphonododecanoic acid [24]. The terminal carboxylic moiety of the monolayer was then activated with carbodiimide and reacted with an amine-terminated oligonucleotide probe. The immobilisation of preformed PNA–silane conjugates was also demonstrated in the same paper.

3.2.2. Attachment by electro-copolymerisation

An array of individually addressable ITO electrodes was used as the transduction element in an integrated analytical device used for the multiplexed detection of E. coli and B. subtilis cells [25]. The choice of an electrochemically driven immobilisation strategy (electrochemical copolymerization of pyrrole and pyrrole–oligonucleotide conjugates) allowed individual positioning of the predefined probes at selected ITO surfaces. Notably, such immobilisation chemistry provided the probes sufficient stability to tolerate the repeated thermal cycling of in situ PCR amplification of the target DNA.

3.3. Silicon oxide electrodes

Contrasting with the widespread use of glass in optical sensing, silicon-based electrodes have found, up to now, limited application in electrochemical genosensors. Ingemann et al. [26] used, for example, an ultrathin silicon dioxide layer as the dielectric gate in a field effect transistors (FETs) DNA microarray (eight transistor gates/chip). The bio-modification of the SiO2 gates involved prior cleaning and activation followed by silanisation with 3-aminopropyltriethoxysilane (APTES). The amino-silanised SiO2 surfaces were then modified using succinic anhydride, which allowed cross-linking of amino-modified oligonucleotide probes.

Gao et al. employed a related approach for modifying arrays of highly ordered n-type silicon nanowires (SiNW), fabricated using a complementary metal-oxide semiconductor (CMOS) compatible technology [27]. The arrays (100 wires/array, with each wire being 5–50 nm wide, several micrometer long) were first cleaned with HF, silanised using trimethoxysilane aldehyde and then interacted with an amino-modified PNA probe. The non-reacted aldehyde moieties were blocked using butylamine and, finally, imine bonds were reduced using sodium borohydride.

3.4. Platinum electrodes

Until recently, platinum electrodes have only found limited application in DNA sensing technologies. One rare example was the work of Guiseppi-Elie and Hang, which used 3-glycidoxypropyl-trimethoxysilane as a coupling reagent to immobilise 5′-amine-terminated oligonucleotides onto the oxidized platinum electrodes of a quartz crystal microbalance (QCM) and the exposed glass surface between the Pt digits of microfabricated electrodes [28].

Contrasting with this rather unpopular past, arrays of platinum microelectrodes are the heart of a breakthrough technology that is likely to revolutionise the current equilibrium between optical and electrochemical detection in molecular diagnostic devices [29]. A general limitation for electrochemical detection has been the technical difficulty to obtain an output from an array of electrodes, due to wiring complexity and requirement for a multichannel reader. Overcoming such a fundamental drawback, the core technology developed by CombiMatrix was based on a specially modified CMOS semiconductor containing 12,544 individually addressable platinum microelectrodes (44μm in diameter). Outstandingly, the semiconductor logic circuitry even allowed to direct the in situ synthesis of different oligonucleotides at thousands of electrodes simultaneously. The probes were grown within a proprietary “porous reaction layer” (low blockage membrane) that coated each microelectrode within 1μm of the surface. Upon digital activation, each feature on the array selectively generated acid by means of an electrochemical reaction. The generated acid, in turn, controlled detritylation of the growing oligonucleotide chain, thus activating it for binding of the next nucleotide. Since a different probe can be synthesized at each microelectrode, this technology enabled design of microarrays of any desired configuration.

Fig. 3 – Pyrene-functionalised oligonucleotide immobilised (self-assembled) onto a highly oriented pyrolytic graphite electrode. Adapted from Ref. [20].
The massive availability of test sites allowed synthesis of hundreds of unique 35–40-mer probes (each in tens of replicates), thus reaching levels of parallelisation that were, till recently, prerogative of fluorescence-based arrays. The advantageous chemistry used for the bio-modification of the Pt microelectrodes allowed the microarrays to be chemically stripped of hybridised targets and re-used. No memory effects or significant loss of sensitivity were observed after re-use of a microarray up to three times.

3.5. Gold electrodes

Immobilisation by self-assembly onto gold electrodes, widely used for both electrochemical and piezoelectric sensing, takes advantage of the strong interaction (chemisorption) which is established between thiolated molecules and this metal surface.

While earlier works described preliminary formation of a SAM of a reactive thiol followed by carbodiimide coupling of phosphate, amino or carboxyl-terminated oligonucleotides, the most recent approaches involved chemical stripping of hybridised targets and re-used. No memory effects or significant loss of sensitivity were observed after re-use of a microarray up to three times.

3.5.1. Electrochemical sensing

3.5.1.1. Chemisorption onto interdigitated microelectrodes arrays. Elsholz et al. [35] described their analytical platform as a chip carrying 16 electrode positions (500 µm in diameter), each consisting of arrays of interdigitated gold electrodes. As the detection mechanism of choice relied on redox cycling the product of an enzymatic reaction (Fig. 4), the comb-like structures were designed so that gaps of just 400 nm separated the 800 nm wide anode and cathode fingers. To demonstrate the multiplexing capability of their commercially available analytical device (“eMicroLISA” automated Array Analyzer), thiol-tethered oligonucleotide probes specific for five pathogens were spotted in triplicate onto the chip using a piezoelectric nanoplotter.

3.5.1.2. Electrochemical lithography. Plaxco’s group exploited alternate chemisorption and electrochemically driven desorption steps to selectively immobilise different capture oligonucleotides onto closely spaced gold electrodes [36].

Inspired by photolithography, in which a light-sensitive resist that covers a surface is selectively removed to expose specific regions to further modifications, the electrodes were first passivated with a self-assembled monolayer of 11-mercapto-1-undecanol. Site-specifically, the monolayer could be then stripped off by electro-oxidation, thus regenerating a bare gold surface available for immobilisation of a specific probe. Interestingly, immobilisation and oxidative desorption

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Fig. 4 – Enzyme- and electro-amplified detection of unmodified sequences: redox recycling the product of an enzymatic reaction. Adapted from Ref. [35].
conditions were so gentle that sequential labelling of multiple, closely spaced electrodes (95 μm separation), was possible. Unlike photolithography or dip-pen nanolithography, the “electrochemical lithography” did not require expensive micromechanical devices, obviating the need for having direct physical access to the electrode surface. However, the length of the process (which was limited by thiol adsorption kinetics and the need for extensive washing steps) probably limited to about 100, the number of electrodes that could be modified in a reasonable time frame.

3.5.1.3. Immobilisation onto sandwiched assemblies. With the aim to improve the electrical communication between a redox enzyme and the electrode surface, Domínguez et al. exploited electrostatic self-assembly as a means to sandwich the anionic horseradish peroxidase between two layers of a positively charged osmium-based redox polymer [38]. Platform for such multi-layer assembling was a gold electrode modified with a negatively charged monolayer of 3-mercaptopropane sulfonic acid. The oligonucleotide probes were then covalently attached through their 3'-phosphate groups to the free amino functionalities of the redox polymer. Embedded into the redox complex, peroxidase exhibited enhanced communication with the electrode, thus efficiently supporting the bio-catalysed reduction of the hydrogen peroxide generated by an oligonucleotide-conjugated glucose oxidase label.

3.5.1.4. Immobilisation onto an electropolymerised film. Garnier et al. used a low-density array of gold electrodes (100 μm in diameter) to develop a DNA chip based on an electropolymerised film of polypyrrole bearing capture oligonucleotides [39]. Two monomers (3-acetic acid pyrrole and 3-N-hydroxyxypthalimide pyrrole) were first electropolymerised onto the gold surfaces. Covalent attachment of the capture probes was then obtained by chemical substitution of the easy leaving ester group N-hydroxyxypthalimide by the amino-substituted oligonucleotides. The system was used for monitoring the interaction between the surface-immobilised probes and the synthetic targets in a label-free and real-time mode.

