Chapter I. Introduction

1.1. DNA sensors and arrays

1.1.1. Introduction and definitions

The detection of specific DNA sequences has always been an important issue in the biomedical field due to its application in DNA sequencing and diagnostics. Thirty years ago, the method by excellence to identify DNA was "sequencing by digestion", which was laborious and time-consuming. Soon thereafter, membrane-supported methods appeared based on "sequencing by hybridisation" (SBH), which were simpler (Wallace *et al.*, 1979 and 1981). However, the most important advance did not appear until the last decade, with the advent of DNA sensors and arrays (Fodor *et al.*, 1991; Pease *et al.*, 1991; and Southern *et al.*, 1992). These two approaches overcame the problems of the previous technologies, such as low hybridisation efficiency, low sequence discrimination, long analysis time and laborious procedures. Additionally, DNA arrays allow simultaneous multi-site detection, which further reduces the analysis time and facilitates genetic analysis. Miniaturised arrays allow small sample volumes and can be mass-produced.

DNA sensors are analytical devices that consist of two components in intimate contact: the biorecognition molecule and the transducer. The biorecognition molecule is immobilised on the transducer and recognises the DNA target, and the transducer is the component that converts the biorecognition event into a quantifiable signal. DNA arrays or chips can be defined as the integration of several DNA sensors in the same device. In other words, DNA arrays are ordered sets of known biorecognition molecules immobilised on precisely defined locations of a solid substrate. In most cases, these biorecognition molecules are oligonucleotides of known sequence and the recognition event is the hybridisation with the complementary sequences. The advantages of this high throughput analysis tool are obvious and represent a drastic reduction in effort, time and costs. Microarrays can be applied to both genomics and proteomics. Whilst genomics is focused on the identification, sequencing and study of the function of genes, proteomics includes the identification and quantification of proteins, and the determination of their function, localisation, modifications and interactions. Proteomics array technology is still one step behind that of the genomics arrays. These are now almost routinely applied in old and new DNA analysis areas that can be loosely divided into four main inter-related groups:

1. **Sequencing.** Gene discovery or identification by sequencing requires arrays modified with probes that include all possible combinations of nucleotides for a determined length. Usually, an n-mer sequence is chosen as the basic identification unit and the array must have 4^n probes, corresponding to all the possible combinations with the 4 bases. The target DNA fragment is broken into smaller pieces labelled and hybridised with the immobilised

oligonucleotides or probes. Detecting the location of hybridised probes and using appropriate analysis, the total sequence can be identified.

2. **Expression analysis.** Gene expression is the process by which gene coded information is converted into the structures present and operating in the cell. Expression analysis includes both identification of differentially expressed genes and quantitation of expression levels, important in growth, metabolism, development, behaviour and adaptation of living systems. In a simple gene expression array, also known as a gene array, the messenger RNA (mRNA) is extracted and reverse-transcribed into more stable complementary DNA (cDNA). These cDNAs are marked with different labels and subjected to SBH on the array. Scanning of the array provides information about the position and intensity of the hybridisation events and, therefore, the level of expression of genes. Expression analysis contributes to find correlations between the genetic profiles of patients and the therapeutic responses to drugs (pharmacogenomics) or to toxic agents (toxicogenomics), which act at the protein level to disrupt or alter protein function. Consequently, microarrays for the analysis of gene expression can be used as powerful tools to understand the action of drugs and toxic agents at the molecular level and to tailor drug design to individuals.

3. **Disease diagnosis and mutation detection.** Microarray technology can be used in viral infection and mutation detection, and in identification of genes that have been upregulated or downregulated (Yershov *et al.*, 1996; Drobyshev *et al.*, 1997; Healey *et al.*, 1997; Head *et al.*, 1999; and Proudnikov *et al.*, 2000). The identification of these pathogens, infectious species and drug-resistant mutants at molecular level is based on SBH technology and can contribute to a better understanding of the disease processes and to diagnosis.

Related with the three areas is **polymorphism identification** (Gentalen and Chee, 1999), which uses SBH technology to detect differences in DNA sequences among individuals. These genetic mutations, commonly referred to as single nucleotide polymorphisms (SNPs), are often diagnostic of particular genetic predisposition towards disease and drug-response, so that can be linked to the genotype and phenotype information of individuals.

This thesis contributes to the development of methods for the construction of DNA arrays and sensors. The development of DNA arrays is already having a profound effect on various aspects of social, economic and scientific activity. When combined with the results of the human genome and other sequencing projects, it is accelerating the changes in these aspects of human activity that together contribute to the new technological revolution based on the combination of silicon technology and biotechnology, which is widely believed that will characterise the XXI century. It is only appropriate that at this point an effort is made to systematically define the steps involved in DNA or biochip array development. Although the limits are not clearly delineated between them, for the sake of a systematic approach these are defined as:

- Probe immobilisation (and characterisation).
- Arraying.
- Biorecognition event detection (and amplification).
- Data analysis (a field that is widely known as bioinformatics).

This thesis makes a contribution to the state-of-the-art of the three first, and for this reason they are further examined in what follows.

1.1.2. Probe immobilisation and characterisation

DNA sensors and chips are made using different types of probes. The most commonly used probes are **single-stranded deoxyribonucleic acid** (ssDNA) sequences or **oligonucleotides**, which are synthesised *in situ* or obtained synthetically with DNA synthesisers and afterwards immobilised on solid substrates. Probe oligonucleotides are usually linear. However, hairpin oligonucleotides with dangling ends are being used with increasing frequency, as they have been observed to display higher rates of hybridisation and larger equilibrium amounts of captured targets than linear probes (Riccelli *et al.*, 2001). Additionally, hairpin-target complexes are thermodynamically more stable. Molecular beacons, scorpions and light-up probes are special types of DNA probes that will be defined later. **Messenger ribonucleic acid** (mRNA) is less commonly used, due to its higher instability compared to DNA. mRNA is obtained from cells and afterwards purified. Consequently, mRNA analysis provides information about gene expression. Moreover, as mRNA is a copy of the DNA coding regions, it can be also used to identify polymorphisms in these coding regions. However, mRNA information in probe form is usually reverse-transcribed into more stable cDNA. **Peptide nucleic acid** (PNA), a probe that has found increasing recent application, deserves special mention. PNA is a linear polymer that, unlike DNA, contains a neutral, achiral backbone of repeating *N*-(2-aminoethyl)glycine units linked by amide bonds, with the purine and pyrimidine bases attached by methylene carbonyl linkages. In other words, it is a sequence made up of derivatives of the four nucleobases found in DNA but without 2'-deoxy-D-ribose residues and phosphodiester bonds, having instead a neutral backbone that reduces the electrostatic repulsion during duplex formation (Chandler *et al.*, 2000). PNA forms hybrids stabilised by hydrogen bonding and base stacking with distances similar to the DNA double helix structure but, unlike DNA/DNA hybrids, PNA/DNA hybrids are resistant to nuclease and protease attack due to the inability of nuclease and proteolytic enzymes to recognise the peptide backbone, have higher thermal stability and their melting temperature (T_m) is approximately 10ºC higher than the corresponding DNA/DNA duplex, and are relatively insensitive to ionic strength, due to the neutral charge of PNA. Additionally, single base mismatched duplexes are less stable than their corresponding DNA/DNA hybrids, a fact that makes them perfectly suitable for specific mutation detection (Jensen *et al.*, 1997 and Schwarz *et al.*, 1999).

1.1.2.1. Probe immobilisation

The most commonly used immobilisation methods for DNA sensors, summarised in Table I.1 and described below, are retention in a polymeric matrix, covalent attachment on a functionalised support, affinity immobilisation, physical adsorption on a solid surface, and monolayer self-assembling. Among these well-known and traditional methods, self-assembled monolayer (SAM) immobilisation is finding more followers, as it provides advantages in terms of simplicity, efficiency, ordered immobilisation and cost.

Table I.1. DNA immobilisation methods.

