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Chapter 26

Thick- and thin-film DNA sensors

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26.1 INTRODUCTION

Recently, an impressive number of inventive designs of DNA-based electrochemical sensing are emerging. These types of sensors combine nucleic acid layers with electrochemical transducers to produce a biosensor and promise to provide a simple, accurate and inexpensive platform for patient diagnosis.

A wide variety of electrodes have been used as detectors or supports/detectors (genosensors) in the electrochemical DNA assays, including carbon paste, glassy carbon, graphite, graphite-epoxy composites, gold, mercury or Hg film on silver electrodes. Moreover, thick- and thin-film electrodes have been used and different DNA assays have been carried out on these substrates, either used as genosensors or detectors. The technologies used in their fabrication allow the mass production of reproducible, inexpensive and mechanically robust strip solid electrodes. Other important features that these electrodes exhibit are related to the miniaturisation of the corresponding device along with their ease of handling and manipulation in a disposable manner.

The use of films as electrodes makes possible numerous experiments that would be difficult or impractical to implement with the conventional bulk electrodes. Discussion here emphasises either ''thin'' ($<5\,\mu m$ thick; usually quite a bit thinner) or ''thick'' ($>5\,\mu m$; usually quite a bit thicker) film electrode materials, consisting of a conductor, either a continuous or a spatially patterned film, most commonly deposited on a suitably prepared insulating substrate. Films consisting primarily of insulators are not considered here, except to the extent that they may be used to form patterned arrays or electrodes with special geometries. A view of applications and properties of film

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electrodes as well as the methodologies used in their fabrication is given by Anderson and Winograd [1].

26.1.1 Genosensors on thick- and thin-film electrodes

As mentioned above, thick- and thin-film electrodes have been used for different DNA assays, either as genosensors or detectors. Here the discussion will be focused on genosensor devices based on hybridisation event; that is, the electrode is used as support/detector of the hybridisation event.

For designing a genosensor, the crucial steps are the choice of the transducer surface and the immobilisation of the single-stranded DNA (ssDNA) probes onto electrode surface. The immobilisation method will determine the sensitivity and reproducibility of the genosensor. Several strategies for the immobilisation of ssDNA have been carried out and will be discussed later. The ssDNA probe immobilised on the transducer surface recognises its complementary (target) DNA sequence *via* hybridisation. The DNA duplex is then converted into an analytical signal by the transducer. Different strategies for electrochemical detection have been performed. One of them is based on label-free electrochemical detection, *via* the intrinsic electrochemical behaviour of DNA, through guanine or adenine nucleotides. However, most of the strategies are based on the use of indicators or labels. The first ones are based on the differences in the electrochemical behaviour of indicators that interact in a distinct extension with double-strand DNA (dsDNA) and ssDNA. The indicators for hybridisation detection can be anticancer agents, organic dyes or metal complexes. The latter strategies include the use of labels such as ferrocene, enzymes or metal nanoparticles.

Before a more extended explanation of the different strategies for the immobilisation of ssDNA probe on the electrodes surfaces and the different strategies for electrochemical detection of the hybridisation event, some aspects of the fabrication of thick- and thin-film electrodes will be given in the next section.

26.1.1.1 Fabrication of film electrodes

There are numerous film-fabrication methods available, depending on the film material. The two most common materials used for the fabrication of thick- and thin-film electrodes used as support/detector in genosensor devices found in the literature are gold and carbon,

although other materials such as platinum, titanium or aluminium are used too but to a minor extent [2–5].

Gold thin-film electrodes (with different thickness ranging between 45 and 500 nm) used in genosensor devices have been mainly prepared by sputtering [6–9] and vacuum evaporation [10–15]. Sputtering method consists of an electrical discharge in a low-pressure gas such as argon at 10–100 mtorr, which is maintained between two electrodes, one of which is the material to be deposited (the target). When the target is made negative, with an accelerating voltage of 1–3 kV, it will be bombarded by energetic argon ions that transfer momentum to the material and cause ejection of atoms or ions, which deposit with relatively high velocity on the substrate.

Vacuum evaporation method is normally carried out in a high-vacuum system (10^{-5}–10^{-8} torr). The high vacuum is necessary to ensure that the mean-free-path of evaporated particles is long enough to reach the substrate, but perhaps more importantly, to maintain cleanliness of the substrate. Evaporation is most commonly initiated by melting the metal onto a conductive support, which may be a filament or boat, usually tungsten or tantalum, followed by further electrical heating of the metal to its melting point. Alternatively, higher-melting metals can be successfully evaporated using electron-beam heating techniques [11,12,14].

The most common substrates used to deposit gold are glass [6,10,12] or various forms of quartz or fused silica [7,8,11,13,14], although an other polyimide substrate named Kapton HN® [9] has been used. In many cases, adhesion of the deposited film to the substrate may be inherently poor. It is extremely desirable in such cases to deposit a thin layer of an intermediate material as titanium [6,8,12] or chromium [10,13] that has better adhesion to the substrate.

Both vacuum evaporation and sputtering methods allow obtaining continuous gold films. To obtain discontinuous films with different geometries, as gold arrays or electrodes with circular geometry with sizes on the order of 10 μm, it is necessary to combine these methods with microlithographic methods. In these methods, the device to be fabricated is coated with a photosensitive polymeric coating, which is baked and then exposed to a light source through a mask or a sequence of masks. The masks are designed using computer-aided design, and are typically produced by covering a metal film (e.g., chromium) on a high-quality glass plate with negative photoresist, exposing and developing the resist and then etching the metal to reveal the desired pattern. Masks may be positive or negative. Positive masks block light whereas

negative masks transmit light. The photoresists can also be classified as positive and negative. The positive photoresist is initially polymerised and is then depolymerised and made soluble where light strikes it, whereas the negative photoresist is initially soluble but rendered insoluble at points that have been photopolymerised by exposure to light.

With respect to thick-film electrodes, the screen-printing technology is the most common method used to fabricate genosensor devices. The electrodes obtained with this technology are usually called screen-printed electrodes (SPEs). Most of them are carbon thick film [16–35], although gold thick films are also used [36,37]. The screen-printing technology has become increasingly popular recently for the fabrication of electrodes and complete cells for applications where a disposable, one-time-use electrochemical measurement is desired. Masking patterns are photographically exposed and developed on an emulsion applied to a fine screen whose mesh is open in zones where ink (gold, carbon, silver or Ag/AgCl) is not to be applied. Pastes or inks are then forced by means of a semiflexible squeegee blade through the masking screen onto the substrate to create the film electrode assembly. The finished assembly is then treated according to the type of electrode desired. In some cases (metal electrodes), the inks are fired to form the electrode. In other cases (carbon films), the electrodes are ready as soon as the solvent has evaporated and the film has dried. The most common substrates used to fabricate these SPEs are aluminium ceramic [16–18], polyester flexible film [19–26,36,37] and polycarbonate sheet [27–31].

26.1.1.2 Strategies for immobilisation of ssDNA probe

A crucial step in the design of a genosensor is the immobilisation of the ssDNA probes onto the electrode surface. The immobilisation method will determine the sensitivity and reproducibility of the genosensor. Several strategies for the immobilisation of ssDNA have been carried out and the strategy selected to immobilise the ssDNA probe depends, in many cases, on the nature of the electrode surface. The strategies for the immobilisation of ssDNA probes on solid surfaces include adsorption at controlled potential usually on pretreated screen-printed carbon surfaces [16–22,29]. However, in these cases, the immobilised ssDNA probes are not totally accessible for hybridisation, resulting in poor hybridisation efficiency. Moreover, in these cases, no blocking step is carried out and the background signals obtained in many cases are important.

However, other systems allow obtaining a sensing phase with more strands of DNA than by direct adsorption on the electrode. Moreover,

the ssDNA probes are oriented in the genosensing phase, leaving the probes accessible for the reaction with their complementary targets. Actually, in most of the gold thin- [6,7,9,10–15,38–40] as well as thick-film electrodes [36,37], the immobilisation of the ssDNA probe is carried out by the well-known chemisorption of thiol groups on gold surfaces. In these cases, the thiolated probes, thiolated peptide nucleic acids (PNAs) [15] or an adequate thiolated primer (used to carry out PCR amplification on electrode surface [10]) form an ordered self-assembled monolayer on the gold surface. In these cases, a blocking step with a sulphur-containing compound is carried out to avoid the background signals. Other strategies are based on the formation of a polymer, through the electropolymerisation of a monomer-modified ssDNA probe [3] on the electrode surface, through the electropolymerisation of a monomer and subsequently covalent attachment of the amino modified ssDNA probe or through the copolymerisation of the monomer in the presence of ssDNA probe [2,24,25,35]. The avidin–biotin interaction to attach biotinylated ssDNA probes on the electrode surface has also been used to obtain genosensing phases [19,23,27,28,30,31,34].

An other interesting strategy is the modification of the surface of the electrodes with multiwalled carbon nanotubes (MWNTs) or single-walled carbon nanotubes (SWNTs) [13,32]. The MWNTs are grown on the electrodes covered with a nickel catalyst film by plasma-enhanced chemical vapour deposition and encapsulated in SiO_2 dielectrics with only the end exposed at the surface to form an inlaid nanoelectrode array [13]. In the other case, commercial SWNTs are deposited on SPE surface by evaporation [32]. The carbon nanotubes are functionalised with ssDNA probes by covalent attachment. This kind of modification shows a very efficient hybridisation and, moreover, the carbon nanotubes improve the analytical signal.