3.5.2. Piezoelectric sensing
While in the case of electrochemical genosensors direct chemisorption of thiol-modified probes onto the gold electrode is one of the most popularly employed methods, in the case of piezoelectric sensing the gold electrode surface is typically modified using more complex immobilisation chemistries (e.g., thiol/dextran/streptavidin modification). Although longer (taking a few days instead of a few hours), the latter strategies provide the immobilised bio-layers of superior analytical performances (robustness for repeated use, higher sensitivity, etc.). Use of an immobilisation chemistry which leads to sensing interfaces resistant to non-specific adsorption phenomena is also of particular importance. Sensitivity and specificity of QCM devices are, in fact, essentially limited by the non-specific adsorption of the constituents of real matrices.

3.5.2.1. Direct chemisorption of thiol-tethered probes. As for electrochemical genosensors, the influence of the interfacial design on efficiency and kinetics of the hybridisation reaction has been widely investigated over the past decade. More recently, Gooding et al. [40] elucidated the effect of the length of the diluent thiol used to create mixed self-assembled monolayers. Wu et al. [41] also demonstrated that the addition of a 12-deoxyxymidine-5′-monophosphate (12-dT) spacer at the 5′-end of the 30-mer immobilised thiolated probe enhanced by twofold the hybridisation efficiency.

3.5.2.2. Attachment of biotinylated probes to (strept)avidin-coated surfaces. Functionalised alkane thiols form stable self-assembled layers on planar surfaces and act, therefore, as ideal linkers to achieve single-point attachment of the DNA probe at either 5′- or 3′-end [42]. A procedure widely employed on QCM devices exploits the well-known affinity between biotin and (strept)avidin. This method relies on four basic steps: formation of a self-assembled monolayer of functionalised alkane thiols onto the gold surface; subsequent binding of a dextran hydrogel; covalent attachment of (strept)avidin; affinity immobilisation of biotin-labelled probes. This immobilisation chemistry, initially introduced for optical (surface plasmon resonance, SPR) sensing, was successfully transferred to piezoelectric transducers by Tombelli et al. [43]. The good performances of such biotinylated probes (immobilised onto dextran-bound streptavidin) have been widely demonstrated for targets of clinical [44–46], food and environmental interest [47,48]. Tombelli et al. [43] also compared the performance of QCM genosensing platforms obtained using either the thiol/dextran/streptavidin chemistry or thiolated probes directly. The two immobilisation procedures exhibited similar analytical characteristics in terms of selectivity, sensitivity and reproducibility.

Several alternative immobilisation methods have also been used to create the streptavidin layer at the sensor surface. One of the most frequently used is the covalent coupling of the free amino groups of streptavidin to carboxyl-functionalised surfaces [49]. This attachment procedure does not influence the ability of streptavidin to bind biotin, thus leaving the protein available for the immobilisation of the biotinylated oligonucleotide probes.

The influence of streptavidin packing density and film structure on binding and orientation of the DNA probe was studied by Knoll and co-workers [49]. How these parameters influenced the hybridisation efficiency of the probe was investigated comparing two different methods for immobilising a streptavidin layer onto the gold surface: direct interaction of streptavidin with a biotin-terminated SAM and covalent attachment of the protein onto an 11-mercaptoundecanoic acid SAM via amine coupling. Streptavidin films assembled onto the biotin-containing thiols appeared to be highly rigid with a well-ordered structure, while the streptavidin film formed via amine coupling was less ordered. Interestingly, the biotinylated probes immobilised onto the well-organised streptavidin layer allowed a higher sensitivity to be obtained.

The same group exploited such biotin/streptavidin/biotin-probe bridges as platforms to detect single-base mutations, insertions and deletions [50]. The gold electrodes of the QCM were first modified with a mixed layer of a biotin-terminated thiol and an OH-terminated thiol; streptavidin was then bound to this film. Using a biotinylated probe, DNA mutations
in oligonucleotide targets were detected by the use of MutS, a particular protein which binds DNA sequences containing mispaired and unpaired basis, but not the perfectly matched duplex.

Use of biotin-doped supported lipid bilayers (b-SPB) on SiO$_2$-coated QCM crystals was also reported by Larsson et al. for immobilising streptavidin first and then biotinylated oligonucleotides [51].

4. Hybridisation reaction

If, on one hand, RNA-based assays benefit from the typically high number of ribosomes per cell, on the other the sensitivity of most genoassays do not generally allow the direct analysis of sequences that occur as a single copy within organisms genome. Detection thus depends on the PCR pre-amplification of the target sequence.

Specific aspects crucial to the pre-analytical and analytical phase will be illustrated in the next sections. Along with use of capture probes with improved selectivity and hybridisation efficiency (see Section 2), specific pre-treatments of the samples can be used to greatly enhance the yield of the heterogeneous hybridisation events and, consequently, the sensitivity of detection. The reasons that make direct analysis of genomic DNAs (i.e., unamplified sequences) extremely difficult will be also discussed.

4.1. Sample pre-treatment

Many analytical protocols require the samples to be pre-treated prior to hybridisation. The aims of such pre-treatments include: (a) the reduction of the complexity of the sample; (b) the separation of possible interfering molecules (such as unincorporated primers and nucleotides); (c) the generation of the target in a single-stranded form (or at least the minimisation of amplicon sister strand re-annealing); (d) the disruption of thermodynamically stable secondary structures of the sequence (which might severely inhibit the interfacial biorecognition process). As a general rule, the number of sample pre-treatments should be kept as small as possible, as they significantly slow down the whole analytical process.

4.1.1. Genomic DNA

Although high levels of fidelity generally characterise the PCR amplification step, this process have always to be carefully optimised. Non-specific annealing of the primers can, for example, lead to the generation of a wrong product (i.e., a false positive amplicon), while the presence of an inhibitor of polymerase can lead to a false negative result. Having the possibility to directly sense genomic DNAs, these drawbacks would be all overcome, and the number of sample pre-treatments reduced, thus significantly facilitating the detection of nucleic acids. Nevertheless, scientists who have attempted the analysis of unamplified genomic DNA have often faced insurmountable difficulties. The huge size of genomic samples constitutes the first of a series of obstacles. Hence, in order to facilitate the interfacial hybridisation of such targets, fragmentation of the samples by sonication [52] or enzymatic digestion [53,54] has been suggested. Although simpler and cheaper, an undesired effect of sonication (which is a random process) might be disruption of the sequence targeted by the surface-immobilised probe. Nevertheless, even envisioning an optimal fragmentation, additional problems arise. If one considers that the genome of many organisms has a size of about $10^9$ to $10^{10}$ bp and that fragments in the order of 500 bp on average would probably be the most suited for hybridisation, as few as a single copy of the target would be “lost” between $10^5$ and $10^6$ non-relevant strands. Diffusion of the fragment containing the target toward the probe-modified surface would be significantly slowed down by the concomitant presence of such sequences and further hindered by their electrostatic repulsion when accumulated in close proximity of the sensing layer. Effective match of the capture probe with the target sequence (the process that leads to duplex formation) is, therefore, a rather unlikely event. One has additionally to consider that, due to the complexity of genomic DNA, several of the obtained fragments could include sequences that present substantial homology with the true target. Yielding non-specific hybridisation with the capture probe, such sequences would be, therefore, responsible for false positive signals. The development of sufficiently sensitive methods represents an additional analytical challenge when levels of selectivity are required that single-nucleotide polymorphisms can be unambiguously discerned. To these authors knowledge, the work of Patolsky et al. still constitute the unique example describing the electrochemical detection of a single-nucleotide polymorphism in unamplified genomic samples [53]. QCM sensing of genomic sequences related to the P35 promoter in transgenic plants (i.e., Nicotiana glauca) was also obtained after fragmentation with restriction enzymes and use of an optimised sample denaturation step [55]. Viscoelastic effects coupled to the increase of mass at the surface of the sensor combined with a denaturation protocol aimed to prolong the lifetime of the target in its single-stranded state could account of this sensitive detection. Similar results were obtained by Evans and co-workers for bacterial DNAs [56].