Method	Technique	Imm. site	Orientation	Access	Advantages	Drawbacks	Ref.
Retention in a polymeric matrix	Entrapment or cross-linking	Random	Random	Low	High amounts of probes	Surface treatment needed Low hybridisation yields	Hasebe,1997
Covalent attachment	EDC reaction	Activated terminal	Ordered	High	High stability	Surface treatment needed Low immobilisation yields	Millan,1993 Caruana,1999
Silanisation – covalent attachment	EDC reaction	Activated terminal	Ordered	High	Possible probe density control	Surface treatment needed Possible non-specific interactions Low immobilisation yields	Joos,1997 Potyrailo,1998 Berney,2000 Balladur,1997 Barendrecht,1990
Affinity	Biotin-avidin/ streptavidin interaction	Biotinylated terminal	Ordered	High	High stability Simplicity	Surface treatment needed Possible non-specific interactions	Lucas,2000
Adsorption	Adsorption	Random	Random	Low	Simplicity	Low hybridisation yields	Krznaric,1986 Cai,1996 Fojta,1996 Oliveira-Brett,1996,1997 Wang,1996,1997 Zhao,1997 Pang,1998 Marrazza,1999 Armistead,2000 Azek,2000
Inclusion in a composite	Entrapment	Random	Random	Low	Possible ordered attachment	Low hybridisation yields	Millan,1994
Direct SAMs	Dative binding	Thiolated terminal	Ordered	High	Simplicity Possible probe density control	Possible non-specific interactions	Okahata,1992 Hashimoto,1994 Caruso,1997b Nakano1997 Napier,1997 Steel,1998 Bardea,1999 Patolsky,1999 Bonn,2000 Ketterer,2000
Indirect SAMs	EDC reaction	Activated terminal	Ordered	High	Possible probe density control	Surface treatment needed Low immobilisation yields	Napier,1997 Steel,1998

Four effects have to be taken into account with probe immobilisation. Firstly, it is necessary that the immobilisation chemistry is stable during subsequent assay steps, which means that the immobilised biorecognition molecules should not desorb from the transducer surface. Secondly, the probes have to be functional after attachment, which means that the immobilisation technique should not change the chemical structure of the probe, as the biorecognition function could be modified and inhibited. Thirdly, biomolecules have to be immobilised with an appropriate orientation and configuration so that base pairing is not restrained. Finally, even with the appropriate orientation, there should not exist steric impediments or lack of accessibility due to the dense packing of the immobilised probes.

a) Retention in a polymeric matrix

The biorecognition biomolecules are entrapped or cross-linked with bifunctional agents, which have been activated chemically, electrochemically, photochemically or thermally (Hasebe *et al.*, 1997). The support matrix for entrapment is formed by polymers that can be organic, inorganic or organometallic, and are immobilised on a surface by chemisorption or physical adsorption. The advantage of this technique is the high amounts of oligonucleotides that are covalently attached. The main disadvantage is that the matrix does not provide any orientation, all the strands being randomly immobilised, which decreases their mobility and accessibility and inhibits the hybridisation event. This problem can be solved linking the oligonucleotides to the polymer by one of their terminals, which increases hybridisation efficiency (Caruana and Heller, 1999) (*see next section*).

b) Covalent attachment

To immobilise the biorecognition molecules on the transducer via covalent attachment it is usually necessary to pre-treat both the oligonucleotide and the surface, in order to introduce the reactive groups necessary for attachment. As the reactive group can be introduced in one of the extremes of the oligonucleotide without inhibiting its hybridisation capability, this approach confers more mobility and better orientation to the immobilised strands. Depending on the transducer surface and the reactive groups available on the oligonucleotide, different strategies have been proposed. In the strategy mentioned in the previous section, the oligonucleotide is EDC-activated and reacted with hydrazide functions of a polymer previously deposited on the electrode surface (Caruana and Heller, 1999). In the covalent immobilisation of oligonucleotides on glassy carbon, this surface can be activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) in order to react with the deoxyguanosine residues of the DNA strand (Millan and Mikkelsen, 1993). Among the different transducer modification methods, silanisation is the most popular. The hydroxide or oxide layer of the substrate, which can be glass (Joos *et al.*, 1997 and Potyrailo *et al.*, 1998), silicon (Berney *et al.*, 2000), silica (Balladur *et al.*, 1997) and metals, is firstly activated with an organosilane, usually introducing amine groups (Barendrecht, 1990). Afterwards, the oligonucleotide strands are attached via EDC. Depending on the

organosilane molecule, the packing density can be controlled. However, electrostatic interactions between the aminated groups on the silanes and the negatively charged DNA are difficult to control, and this can result in non-orientated immobilisation and non-specific adsorption of possible target sequences in the hybridisation step.

c) Affinity

This immobilisation technique is based on the interaction between biomolecules, one being immobilised on the transducer surface and the other one being attached to one of the extremes of the oligonucleotide. The avidin/streptavidin-biotin affinity system is the most commonly used due to the high affinity complexation constant (K_d = 10^{-15}M) (Ebersole *et al.*, 1990; Abel *et al.*, 1996; Kolakowski *et al.*, 1996; Caruso *et al.*,1997a; Okahata *et al.*,1998; and Trabesinger *et al.*,1999). This binding force is almost equal to that of a covalent bond and can be only broken under drastic conditions. This high binding force combined with the simplicity of the procedure, makes the technique very attractive to immobilise DNA strands. However, the attraction between the positively charged lysines or arginines on the large surface of streptavidin (70kDa) and the negatively charged backbone of DNA may cause non-specific adsorption, compromising the sensitivity and selectivity of the sensor (Lucas and Harding, 2000).

d) Adsorption

In this immobilisation technique, DNA is attached by irreversible adsorption via the multiple interactions between the transducer surface and the phosphate backbone. The adsorption can be performed on different substrates, such as gold (Zhao *et al.*, 1997 and Pang and Abruña, 1998), indium-tin oxide (ITO) (Armistead and Zhorp, 2000) and screen-printed electrodes (Marrazza *et al.*, 1999 and Azek *et al.*, 2000). However, glassy carbon electrodes are more commonly used (Oliveira-Brett, 1996 and 1997). The electrodes can be covered by a drop of probe oligonucleotide and allowed to dry, or immersed in a DNA solution. In this last strategy, the oligonucleotide can be pre-concentrated on the electrode by applying a positive potential for a pre-determined time (dependent on the DNA concentration). This potential makes the electrode positively charged, improving the interaction with the negatively charged DNA backbone (Cai *et al.*, 1996; and Wang *et al.*, 1996a, b, c, 1997a, b, c). The main advantage of this method is the simplicity, but it has the inconvenience of non-ordered immobilisation and multiple-point attachment, which inhibits the recognition event. In addition to this, the immobilisation is not very stable, as depending on the hybridisation conditions the nucleic acid may be desorbed from the electrode surface.

e) Inclusion in a composite

In this immobilisation technique, the biomolecule is mixed with a matrix material, such as graphite powder, and the carbon paste mixture is introduced into an electrode body and pressed. This methodology is widely used in enzyme sensors, but it is not very commonly used in DNA sensors

due to the limited accessibility of the immobilised strands. However, in order to give more mobility to the strand, oligonucleotides can be attached to the surface of this composite paste (Millan *et al.*, 1994).

f) SAMs formation

Self-assembled monolayers (SAMs) are molecular layers formed on a surface when it is immersed in a solution containing molecules that specifically interact with this surface. Although different molecules can be immobilised (silanes, carboxylic acids, pyridines, sulphites and thiols) on different surfaces (gold, silver, platinum, copper, mercury and glass), thiols are the most commonly used especially in conjunction with gold surfaces. The stability and organisation of the SAMs depend on the forces of attraction between the immobilised molecules, the interaction between terminal groups and their local environment, and the binding force between the surface and the binding group. Oligonucleotide SAMs can be formed directly on the surface (Okahata *et al.*, 1992; Hashimoto *et al.*, 1994; Caruso *et al.*, 1997b; Nakano *et al.*, 1997; Napier and Thorp, 1997; Steel *et al.*, 1998; Patolsky *et al.*, 1999; Bonn *et al.*, 2000; and Ketterer *et al.*, 2000), when the oligonucleotides contain a pendant thiol group, or can be attached to reactive and previously formed SAMs via EDC (Napier and Thorp, 1997; and Steel *et al.*, 1998). The direct strategy reduces the number of steps required for immobilisation and avoids the EDC reaction, which usually results in considerably lower immobilisation efficiency. However, the indirect strategy (SAMs + EDC) is an alternative when non-saturated monolayers are desired.