Finally, there are DNA arrays, whose electrical detection of hybridisation event is based on changes in electrical resistance or in capacitance between neighbouring electrodes, where the immobilisation of the ssDNA probe is carried out on the gaps between electrodes (not on the surface of the electrodes). These arrays are constructed on silicon substrate and the gaps between electrodes are functionalised using glycidosypropyltrimethoxysilane [41] or 7-octenyltrimethoxysilane [5] and then amino modified ssDNA probes are covalently attached. Other authors modified the gap between electrodes using 3-mercapto-propyltrimethoxysilane [42]. One end of this chemical compound is used to silanise the substrate surface while the thiol end is used to bind

gold nanoparticles that are bound to thiolate ssDNA capture probes. The presence of nanoparticles allows modifying the gap with ssDNA probes as well as enhancing the current between the electrodes and subsequently the sensitivity of the sensor.

26.1.1.3 Strategies followed for electrochemical detection

Different strategies for electrochemical detection have been performed. One of them is based on label-free electrochemical detection, *via* the intrinsic electrochemical behaviour of DNA, through guanine or adenine nucleotides. However, most of the strategies are based on the use of indicators or labels. The first ones are based on the differences in the electrochemical behaviour of indicators with dsDNA and ssDNA. The indicators for hybridisation detection can be anticancer agents, organic dyes or metal complexes. The latter strategies are the use of labels as ferrocene, enzymes or metal nanoparticles. Table 26.1 shows the most common methods followed to detect the hybridisation event using thick- and thin-film electrodes found in the literature.

Moreover, Fig. 26.1 displays general schemes followed to detect the hybridisation event. The most common schemes used in the detection of hybridisation event are schemes a–c. Schems d_1 and d_2 have been recently developed. The scheme used to detect the hybridisation event depends mainly on the method employed to obtain the analytical signal. Thus, scheme a is used when label-free methods, with or without indicators, are employed to obtain the analytical signal. In this scheme the unlabelled ssDNA target is hybridised with capture probe immobilised on the electrode surface and then the detection is carried out *via* guanine oxidation or *via* the oxidation or reduction of the indicator. Schemes b and c are performed when labels are used to obtain the analytical signal. In the former scheme (scheme b) the labelled ssDNA target is hybridised with capture probe immobilised on the electrode surface and then the detection is carried out in an adequate manner. For this purpose, the labelling of ssDNA target is carried out by PCR amplification using a labelled primer or by the use of labelling kits, e.g., the Universal Linkage System (ULS). In the latter scheme (scheme c), called ''sandwich'', a first hybridisation step is carried out between the unlabelled ssDNA target and capture probe immobilised on the electrode surface followed by a second hybridisation with a labelled synthetic oligonucleotide (detector probe) complementary to other region of ssDNA target (different to capture probe). Finally, the detection of the label is carried out in an adequate manner. The main advantage of this scheme is that DNA-labelling procedures are avoided.

TABLE 26.1

Methods for detection of hybridisation event based on label-free, with or without indicators, and based on different labels

Methods	Tracers/labels	Strategy	Signal	Detection limit	References
Label-free (without indicators)		Fig. 26.1a	Guanine oxidation	90 ng/mL, 3 μg/mL, 30 μg/mL, 0.15 μg/mL	[16,22,29,32]
	$Os(bpy)_3^{2+}$		Electrocatalytic 8-G or 5-U oxidation	400 fM	[10]
	$Ru(bpy)_3^{2+}$	Fig. 26.1a	Electrocatalytic guanine oxidation	1000 target molecules	[13]
Label-free (with indicators)	$Co(bpy)_3^{3+}$ (intercalator)	Fig. 26.1a	Co^{3+} reduction	50 ng/mL	[18]
	Daunomycin (intercalator)	Fig. 26.1a	Indicator oxidation	1 μg/mL	[19,20]
	FND (ferrocenyl derivate) (intercalator)	Fig. 26.1a	Ferrocene oxidation		[48]
	Methylene blue (interaction with guanines)	Fig. 26.1a	Electrocatalytic		[38]
	Methylene blue (interaction with guanines)	Fig. 26.1a	Indicator reduction	2.4 μg/mL	[21]

TABLE 26.1 (*continued*)

Methods	Tracers/labels	Strategy	Signal	Detection limit	References
	Hoescht 33258 (minor groove binder)	Fig. 26.1a	Indicator oxidation	10^4 copies/mL, 10^{11} copies/mL	[6,15]
Electroactive labels	Pt (II) complex (ULS system)	Fig. 26.1b	Electrocatalytic	0.7 nM	[31]
	Ferrocene	Fig. 26.1c	Ferrocene oxidation		[39,40]
	Ferrocene	Fig. 26.1d$_1$	Ferrocene oxidation	10 pM	[43]
	Ferrocene		Ferrocene oxidation		[44]
	Colloidal gold	Fig. 26.1b	Silver enhancement and changes in electrical resistance		[41]
	Colloidal gold	Fig. 26.1b	Silver enhancement and changes in capacitance	0.2 nM	[5]
	Colloidal gold	Fig. 26.1b	Changes in electrical resistance	1 fM	[42]
	Colloidal gold	Fig. 26.1c	Redisolution of colloidal gold in HBr/Br_2 and Au^{3+} detection		[33]

Enzymatic labels	Glucose oxidase	Fig. 26.1c	Electrocatalytic using Os complex as mediator	1, 0.5 fM	[11,12]
	Preoxidase	Fig. 26.1b	Substrate OPD	0.3 pM, 0.6 fM	[3,26]
	Preoxidase	Fig. 26.1d$_2$ and c	Electrocatalytic reduction of H_2O_2 using Os complex as mediator	200 fM	[23–25]
	Peroxidase	Fig. 26.1c and b	Substrate TMB	1 pM, 16 copies	[27,28]
	Preoxidase	Fig. 26.1c	Substrate ADPA	16 μg/mL	[34]
	Alkaline phosphatase	Fig. 26.1c	Substrate *p*-aminophenyl phosphate and reduction of Ag^+ to Ag. Oxidation peak of Ag	100 aM	[14]
	Alkaline phosphatase	Fig. 26.1b	Substrate α-naphthyl phosphate	1 nM	[36]
	Alkaline phosphatase	Fig. 26.1c	Substrate BCIP/NBT coupled with $Fe(CN)_6$	1.2 pM	[37]
	Alkaline phosphatase	Fig. 26.1b	Substrate 3-indoxyl phosphate	5, 16 pM	[9,30]

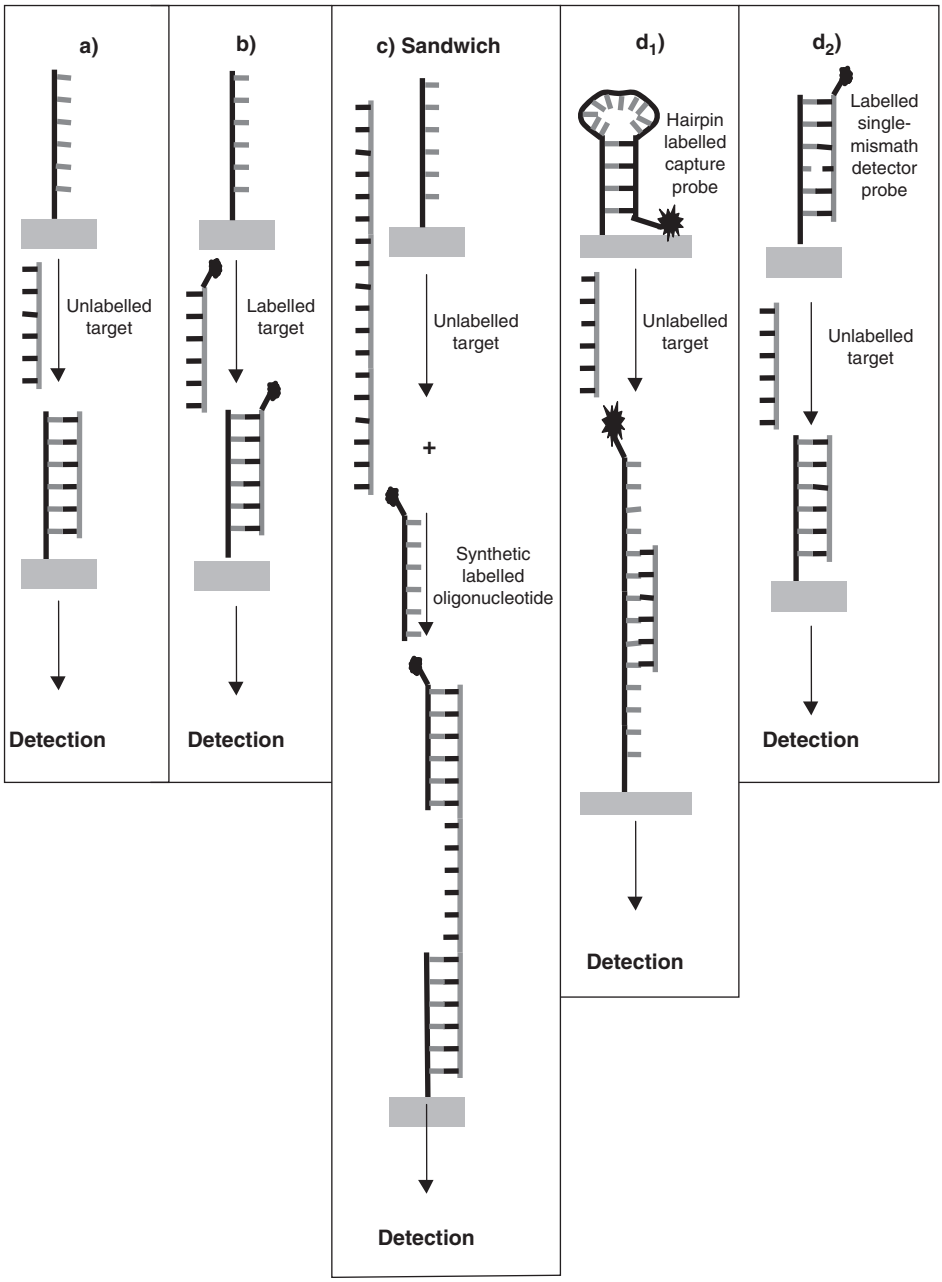

Fig. 26.1. Schemes followed to detect the hybridisation event. Scheme (a) for label-free methods with and without indicators. Schemes (b) and (c) for methods that use labels. Schemes (d_1) reagentless and (d_2) quasi-reagentless.