The double role of the PCR amplification thus clearly appears from the above-mentioned considerations. Firstly, it helps reducing the complexity of the target DNA; secondly, it increases the concentration of the sample.

A case where the amount of genomic sample is unusually abundant is represented by highly repeated (microsatellite) sequences. In contrast to single copy targets per haploid genome, microsatellite sequences appear in high numbers of copies, thus making their detection significantly more accessible. Minunni et al. [54] reported, for example, the detection of microsatellite bovine DNAs using a QCM device. Again, the possibility to successfully detect such a genomic sample using QCM mainly relied on the development of an accurate sample pre-treatment (fragmentation with restriction enzymes and proper thermal denaturation). Similarly, Mascini et al. detected this microsatellite bovine genomic DNA through a label-free electrochemical genoassay [57].

4.1.2. PCR-amplified samples

Analysis of PCR amplicons is definitely simpler than analysis of genomic DNA. However, even when dealing with PCR-amplified sequences, detection can be anything but straightforward. Inconsistent results along with a consider-
able number of false positive and false negative responses were, for example, found by Barlaan et al. [10] when working with relatively long (533 bp) amplicons. Shorter PCR products (200 and 350 bp) gave much better results.

Amplified sequences are usually in a double-stranded form that have to be thermally or chemically denatured. Even doing so, the strand complementary to the amplified target (still present in solution) competes with the immobilised capture probe for hybridising the target itself. Upon decreasing the amount of the single-stranded sequence available for surface hybridisation, this phenomenon is thus responsible for generally lower hybridisation yields.

In order to overcome this problem three main strategies have been developed. The first one relies on use of asymmetric PCR [15,25], a process that naturally leads to samples in which an excess of the single-stranded target is present. The main disadvantage of this method is, however, that amplification of the target proceeds linearly with the number of PCR cycles instead of exponentially as in the case of usual PCR protocols [58]. Relatively lower amplification factors are therefore obtained when using this strategy.

Single-stranded targets can also be obtained by chemical denaturation of the duplex followed by affinity binding of the biotinylated interfering strand [59] or through specific enzymatic digestion of the undesired sequence [60]. Both methods are, however, labour-intensive and time-consuming.

A simple, rapid and cost-effective alternative to these methodologies is represented by the use of the so-called “helper” or “blocking” oligonucleotides [9,35,61]. When considering heterogeneous hybridisation reactions, the accessibility of the target sequence by the oligonucleotide probe is one of the key conditions determining the overall hybridisation efficiency. The helper oligonucleotides are unmodified oligos which, in the most efficient format, are hybridised to the sample upstream and downstream the site targeted by the capture probe (Fig. 5). The resulting single-stranded bulge is thus easily accessible to the immobilised probe, determining substantially higher heterogeneous hybridisation yields. Similar unmodified “blocking” oligos were also used as a general means to inhibit re-annealing of double-stranded amplicons [15,47,48,61], to break thermodynamically stable secondary structures of the target [35] and even to control the extent of chemical fragmentation of RNA targets by forming relatively short stretches of cleavage-resistant RNA/DNA duplexes [35]. Notably, helper oligonucleotides can be added to the sample solution just prior to its thermal denaturation. Hence, heterogeneous hybridisation of the target and binding of helper oligonucleotides occurs in a single step, eliminating the need to perform successive rounds of hybridisation. All these characteristics thus render use of this strategy particularly amenable to solve a range of hybridisation-related problems.

### 4.2. Passive vs. active hybridisation

The hybridisation reaction can be carried out in both a passive and an active mode. Passive hybridisation takes place when temperature, buffer composition (salt concentration, formamide, etc.) and washes are used to control the stringency and rate of the reaction. These and other experimental variables were, for example, recently explored [11,62] to improve the selectivity of single-base mutation detection.

In contrast to the passive approach, active hybridisation uses electric fields on a microelectronic device to regulate nucleic acids transport, hybridisation and stringency [10]. This technology, developed and implemented by Nanogen Inc. for arrays of 100 individually addressable microelectrodes, is reported to possess several advantages over passive methodologies. An electric field is firstly used to address different probes at specific sites of the microarray. The applied positive potential can also be used to increase the concentration of the target locally at the functionalised electrodes. Hence, while passive hybridisation is limited by diffusion, electrochemical preconcentration of the target can ultimately lead to increased hybridisation rates and efficiencies. Finally, reversing of the potential (i.e., the application of negative voltages) imparts to the system an extremely high stringency for mutation detection based on the preferential destabilisation of mismatched hybrids by means of purely electrostatic (repulsive) forces.

![Fig. 5 – Use of “helper” oligonucleotides to facilitate the heterogeneous hybridisations of targets prone to fold into thermodynamically stable secondary structures. Adapted from Ref. [35].](image-url)
5. Hybridisation detection

Once the target DNA has been captured onto the sensor surface, a range of different approaches can be used for transducing the biorecognition event. The transducing principles can be broadly divided into reagentless, label-free and label-based schemes.

Reagentless sensing concepts are the simplest, as nothing but the sample solution itself is needed to perform the analysis. Reagentless methods are not necessarily label-free. However, when both conditions are met (i.e., the method is reagentless and label-free), one can take advantage from the fact that undesired effects, such as steric impediments to the hybridisation reaction due to the reporter molecule, are completely absent. Such schemes have thus attracted intense research efforts due to their promise to provide analytical information in a faster, cheaper and safer mode compared to other strategies. As an additional advantage, transduction in the reagentless mode can also give access to the kinetics of the biorecognition event. Piezoelectric transduction is essentially reagentless and label-free, with only a limited number of exceptions being described. Indeed, much more difficult is to draw net boundary lines between the concepts reagentless, label-free and label-based when considering electrochemical transducers. Those methods that use solution-phase reagents (e.g., metal complexes or organic dyes) as markers of the hybridisation process will be referred to as label-free. Label-free approaches typically rely on the measurement of changes in the electrical characteristics of sensing layer, before and after the hybridisation reaction. By contrast, when organic and organometallic electroactive compounds, nanoparticles, catalytic and redox enzymes are permanently bound (e.g., covalently or via (strept)avidin–biotin interactions) to one of the constituents of the surface-tethered duplex, the method will be considered as label-based. Sensitivity and reliability of label-based approaches are often still unrivalled, as also witnessed by the choice of these methodologies for the electrochemical microarray platforms now on the market.

As one of the most attractive features of electrochemical and piezoelectric sensing, electrochemical reactions on one hand and mass changes on the other, give an electronic output directly, which makes the signal transduction equipment particularly compact and relatively inexpensive. Those mature technologies which have led to commercially available DNA sensors will be discussed within a dedicated paragraph.

5.1. Reagentless methods

5.1.1. Fully electronic detection

With the introduction of the differential transfer function (DTF) method, Ingebrandt et al. substantially improved the general reliability of the label-free DNA hybridisation detection based on silicon FET microarrays [26]. Enabling fast and fully electronic readout of ex situ hybridisations, such a method relied on the intrinsic charge of the DNA molecules and/or on changes of the interfacial impedance that followed binding of the target sequence. Ingebrandt’s approach overcame the drawbacks of the systems that used a time-resolved dc readout, where the FET signal suffers from sensor drift, temperature drift, changes in electrolyte composition or pH, influence of the reference electrode. Channels modified with a fully non-complementary probe and three and two bases mismatched capture oligonucleotides provided reference signals that were subtracted from the outputs generated in the presence of single-base mismatched or perfectly matched probes. The resulting differential readout of the transfer-function cancelled out any signal change due to non-specific binding of the target DNA, thus allowing easy detection of the SNP in low ionic strength buffers when using low ac frequencies. The sensitivity of the method (μM range) required, however, further improvement.