As mentioned above, SAMs are commonly used in DNA sensor formats, as they confer ordered, stable, simple, easy and cost effective immobilisations. However, the packing density has to be controlled if optimum hybridisation efficiency is to be achieved. Too densely packed monolayers (although sometimes difficult to achieve, due to the repulsion between negatively charged probes) can be avoided using mixed monolayers, formed by the same method as pure monolayers but using oligonucleotides and spacers such as mercaptopyridine, mercaptoethanol or mercaptohexanol (Bardea *et al.*, 1999). These spacers not only block the free remaining sites, called pinholes, that could have been formed during SAM formation, but also compete with the thiol-oligonucleotides, separating them, facilitating the transport of complementary strands and increasing hybridisation efficiency.

1.1.2.2. Probe immobilisation characterisation

During the development of a biosensor or biochip, it is necessary to characterise the immobilisation efficiency and immobilised probe functionality to rationally design sensors and arrays. The techniques used for the characterisation of the probe immobilisation in DNA sensors are reviewed below.

a) Piezoelectric methods

The quartz crystal microbalance (QCM) is the most commonly used piezoelectric or microgravimetric device. This device consists of a piezoelectric quartz crystal with a determined frequency of oscillation under an applied potential. When the oligonucleotide is immobilised on the surface, the frequency of oscillation changes proportionally to the immobilised mass. The dependence of the frequency modulation with the mass change is described by the Sauerbrey equation (1959):

$$\Delta F = -2.3 \times 10^{-6} F^2 \Delta M / A \qquad \text{(Eq. I.1)}$$

ΔF being the change in the frequency (MHz), F the characteristic resonant frequency of the crystal (MHz), ΔM the mass deposited (g) and A the area of the quartz crystal (cm^2). The advantages of this technique are not only the high sensitivity and low limit of detection, but also the labelless operation. Additionally, frequency changes can be followed in real time. Caruso *et al.* (1997a, b) and Okahata *et al.* (1998) immobilised oligonucleotides directly on the gold quartz crystals, by forming a direct SAM, and also used biotin-oligonucleotides to immobilise them on streptavidin-modified gold quartz crystals. By real-time frequency measurement, they compared the two methods in terms of immobilisation kinetics. As previously mentioned, the immobilisation step is a key factor in the performance of the sensor, as it has an influence on the hybridisation kinetics. In this direction, Zhou *et al.* (2001) compared different immobilisation methods (direct chemical bonding, avidin-biotin interaction and electrostatic adsorption on polyelectrolyte films) and different immobilisation architectures (oriented oligonucleotide monolayers and multilayers created by self-assembling of alternating DNA and polymers). They observed that biotinylated DNA films provided fast sensor responses and high hybridisation efficiencies, due to the spacer group that conferred better accessibility, and that multilayered films increased the sensor sensitivity, indicating that the complementary DNA can penetrate into the multilayered sensing film, but also increased the sensor response time because of the more difficult transport of the complementary sequence.

b) Electrochemical methods

The oligonucleotide immobilisation efficiency can be determined using different electrochemical techniques. The oldest method is based on the detection of the direct oxidation of guanine by chronopotentiometry (Cai *et al.*, 1996). This technique requires the presence of guanines in the sequence, which restricts its common use. Additionally, as the direct oxidation requires a rather high potential and the reproducibility of the analysis is usually a difficult issue, mediators such as $Ru(bpy)_3^{3+}$ are commonly used as catalyst that enhance the Faradaic currents and can allow one to detect attomole quantities of immobilised DNA (Napier *et al.*, 1997; Ontko *et al.*, 1999; and Armistead and Thorp, 2000). Apart from chronopotentiometry, DNA immobilisation can be characterised by cyclic voltammetry (CV), linear sweep voltammetry (LSV) or alternating current

(AC) impedance spectroscopy of redox complexes, like $Co(bpy)_3^{3+}$, $Co(phen)_3^{3+}$ and $[Fe(CN)_6^{4-/3-}]$, or dyes, like Hoechst 33258, that present different electrochemical behaviour depending on the absence or presence of ssDNA on the electrode surface (Millan and Mikkelsen, 1993; Millan *et al.*, 1994; Hashimoto *et al.*, 1994; and Zhao *et al.*, 1997 and 1999).

SAMs can also be characterised by electrochemical methods. Cyclic voltammetry allows the observation of the reduction of the thiol-gold bond at approximately -1V (*vs.* Ag/AgCl) (Yang *et al.*, 1996 and 1997a; Imabayashi *et al.*, 1997; and Madoz *et al.*, 1997). This is a destructive technique because the thiol-gold bond is broken during reduction. Cyclic voltammetry can also be used to determine the effect of an immobilised monolayer on the electrochemical behaviour of a redox compound, which has been previously adsorbed or attached to it. Surface coverage measurements can provide data about the formation, capacitance and packing density of the DNA SAM (Chidsey *et al.*, 1990; Katz and Solov'ev, 1990; Hickman *et al.*, 1991; Miller *et al.*, 1991; Katz *et al.*, 1992; Acevedo *et al.*, 1994; Bretz and Abruña, 1995 and 1996; Cheng and Braiter-Toth, 1996; Maskus and Abruña, 1996; and Tirado and Abruña, 1996).

c) Optical methods

Immobilisation of ssDNA has been characterised by several spectroscopy techniques, such as *in situ* Ultra-Violet/visible (UV/vis), reflection spectroscopy, X-ray photoelectron spectroscopy (XPS), scanning tunnelling microscopy (STM), Raman spectroscopy and surface-enhanced Raman scattering (SERS), each one of them providing complementary structural information (Zhao *et al.*, 1999). Surface plasmon resonance (SPR) is an optical technique based on the change in the refractive index of a surface when a biomolecule is immobilised or when an affinity interaction occurs. The main advantages are that it does not require any label and that measurements can be carried out in real time (Caruso *et al.*, 1997b). Fluorescence can also be used to characterise probe immobilisation, but the oligonucleotide has to be tagged with a fluorescent label, and thus labelling efficiency and lifetime have to be taken into consideration.

1.1.3. Arraying

In making the step from DNA sensors to arrays, the ability to spatially direct the immobilisation of distinct probes is essential. Therefore, the resolution of the DNA immobilisation technique is crucial for arraying and miniaturisation. Microarraying technologies thus have to fulfil several requirements: easy implementation, robustness, consistency, automation, high speed of fabrication, versatility, repeatability, regularity, uniformity, accuracy, high precision, high resolution, high array density, durability and cost effectiveness. This long list makes it difficult to find one technique that fulfils all the conditions. In this competitive field, technologies are quickly consolidated and reduced to practice. For this reason, technology breakthroughs are usually represented by manufacturing

systems used by different companies. Table I.2 summarises some of the systems commonly used in the area as well as their advantages and drawbacks.

Table I.2. Leader companies and systems in microarraying technology.