Finally, in schemes d_1 and d_2 the label is on the electrode surface. The first scheme is called reagentless and the second one is named quasi-reagentless. In the first one, the unlabelled ssDNA target is hybridised with a ferrocene-labelled hairpin capture probe immobilised on the electrode surface [43]. In the hairpin form, the redox-active ferrocene is close to the electrode surface and undergoes oxidation. Hybridisation of the loop region to the target disrupts the hairpin, increasing the distance from the redox-active ferrocene to the electrode surface and thereby lowering the electrochemical signal. The impressive characteristic of the response is the dynamic range, which spans six orders of magnitude of the DNA concentration. Although this broad dynamic range is useful in some applications, the sensitivity of the method (the percentage change per increment of input concentration) is correspondingly less marked, making this scheme more suitable for applications such as the analysis of single nucleotide polymorphisms (SNP) or the detection of pathogens, in which reading small changes in input concentration is less important (than it is in expression analysis).

In the second scheme (scheme d_2), the HRP labelled oligonucleotide containing one base mismatch is suboptimally prehybridised with the capture probe and is displaced upon introduction of the unlabelled fully complementary ssDNA target, producing a decrease in analytical signal [23]. This scheme is called quasi-reagentless by authors because the label used is an enzyme that needs an adequate substrate to obtain an analytical signal.

In label-free systems, the nucleobases inside dsDNA are oxidised to a lesser extent than those in the resulting ssDNA and a decrease in the analytical signal after the hybridisation should be expected. The analytical signal diminishes relative to the signal from the ssDNA probe as the duplex is formed but at the same time as new electroactive bases are added within the target, its contribution to the increase in the analytical signal must be taken into account. Because of these opposite trends, nonlinear calibration curves are often obtained. The alternative is to use guanine-free probes. In these probes guanine is substituted for inosine, which binds preferentially to cytosine and its oxidation process is far removed from that of guanine [22,29]. The signal now appears on hybridisation and background signals are negligible. An other strategy to increase the differences between analytical signals obtained for ssDNA and dsDNA based on guanine oxidation has been developed recently [32]. It consists of the use of a single-strand DNA-binding protein (SSB). This protein binds selectively to ssDNA. The guanine oxidation is hampered when ssDNA probe is bound to SSB but when

the hybridisation event is carried out, the SSB does not bind to dsDNA and the guanines oxidation is recorded. Methods based on oxidation of bases are attractive because they are label-free, rapid and the most simple (Fig. 26.1a). Oxidation of guanine at thick- and thin-film electrodes requires high-applied potentials and the associated high background currents must be subtracted to reach competitive detection limits using potentiometric stripping analysis. Another strategy employed to increase signal-to-noise ratio is the use of ruthenium or osmium complexes that act as mediators of the oxidation of guanine, increasing the rate of electron transfer between the nucleic acid and the electrode and enhancing the oxidation signal.

The use of indicators is an other common method to detect the hybridisation event. Electrochemical hybridisation indicators are electroactive compounds with different affinities for ssDNA relative to dsDNA. Three modes of binding of small molecules to DNA are normally described: electrostatic interaction, groove binding and intercalation. Because grooves are formed only within the double helix, groove-binders as Hoescht 33258 have greater affinity for dsDNA than ssDNA and thereby when hybridisation event takes places the oxidation peak of the indicator increases. Other indicators, such as daunomycin, cobalt complex and ferrocenyl-naphtalene diimide (FND), act as intercalators. Shifts in the peak potential or area of the oxidation process of daunomycin on dsDNA when compared to that obtained with ssDNA have been successfully used as electrochemical signals [19,20]. The treading intercalator FND has higher duplex affinity and binding stability and the ferrocene oxidation increases when duplex DNA is formed. With respect to methylene blue (MB), an other indicator, this compound gives lower signals for duplex DNA than those obtained for ssDNA, because the specific interaction of MB with guanine bases is prevented in the duplex and then the reduction peak of MB decreases.

The main advantage of using hybridisation indicators is that DNA-labelling procedures are avoided, but the weak point is that discrimination is not usually high enough and should be improved.

Other methods of detection of hybridisation event consist of the use of labels. These labels can be divided into electroactive and enzymatic labels. The schemes commonly used for detection are scheme b or c. The scheme selected to detect the hybridisation event depends on whether the target is labelled with a marker by PCR amplification or using a labelling kit, or whether a labelled synthetic detector oligonucleotide is used. These methods are usually the most sensitive, but the need of a labelling step makes them more complex, tedious and

expensive although the biotechnology companies already offer, at reasonable prices, oligonucleotides labelled with the most commonly used labels, including biotinylated oligonucleotides.

The electroactive labels used in the thick- and thin-film genosensors found in the literature are ferrocene, colloidal gold and platinum (II) complex. The ferrocene moiety is incorporated into a detector probe through the use of a modified adenine residue that has a ferrocene substitution on the ribose ring [39,40] or through the coupling of a ferrocene derivative (ferrocene carboxylic acid) to amino-terminated DNA molecules [44]. The reversible oxidation process of ferrocene is detected using different electrochemical techniques such as cyclic voltammetry, alternating current voltammetry or chronocoulometry.

With respect to colloidal gold, the nanoparticles are usually bound to thiol-terminated DNA molecules [33,41,42], although in other cases [5] the nanoparticles are linked indirectly through biotin–anti-biotin bridges. The detection of gold nanoparticles can be carried out by measuring the changes in the electrical resistance or capacitance between electrodes usually after a silver enhancement procedure or by anodic stripping voltammetry of the gold (III) obtained after a treatment with an oxidant Br_2/HBr mixture.

A platinum (II) complex label has been used by our group [31]. The oligonucleotide targets used are labelled using the Universal Linkage System (ULS®). This kind of labelling consists in the use of a square-planar complex of platinum (II) called BOC-ULS. One of the ends of this complex finishes in a BOC group (*tert*-butoxycarbonyl group) that can be substituted by a molecule (the label, such as fluorescein, digoxigenin, biotin, etc.). The complex ''platinum-label'', so obtained, is monofunctional, that is, the other end of the complex finishes in one Cl, through which the attachment of the complex to the oligonucleotide takes place. The Cl is substituted by the N7 position of the guanines of the oligonucleotides in a very simple labelling reaction that takes not more than 35 min. The analytical signal is obtained from platinum (II) complex, which is deposited on the electrode surface. In the presence of platinum on the electrode surface and after fixing an adequate potential in acidic medium, the protons are catalytically reduced to hydrogen. The current generated by this catalytic reduction can be measured and increases with platinum concentration and consequently with labelled target concentration following scheme b.

The methods based on the use of enzymatic labels are without doubt among the most sensitive. Moreover, because routine laboratories are usually working with enzymatic labels, this might make them the most

attractive labels for implementation of DNA diagnosis in such laboratories. The signal normally arises from a redox process of a product of the enzymatic reaction (Table 26.1). However, in other cases the analytical signal is not based on the redox process of the enzymatic product. Thus, Kwak *et al*. [14] use a biometallisation process where a nonelectroactive substrate (*p*-aminophenyl phosphate) is enzymatically converted into the reducing agent *p*-aminophenol that reduces Ag^+ ions leading to deposition of the metal onto electrode surface. In this case, the oxidation peak of deposited Ag is recorded. Mascini *et al*. [37] use the alkaline phosphatase substrate BCIP/NBT that after the enzymatic reaction generates an insoluble and insulating product on the sensing phase that blocks the electrical communication between the electrode surface and the $Fe(CN)_6^{3-/4-}$ pair. In this case, faradaic impedance spectroscopy is finally used to detect the enhanced electron-transfer resistance.

The enzymes can be linked directly to the DNA strand [3,23–25] or indirectly through biotin–avidin [9,11,12,14,26,36,37], through fluorescein–anti-fluorescein [27,28,30] or digoxigenin–anti-digoxigenin [34] bridges.

Among all enzymes, peroxidases are the most widely used and commercial kits for labelling and are already available [28].

26.1.2 Pretreatments followed with real samples

Before the DNA from real samples are tested with genosensors, the DNA must be isolated from the samples. The DNA-isolation procedures followed depend on the type of sample (tissues, blood, etc.). First of all, the DNA must be extracted from the cells, after a twice centrifugation and lysis procedure. There are several protocols for extraction of DNA. The most common are the salting out [45,46] and phenol–chloroform extraction [47], although commercial extraction kits (e.g., QIAmp Kit from Qiagen or Invisorb kit form Invitek) are used too.

After that, when the amount of DNA is very small and the sensitivity of the genosensor is not enough to detect the DNA contained in the sample, an amplification step using the polymerase chain reaction (PCR) is usually carried out. Although there are several PCR protocols such as normal PCR, asymmetric PCR (A-PCR), long PCR, reverse transcriptase PCR (more used in RNA amplification), etc., the most commonly used is normal PCR. This procedure is performed in thermocyclers, which apply a cycling temperature protocol that comprises: a denaturation step, a primer annealing step and a primer

extension step by polymerase enzyme. These steps are cycled several times.