Gao et al. described real-time and label-free electrical detection of DNA at femtomolar levels using an array of highly ordered SiNW [27]. A monolayer of neutral PNA capture probes, assembled onto the individual SiNWs via silane chemistry, acted as the biosensing interface. When exposed to the negatively charged target sequence, the PNA-functionalised SiNWs showed a concentration-dependent increase of resistance. As with other SiNW biosensing devices, the sensing mechanism could be understood in terms of the change in charge density at the SiNW surface after hybridisation, the so-called “field effect”. The reason for using PNA instead of DNA probes was twofold. Due to the neutral character of the PNA backbone, hybridisation could take place in low ionic strength buffers, thus minimising the background contribution from dissolved salt ions while emphasising the hybridisation-related resistance changes. The SiNW array also satisfactorily discriminated mismatched target DNAs, with the 1 bp mismatch yielding only 15% of the signal observed for the perfectly matched target. Besides being ultrasensitive and selective, an additional advantage of the SiNW biosensor array described by Gao was the capability to detect the hybridisation events in situ, in real-time and in a reagentless and label-free mode. Nonetheless, as pointed out by the authors themselves, the analytical signal intensity (in terms of signal/unit concentration) was very low, while being superimposed on a high background.

5.1.2. Electronic beacons

Jenkins et al. developed a reagentless assay for the detection of Agrobacterium tumefaciens strain C58 using a mixed monolayer of a ferrocene-labelled hairpin probe and β-mercaptoethanol assembled onto the gold surface of disposable screen-printed electrodes [12]. Upon hybridisation, the hairpin probe underwent a conformational change which displaced the redox-active label from the electrode surface. This process resulted in a diminished efficiency for electron transfer from the label, which was detected as a drop in peak redox current measured by cyclic voltammetry (Fig. 1).

The unexpectedly high electrochemical signals allowed the oligonucleotide target to be quantified over seven orders of magnitude (100 fM to 1 μM). However, when performing the assay at room temperature, the hairpin probes yielded non-specific hybridisation with a number of oligonucleotide sequences which presented a certain degree of homology with the target. Notably, the perfectly matched target was distinguished from the single-base mismatched sequence by
recording in situ melting profiles. While increasing the temperature of the hybridisation solution, the ferrocene oxidation signals were periodically measured, evidencing a \( \Delta T_m \) of about 5 °C between the target and the SNP-containing sequence. Such results were consistent with the behaviour theoretically predicted for such oligonucleotide duplexes.

Using a methylene blue-modified hairpin probe covalently attached to a gold electrode, Ricci et al. [14] characterised how signalling properties, specificity, and response time of such systems depended on probe surface density, target length, and other aspects of interfacial molecular crowding. The highest signal suppression (i.e., the highest sensitivity) was obtained with the highest probe density investigated; however, the response time also increased at high probe coverages. The kinetics of the redox process was further investigated using ac voltammetry. This allowed to suggest that the signalling mechanism of electrochemical hairpins might essentially rely on hybridisation-induced changes in the rate with which the redox moiety collides with the electrode and transmits electrons.

Most of the methods based on the use of redox-labelled hairpin probes typically rely on a “signal-off” mechanism. Thus, they suffer from the need to measure small variations (diminutions) of the signal, when the background value is the highest possible. With the aim to overcome such a key drawback of hairpin probes, Grinstaff and co-workers [32] developed an alternative sensing scheme in which a thiol-tethered capture probe and a ferrocene-labelled reporter sequence were interconnected through a flexible poly(ethylene glycol) spacer. This system exploited the conformational change that occurred when the surface-immobilised triblock assembly bound to the target strand. When the macromolecular assembly and the mercaptopropionic acid spacer were co-immobilised onto the gold electrode, the 5′-terminal redox label was electrochemically inaccessible because electrostatically repelled far from the anionic surface. Upon binding of the target DNA, folding of the oligo-PEG-oligo macromolecule decreased the distance between the ferrocene moiety and the electrode, thus affording an electrochemical signal.

5.1.3. Polypyrrole electrochemistry

Garnier et al. achieved reagentless, label-free and real-time electrochemical sensing of DNA sequences using capture oligonucleotides covalently immobilised onto an electropolymerised thin film of polypyrrole [39]. Such a conjugated polymer film possessed an electrochemical signature which underwent a modification upon hybridisation of the grafted probes with their complementary targets. This effect was related to the increase of steric hindrance which followed hybridisation at each probe immobilisation site. Because of the bulkiness of the hybridised DNA pending along the polypyrrole chains, the structural reorganisation of the macromolecular polymer (which normally imposes the complete planarity of the whole pyrrole system) was forbidden. This was reflected into a modification of the electrochemical properties of the polymer film, thus offering direct measurement of the hybridisation kinetics. Interestingly, the authors showed that the quantity of probes immobilised onto the polypyrrole governed the detection threshold of the method. By tuning the amount of the surface-attached capture oligonucleotides to the quantity of target present in solution, a detection limit of about 1 pM was obtained.

5.1.4. Alternative approaches in QCM sensing

Till recently, most of QCM studies have been conducted using continuous resonance devices. However, in the past few years discontinuous resonance methodologies, mostly the QCM-D technique, have shown their potential for monitoring the hybridisation reaction. In comparison with traditional variants, the key feature of QCM-D technique is that, in addition to changes in resonant frequency, \( f \), it also provides simultaneous measurement of energy dissipation, \( D \), induced by interfacial reactions [63]. If, on one hand, the resonance frequency \( f \) is related to the mass \( m \) bound at the sensor surface, on the other the energy dissipation \( D \) depends on the viscoelastic properties of the interface.

The measurement of the energy dissipation (the so-called \( D \) factor) has become popular to indicate the energy loss of the viscous (immobilised) bio-layer against the total vibrational energy of the QCM plate. The \( D \) factor has been reported to indicate the energy loss between viscous molecules and the media in cases of heterogeneous DNA hybridisation [51], adsorption and subsequent structural modification of large vesicles [64] and proteins [65]. The information contained in combined energy dissipation and frequency measurements has an added value since it gives much richer information than a mass measurement alone. Of particular value is that structural changes of surface-bound molecules (e.g., upon antigen–antibody binding or vesicle decomposition into a supported lipid bilayer) yield signals that, on the basis of existing theories, can be converted into well-known quantities such as film thickness, shear viscosity and elastic modulus of the adlayers [66].

Use of QCM-D to study nucleic acid interactions and to compare different immobilisation procedures and probes (i.e., DNA and PNA probes) has been reported by Höök et al. [66]. Combined measurements of dissipation energy and frequency changes by using a 27-MHz piezoelectric quartz crystal have also been recently reported for monitoring conformational changes of proteins, single-stranded and double-stranded DNAs [67].

5.2. Label-free methods (solution-phase metal complexes and organic dyes)

Indicators such as cationic metal complexes [e.g., \( \text{Ru(NH}_3\text{)}_6^{3+} \), \( \text{Fe(CN)}_6^{3-/4-} \), Co(phen)$_2^{3+}$ and Ru(bpy)$_3^{2+}$], or organic compounds, like Hoechst 33258, daunomycin and methylene blue, recognise the hybridised DNA binding to the grooves of the helix, selectively oxidising the guanine moiety, intercalating selectively and reversibly into the double-stranded DNA. Being their use for electrochemical sensing investigated since the pioneering work of Millan and Mikkelsen in the early 1990s [68], most of them have already been comprehensively described in Ref. [6] and other reviews. Research on these classes of compounds is, however, still open, with recognition mechanisms spanning from the simple yet effective electrostatic attraction/repulsion to the more complex intercalation, in simple and threading mode.
Among the different electrochemical techniques, electrochemical impedance spectroscopy (EIS) has shown to be an effective and sensitive tool for the characterization of bio-functionalised electrodes and for monitoring interfacial biocatalytic reactions [53]. Knoll and co-workers [16] employed such a powerful technique for in situ characterisation of PNA/DNA hybridisation kinetics. Impedimetric sensing relied on the dramatic change of electrical charge at the electrode surface that resulted from hybridisation of the polyanionic target DNA to the neutral PNA probe. Buffered solutions containing both the unmodified target and the anionic redox indicator Fe(CN)$_6^{3-}$/$^{4-}$ were interacted with the PNA/MCH modified electrodes. As hybridisation proceeded, increasing amounts of the negatively charged target were specifically accumulated at the electrode surface. The resulting electrostatic repulsion between the redox-active molecules and the polyanionic backbone of the targets thus led to increasing charge transfer resistance ($R_C$) values. By recording the change of $R_C$ with time, EIS allowed in situ characterisation of the hybridisation kinetics of PNA/DNA duplexes. Association and dissociation kinetics of a fully matched and a single-base mismatched duplex were obtained.