Company	Technique	Probe	Spatial resolution	Detection	Advantages	Drawbacks	Application
Synteni / Incyte Pharmaceuticals Inc.	Ink-jetting on glass	500nt DNA samples PCR fragments	10000 spots on 3.6cm^2	Fluorescence Radioisotopic	Off synthesis	Low density Low cost	Expression profiling, gene identification, diagnostics, polymorphism analysis
Hyseq	Pin deposition on membranes	5nt oligos 500-2000nt DNA samples	1024 spots on 1.15cm^2 64 spots on 0.6cm^2	Fluorescence Radioisotopic	High density Off synthesis	Low cost	Expression profiling, gene identification, diagnostics, sequencing, polymorphism analysis
Affymetrix	Photolithography with activated nucleotides	25nt oligos	9000 spots on 1.6cm^2	Fluorescence	High density	High cost *In situ* synthesis	Expression profiling, diagnostics, polymorphism analysis
Clinical Micro Sensors	Pin deposition on electrodes or Photolithography with activated oligonucleotides	Small DNA/RNA fragments	36 targets on a chip	Impedance	Off synthesis Low cost	Low density Low cost or High cost	Expression profiling, gene identification, diagnostics, sequencing, polymorphism analysis
Nanogen	Electronically-driven binding of biotin on streptavidin-modified agarose	20nt oligos	99 locations on 2mm^2	Fluorescence	High density Off synthesis	Low cost	Expression profiling, gene identification, diagnostics, short tandem repeat identification

Immobilisation can be carried out on many different substrates: glass, agar, gels or membranes (nitrocellulose or nylon). Usually, they have to be previously treated in order to introduce the functional groups necessary for the biomolecule attachment. The probes can be either synthesised *in situ* (like in the photolithographic technique) or pre-made and afterwards immobilised. Although *in situ* construction of arrays by lithography is advantageous in terms of resolution, this technique implies lack of quality control of the sequences. On the other hand, techniques that use pre-made oligonucleotides allow the previous testing of the sequence, in order to see if there are some errors that could produce false responses. Below, different arraying methods will be reviewed.

a) Ink-jetting

Ink-jetting is a non-contact printing technology, where the DNA probes are dispensed in small volumes on a slide without touching it, reducing the risk of possible contamination. During ink-jetting, the DNA sample is withdrawn from the source, introduced into the print head, moved to the pre-determined site of deposition, and deposited through ejection onto the surface. Usually, this

surface is pre-treated in order to covalently attach the DNA. There are two different types of ink-jet printing: piezoelectric, where a piezoelectric crystal biased by a voltage squeezes the capillary containing the sample and causes its ejection (Incyte Pharmaceuticals), and syringe-solenoid, where the microsolenoid dispenses low volumes ejected by a syringe pump (Cartesian Technologies). Whilst in the syringe-solenoid technique the minimum dispense volume is in the order of 4-8nL, with a spot size of 250-500μm and a density of 200-400 spots cm^{-2}, in the piezoelectric technique the spot volume is on the order of 50pL, with a spot size of 125-175μm and a density of 500-2500 spots cm^{-2}. Moreover, the delivery speed is 10-50 spots sec^{-1} with the syringe-solenoid technique and 100-500 spots sec^{-1} with the piezoelectric device. However, despite the larger volumes and the lower array densities, the reliability of the syringe-solenoid technique is higher and the equipment is cheaper and more robust. In general, despite the advantages of ink-jet arraying, the main disadvantages are the air bubbles, which reduce the repeatability and reliability of the system; the difficult sample changing; maintenance problems related to the obstruction of the inlet tubing and capillaries or syringes; excessive splashing; clogging of the nozzle; and poor uniformity of the deposit, which can cause cross-over contamination between probes. The ink-jet technology has been used for the *in situ* synthesis of oligonucleotides of 40-50 bases in length on an array (Castellino, 1997). In this case, the printer dispenses one of the four bases onto a coated surface, where it is anchored by standard chemistry. Following washing and deprotection, the next dispense adds the following base and the cycle is repeated until the complete sequence is immobilised. Nevertheless, this strategy, although feasible, is laborious, requires many washing steps and lacks control of the quality of the sequence.

b) Pin deposition

Pin deposition is a contact printing technique, as there is direct contact between the dispenser and the surface. Pin tools are immersed in the DNA probe source, which results in the adherence of small volumes onto the tip of the pins. When the pin touches the surface, a drop is transferred from the tip to the sensor surface. The number and the diameter of the spots, between 50 and 360μm, depend on surface and solution properties, the velocity of the pin, the diameter of the tip and its geometry. Different pin geometries are available: tweezers, split tip, micro spotting pin (Hyseq and TeleChem International) or Pin-and-Ring™ (Genetic MicroSystems). As a practical example, Zammatteo *et al.* (2000) used different strategies to covalently attach DNA onto glass surfaces. After comparison between amino-silane, carboxylic acid and aldehyde-covered glass slides, they chose the aldehyde-modified surfaces to build, using 250-μm pins, DNA microarrays for human cytomegalovirus (HCMV) detection. Apart from this functionalised glass substrate, many authors have also used glass modified with poly-L-lysine[6] or polyacrylamide gel (Guschin *et al.*, 1997) as substrate. Usually, the dispensed volume is in the range of 0.5-2.5nL. This technique allows 400-625 locations on an area of $1cm^2$, a density that depends on the spatial resolution of the robotics and the dispersion of the deposit. The delivery speed is 64 spots sec^{-1}, slower than the non-contact

printing techniques. However, pin deposition is more robust, simpler and cheaper. Nevertheless, a common disadvantage to all the printing techniques is the splashing or the poor uniformity of the deposit. The variability in spot size and in probe concentration diminishes the reliability on the measurement, and the variability in spot location produces cross-contamination, also generating false measurements.

c) Polypyrrolisation

Polypyrrolisation is based on the co-polymerisation of pyrrole and oligonucleotides bearing a pyrrole moiety introduced via phosphoramidite chemistry at their 5' termini. The different pyrrole-modified oligonucleotide sequences can be electrochemically immobilised on specific sites by sequentially switching on the different electrodes. These arrays have been demonstrated, going from 4 working electrodes and using radioisotopic detection (Livache *et al.*, 1994 and 1995) to 10 and even 48 electrodes (4 x 12-electrode matrix in a 50 x 50-μm^2 chip), and using fluorescence microscopy detection (Roget *et al.*, 1995 and Livache *et al.*, 1998). This arraying technique presents the drawbacks of wet chemistry, which increases the irreproducibility of the system, and the necessity of a washing step after each polypyrrole synthesis to avoid cross-contamination with residual oligonucleotides, which makes the arraying procedure time-consuming. However, the advantage of this method is the low instrumentation costs for the manufacturing of the array unlike other methodologies involving photolithography or robotic deposition. Guedon *et al.* (2000) used a strategy to create a polypyrrole-based DNA sensor in which they combined polypyrrolisation and spotting, strategy called "electrospotting". In this arraying strategy, they filled a pipette tip incorporating a platinum wire with pyrrole-oligonucleotide and moved it to a precise location on a gold layer used as the electrode. After depositing a droplet of solution, an electrochemical pulse allowed the synthesis of the polypyrrole film. By successive co-polymerisations with different oligonucleotides, they constructed a 500-µm diameter four-spot oligonucleotide array. This "electrospotting" process allows an easy and rapid preparation of oligonucleotide matrices onto a gold substrate without the need for multi-step synthesis, but with loss of the possible resolution.

d) Photolithography

Affymetrix was the pioneer in applying photolithography to microarrays (Fodor *et al.*, 1991). Their first chip was an array of 106 probes on $1cm^2$. The photolithographic DNA arrays are fabricated by *in situ* synthesis of oligonucleotides on Si wafers. The pattern of exposure to light through a mask determines which regions of the support are activated for chemical coupling. Activation by light is due to the removal of photolabile protecting groups from selected areas. After deprotection, activated nucleic acid monomers are exposed to the entire surface, but reaction takes place only with regions that were addressed by light in the previous step. The substrate is then illuminated by a second mask, which activates other sites for reaction with the following nucleic acid monomers. The procedure is repeated until the whole oligonucleotide sequence is synthesised. Pease *et al.*