One of the primers used in PCR amplification can be conjugated with a label if the strategy used on the genosensor device is the one explained in scheme b of Fig. 26.1.

After PCR amplification, several authors carry out a purification step of PCR products before they are tested with genosensor devices in order to diminish the PCR blanks [5,15,22,27,36,48]. For this purpose, commercial purification kits are used (e.g., Qiaquick from Qiagen). This purification step removes the primers, dNTPs, Taq polymerase and salts from the PCR products.

As DNA extracted from the sample, with or without PCR amplification step, usually is a double-strand, the hybridisation of the target strand with the capture probe immobilised on genosensor device cannot occur. Then, it is necessary to obtain ssDNA targets previously to test with genosensor device. Several strategies are followed to achieve ssDNA targets.

- *Thermal denaturation*. It is the most commonly used strategy [6,12,13,15,20–22,29,34–36,42]. It consists of denaturing the dsDNA thermally by using a boiling water bath for sometime and then immersing the tube containing the denaturalised DNA in an ice-water bath for a short time in order to retard the re-annealing of the strands. Although the denaturalisation is achieved, one drawback of this procedure is that the sister strand can compete with capture probe by target strand.
- *Asymmetric PCR*. Some authors used this procedure to obtain ssDNA targets [39,40,48]. It consists of a PCR amplification with one of the primers in excess compared with the other one. In this way, the target strand of interest is in excess compared with the sister strand, thus favouring binding at the capture probe as opposed to sister strand reannealing. When the amount of DNA from the sample is enough to detect on the genosensor device without a PCR amplification, this procedure is not used.
- *Alkaline treatment*. Some authors have used this procedure [26,27,48]. It consists of adding to DNA an alkaline solution (e.g., 0.5 M NaOH solution). An increase in pH affects the ionisation of the functional groups of bases implied in the hydrogen bonds, thus decreasing the number of these bonds between the strands. It is a rapid and simple method.

– *Lambda-exonuclease treatment*. This procedure is used by Alderon Biosciences [28]. Exonucleases are enzymes that hydrolyse the terminal phosphodiester bonds of DNA strands obtaining mononucleosides monophosphate or biphosphate. In this case a 5′-exonuclease that hydrolyses the 5′-end phosphodiester is used. In this way, the strands are destroyed. To achieve the total hydrolysis of the sister strand, the target strand must be protected, for example, by a label at its 5′-end. Therefore, the PCR amplification is carried out using one of the primers biotinylated at the 5′-end and the other one modified at its 5′-end with a phosphate group. The extension of these primers give rise to the 5′-biotinylated target DNA strand and a 5′-phosphate sister DNA strand, which is hydrolysed by 5′-exonuclease.

26.1.3 Experimental conditions for hybridisation reaction

The hybridisation event is affected by the concentration of DNA target, concentration and size of capture probe, temperature, hybridisation time and hybridisation buffer composition (pH, ionic strength, denaturalising chemical agents, etc.). The control of the experimental variables that affect the hybridisation event is very important in order to obtain an efficient and selective hybridisation. The detection of SNP or genetic mutations requires an efficient discrimination between mismatched and complementary strands. In most of the cases, the selectivity relies on the operating conditions of the assay as hybridisation buffer composition, hybridisation time and hybridisation temperature.

One way to obtain discrimination between mismatched and complementary strands is the use of high temperatures during the hybridisation reaction. The difference in the melting curves of the target from perfectly matched and mismatched capture probes can allow to define a specific temperature to discriminate perfect matches and mismatches. However, the employment of high temperatures during hybridisation step requires precise temperature control, which is difficult to achieve and is expensive [48].

A simple manner to obtain discrimination between mismatched and complementary strands is to use more stringent conditions: varying the saline concentration (ionic strength) of the hybridisation buffer or adding chemical agents that destabilise the DNA duplex as formamide or urea. The presence of ions in DNA solutions stabilises the DNA

duplex because they diminish the electrostatic repulsion between phosphate groups of the strands and therefore facilitate the approximation between both strands. If the ionic strength is decreased, only complementary hybrids can be formed. Chemical agents with amino and carbonyl groups in their structure such as formamide or urea present in the hybridisation solution compete with nucleotide bases for hydrogen bonds formation, facilitating the DNA duplex destabilisation and strands separation and achieving discrimination between mismatched and complementary strands. The addition of these chemical agents to obtain more stringent conditions and therefore the discrimination of single-mismatched strand has been used by several authors [9,30,31,36].

However, there are other methods to detect SNP where nonstringent conditions are used. One of them is the one developed by Huang *et al*. [27]. The strategy followed in this case is the use of a hairpin-forming detector probe complementary to the target combined with an unlabelled hairpin-forming competitor probe complementary to mismatched strand in a sandwich format (scheme c of Fig. 26.1). Using the hairpin-forming detector probe, the mismatched target-probe hybrids produce significantly lower signals than that generated by the perfectly matched target-probe hybrids. Moreover, the addition of a hairpin-forming competitor probe containing sequences complementary to mismatched targets increases the sensitivity and specificity of the system by decreasing the formation of mismatched target-probe hybrids.

Other method has been developed by Amano *et al*. [48] to identify genetic mutations on the lipoprotein lipase gene. They combine the use of DNA ligase enzyme and A-PCR with one of the primer (called special primer by authors) containing a Tag sequence at its 5′-end. The Tag sequence is complementary to the genomic region between the mutation point and the special primer. Since the A-PCR products contain self-complementary sequences, they form a self-loop. The capture probes are attached to the electrode surface by their 5′-end. The wild-type probe (W probe) has the same nucleotide as the wild-type genome at the 3′-end and the mutant-type probe (M probe) has the same nucleotide as the mutant-type genome. When the A-PCR product is hybridised with the W probe or the M probe, the ligase enzyme recognises the matched or unmatched nucleotide pair at the 3′-end of the probe. If the 3′-end nucleotide is complementary (or matched), the enzyme repairs the nick between the 3′-end of the probe and the 5′-end of the A-PCR product. As a result, the probe and the A-PCR product become one molecule. However, if the last nucleotide is not

complementary (or is mismatched), the enzyme does not repair the nick and the probe and the A-PCR product remain as two different molecules. After a denaturation step, if the enzyme repairs the nick, the probe and the A-PCR product are not separated and then FND (intercalator) is detected; but if the enzyme does not repair the nick, the probe and the A-PCR product are separated and then FND is not detected.

26.2 APPLICATIONS

In this section several examples of genosensors based on hybridisation event, which have been constructed on thick- and thin-film electrodes, will be described. Two of them have been designed to identify the nucleic acids determinants exclusively present on the genome of the pathogen *Streptococcus pneumoniae* [30,31], whereas an other one has been designed to detect a 30-mer SARS (severe acute respiratory syndrome) virus sequence ([9], Procedure 36 at CD accompanying this book). Although in most of them alkaline phosphatase and 3-indoxyl phosphate are used as label and as enzymatic substrate, respectively, other label, a platinum (II) complex, will be presented and its detection discussed [31]. In all cases, synthetic target oligonucleotides as well as three-base mismatch and one-base mismatch strands of the pathogen *Streptococcus pneumoniae* or SARS virus are tested using these genosensor devices. Finally, a genosensor device to detect TNFRSF21 PCR products will be presented (Procedure 37 in CD accompanying of this book).

26.2.1 Enzymatic genosensor on gold thin-films to detect a SARS virus sequence

A DNA hybridisation assay with enzymatic electrochemical detection is carried out on a 100 nm sputtered gold thin film that allows working with small volumes. Reducing the cell volume has several advantages [49]. The first one is the decrease in the diffusion distances required for analytes to reach their surface-bound receptor partners. Moreover, in the case of enzymatic detection, the product dilution, a critical factor in achieving low detection limits, diminishes. A simple, cheap and easy-to-handle homemade device that permits to perform simultaneous hybridisation procedures and sequential detection of more that 20 assay sites is presented.

The sequence chosen as target is included in the 29751-base genome of the SARS (severe acute respiratory syndrome)-associated coronavirus [50]. This is the causative agent of an outbreak of atypical pneumonia, first identified in Guangdong Province, China, that has spread to several countries. The sequence corresponds to a gene that encodes the nucleocapsid protein (422 amino acids), concretely a short lysine rich region that appears to be unique to SARS and suggestive of a nuclear localisation signal. A 30-mer oligonucleotide with bases comprises between numbers 29,218 and 29,247, both included, has been chosen.

26.2.1.1 Fabrication of gold thin-film electrodes and three-electrode potentiostatic system

A kapton slide is cleaned with ethanol and after being dried, it is covered with gold by a sputtering process. Gold atoms are deposited (from the cathode) over kapton (placed on the anode) in a vacuum chamber filled with argon. Gold layer thickness is controlled with the time and the intensity of the discharge. For a 100 nm thick layer a 35 mA discharge is applied over 220 s. Then, a conductor wire is attached to the centre of one of the sides by means of an epoxy resin (CW2400), obtained from RS Components, that is cured at room temperature.

The working area is limited by self-adhesive washers of 5 mm of internal diameter (19.6 mm^2 of internal area). The total area of the gold surface lets approximately 23 washers to stick. The gold film is placed on a support where a crocodile connection is fixed.