Steichen et al. [17] also used a 16-mer PNA probe and ac faradic admittance for monitoring the hybridisation reaction. In contrast to Knoll’s paper, these authors used the highly charged Ru(NH$_3$)$_6^{3+}$ cation as the redox probe. Transduction thus relied on the evaluation of the amount of Ru(NH$_3$)$_6^{3+}$ electrostatically associated to the anionic backbone of the hybridised target DNA. When compared to an analogous DNA probe (also 16-mer), the PNA exhibited higher levels of selectivity against a single-base mutant target. Analysis of two PCR-amplified sequences from the 23S rRNA gene of H. pylori (100 and 400 bp fragments, respectively) evidenced a higher sensitivity for the longer amplicon, as it possessed much more binding sites (phosphate groups) for the Ru complex.

Following an analogous strategy, Kerman et al. [15] used an 11-mer PNA probe immobilised onto a GCE and the Co(NH$_3$)$_6^{3+}$ redox probe to detect a single-base mutation within the gene encoding the human alcohol dehydrogenase (ALDH). The selectivity of the electrochemical method directly derived from the selectivity of the PCR protocol used to amplify the real samples. Selective amplification of either the wild-type or mutant ALDH target was obtained by means of a “PCR clamping” strategy. This method was based on the ability of PNA/DNA complexes to block the formation of a PCR product when a PNA (also introduced into the PCR mix) was targeted against one of the PCR primer sites. Interestingly, this blockage event allowed selective amplification-suppression of target sequences that differed by only one base pair. Once generated, the double-stranded amplicons were thermally denatured and then interacted with the PNA capture probe immobilised at the surface of a GCE. After accumulation of the Co(NH$_3$)$_6^{3+}$ redox probe, differential pulse voltammetry was used for the electrochemical interrogation of the surface.

Gorodetsky and Barton demonstrated that reduction of DNA-bound intercalators (e.g., methylene blue) at highly oriented pyrolytic graphite electrodes (HOPG) modified with pyrene-functionalised duplexes also proceeded being mediated by the DNA helix [20]. The signal from the intercalator was attenuated in the presence of the single-base mismatches CA and GT. As on gold, this sensitivity to single-base mismatches was enhanced when methylene blue reduction was coupled in an electrocatalytic cycle with ferricyanide. The extended potential range afforded by the HOPG surface also allowed to investigate the electrochemistry of previously inaccessible metallointercalators [Ru(bpy)$_3$dpz$^{2+}$ and Os(phen)$_2$dpz$^{2+}$] which proved to be suitable labels for DNA-mediated charge transfer reactions.

Following the concept of long-range charge transfer through a DNA duplex developed by Barton’s group, Wong and Gooding [33] developed an improved and real-time electrochemical genoassay to detect single-base pair mismatches. Gooding’s approach overcame the one limitation of Barton’s technology, namely the need to create closely packed arrays of DNA molecules to ensure the electrochemical response was due to charge transfer through the double helix only. The DNA recognition interface consisted of a mixed monolayer of loosely packed thiolated oligonucleotide probes and mercaptohexanol immobilised onto a gold electrode surface. Such a design of the sensing layer thus ensured a higher hybridisation efficiency at the transducer–solution interface. An anionic intercalator, anthraquinonemounsulfonic acid (AQMS), was also selected. Hence, the electrostatic repulsion between the anionic AQMS molecules and the hydroxyl-terminated MCH layer limited the direct interaction of the redox reporter with the metal surface. In situ and real-time monitoring of the hybridisation events was allowed, as AQMS and the targets (perfectly matched and mismatched) coexisted within the solution hybridised at the electrode surface. Interestingly, the electrochemical behaviour AQMS changed according to its surrounding environment, with the freely diffusing compound being reduced at lower potentials than the intercalated one. This shift in the reduction potential of AQMS actually provided the means for monitoring the hybridisation event in situ (as no washing steps were required) and in real-time. Confirming the good selectivity of the assays based on long-range charge transfer, such an improved detection scheme allowed clear differentiation of the perfectly matched target from C-A or G-A mismatched sequences. Compared to previous works, the operational simplicity of this in situ assay constituted the major advantage.

Tansil et al. [69] described synthesis, characterisation, and analytical applications of a novel imidazole-substituted naphthalene diimide (PIND) threading intercalator functionalised with electrocatalytic redox moieties. Two Os(bpy)$_3$Cl$_2$ complexes were grafted onto each PIND unit through coordinative bonds with the imidazole groups, thus yielding a [Os(bpy)$_3$Cl]–PIND–[Os(bpy)$_3$Cl]– compound (PIND-Os). Interestingly, the naphthalene diimide bound to the double helix in a “classical” threading intercalation mode, while the two Os(bpy)$_3$Cl$^+$ pendants interacted with DNA via electrostatic interaction, thus reinforcing the intercalation by “locking up” the naphthalene diimide group in place. The experimental results suggested that PIND-Os was highly selective to ds-DNA, while presenting a higher binding constant with respect to its parent compound PIND. The combination of the Os-labelled threading intercalator with a sacrificial electron donor (ascorbic acid) generated highly enhanced electrocatalytic currents, thus providing a platform for ultrasensitive detection of DNA.
5.3. Label-based methods

Shen and co-workers [70] developed a novel strategy to detect single-point mutations by combining a DNA ligase assay with a reverse molecular beacon concept. A detection probe containing a phosphoryl group at the 5′-end, a ferrocene tag at the 3′-end, and a six-base sequence close to the 3′-end complementary to the 5′-end region of the thiol-tethered capture probe was used. After “sandwiching” the target DNA between the surface-immobilised capture probe and the detection probe, a ligation reaction was carried out in the presence of *E. coli* DNA ligase. As a result of the subsequent thermal denaturation (which dissociated the target sequence from the surface), the ligated assembly assumed a molecular beacon-like hairpin structure which brought the ferrocene label in closer proximity to the electrode surface. By this method, the target DNA could be determined down to the picomolar range. Additionally, because of the high fidelity of *E. coli* ligase, the genosensor exhibited excellent capability to discriminate single-base mutant targets. In contrast to existing methods based on the conformational change of redox-labelled oligonucleotides, Shen’s strategy offered several substantial advantages, such as negligible background current and “signal-on” mechanism.

Flechsig and Reske extended the simple and effective technology developed by Palecek and co-workers for labelling thymine-rich sequences to develop a heterogeneous hybridisation assay [36]. Specifically, the method relied on the covalent modification of the target sequence using [OsO₄(bipy)]. This complex preferentially reacted with thymine bases forming a diester of osmium(VI) acid through the oxidation of the C=C double bond of the pyrimidine ring. Interestingly, the reaction of the osmium tetroxide complex was strand-selective, proceeding at a reasonable rate only when the oligos were in their single-stranded form. The Os-modified strands are known to lose their hybridisation capability. However, with the simple requirement of masking the hybridisation sites of the sequences using short “protective” oligonucleotides, this method allowed convenient and easy synthesis of redox-labelled strands.

Interesting aspects of this approach are as follows. The labelling reaction results particularly effective when the sequences are provided of a non-hybridising poly-T tail, and multiple redox markers can be simultaneously introduced. Additionally, if compared to other metal complexes (e.g., ferrocene derivatives), one can take advantage of the two electrons reaction of the reversible osmium couple [Os(VI/IV)] to further improve the sensitivity of the assay. As the last consideration, the redox potential of the Os label is extremely facile to be tuned, by simply changing the organic ligand into the complex. This strategy thus offers the possibility to develop a series of redox-labelled signalling probes each with a different formal potential and, therefore, suitable for use in a multiplexed assay.