(1991) improved the initial configuration and fabricated a 1.28 x 1.28-cm^2 array of 256 different tetranucleotides in 16 chemical reaction cycles, requiring 4 hours to complete. Now, their Genechip® allows the immobilisation of 9000 oligonucleotide sequences up to 30 bases in length on a glass area of 1.6cm^2. The number of compounds that can be attached by this technique is limited only by the number of synthesis sites that can be addressed with appropriate resolution. In this case, the physical limit is the radiation wavelength used for patterning. The high arraying density is the main advantage of this technique, as none of the techniques used by other companies can achieve such resolution. However, the main drawback is the *in situ* synthesis and the consequent inability to control the sequence quality. This makes the existence of a high number of redundant sites necessary. The errors in synthesis can be due to problems with the alignment of the photolithographic masks, the removal of photoprotecting groups or the phosphoramidite coupling. In order to overcome the problem of the lack of quality control, Beier and Hoheisel (2000) developed a method to check the quality of each individual DNA microarray position after the synthesis, using removable fluorescent tags. With this method, only full-length oligonucleotides were labelled. However, any previously failed deprotection leaving protected molecules could be deprotected in a succeeding step, resulting in internally deleted oligonucleotides still capable of incorporating the fluorophore, and making the hybridisation with known sequences necessary for the identification of false sequences.

e) Affinity and capture with electronic addressing

This technique has been developed by Nanogen. In principle, it is based on the immobilisation of biotinylated oligonucleotides on streptavidin sites of the array. Because DNA oligonucleotides are negatively charged, the application of an electric field favours their migration, accumulation and immobilisation to determined locations on the array (and once immobilised, the high streptavidin-biotin affinity makes the coupling essentially irreversible). In addition, these sequences can be moved away from negatively charged sites if no immobilisation is desired (Sosnowski *et al.*, 1997 and Radtkey *et al.*, 2000). This approach allows the simultaneous use of a variety of probes of different content, length and chemical composition on the same chip. Additionally, as the electric field control can also be used in the hybridisation step, the approach has significant advantage over passive arrays, where the hybridisation is limited by diffusion.

f) Selective electrodeposition

This thesis is examining a new approach for oligonucleotide arraying (Campàs and Katakis, 2002). It is based on the selective electrodeposition of biorecognition nanomodules on photolithographically defined electrodes. These biorecognition nanomodules consist of oligonucleotide molecules conjugated to colloidal gold particles. The selective deposition of these biorecognition nanomodules on electrodes held at an applied potential, permits the location of specific oligonucleotide sequences on determined electrode locations. This means that the

technique has lithographic resolution, only limited by the photolithographic limit needed to pattern the electrodes and consequently, it can be competitive with the present techniques used by the leading DNA array companies. Additionally, this approach is generic, since it can be used to construct other biochip arrays such as arrays of enzymes, antibodies, aptamers, and any other chemical of biochemical recognition elements. This deposition method, although it uses wet chemistry, is fully compatible with manufacturing and testing procedures common in the microelectronics industry, making large-scale fabrication possible. The selective deposition can be performed in short times, therefore it is conceivable that the streaming of the process can be effected at speeds that match even the most extensive contact and non contact printing heads. The main disadvantage of the method is that there is about 10% non-selective deposition between sites. This fact makes the technique unsuitable for extensive gene arrays. However, it makes it perfectly competitive for applications where 2-25 sites are needed for common diagnostics allowing the use of sample volumes from 20 to 200nL. These characteristics make the technique suitable for most common affinity or enzymatic diagnostic tests.

1.1.4. Hybridisation detection and amplification in DNA sensors and arrays

The sensitive and discriminating detection of the hybridisation event is an important feature of the successful DNA sensor or array. The biorecognition event in DNA sensors and arrays is based on the pairing affinity between complementary DNA sequences. The immobilised DNA probe recognises its complementary sequence, if the target is present in the sample. The transducer converts the biological interaction into a measurable signal, proportional to the degree of hybridisation and, consequently, to the amount of target in the sample. Whereas in direct detection techniques, the target DNA does not need to be labelled, in indirect detection techniques, labelling is a requirement to translate the hybridisation event into a signal. Label-free strategies are desirable as they facilitate reduced analysis times, and there are no undesirable effects from the label, such as steric impediments and instability of the label. However, the instrumentation cost and operational requirements of label-free techniques tend to be elevated, therefore electrochemical and fluorescence label-dependent techniques are also broadly used. In fact, most DNA chips use fluorescence methods for detection.

1.1.4.1. Hybridisation detection techniques

Several reviews describe the different transducers and detection methods used in DNA sensors (Mikkelsen, 1996; Palanti *et al.*, 1996; Yang *et al.*, 1997b; and Pividori *et al.*, 2000). Table I.3 summarises the most commonly used techniques that are briefly examined in what follows.

a) Piezoelectric methods

Piezoelectric sensors provide label-free detection. Piezoelectric transducers demonstrate high sensitivity, as they are capable of measuring sub-nanogram levels of mass change. The

transduction is based on the change in the frequency of the piezoelectric crystal when the target complementary sequence hybridises with the immobilised probe, the frequency of oscillation of the piezoelectric crystal changing proportionally to the hybridisation efficiency (Ebersole *et al.*, 1990; Caruso *et al.*, 1997a, b; Okahata *et al.*, 1992 and 1998; Ketterer *et al.*, 2000; and Bardea *et al.*, 1999). This technique allows real-time measurements, which is of great interest in DNA sensing, facilitating the determination of association constants and binding and dissociation rate constants (Okahata *et al.*, 1998 and Zhou *et al.*, 2001). Additionally, the effect of mismatches in the target sequences, hybridisation temperature or ionic strength of the hybridisation media on the kinetic parameters can be studied. Summarising, the advantages of this technique compared to fluorescence techniques are: non-requirement of probe treatment, possibility to obtain absolute binding amount and in real time, relatively rapid measurement, re-usability (removing the target oligonucleotide by heating or alkali treatment) and relatively inexpensive instrument.

Although it is not very common, piezoelectric devices can be found in a multi-array format. Tatsuma *et al.* (1999) developed four-channel QCMs, each channel giving an independent measurement. Although this is in an early step, the applicability to genetic disease diagnosis seems to be possible.

Table I.3. DNA hybridisation detection techniques.

Transducer	Techniques	Label	Real-time measurement	Multi-analysis	Ref.
Piezoelectric	Piezoelectric	Not needed	Yes	Possible	Ebersole,1990 Caruso,1997 Okahata,1992,1998 Bardea,1999 Tatsuma,1999 Ketterer,2000 Zhou,2001
Electrochemical	Chronopotentiometry Linear sweep voltammetry Cyclic voltammetry Impedance	Cationic complexes Redox compounds	Possible	Possible	Millan,1994 Palanti,1996 Marrazza,1999 Wang,1996a,1997 Napier,1997
Electrochemical	Chronoamperometry	Redox enzymes	Possible	Possible	Caruana,1999
Electrochemical	Capacitance	Not needed	Yes	Possible	Berney,2000
Optical	Colourimetry	Enzymes	Possible	Possible	Jablonski,1986 Li,1987 Kaway,1993
Optical	Surface plasmon resonance	Not needed	Yes	Possible	Jensen,1997 Georgiadis,2000 Heaton,2001
Optical	Surface plasmon resonance imaging	Not needed	Yes	Yes	Guedon,2000 Nelson,2001
Optical	Electrochemical chemiluminescence	Ruthenium chelate	No	Difficult	Blackburn,1991 Gudibande,1992 Kenten,1992 Yu,1995 Hsueh,1996
Optical	Fluorescence	Fluorophores	Yes	Possible	Abel,1996 Piunno,1995 Trabesinger,1999 Svanvik,2000b