Reference and auxiliary electrodes are coupled in a micropipette tip. The reference electrode consists of an anodised silver wire introduced in a tip through a syringe rubber piston. The tip is filled with saturated KCl solution and contains a low-resistance liquid junction. The platinum wire that acts as auxiliary electrode is fixed with insulating tape. For measurement recording the tip is fixed on an electrochemical cell Metrohm support allowing horizontal and vertical movement (see Fig. 36.1 of Procedure 36 in CD accompanying this book).

26.2.1.2 Genosensor design

A complementary strand to the chosen SARS sequence is labelled with a thiol group and immobilised on the gold surface. The target (30-mer oligonucleotide with a sequence included in the SARS-coronavirus) is conjugated to biotin and hybridised with the probe. Addition

of AP-labelled streptavidin allows enzymatic detection through the electrochemical signal of the indigo carmine (IC), enzymatic product of 3-indoxyl phosphate (see more details for genosensor construction in Procedure in CD accompanying this book). This substrate was proposed by our group [51] as a suitable substrate based on the favourable processes that IC, a soluble derivative of the product generated (indigo blue), presented. Moreover, compared with other substrates, kinetic constants resulted more favourable. The electrochemical behaviour of IC on gold electrodes has been studied [52].

26.2.1.3 Results

Two steps, the probe immobilisation and the blocking steps, in the design of the genosensor are very important. In the immobilisation step the presence of the thiol group in the probe and the effect of the evaporation during the immobilisation process has been studied. The favourable immobilisation of DNA through its thiol group is observed in Fig. 26.2. Signals were obtained for unmodified DNA and SH-DNA (both biotinylated) following similar procedures. Although ssDNA adsorbs on gold substrates, it adopts a coiled configuration, and therefore cannot form an ordered structure [53]. An almost negligible adsorption of unmodified DNA is presented, that is very favoured by the insertion of a thiol group. Moreover same results were obtained when DNA immobilisation took place at 4°C for 12 h or at 37°C for 20 min. Moreover, the evaporation is a critical condition in the immobilisation of SH-DNA. This effect is shown in Fig. 26.3. It can be observed that the enhancement of the signal with immobilisation time was greater when

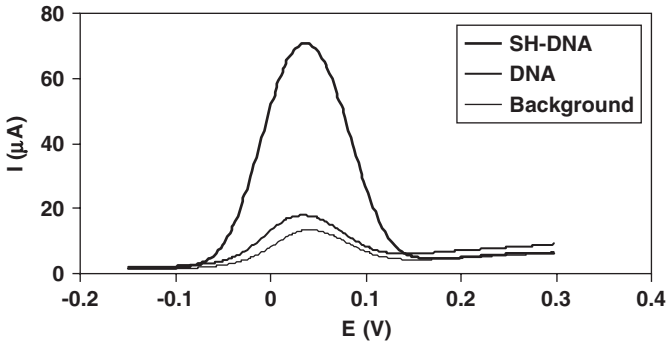

Fig. 26.2. Signals recorded for biotinylated SH-DNA, biotinylated DNA and background. $C = 1\,\mu M$, $V_{drop} = 5\,\mu L$, $T_{immob} = 4^{\circ}C$, $t_{immob} = 12\,h$. Reprinted from Ref. [9], Copyright 2005, with permission from Elsevier.

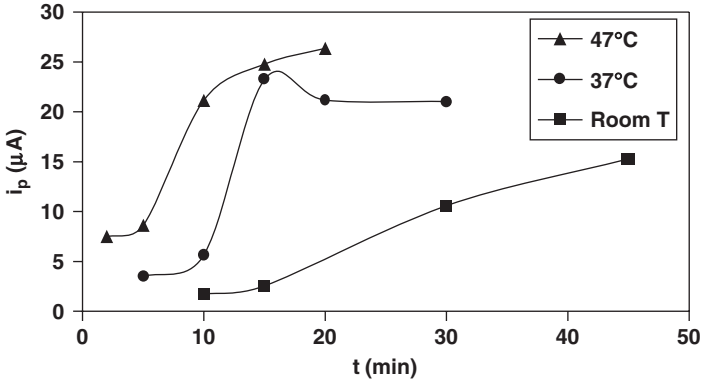

Fig. 26.3. Influence of temperature on DNA immobilisation. $C = 1\,\mu M$, $V_{drop} = 5\,\mu L$. Reprinted from Ref. [9], Copyright 2005, with permission from Elsevier.

evaporation occurred, which corresponds to 30, 15 and 10 min for room temperature, 37 and 47°C, respectively. When the immobilisation took place at 4°C overnight (12 h, attaining evaporation as well) the signal was similar to that obtained at 37°C (27 vs. 29 μA, respectively). Therefore, both methodologies could be employed. The repeatability of the signals obtained for both immobilisation procedures was also similar, obtaining an RSD of 9% and 8% for five measurements when immobilisation was carried out at 37°C for 20 min and 4°C for 12 h, respectively. Considering five different gold films, precision was analogous, with an RSD of 10%. Despite the evaporation that favours DNA adsorption, this effect is the contrary to what occurred when the hybridisation step is carried out, where the evaporation diminishes the hybridisation signal.

Blocking the surface is one of the most important steps to minimise and control non-specific adsorption. Two main types of agents were considered: proteins and sulphur-containing compounds. The signal/background ratio (S/B), and therefore the blocking capacity, was observed for each compound. Among blocking agents assayed, better results were obtained with albumin and 1-hexanethiol. A comparison between the signals obtained for background and immobilised DNA (double labelled with biotin and thiol) when BSA and 1-hexanethiol were employed as blocking agents is shown in Fig. 26.4. It can be observed that the background with albumin is negligible and the S/B ratio is high (22.4), but the DNA signal is better defined for 1-hexanethiol where the capacitively current approaches zero. Moreover, when the

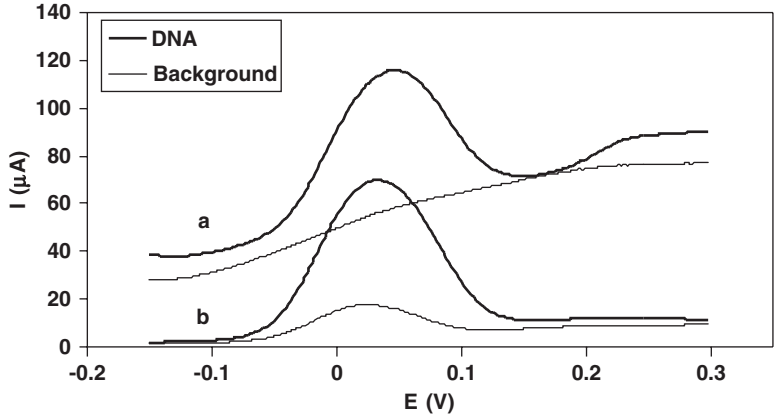

Fig. 26.4. Signals and backgrounds obtained when (a) albumin and (b) 1-hexanethiol are employed as blocking agents. $C_{\text{blocking agent}} = 2\%$, $C_{\text{DNA}} = 1\,\mu\text{M}$, $V_{\text{drop}} = 5\,\mu\text{L}$, $T_{\text{immob}} = 4^{\circ}\text{C}$, $t_{\text{immob}} = 12\,\text{h}$. Reprinted from Ref. [9], Copyright 2005, with permission from Elsevier.

hybridisation step was carried out the S/B ratio obtained for albumin was much lower than that obtained with a double-labelled DNA strand. This is probably caused by the steric hindrance, due to the large size of albumin, which hinders hybridisation.

Repeatability is checked under the experimental conditions explained in Procedure 36 in CD accompanying this book, 1 h of hybridisation and a $2 \times$ SSC buffer containing 50% of formamide. The value of the RSD was 11% for nine measurements.

A complete study on the selectivity of DNA hybridisation has been carried out. It is of relevant interest in the study of SNP as well as for the study of virus mutation. After testing many stringency conditions, it was observed that carrying out the interaction between strands in a medium containing 50% of formamide during 1 h was enough for achieving a high degree of discrimination for all the mutated strands tested.

Studies with mutated (base substitution) 30-mer synthetic oligonucleotides revealed differences for 3, 2 and 1 mismatched strands. The type of interaction that disappears and the possible new interactions that are generated when a base substitution occurs are of importance. One mismatch is detected even if it is located extreme far from the electrode, being the attenuation of the signal lower than when the substitution occurs near the electrode surface (Fig. 26.5). Moreover,

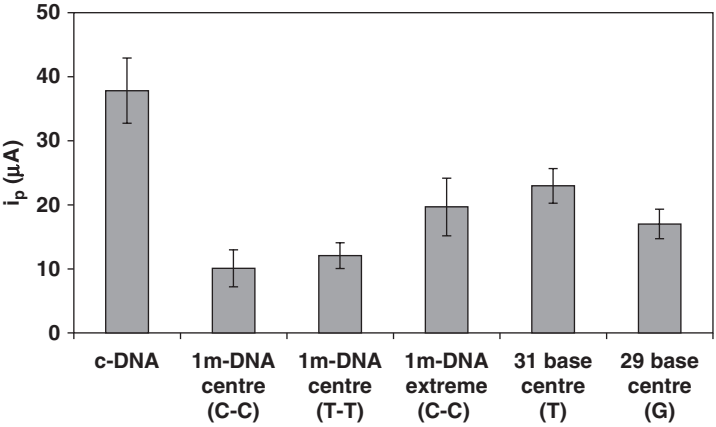

Fig. 26.5. Comparison between signals for the complementary and point mutated strands: substitutions at the centre or 3′-extreme, insertion and deletion. $C_{\text{target}} = 2.5\,\text{nM}$, $V_{\text{target}} = 20\,\mu\text{L}$, $2 \times \text{SSC}$ pH 7 buffer with 50% formamide, $t_{\text{hybr}} = 60\,\text{min}$. Data are given as average $\pm$ SD ($n = 3$).

mutations that involve deletions and insertions are differentiated from the fully complementary strand. However, although destabilisation of the duplex occurs, lower discrimination than with a substitution mutation is obtained. As a higher number of interactions is present, higher signals are obtained for the base insertion mutation. Hybridisation studies carried out between 30-mer strands were compared with those for 40-mer oligonucleotides. The length of the strand influences the hybridisation in such a way that higher signals are obtained and lower discrimination is achieved when the number of oligonucleotides increases. Experiences with crossed strands (immobilised 40-mer strand that hybridises with a 30-mer target and vice versa, hybrids between immobilised 30-mer strand and a 40-mer one) revealed the importance of an oligonucleotide tail in the immobilised strand for favouring the mobility and therefore the hybridisation. On the other hand, hybridisation is disfavoured when an immobilised strand has to react with another that contains a higher number of oligonucleotides in such a way that a rest near the electrode is present (30–40) (Fig. 26.6).