5.4. Signal amplification

One of the limiting factors for the development of DNA sensors and arrays is the sensitivity. When the specific target gene is present as a single copy in the organism genome, the amount of DNA that has to be detected is, in fact, at the attomolar to femtomolar level. To date, as the possibility to directly sense such low concentrations via heterogeneous hybridisation to surface-immobilised probes has not reliably achieved (see Section 4.1.1 for more details), PCR amplification partially solves the problem, increasing the amount of target DNA to be identified. Intense research efforts are, nevertheless, devoted to further improve the sensitivity of the analytical protocols. Interestingly, the most efficient signal amplification paths are now approaching sub-femtomolar detection limits. The successful use of these ultrasensitive bioelectronic detection schemes requires, however, proper attention to non-specific adsorption issues, as they commonly control the sensitivity of such bioaffinity assays [5,71].

5.4.1. Nanoparticles

Use of nanoparticle labels has proved to be particularly advantageous, due to the fact that single biorecognition events are typically translated into the specific accumulation of a multitude of detectable atoms (or ions) at the electrode surface. Gothelf’s group described a multiplexed metal sulphide nanoparticle-based electrochemical detection scheme that provided sensitivities down to the femtomolar level [72]. Semiconductor Cds, ZnS, and PbS nanoparticles were synthesised and then conjugated with distinct 5′-thiolated reporter sequences. A competitive hybridisation assay was subsequently adopted. The oligonucleotide-conjugated nanoparticles were first hybridised to specific capture probes arranged on the surface of a gold electrode. Addition of a defined target sequence (which acted as a competitor, being complementary to one of the oligos used for labelling the nanoparticles) led to selective dissociation of the corresponding metal sulphide from the surface. Transduction of the biorecognition event was finally accomplished by means of acidic dissolution of the remaining nanoparticles and subsequent detection of the metal signals by anodic stripping voltammetry. Compared to previously reported sandwiched assays, the competitive assay described by Gothelf and co-workers was, in principle, reagentless. However, the long time required for competition (5–6 h) discourages routine use of such an analytical protocol.

5.4.2. Enzymes

The successful and widespread use of enzymes as labels in affinity bioassays is also essentially due to their ability to convert single hybridisation events into a multitude of detectable molecules. Enzymes of choice are usually alkaline phosphatase (AP), horseradish peroxidase (HRP) and oxidases (such as glucose oxidase [GOx]), all having the common advantage of being relatively stable, cheap and to possess generally high turnover rates. Such enzymes are typically used as avidin (or related proteins) conjugates and are, therefore, coupled to the duplex (that must be biotinylated, for example using a biotin-labelled signalling probe) by taking advantage of the avidin–biotin affinity reaction. The most common enzymes are commercialised as avidin, streptavidin, neutravidin or extravidin conjugates. Hence, the selection of the most suited for a given assay must be done carefully. For example, when comparing avidin–alkaline phosphatase and streptavidin–alkaline phosphatase conjugates, Carpini et
al. [73] found the former to give much higher levels of non-specific signals, due to the higher isoelectric point of avidin compared to streptavidin.

Besides these commercially available enzymatic conjugates (which are exceptionally versatile, easy to use and relatively cheap), direct use of oligonucleotide-enzyme conjugates has also been reported. The synthetic pathway described by Dominguez et al. [38] for labelling of GOx with synthetic oligonucleotides relied on the oxidation of the glycosidic residues of the enzyme and their covalent binding with 5′-amino-terminated oligos. Upon use of such GOx-conjugated signalling probes in a sandwich hybridisation assay and addition of glucose, the H2O2 generated by the enzymatic reaction readily diffused through a multilayer composed by an Os-based redox polymer and HRP sandwiched onto a gold electrode. The resulting electrocatalytic current (evaluated by amperometry) was used as the analytical signal.

Possible drawbacks of these oligonucleotide–GOx conjugates included lower hybridisation kinetics and labour-intensive synthesis and purification. However, this way of labelling the reporter probe coupled with use of different oxidases could represent a route for designing affinity-based multidetection systems with no need for spatial resolution. Also, their potential for use as building blocks to assemble more sensitive analytical devices will be subsequently described.

Metfies et al. reported the detection of the toxic algae A. ostenfeldii, responsible for shellfish poisoning, using a digoxygenin/anti-digoxygenin–HRP conjugate labelling scheme [9]. Algal rRNA was detected by sandwich-hybridisation in the presence of a digoxygenin-labelled reporter oligonucleotide. Labelling with horseradish peroxidase was achieved using a HRP-conjugated anti-digoxygenin antibody. Catalysing the reduction of hydrogen peroxide to water, the enzyme was first oxidised. The reduced form of HRP was, however, readily regenerated by p-aminodiphenylamin, which acted as a mediator. Reduction of the oxidised mediator at −150 mV vs. Ag/AgCl provided the electrochemical readout.

Miranda-Castro et al. exploited the features of a surface-immobilised hairpin probe for the selective identification of Legionella pneumophila sequences [13]. A thiolated hairpin, immobilised onto a gold electrode surface, was combined with a sandwich-type hybridisation assay in the presence of a biotinylated signalling probe and streptavidin–alkaline phosphatase as the reporter enzyme. The sensor allowed selective discrimination between L. pneumophila and L. longbeachae (52-mer sequences) with a detection limit of 340 pM of L. pneumophila DNA. Direct comparison of the analytical performance of this assay with that of an analogous test based on the use of linear oligonucleotide probes, clearly demonstrated the superior sensitivity and selectivity of the hairpin oligonucleotide.

5.4.3. Enzyme multilabelling (dendritic-like architectures)
Traditionally, a single enzyme label accounted for each hybridisation event. However, more sensitive detection concepts exploited the transduction of the hybridisation reaction through multi-labelling strategies.

The basic idea behind one of such approaches is to take advantage from the considerable length of the real samples (PCR products) to introduce multiple labels onto the target sequence [35,74]. In Del Giallo et al. binding of more than one biotinylated oligonucleotide per target sequence offered multiple anchoring points for a streptavidin–alkaline phosphatase conjugate. Simultaneous use of two biotinylated probes led to +82% enhanced signals, while a +126% (relative to the case of a single signalling probe) was observed when using three biotin-labelled sequences. Such a non-linear growth of the signal with the number of labels was explained as a consequence of the steric hindrance of streptavidin–alkaline phosphatase, the footprint of which is in the order of 134 nm². The steric and electrostatic interference from the conjugates bound to neighbouring duplexes was likely to further inhibit the simultaneous binding of three enzymatic labels per target strand.

Each of the oligonucleotide-conjugated GOx labels developed by Dominguez et al. [38] carried an average of three oligos. The authors thus suggested that, through successive rounds of hybridisation and labelling, the resulting enzyme-rich architectures could provide a means to significantly enhance the sensitivity of their genoassay. This attractive concept was, however, only partially demonstrated, hybridising a biotinylated sequence onto the primary GOx-oligonucleotide label and using an avidin-modified GOx as the secondary enzymatic conjugate.

Mascini and co-workers [71] also described a genosensing scheme based on a dendritic-like signal amplification path. The analytical strategy relied on labelling the biotinylated hybrid obtained at the electrode surface by alternate exposure to streptavidin and biotinylated alkaline phosphatase, thus self-assembling nanoarchitectures rich in enzyme labels. Compared to the commercially available streptavidin–alkaline phosphatase conjugates, a single generation of the streptavidin–biotinylated alkaline phosphatase assembly readily allowed a 15–20-fold enhancement of the electroanalytical signals. Interestingly, after the first binding event, each biotinylated alkaline phosphatase still possessed several free biotins that could efficiently cross-link a second “layer” of streptavidin. By sequentially repeating such a self-assembling process, one could in principle generate dendritic-like nanoarchitectures with the desired content of alkaline phosphatase labels. Indeed, the experimental results demonstrated that the response increased linearly with the number of protein–enzyme generations, confirming that even the α-naphthol produced by alkaline phosphatase molecules bound on the top of the network could easily diffuse through the protein layers becoming electrochemically accessible.