b) Electrochemical methods

Electrochemical transduction is very useful due to its simplicity, low instrumentation costs and high sensitivity, which is comparable to fluorescence techniques. There are several electrochemical methods to detect DNA hybridisation, the use of electroactive hybridisation indicators being the most commonly used. These indicators are cationic metal complexes, like $Co(bpy)_3^{3+}$, $Co(phen)_3^{3+}$ and $Ru(bpy)_3^{3+}$, or organic compounds, like Hoechst 33258 and daunomycin, that recognise the DNA helix and intercalate selectively and reversibly into double-stranded DNA (dsDNA) (Millan *et al.*, 1994; Palanti *et al.*, 1996; Wang *et al.*, 1996a, 1997a, b, c; Napier *et al.*, 1997; and Marrazza *et al.*, 1999). This technique, although useful for obtaining general information, is not very suitable for mutation discrimination, which makes it inappropriate for diagnosis applications. Capacitance can also be used to detect specific label-free sequences, as hybridisation induces charge effects, altering the dielectric properties of the biolayer (Berney *et al.*, 2000). However, most electrochemical detection techniques include enzymatic labels. Enzymes are usually conjugated to intercalators or to avidin or streptavidin, which recognises its corresponding biotin-modified oligonucleotide, and results in an electrochemical response proportional to the hybridisation efficiency. Due to the high sensitivity of electrochemical techniques, the detection limits are always lower compared to the traditional colourimetric hybridisation assays in microtiter plates. In an advantageous strategy, Caruana and Heller (1999) labelled the target oligonucleotide directly with soybean peroxidase (SBP), a thermostable enzyme able to work at the temperatures needed in the hybridisation step. When hybridisation occurred, the enzyme was brought close to the surface, reacting with a previously deposited redox “wire” and resulting in an amperometric current proportional to the amount of hybridised strands. As no current was obtained from the enzyme-modified oligonucleotide in solution (due to the large distance between this enzyme and the redox polymer immobilised on the electrode surface), no washing step was needed and the format provided measurements in real time.

Although fluorescence is the most common detection choice for DNA arrays, some companies use electrochemical techniques. The work of Clinical Micro Sensors deserves special attention, as they have developed a versatile platform for the electronic detection of nucleic acids on microarrays (Tatsuma *et al.*, 1999). Each biochip has several electrodes, each one with a different DNA capture probe that recognises its complementary sequence. When the target hybridises with the oligonucleotide probe, a ferrocene-labelled DNA sequence (signalling probe) also hybridises with the target oligonucleotide. This implies that there is no need to incorporate a label into the target. The double hybridisation event is detected by the change in impedance. Carefully choosing the capture and signalling probes, multiple-target detection is possible, even at room temperatures. This method is not inhibited by common components of blood, serum, saliva, plasma and urine, and is compatible with PCR amplification. Moreover, since no washing is necessary, the detection works in real time. Clinical Micro Sensors have successfully applied their technology to sequence-specific

detection of amplicons, mismatch discrimination for the characterisation of single nucleotide polymorphisms, and gene expression monitoring. Additionally, the low manufacturing costs make the development of a disposable chip possible. Currently, Clinical Micro Sensors is developing the integration of specimen preparation with a new amplification method (to obviate PCR) in the same system to develop a portable device.

c) Optical methods

In DNA sensors, to use optical techniques as hybridisation detection methods, it is necessary to label the oligonucleotide with an indicator dye, a fluorophore or an enzyme that will produce a coloured product. The most commonly used techniques are based on Ultra-Violet (UV), visible (vis) or Infra-Red (IR) absorption, fluorescence and surface plasmon resonance (SPR). Below, some optical DNA sensors are reviewed.

In colourimetric DNA sensors, the enzyme is directly linked to the oligonucleotide or to antibodies, which in an affinity reaction interact with the antigen-labelled oligonucleotides. In these assays, a washing step is necessary to remove the non-hybridised oligonucleotides or the antibody-enzyme in excess, respectively. The enzyme converts its substrate into a coloured product so the change in the absorbance is directly proportional to the hybridisation efficiency (Jablonski *et al.*, 1986; Li *et al.*, 1987; and Kaway *et al.*, 1993). It is also possible to obviate the use of enzymes, by taking advantage of the absorbance of some intercalators that recognise dsDNA.

Surface plasmon resonance (SPR) is much more sensitive and moreover, it does not require any label and allows real-time measurements. In DNA sensors, the refractive index of the sensing layer changes depending on the amount of DNA in proximity with the surface. The SPR system is particularly useful for the determination of binding and dissociation kinetics, and it has been demonstrated to be sensitive enough to detect the presence of mismatches (Jensen *et al.*, 1997 and Georgiadis *et al.*, 2000). SPR can also be combined with electrochemical techniques. In this direction, Heaton *et al.* (2001) used the electric field to easily control the electrostatic forces on a surface-bound oligonucleotide monolayer. They used this field in a reversible manner to increase or decrease the rate of oligonucleotide hybridisation. Additionally, they demonstrated that a repulsive potential preferentially denatures mismatched DNA hybrids within a few minutes, while leaving the fully complementary hybrids largely intact. This sequence selectivity imparted an extremely high stringency for mutation detection based purely on electrostatic effects. However, unlike Clinical Micro Sensors, they used the strategy for DNA sensors and not for DNA arrays.

A slightly modified SPR system, called “SPR imaging”, can be used in array formats. “SPR imaging” is based on the same SPR principle, with the exception that the metal surface is imaged on a CCD camera via an imaging lens. This slight modification allows the sensing on several areas of the gold surface at the same time. Additionally, the detection is only limited by a spatial resolution of a few

micrometers. Guedon *et al.* (2000) used this system to detect the hybridisation of target sequences to four-spot polypyrrole oligonucleotide matrices and the sensitivity was a few pg mm^{-2}. Nelson *et al.* (2001) applied "SPR imaging" to the multiple detection of sequence-specific ribosomal RNA (rRNA), which is not very common and has applicability in the identification of microbial populations.

Electrogenerated chemiluminescence (ECL) is a highly sensitive technique that combines both optics and electrochemistry. It has mainly been used to detect and quantify PCR products as it increases the sensitivity and reduces routine analysis time (Blackburn *et al.*, 1991; Kenten *et al.*, 1992; Gudibande *et al.*, 1992; Yu *et al.*, 1995; and Hsueh *et al.*, 1996). In this technique, a chemiluminescence reaction is initiated by an electrical stimulus generated from the label of the complementary strand, which is a ruthenium chelate, tris(2,2'-bpy)ruthenium (II) (TBR). This technology benefits from simplicity, short analysis times, low detection limits, wide dynamic range for label quantification, and extremely stable labels.

Fluorescence is the optical technique with the highest sensitivity and lowest background noise, as weak signals are easily detected against a dark background. Fluorophores, such as acridine orange (AO) and fluorescein-isothiocyanate (FITC), are attached to the target oligonucleotide, allowing real-time monitoring of the hybridisation event and subsequent evaluation of association and dissociation kinetics (Trabesinger *et al.*, 1999). Fiber-optic sensors, based on fluorescence excitation and detection in the evanescent field of a quartz fiber, coupled to fluorescence microscopes and/or photomultipliers provide ways to detect the hybridisation of oligonucleotides. The complementary strand can be labelled with a fluorophore that in the case of biorecognition increases the emission signal (Abel *et al.*, 1996), or the double helix can be recognised by an intercalating dye, such as ethidium bromide (EB) (Piunno *et al.*, 1995), cyanine dimer YOYO or PicoGreen (Kleinjung *et al.*, 1997).