Discrimination is seen over a wide interval of concentrations (Fig. 26.7). Signals are linear for both complementary and one-base mismatched strands, between 0.01 and 1 nM. Detection limits of 5 and 70 pM were, respectively, obtained.

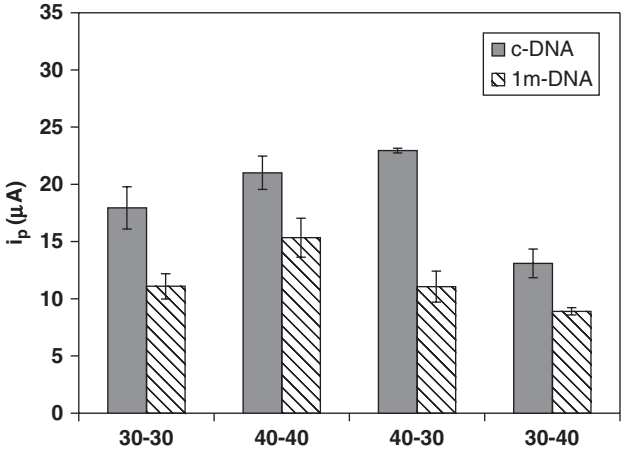

Fig. 26.6. Results obtained for immobilised and complementary (c-DNA) or one-base mismatched (1 m-DNA) target strand hybridisation, combining different lengths. $C_{\text{target}} = 2.5\,\text{nM}$, $V_{\text{target}} = 20\,\mu\text{L}$, $2 \times \text{SSC}$ pH 7 buffer with 50% formamide, $t_{\text{hybr}} = 60\,\text{min}$. Data are given as average $\pm$ SD ($n = 3$).

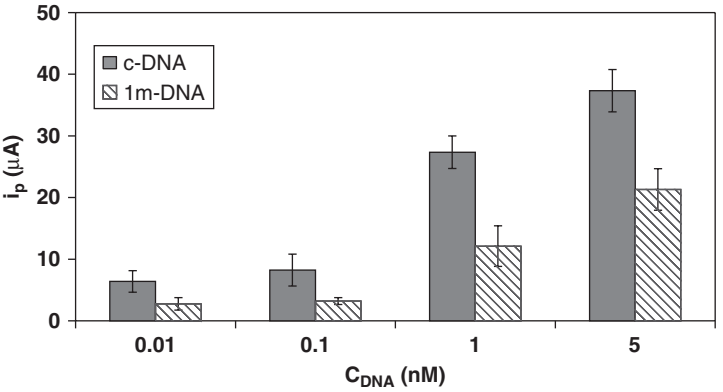

Fig. 26.7. Effect of the concentration on the selectivity. Signals for the complementary (c-DNA) and the one-mismatched strand (C–C at the centre, 1 m-DNA). $V_{\text{target}} = 20\,\mu\text{L}$, $2 \times \text{SSC}$ pH 7 buffer with 50% formamide, $t_{\text{hybr}} = 60\,\text{min}$. Data are given as average $\pm$ SD ($n = 3$).

26.2.2 Genosensors on streptavidin-modified thick-film carbon electrodes to detect *Streptococcus pneumoniae* sequences

This section outlines the development of genosensors on screen-printed carbon electrodes (SPCEs) for the identification of nucleic acid

determinants exclusively present in the genome of the pathogen *Streptococcus pneumoniae*. Orientation of the strands in the sensing phase is achieved by modifying the surface of the electrode with streptavidin by physical adsorption followed by the immobilisation of biotinylated oligo probes. The physical adsorption of streptavidin must be performed at a constant temperature above the room temperature. Moreover, the electrode surface must be previously electrochemically pretreated at an anodic potential in acidic medium to improve its adsorptive properties. In this way, reproducible, sensitive and stable sensing phases are obtained [54]. The biotinylated oligo nucleic acid probes used in this work target the pneumolysin (ply) gene. This target is randomly labelled with the Universal Linkage System (ULS®). This labelling system consists of the use of a platinum (II) complex that acts as a coupling agent between DNA strands and a label molecule, usually fluorescent. This platinum complex is a monofunctional derivate of cisplatin (a potent anticancer agent used in the treatment of a variety of tumours) that binds to DNA at the N7 position of guanine with release of one Cl^- ion per molecule of the complex. The label molecule used in this study was fluorescein (FITC). Electrochemical detection is achieved using two strategies. One of them is carried out using an anti-FITC alkaline phosphatase-labelled antibody and 3-indoxyl phosphate (3-IP) as enzymatic substrate of AP. The resulting enzymatic product is indigo blue, an aromatic heterocycle insoluble in aqueous solutions. Its sulfonation in acidic medium gives rise to IC, an aqueous soluble compound that shows an electrochemical behaviour similar to indigo blue. Both 3-IP and IC have already been studied on SPCEs [55,56]. However, although these genosensors are stable and sensitive devices for the detection of specific nucleic acid fragments, the need of two additional steps to obtain the analytical signal resulted in a large time-consuming analysis. This fact can be avoided using the second strategy for detection. In this case the analytical signal is directly obtained from platinum (II) complex, which is deposited on the electrode surface. In presence of the platinum on the electrode surface and after fixing an adequate potential in acidic medium, the protons are catalytically reduced to hydrogen. The current generated by this catalytic reduction can be measured and increases with platinum concentration and consequently with labelled target concentration.

Data presented here demonstrate the potential applicability of SPCEs genosensors in the diagnosis of a human infectious pulmonary disease. These electrochemical genosensors are stable and sensitive devices for the detection of specific nucleic acid fragments. Moreover,

these devices allow the detection of a one-base mismatch on the targets if adequate experimental conditions are used.

26.2.2.1 Genosensor design

- Electrode pretreatment: 50 μL of 0.1 M H_2SO_4 are dropped on the SPCEs and an anodic current of +3.0 μA is applied for 2 min. Then, the electrodes are washed using 0.1 M Tris buffer pH 7.2.
- Adsorption of streptavidin: an aliquot of 10 μL of a 1×10^{-5} M streptavidin solution is left on the electrode surface overnight at 4 °C. Then, the electrode is washed with 0.1 M Tris buffer pH 7.2 to remove the excess of protein.
- Blocking step: free surface sites are blocked placing a drop of 40 μL of a 2% (w/v) solution of BSA for 15 min followed by a washing step with 0.1 M Tris pH 7.2 buffer containing 1% of BSA.
- Immobilisation of oligonucleotide probes onto the electrode surface: 40 μL of 3′-biotinylated oligonucleotide probes (0.5 ng/μL) is left on the electrode surface for 15 min. Finally, the electrodes are rinsed with 2 × SSC buffer pH 7.2 containing 1% of BSA.

26.2.2.2 Hybridisation step

Hybridisation is performed at room temperature placing 30 μL of FITC labelled oligonucleotide target solutions in 2 × SSC buffer pH 7.2, containing 1% of BSA, on the surface of the genosensor for 45 min and then rinsing with 0.1 M Tris pH 7.2 buffer containing 1% of BSA. The methodology used to detect one-base mismatch strands is similar, but in this case 25% formamide is included in the hybridisation buffer.

26.2.2.3 Analytical signal recording

Two strategies are performed to detect the hybridisation event: enzymatic detection and electrocatalytic detection. The following steps are carried out:

Enzymatic detection

- Reaction with anti-FITC AP conjugate (Ab-AP): an aliquot of 40 μL of Ab-AP solution (1/100 dilution) is dropped on the genosensor device for 60 min. After a washing step with 0.1 M Tris buffer pH 9.8, containing 1% BSA, is carried out.
- Enzymatic reaction: an aliquot of 30 μL of 6 mM 3-IP is deposited on the electrode surface for 20 min. After that, the reaction is stopped

adding 4 μL of fuming sulphuric acid and 10 μL of ultra-pure water. In this step, the corresponding indigo product is converted to its parent hydrosoluble compound IC.
- Analytical signal recording: the SPCEs are held at a potential of −0.25 V for 25 s, and then, a cyclic voltammogram is recorded from −0.25 to +0.20 V at a scan rate of 50 mV/s. The anodic peak current is measured in all experiments.

Electrocatalytic detection
A 50 μL portion of 0.2 M HCl solution is dropped on the electrode surface and the electrode is held at a potential of +1.35 V for 1 min. Then, the chronoamperometric detection is performed at −1.40 V, recording the electric current generated for 5 min.

Figure 26.8 shows the scheme of the genosensor device and the analytical signals obtained with electrocatalytic detection (Fig. 26.8A) and enzymatic detection (Fig. 26.8B).