5.4.4. Ferrocene–streptavidin conjugates
Knoll and co-workers [75] employed a modified streptavidin (Strept) labelled with multiple ferrocene (Fc) units to amplify electrochemical and SPR response of short biotinylated sequences. Interaction of the surface-immobilised PNA probes with the biotinylated targets resulted in hybrid duplex formation. Subsequent labelling with the Fc-conjugated Strept, allowed SPR and electrochemical monitoring of the hybridisation events. The amount of hybridised target was estimated by cyclic voltammetry, chronocoulometry and square wave voltammetry. Due to the long linker between the Fc groups and the Strept (six glycine arms), electron transfer between the metal complex and the underlying gold electrode occurred...
efficiently. Additionally, each Strept molecule carried at least nine Fc moieties. As a result, a detection limit of 10 pM was observed for the 12-mer oligonucleotide target.

5.4.5. Polymeric osmium complex

Liu and Anzai [76] developed a high performance redox indicator based on a polymeric chain bearing numerous Os complexes. When interacted with the surface-immobilised duplex, the poly(4-vinylpyridine) labelled with [Os(5,6-dimphenyl)2Cl]2−, exhibited 1000 times higher sensitivity than the monomeric analogue, [Os(5,6-dimphenyl)3]2+. This was explained as a probable consequence of the polymeric structure of the indicator. Unlike monomeric labels, intercalation of the first Os complex into the double helix facilitated the interaction of other complexes adjacent in the same polymeric chain. Since about 120 Os residues were connected to a single polymer, the improved sensitivity allowed detection of as few as 1 pM of a 25-mer synthetic target.

5.4.6. Oligonucleotide-loaded gold nanoparticles and [Ru(NH3)6]3+

Following the concepts based on the quantitation of the [Ru(NH3)6]3+ electrostatically associated to the anionic backbone of DNA, Fan and co-workers [77] described a novel approach which exploited the signal amplification feature of gold nanoparticles (AuNPs). The DNA sensor was based on a “sandwich” detection strategy, which involved hybridisation of the target sequence between the surface-immobilised capture probe and a reporter sequence-loaded gold nanoparticle. Hybridisation thus brought the AuNPs proximal to the electrode surface. Since a single nanoparticle carried hundreds of reporter oligonucleotides, the chronocoulometric interrogation of [Ru(NH3)6]3+ allowed amplification of the target signal by two to three orders of magnitude. As a result, the genosensor could detect as low as femtomolar concentrations of the target. Additionally, because of the sharp melting profiles of such nanoparticle-loaded sequences in low ionic strength buffers, an excellent selectivity against single-base mismatches was demonstrated.

5.4.7. Enhancement of gold nanoparticles by electrocatalytic deposition of silver

Yeung et al. demonstrated the multiplexed detection of E. coli and B. subtilis cells implementing sample preparation, DNA amplification and electrochemical detection in a single-microdevice [25]. Detection of the asymmetric PCR amplification products was accomplished by labelling the targets with streptavidin-conjugated gold nanoparticles (5 nm), electrocatalytic deposition of silver (from a AgNO3 solution) and chronopotentiometric quantitation of Ag at ITO electrodes. As few as 100 cells sample−1 could be detected using such an integrated DNA biochip.

5.4.8. Bio-metallization

Hwang et al. [30] demonstrated the amplified detection of a target DNA through the bio-catalysed deposition of silver (bio-metallization). In this method, the target DNA and a biotinylated detection probe hybridised to a 16-mer capture probe tethered onto a gold electrode. Upon binding of a neutravidin-conjugated alkaline phosphatase label, the non-electroactive substrate of the enzyme, p-aminophenyl phosphate, was converted into a reducing agent, p-aminophenol. The latter, in turn, reduced Ag+ ions also present in the enzymatic substrate solution, thus leading to deposition of the metal onto the electrode surface and the DNA backbone. Linear sweep voltammetry was finally used for stripping the accumulated silver, with the corresponding current taken as the analytical signal.

In contrast to previously developed methods where the sensitivity depended on the size of the metal nanoparticle tracer, the sensitivity of this protocol essentially depended on the condition of the enzymatic reaction only. As a result, the stripping signal of the metal was amplified by the enzyme until alkaline phosphatase was completely buried into the precipitated silver. The bio-metallization protocol also took advantage from detecting the signal of a compound which did not diffuse away from the electrode surface but, rather, continuously accumulated onto the sensing layer as the reaction proceeded. This analytical strategy, coupled with careful design of the capture oligonucleotide, allowed sensitive and selective detection of as few as 100 aM (10 zmol) of the 31-mer perfectly matched target, while efficiently discriminating a single A-C mismatch.

5.4.9. Streptavidin-conjugated nanoparticles for amplified piezoelectric sensing

Mao et al. [78] described a QCM biosensing strategy based on the use of streptavidin-conjugated Fe3O4 nanoparticles labels. A thiolated probe specific for E. coli 0157:H7 eaeA gene was hybridised with the biotinylated target (both a synthetic oligonucleotide and a PCR product). The amplification features of the streptavidin-conjugated nanoparticles (average diameter = 145 nm) allowed detection of as low as 2.7 × 102 colony forming units mL−1 of E. coli 0157:H7 cells.

5.5. Commercially available tools

In the past few years the most promising electrochemical detection concepts have also been implemented into commercially available genosensing platforms. Their features will be discussed in the following paragraphs. While some of these products were not successful and rapidly disappeared, some other are likely to play a significant role in the future molecular diagnostics market.

5.5.1. Electrocatalytic oxidation of guanine

Oxidation of guanine residues using Ru(bpy)32+ as the mediator provided a sensitive and simple method for detecting unmodified nucleic acids at ITO electrodes. Originally developed by Armistead and Thorp [23], this concept involved use of immobilised synthetic oligonucleotide probes in which guanine was substituted with the non-physiological base inosine, in order to minimize the background signal. After hybridisation of the sample and an appropriate washing step, ruthenium tris(2,2’-bipyridine) was added and cyclic voltammetric scans performed. In the absence of surface-confined guanines (i.e., when the capture oligonucleotide did not find its complementary sequence in solution), each mediator molecule was oxidised only once. On the contrary, when the guanine-containing target was hybridised at the surface,
the oxidised form of the metal complex was able to abstract an electron from such purine moieties, thus regenerating the Ru(II) form. The regenerated reduced mediator was again oxidised at the electrode, thus completing a catalytic cycle (Fig. 6).

This technology, implemented in an array of 96 working electrodes (200-μm diameter) at Xanthon Inc., was supposed to be commercialised with the name of Xanthon Xpression Analysis System. Unfortunately, for a number of economical and technical reasons (including difficulties in manufacturing and modifying the ITO chips by the DNA probes) the company has not survived. The technology was subsequently acquired by Motorola.

5.5.2. DNA-mediated charge transport

The ability of the DNA duplex to conduct electrical currents was first exploited by Barton and co-workers [79], who developed an elegant electrochemical assay format to detect single-nucleotide polymorphisms. Chips of gold electrodes were used as platform for the covalent immobilisation of synthetic oligonucleotide probes corresponding to mutational “hot spots”. After hybridisation with clinically relevant samples, the redox-active intercalator methylene blue (MB), was interacted with the surface-tethered hybrids along with ferricyanide. Upon applying a negative potential, the intercalated MB underwent electrochemical reduction. The original form of the intercalator was then re-generated (by-oxidation) by the solution-phase ferricyanide, thus entering a catalytic cycle (Fig. 7). Interestingly, a mismatch located within the immobilised duplexes caused a striking decrease in the electrochemical accessibility of the redox-active intercalator. Mismatch detection could be accomplished irrespectively of DNA sequence and mismatch identity, being depended on electronic coupling within the base pair stack, rather than the thermodynamics of base pairing.