Fluorescence techniques can also be applied to homogeneous assays by using molecular beacons, scorpions and light-up probes. Molecular beacons are probes with a fluorescent moiety at one end and a quenching moiety at the other. When they are not hybridised with the target, they form a hairpin or stem-and-loop structure so that the termini are close and quenching occurs. In the presence of the target the hairpin opens because the loop portion of the molecule is complementary to the target, the two ends are then separated, and fluorescent signal results. Molecular beacons have been widely applied to real-time DNA-RNA hybridisation detection in living cells (Sokol *et al.*, 1998), to single base-pair mutation detection in PCR systems (Giesendorf *et al.*, 1998 and Chen *et al.*, 2000) and to DNA-protein binding assays (Stühmeier *et al.*, 2000). Molecular beacons have also been immobilised onto solid supports in order to apply their advantages to the DNA array format (Liu *et al.*, 2000 and Steemers *et al.*, 2000). When using molecular beacons as probes, the main advantage is that no labelling of the target is required. An interesting approach is described by Frutos *et al.* (2002), who did not use molecular beacons, but rather bimolecular beacons. In this

case, they immobilised a fluorophore-labelled probe that was hybridised with a quencher-containing sequence that was complementary except for an artificial mismatch. Hybridisation with the perfectly complementary target brought about a displacement of the mismatched sequence and a consequent increase in fluorescence. Scorpions are similar to molecular beacons, but their structure promotes a unimolecular probing mechanism instead of the common bimolecular mechanism because their amplicon specific region is attached to a PCR primer. This unimolecular mechanism makes them faster and more efficient. Additionally, scorpion primers are selective enough to give high sensitivity and to detect single-base mutations (Whitcombe *et al.*, 1999 and Thelwell *et al.*, 2000). Light-up probes are another kind of probes also used in DNA fluorescence detection in homogeneous solutions. A light-up probe is a PNA to which a dye is tethered. Upon probe hybridisation the dye binds to the target DNA, which results in a large enhancement of the dye fluorescence. These probes have several advantages as it is sufficient to measure the increase in fluorescence intensity, instead of measuring the change in the fluorescence intensity distribution (measured when energy transfer probes are used), they do not change conformation (unlike molecular beacons), and they hybridise faster and stronger than oligonucleotide probes, due to the lack of negative charges on their backbone (Ortiz *et al.*, 1998; Nikiforov *et al.*, 1999; and Svanvik *et al.*, 2000a, b).

1.1.4.2. Amplification systems

Even with all the different detection techniques, the main limiting factor for the development of DNA sensors and arrays is the sensitivity. For a viral infection, for example, the amount of DNA that has to be detected is at femtomolar (10^{-15}M) or attomolar (10^{-18}M) level. To achieve these low detection limits reliably it is possible to increase the amount of sample or to amplify the signal although, in reality, many of the approaches do both at the same time. Polymerase chain reaction (PCR) is the most commonly used technique to increase the amount of DNA in a sample. However, this procedure is time consuming. Nowadays, PCR is being integrated into sensors and arrays, in order to minimise assay time. On the other hand, signal amplification methods, like Rolling Circle Amplification (RCA), labelling or multi-labelling of the oligonucleotides with enzymes or fluorophores, Tyramide signal amplification (TSA), modified-liposomes or electrochemical amplification can be incorporated in the detection system. In the following, PCR and signal amplification methods are described.

a) Polymerase chain reaction (PCR)

PCR is the technique most commonly used for sample amplification. During PCR, the amount of DNA is exponentially amplified by repetitive cycles. PCR is also used as a method to label the target with antigens, fluorophores and labile groups, in the case that the detection technique used to measure hybridisation is not label-free (Zhu and Waggoner, 1997). Attempts have been made to

reduce the amplification time, by using small volume PCR chambers (Giordano *et al.*, 2001) or integrating the PCR step in the detection chip (Tillib *et al.*, 2001).

b) Rolling circle amplification (RCA)

RCA is a hybridisation signal amplification technique that uses padlock probes (circularising oligonucleotides). Once the probe is immobilised, the complementary target sequence is added and it hybridises. Afterwards, a second probe is added. It hybridises with part of the target and is linked to the first probe by a DNA ligase. Then, the target is removed and the padlock probe is added. In the presence of a strand-displacing DNA-polymerase, the primer is extended and, after one complete revolution of the circularised probe, the primer is displaced itself at its 5' termini. Continued polymerisation and displacement generates a single-stranded concatameric (repetitive) DNA copy of the original probe. To detect hybridisation, fluorescent complementary tags are added that hybridise at the multiple repeated sites in the elongated DNA sequence. This kind of amplification is rapid, technically simple and enables the detection of infrequent mutations in the presence of a large excess of wild-type DNA (Lizardi *et al.*, 1998 and Hatch *et al.*, 1999). RCA is a generic amplification technique, as it can also be used in antibody/antigen assays. Schweitzer *et al.* (2000) and Wiltshire *et al.* (2000) combined RCA with immunochemistry: "immunoRCA". In this technique, an oligonucleotide primer is covalently attached to an antibody. Once bound to the antigen and in the presence of circular DNA, DNA polymerase and nucleotides, amplification results in a long chain with repetitive sequences that remain attached to the antibody and that allow the antigen detection.

c) Branched DNA

Another way to amplify the hybridisation response is using branched DNA (bDNA), which is a hyperpolymeric DNA. Depending on the superstructure it is possible to find different types of bDNA: cascades, silvanes, arboranes or dendrimers. Their characteristics are uniformity, homogeneity, controlled composition and relatively large size. However, it is also possible to incorporate strands with different lengths, different orientations and even different sequences by using different blocking groups. The amplifier bDNA is commonly used after two hybridisations: a first hybridisation between the immobilised probe and the target, and a second hybridisation between the target and some preamplifiers. After this, amplifier bDNA structures are hybridised to the preamplifiers. The bDNA has multiple single-stranded arms either available for consecutive conjugation reaction with labels or for further hybridisation with labelled sequences (Collins *et al.*, 1997; Shchepinov *et al.*, 1997; Iqbal *et al.*, 1999; and Stears *et al.*, 2000). The use of bDNA amplifies the hybridisation signal and lowers the detection limit. A special case of dendritic amplification of DNA is the one based on the oligonucleotide-functionalised gold-nanoparticles used in piezoelectric techniques (Patolsky *et al.*, 2000a and Zhao *et al.*, 2001). These nanoparticles contain oligonucleotide fragments that hybridise with the target once it has already hybridised with the immobilised probe, amplifying the frequency

change. Additionally, a second amplifying step can be included by the use of a second gold-nanoparticle able to hybridise with the oligonucleotide fragments of the first one. Appropriately choosing the nanoparticle size, a detection limit of 10^{-14}M can be achieved.

d) Tyramide signal amplification™ (TSA™)

TSA™ is a signal amplification system that increases the sensitivity without loss of resolution or increase in background noise. After hybridisation between the immobilised probes and complementary oligonucleotides labelled with the epitopes biotin or dinitrophenol (DNP), streptavidin- or anti-dinitrophenol-HRP conjugates are added. Cyanide-tyramide fluorescent reagent is then added, thereby providing a fluorescent signal. In the presence of hydrogen peroxide, the HRP oxidises the phenolic ring of these cyanide-tyramide conjugates. This oxidation produces highly reactive free radical intermediates that subsequently form covalent bonds with tyrosine residues of nearby proteins used to block the immobilisation surface. In a short period, multiple depositions are possible through HRP-catalysed substrate conversion. The main advantages of this indirect labelling method are the simplicity, low cost, the 10- to 100-fold signal amplification over direct fluorescence techniques, the non-contamination of the target, and the possibility of multi-target detection by incorporating various fluorophores. Furthermore, it can be used in slide-based immunohistochemistry, *in situ* hybridisation assays and nucleic acid microarrays. TSA technology rivals *in situ* PCR particularly for detection of single copy viral nucleic acid because reproducibility and reliability are much better, and because it does not provide false positive from artefacts or back-diffusion of amplicons. However, the main drawback is the low precision for comparative analysis because of the different labelling efficiencies and protein binding affinities of the epitopes (Adler *et al.*, 2000). TSA can be also used with electrochemical detection techniques, especially impedance spectroscopy (Patolsky *et al.*, 1999).

e) Functionalised liposomes

Patolsky *et al.* (2000b) amplified oligonucleotide recognition events by using functionalised liposomes. There are two different approaches, although both are based on inhibition of the electron transfer between the redox probe $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ and the electrode surface where the probe is immobilised. In the first approach, the liposomes are functionalised with oligonucleotides, which make them negatively charged. Before hybridisation, electron transfer from the redox probe in solution to the electrode is possible and measurable in terms of Faradaic impedance spectroscopy. However, after hybridisation, the negatively charged liposome electrostatically repels the redox probe, inhibiting the electron transfer. In the second approach, the liposomes are functionalised with biotin. Before hybridisation, the redox probe can interact with the electrode. After hybridisation with biotinylated oligonucleotides, avidin molecules are added to the system. These molecules not only interact with the hybridised biotinylated oligonucleotides, but also with the multiple biotinylated liposomes, which in turn interact with other functionalised liposomes.