26.2.2.4 Results
The experimental conditions used to modify the electrode surface with streptavidin by physical adsorption have been studied in detail in a previous work [54], using biotin conjugated with alkaline phosphatase and 3-indoxyl phosphate (3-IP) as enzymatic substrate. The electrode surface must be pretreated by applying an anodic constant current (+3.0 μA) in 0.1 M H_2SO_4 for 2 min to improve its adsorptive properties. The use of this electrochemical pretreatment resulted in an increase in the hydrophilicity of the transducer, allowing the adsorption of streptavidin through hydrophilic and electrostatic attraction. In order to avoid repeatability problems associated to the use of streptavidin, the adsorption of streptavidin on the electrode must be performed at 4°C overnight. Doing this, repeatable signals are obtained and the streptavidin coated SPCEs are stable for months if they are stored at 4°C. Moreover, the significance of the attachment of biotinylated oligonucleotide probes through the streptavidin/biotin interaction has been tested in a previous work [30]. When a double-labelled (biotin and fluorescein) poly-T was attached to the electrode surface through the streptavidin/biotin interaction, the peak currents were much higher than those obtained when it was accumulated on the electrode surface by physical adsorption. This fact means that streptavidin/biotin interaction allows to attach and orient the oligonucleotide strands on electrode surface, whereas the direct adsorption of the oligonucleotide on

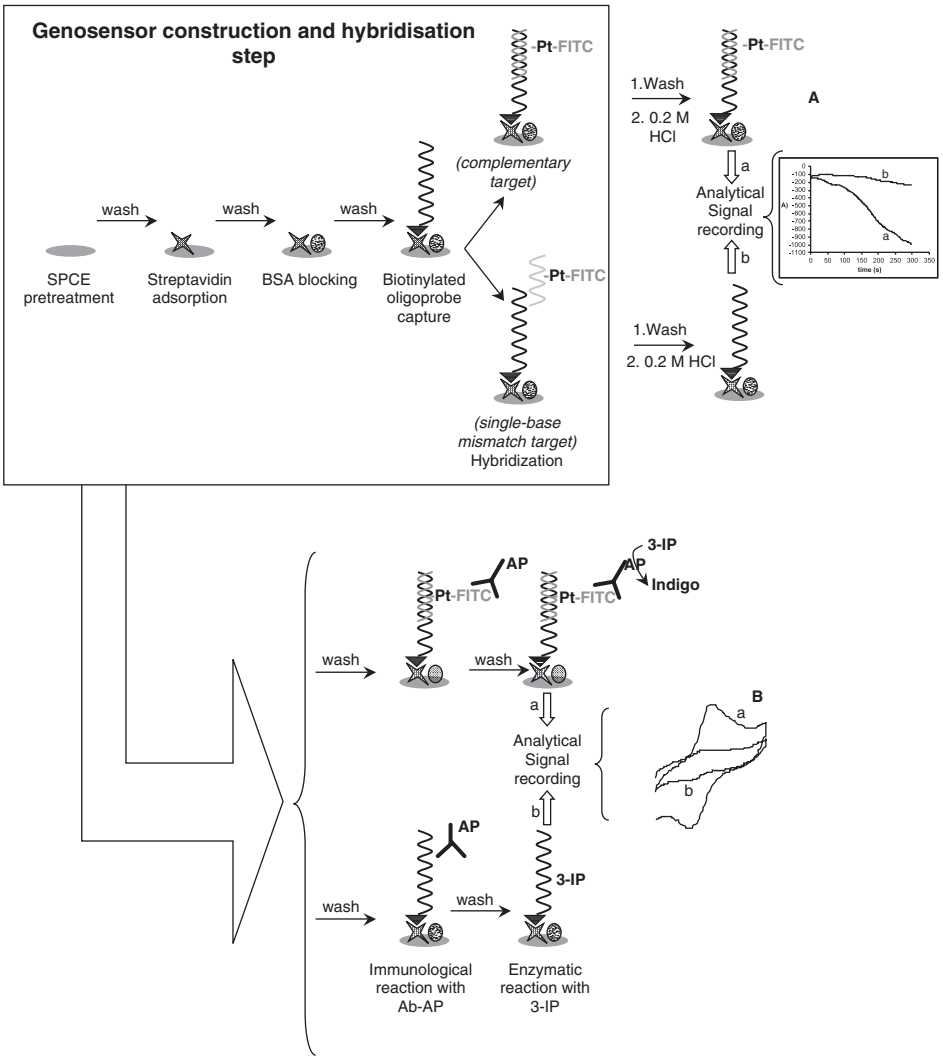

Fig. 26.8. Schematic representation of the analytical procedure followed for the construction of the genosensor and the detection of a complementary target and a single-base mismatch target. (A) Electrocatalytic and (B) enzymatic detection.

the electrode surface results in very poor manner. Using this method of immobilisation of the oligonucleotide probes, the genosensor devices are stable for a year if they are stored at 4°C.

The ply genosensor has been used for detecting oligonucleotide sequences containing a one- or three-base mismatch. Three different

concentrations of complementary ply, plymism1 and plymism3 targets were assayed and three genosensors were used for each concentration. Figure 26.9 displays the results obtained with both enzymatic and electrocatalytic detection. For the three concentrations assayed, the analytical signal obtained for the three-base mismatch oligonucleotide sequence is almost the background signal, indicating that three-base mismatch ply targets can be perfectly discriminated from the complementary ply target. For the one-base mismatch oligonucleotide

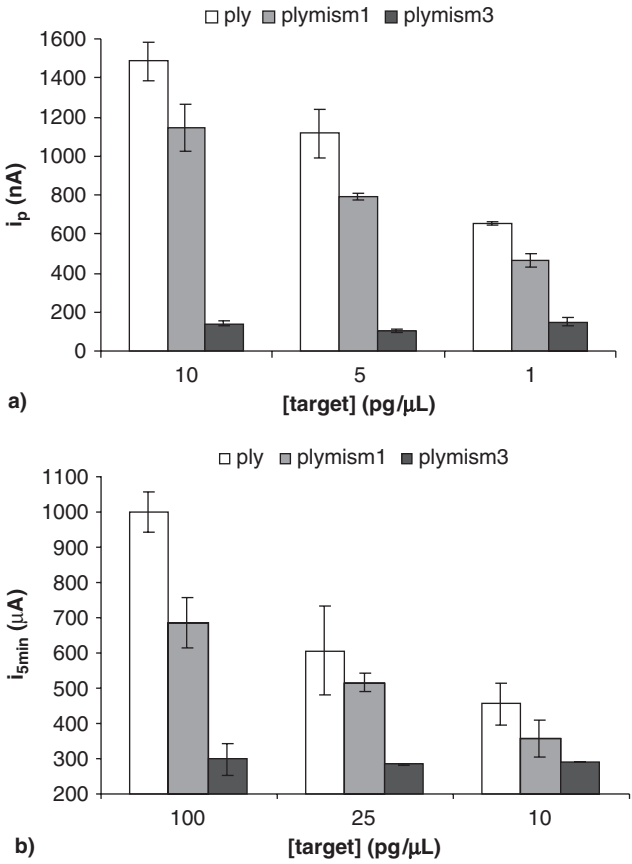

Fig. 26.9. Ply genosensor response to the complementary target (ply, white bars), the single-base mismatch target (plymism1, grey bars) and the three-base mismatch target (plymism3, black bars) for different concentrations. Data are given as average $\pm$ SD ($n = 3$). (a) Enzymatic and (b) electrocatalytic detection. Figure (a) reprinted with permission from Ref. [30]. Copyright 2004, American Chemical Society and Figure (b) reprinted with permission from Ref. [31]. Copyright 2005, American Chemical Society.

sequence, the analytical signals obtained only decrease about 30% with respect to those obtained for the complementary target.

In the optimised experimental conditions the ply genosensor has been tested for different concentrations of the complementary oligonucleotide target. In the case of the enzymatic detection, a linear relationship between peak current and concentrations of complementary ply target has been obtained between 0.1 and 5 pg/μL, with a correlation coefficient of 0.9993, according to the following equation:

$$i_p\ (\text{nA}) = 91 + 220[\text{ply target}]\ (\text{pg}/\mu\text{L})$$

Thus, these genosensors can detect 0.1 pg/μL, which is 0.49 fmol of ply target in 30 μL.

In the case of the electrocatalytic detection, a linear relationship between the recorded current and the logarithm of the concentration of ply target is obtained for concentrations between 5 and 100 pg/μL, according to the following equation:

$$i_{5\ \text{min}}(\mu\text{A}) = 510\ \log\{[\text{ply}]\ (\text{pg}/\mu\text{L})\} - 22; \quad r = 0.997\ (n = 5)$$

These genosensors can detect 5 pg/μL (24.5 fmol in 30 μL) of complementary ply target, using the electrocatalytic detection.

To improve the selectivity of the ply genosensor, more stringent experimental conditions are tested. A concentration of 25% formamide is added to the hybridisation buffer. It is well known that this molecule hampers the hybridisation reaction. In these more stringent conditions and using the enzymatic detection, a linear relationship between peak current and concentration of oligonucleotide target is obtained for concentrations between 0.25 and 5 pg/μL, according to the following equation:

$$i_p\ (\text{nA}) = 29 + 175[\text{ply}]\ (\text{pg}/\mu\text{L}); \quad r = 0.9992\ (n = 5)$$

Genosensors can detect about 1.2 fmols of complementary target in 30 μL in these more stringent experimental conditions.