GeneOhm Sciences Inc. implemented such an elegant principle of detection as the core technology of a microchip-based platform (GeneOhm’s ePlex™ electrochemical array). However, according to our recent visit to GeneOhm’s website (www.bd.com/geneohm/english/), electrochemical genosensing is not in the products list anymore.

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**Fig. 6 – Electrocatalytic oxidation of guanine by means of Ru(bpy)₃²⁺:** (A) simple reaction in the presence of the inosine-modified probe only; (B) electrocatalytic cycle in the presence of the hybridised (guanine-containing) target. Adapted from Ref. [23].

**Fig. 7 – Exploiting DNA-mediated charge transport for the detection of single-base mismatches.** (A) Perfectly matched duplex. When applying a negative potential, the intercalated MB underwent electrochemical reduction, generating leucomethylene blue (LB). MB was then re-generated through the chemical oxidation of LB by the solution-phase ferricyanide. (B) Mismatched duplex. Any mismatch located within the immobilised duplexes inhibited the whole process, being the first electro-reduction step impeded. Adapted from Ref. [79].
5.5.4. Minor groove binder (Genelyzer™ eBiochip Systems GmbH (Germany)).

Besides simple enzymatic, dendritic and nanoparticle-based amplification routes, further amplification of the analytical signal can be obtained by pure electrochemistry, e.g., redox cycling the product of an enzymatic reaction (Fig. 4). Based on this concept, Elsholz et al. demonstrated the species-specific identification and quantitation of 16S rRNAs of five pathogens using a low-density electrical microarray [35]. Recognition of the rRNA samples was accomplished through a sandwich hybridisation scheme in the presence of biotinylated signalling probes, followed by labelling of the surface-confined hybrid with an extravidin–alkaline phosphatase conjugate. The enzymatic hydrolysis of the electro-inactive $p$-aminophenyl phosphate ($p$-APP) produced $p$-aminophenol ($p$-AP) which was detected by amperometry using a 16 channels multipotentiotstat. Redox recycling of the $p$-AP product was obtained polarising the cathodic fingers of the interdigitated array of electrodes at $-150 \, \text{mV}$ (vs. Ag/AgCl) while the anodic ones were polarised at $+350 \, \text{mV}$. The smart design of the interlocking comb-like structures combined the advantageous features of ultramicroelectrodes (which experience enhanced lateral diffusion compared to macroscopic electrodes) with an efficient redox regeneration of the electroactive product (which underwent oxidation and reduction more than 10 times on average). A detection limit of 0.5 ng ml$^{-1}$ of unamplified total RNA from E. coli was determined using a 15 min hybridisation time.

This technology, also adapted for the identification and quantitation of proteins and haptons, is commercialised by eBiochip Systems GmbH (Germany).

5.5.5. Ferrocene-labelled signalling probes (CMS eSensor™)

In 2001 Motorola reported on CMS eSensor™ chips, low-density arrays for the bioelectronic detection of DNA sequences. This technology, now acquired by Osmetech (USA), rests on the generation of an electrochemical signal through the reversible oxidation and reduction of a ferrocene complex, used as an electrochemical label for the nucleic acid targets. eSensor™ chips have up to 36 individually addressable gold electrodes affixed to printed circuit boards. The electrodes are modified with a mixed monolayer of thiol-tethered capture probes (25-mer) and alkylthiol molecules (Fig. 8). Such a modification of the electrode surfaces not only inhibits any non-specific binding but also blocks electrochemical signals from both unbound redox labels and extraneous redox compounds. Bioelectronic detection proceeds via a sandwich hybridisation assay where the target is simultaneously bound by the capture probes on the electrode surface and the signalling probes labelled with ferrocene derivatives. The signalling probes are designed to be complementary to a portion of the target different from, but adjacent to, the region bound by the capture probe. When the target hybridises to both capture and signalling probes, the ferrocene moieties are brought into sufficient proximity to the electrode surface, thus becoming electrochemically accessible for detection. Use of ac voltammetry for interrogating the sensors allows for repeated collection of the electrons from the ferrocene labels. Hence, this detection method is not truly electrocatalytic, but does have a built-in signal amplification that results from the interrogation step [81]. The generated current is detected using an electronic detection platform called the eSensor™ DNA Detection System, which can analyze 36 electrodes at a time.

5.5.6. HRP enzyme label (CombiMatrix ElectraSense™)

A major breakthrough in electrochemical DNA biosensors has been achieved by CombiMatrix (USA) which developed arrays of 12,544 individually addressable microelectrodes and demonstrated their use for genotyping and gene expression analysis [29]. Thousand of different oligonucleotide probes, immobilised onto porous membranes, specifically captured their biotinylated targets from the hybridisation solution. After labelling of the hybrids using an extravidin–horseradish peroxidase conjugate, the enzyme catalysed the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB). Due to the close proximity of the HRP label to the Pt microelectrode surface, the oxidised TMB was readily reduced, thus generating the electrical output. All 12,544 electrodes were sequentially interrogated in approximately 25 s using a specific device (ElectraSense™ Reader). Such a fast reading time ($<2\,\text{s electrode}^{-1}$) ensured that HRP retained a high level of activity throughout measuring of the whole microarray. The performance of the ElectraSense™ platform was also compared to that of the standard fluorescent detection. Good consistency (and similar detection limits, about 1 pM) was observed between these techniques. However, the opera-
tional convenience, the lower cost and the high potential for further improvements make the ElectraSense™ platform favoured over the conventional fluorescent detection. Notably, the authors suggested that the density of spots onto the CombiMatrix chip could be realistically increased from the actual 17,778 up to $1.5 \times 10^6$ electrodes cm$^{-2}$.

6. Conclusions and future prospects

The rapid progresses of DNA-based biosensors highlighted by the elegant sensing concepts summarised by this review, suggest the major impact these technologies may have upon detection of pathogens, genetic mutations and targets of pharmacogenomic and industrial interest (e.g., transgenic crops) in the near future.

Electrochemical and piezoelectric methods thus emerge as simple, accurate and inexpensive means for a range of diagnostic applications. Electrochemical genosensing schemes appear particularly suitable to accomplish this task. Such methodologies have demonstrated the potential to allow for very low detection limits when all of the analyte is efficiently delivered to the electrode surface and the heterogeneous hybridisation reaction proceeds with reasonable yields. Having established that detection cannot presently preclude from prior PCR amplification of the sample (because of the low abundance and extreme complexity of most of the unamplified targets), other important hurdles remain. Improved probe designs and sample pre-treatments are both crucial for allowing efficient biorecognition events to occur at the transducer–solution interface. Use of synthetic DNA analogues like PNA or LNA oligonucleotides will probably lead to unprecedented levels of selectivity, crucial for a reliable screening of clinically relevant SNPs and closely related pathogenic species. Systematic use of helper oligonucleotides is also likely to provide a general and inexpensive means to minimise any interference associated to thermodynamically stable secondary structures of the target sequence. The examples of “active” (electrochemically driven) hybridisation also provide an alternative route to significantly enhance the hybridisation rates and yields, along with the selectivity of detection.

Of great significance, the outstanding technological advance afforded by CombiMatrix chips have finally answered the question of whether electrochemical detection may rival the level of parallelisation of fluorescence-based arrays. Solving the major engineering challenge of individually address thousands of electrodes at a time, such electrical detection scheme also possesses the potential to be miniaturised beyond the present limits imposed by optical readouts. This, in turn, could ultimately led to an increased number of DNA sequences that can be simultaneously interrogated beyond the present limits imposed by optical readouts. Hence, despite the versatility and advances made with optical methods for nucleic acid detection, there is reason to believe that electrochemical methods may ultimately be preferable for distributed use in the field or even in microarray applications.

REFERENCES