In the case of electrocatalytic detection, a linear relationship between the recorded current and the logarithm of the concentration of oligonucleotide target is obtained for concentrations between 50 and 1000 pg/μL, according to the following equation:

$$i_{5\ \text{min}}\ (\mu\text{A}) = 360\ \log\{[\text{ply}]\ (\text{pg}/\mu\text{L})\} - 275; \quad r = 0.991\ (n = 5)$$

Using this strategy of detection, the genosensors can detect about 245 fmol of complementary target in 30 μL in these more stringent experimental conditions.

As expected, the sensitivity decreases in these stringent experimental conditions for both enzymatic and electrocatalytic detection but the detection of one-base mismatch on an oligonucleotide sequence can be performed for any concentration assayed (Fig. 26.10).

Although the sensitivity of the electrocatalytic detection is 50-fold (under non-stringent conditions) and 200-fold (using 25% formamide in the hybridisation solution) lower than that obtained with the enzymatic

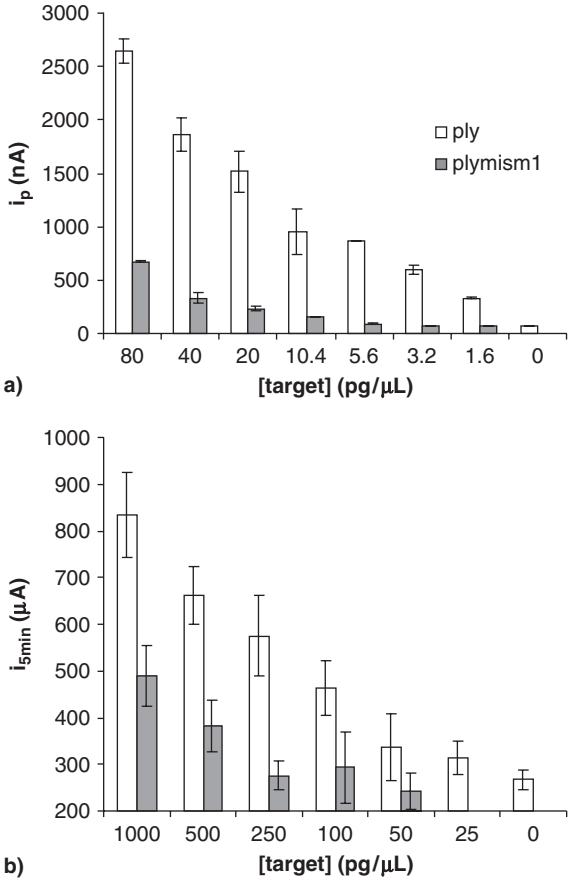

Fig. 26.10. Ply genosensor responses for different concentrations of complementary target (ply, white bars) and the single-base mismatch target (plymisms1, grey bars) when 25% formamide is included in the hybridisation buffer. Data are given as average $\pm$ SD ($n = 3$). (a) Enzymatic and (b) electrocatalytic detection. Figure (a) reprinted with permission from Ref. [30]. Copyright 2004, American Chemical Society and Figure (b) reprinted with permission from Ref. [31]. Copyright 2005, American Chemical Society.

detection, the analysis time is considerably shorter, because the analytical signal is achieved directly from the platinum complex whereas in the enzymatic detection two additional steps are necessary to obtain the analytical signal: the reaction with antibody anti-fluorescein and the enzymatic reaction. Thus, the overall analysis time of this chronoamperometric method is about the half than that resulting from the enzymatic method.

26.2.3 Genosensor on streptavidin-modified screen-printed carbon electrodes for detection of PCR products

The genosensor device described in this section can detect a 110 bp region of the TNFRSF21 gene (tumour necrosis factor receptor superfamily, member 21 precursor), bases comprise between 1859 and 1968, both included. This gene is previously inserted in the plasmid expression vector pFLAGCMV1, and amplified by PCR. Then, the PCR product is detected on the genosensor.

The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor has been shown to induce cell apoptosis. Through its death domain, this receptor interacts with TRADD protein, which is known to serve as an adaptor that mediates signal transduction of TNF receptors. Knockout studies in mice suggested that this gene plays a role in T-helper cell activation, and may be involved in inflammation and immune regulation.

26.2.3.1 Genosensor design

The genosensor device consists of an SPCE where a 20-mer oligonucleotide probe complementary to target is immobilised through the streptavidin/biotin reaction. Orientation of the strands in the sensing phase is achieved by modifying the surface of the electrode with streptavidin by physical adsorption followed by the immobilisation of biotinylated oligo probes. The physical adsorption of streptavidin must be performed at a constant temperature above the room temperature. Moreover, the electrode surface must be previously electrochemically pretreated at an anodic potential in acidic medium to improve its adsorptive properties. The biotinylated oligo nucleic acid probes used in this work target 20-mer of the 110 bp of the PCR product obtained from TNFRSF21 gene. The detection is carried out using an AP labelled antibody anti-FITC and 3-IP as substrate (enzymatic detection explained in last section). A more detailed explanation of the genosensor construction is given in Procedure 37 in CD accompanying this book.

26.2.3.2 PCR amplification and preparation of PCR products for genosensor detection

Two PCR amplifications are carried out. In one of them, an FITC labelled 5′-primer is used for DNA amplification and in the other one the 5′-primer used in the amplification reaction is not labelled. In the latter case the unlabelled PCR products obtained are labelled with fluorescein using a Universal Linkage System, explained in Section 26.2.2. The reactions of amplification are carried out in a thermocycler using a 3′-primer and a 5′-primer with an adequate sequence, Taq polymerase and dNTPs mixture. After an initial denaturation step, 35 cycles of denaturation-annealing-extension are carried out (see more details in Procedure 37 in CD accompanying this book). The unlabelled PCR products obtained, when unlabelled 5′-primer is used, are labelled with Fluorescein ULS® labelling kit before they are checked with the genosensor device (see more details about the labelling procedure in Procedure 37 in CD accompanying this book.

After that, the FITC labelled PCR products must be pretreated before their detection on the genosensor device. The double stranded amplified DNA is denaturated thermally by using a boiling water bath for 6 min at 95°C, then the tube containing the PCR products is immersed in an ice-water bath for 2 min in order to retard strands re-annealing. Finally, an aliquot of 30 μL is placed on the genosensor device.

26.2.3.3 Results

When the PCR products are labelled using the FITC labelled 5′-primer the analytical signals are higher and more reproducible than those obtained using the ULS labelling kit. Moreover, the PCR blank is higher in the latter case (see Fig. 37.2 of Procedure 37 in CD accompanying this book).

For PCR products labelled using the FITC labelled 5′-primer, a linear relationship between the peak current and the logarithm of the number of copies of plasmid DNA is obtained between 2×10^3 and 2×10^5 copies of plasmid DNA, according to the following equation:

$$i_p \text{ (nA)} = 204 \log \text{ (copies plasmid DNA)} - 354, \quad r = 0.9998, \; n = 4$$

The limit of detection, calculated as the copies of plasmid DNA corresponding to a signal which is the PCR blank signal plus three times the standard deviation of the PCR blank signal, results to be 360 copies of plasmid DNA.

26.3 CONCLUSIONS

As it has been shown in previous sections, the use of thick- and thin-film electrodes as supports for genosensor devices offers enormous opportunities for their application in molecular diagnosis. The technologies used in the fabrication of both thick- and thin-film electrodes allow the mass production of reproducible, inexpensive and mechanically robust strip solid electrodes. Other important advantages of these electrodes are the possibility of miniaturisation as well as their ease of manipulation in a disposable manner and therefore the use of small volumes. This is an important issue that makes this methodology for detection of DNA more attractive.

Very sensitive methods are always required for DNA sensing. Although enough sensitivity to avoid PCR amplification has been achieved by use of enzymatic labels [12,14,26,28] or metal tags [42], most of the assays routinely start with a PCR or other biochemical amplification. Moreover, although label-free formats are used, most of the strategies followed to obtain the analytical signal involve several washing steps and need the use of labelled reagents (or labelling procedures) or indicators, which complicates the assay performance. Motorola Science has commercialised the eSensor [39,40], which detects the hybridisation of the target by a sandwich assay without clean-up steps. Although this sensor requires the addition of reagents, they are included in the microsystem as dry reagents dissolved by the sample. An important drawback is the need of carrying out DNA isolation and purification before testing the DNA sample with genosensor devices due to the inherent complexity associated with the biological sample. The real goal, for specific pathogen detection, is the development of an assay involving rapid DNA isolation with the detection of few copies in half an hour or less. With these electrochemical genosensors this goal is reliable, but it has not yet been achieved.

Optical gene chips dense arrays of oligonucleotides have been successfully applied to detect transcriptional profiling and SNP discovery, where massively parallel analysis is required. However, the fluorescence-based readout of these chips involves not only highly precise and expensive instrumentation but also sophisticated numerical algorithms to interpret the data, and therefore these methods have been commonly limited to use in research laboratories. In this way, thin-film arrays of 14, 20, 25, 48 and 64 electrodes have already been fabricated [12,15,39,40,44,48], using lithographic techniques. Readout systems for these arrays based on electrical detection have also been developed.

Moreover, a thick-film sensor array suitable for automation combined to readout based on intermittent pulse amperometry (IPA) has been commercialised by Alderon Biosciences [27,28]. These genosensors and the readout instruments provide a simple, accurate and inexpensive platform for patient diagnosis.

It is more than probable that arrays for 50–100 DNA sequences will be needed for some clinical applications. Although it is not difficult to design electrode pads with reproducible dimensions of a micron or less, the electrochemical readout requires mechanical connections to each individual electrode. Therefore, the construction of very large, multiplexed arrays presents a major engineering challenge. Electronic switches in the form of an on-chip electronic multiplexer may provide a possible solution for this problem.

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