This large structure inhibits the accessibility of the redox couple to the electrode surface due to steric effects.

f) Electrochemical amplification

Electrochemical amplification is based on enzyme redox cycles. There are many different strategies, which combine different enzymes and transduction chemistries. One example is the cycle that uses diaphorase and dehydrogenase enzymes (Tang and Johansson, 1995). The trigger for the system is alkaline phosphatase, which could be the label of the target. This enzyme reacts with NADPH producing NADH that is oxidised by diaphorase, and the produced NAD^+ is reduced again by a dehydrogenase enzyme (alcohol dehydrogenase, glycerol dehydrogenase, etc.). The diaphorase enzyme can be then reoxidised by a redox mediator, which is in turn oxidised on the electrode surface, providing an electron flux. Because the dehydrogenase is continuously producing NADH, each molecule of NADPH can give rise to many reduced mediator molecules that amplify the signal when oxidised on the electrode. In another example, tyrosinase and dehydrogenase enzymes are used (Bauer *et al.*, 1996 and 1998). In this case, the substrate for alkaline phosphatase is phenyl phosphate, which is converted to phenol. The phenol is oxidised to catechol by the tyrosinase, which also converts the catechol to *o*-quinone. The *o*-quinone is then recycled to catechol by glucose dehydrogenase. In this amplification scheme, the O_2 consumption by tyrosinase on a Clark-type electrode is measured.

1.1.5. Stringency, imaging systems and bioinformatics

It is not within the scope of this work to provide insights to the reliability of hybridisation events and signals. On a purely informative level the possible hybridisation and detection artefacts are mentioned here. These possible artefacts together with the formidable amount of data that is generated with DNA arrays have given rise to bioinformatics that is also briefly reviewed.

The basic requirement for a functional array system is the ability of all different probes to hybridise to their target sequences with high specificity at a single temperature. Since all the different immobilised oligomers are under the same stringency conditions, signal generation may be observed even if there is not an exact binding match. This is particularly relevant for mutation discrimination. In general, it is possible to find hybridisation conditions that give quite strong signals for hybridisation and weak signals for mismatches. For example, since A and T bind more weakly than G and C, sometimes a false GC match can give a stronger signal than a true AT match. Nanogen has solved this problem by the combination of electrophoretic propulsion and electronic stringency control (Sosnowski *et al.*, 1997 and Radtkey *et al.*, 2000). Using this technique, hybridisation stringency conditions can be controlled electrochemically, approaching the analyte to the immobilised probes and repulsing the non-complementary or low-affinity sequences by

reversing the potential. In addition to this advantage, this technique accelerates the transport of DNA and concentrates it to the corresponding locations, thereby increasing the hybridisation rates.

The most extensively used detection method is fluorescence (Pease *et al.*, 1991; Yershov *et al.*, 1996; Drobyshev *et al.*, 1997; Guschin *et al.*, 1997; Healey *et al.*, 1997; Sosnowski *et al.*, 1997; Livache *et al.*, 1998; Proudnikov *et al.*, 1998 and 2000; Gentalen and Chee, 1999; Vo-Dinh *et al.*, 1999; and Tillib *et al.*, 2001). There are two different detection systems for fluorescence that have simplified data analysis and reduced costs: scanners that use lasers to illuminate one pixel at one determined time and that are coupled to photomultiplier tubes detectors, and charge-coupled devices (CCD) with continuous light source for excitation. CCD systems allow simultaneous acquisition of large images but they have the drawback of using broadband xenon bulb technology and spectral filtration for excitation, which makes the effective separation of excitation and emission light more difficult. Some CCD-based systems, in order to solve the problem of limited light collection, integrate the signal over a significant amount of time to allow collection of enough emitted photons to create an acceptable image. However, the time required for this operation is usually longer than the time that a scanning system uses to capture a comparable area. On the contrary, the laser scanning systems use defined excitation wavelengths, which provide many more excitation and emission photons generated and collected for each pixel in a given amount of time, improving the sensitivity of the detection system.

As mentioned, arraying technology, stringency control, and imaging accuracy and time have to continue to improve to efficiently take advantage of the data generated by the new generations of biochips.

Related to data acquisition is data analysis, giving rise to another branch of expertise that has had to satisfy the demands originated by the high amounts of data obtained with biochips. Bioinformatics develop algorithms that, on one hand, are fed with the construction characteristics of the biochips and, on the other hand, take advantage of the vast and expanding genomic and expression databases. Tools are therefore being developed by the same companies and institutions that produce DNA arrays, as a result of the necessity to extract results from the huge datasets that microarray hybridisations generate. These companies develop their own software for their own array schemes, as for example Synteni's GemTools and Affymetrixís LIMS. Bioinformatics includes relational databases, data storage systems, data analysis software (which requires the development of the appropriate algorithms for handling DNA and protein sequences, with results validated against reference data and expected behaviour), mathematics and graphing packages, and interpretation and visualisation programs (as for example evolutionary trees and metabolic, signalling and transport pathway diagrams). Although these bioinformatic tools require large capacity servers linked to workstations, the state-of-the-art of informatics and the continuously improved computational capabilities are rapidly making the use of biochips easier. The importance

of bioinformatics technology in the development and use of biochips is just one more demonstration of the multidisciplinary effort required to associate the data obtained to the medical, pharmacological, toxicological and environmental information and progress in these areas.

1.1.6. Conclusions and future directions

Development of DNA sensing devices is the main focus of many research groups and high technology companies. The extensive work done in this field is particularly due to the broad versatility of these DNA sensing devices. From probes to transducer substrates, from immobilisation to characterisation and detection methods, from single to multi-analyte formats, this wide range of possibilities makes the research field very diversified and competitive.

DNA chips are rapidly replacing other DNA analysis techniques, due to the obvious advantages in terms of potential applicability in rapid DNA sequencing, expression analysis and other high throughput applications. Although research is still focused on probe immobilisation for DNA sensors and new strategies appear every day, it cannot be forgotten that the main focus is now on DNA chip arraying and detection. None of the arraying techniques described above is ideal but the best resolution is achieved by the photolithographic technique developed by Affymetrix. However, despite its high resolution, it lacks probe sequence quality control, an advantage that the other arraying techniques described can provide. Pin deposition and ink-jetting are very useful printing techniques as they provide short arraying times, although they suffer from both poor uniformity of the deposits and operational problems. The arraying technique based on capture with addressing is advantageous in terms of integration, as the control by the electric field can be applied both to immobilisation as well as hybridisation. This technique, based on the streptavidin/biotin interaction for immobilisation, uses the electric field to attract the negatively charged probes and, subsequently, to accelerate the transport of complementary DNA and repel the mismatched sequences. The present work introduces a new technique based on the selective electrodeposition of biorecognition nanomodules, consisting of colloidal gold particles modified with oligonucleotide probes. This strategy allows high resolution and fast selective deposition, is an integrated and generic approach, benefits from low manufacturing costs, is compatible with the microelectronics industry and, once optimised, can lead to miniaturisation.

Regarding the detection of hybridisation, the technique most commonly used in DNA arrays is fluorescence. In this field, new probes such as molecular beacons, scorpions and light-up probes, although not still fully developed for their use in solid-supported DNA arrays, seem to be powerful tools for sensitive, fast and label-free hybridisation measurements. The approach developed by Clinical Micro Sensors, based on electrochemical detection, is especially interesting due to several advantages: no need for a label, multiple target detection possible even at room temperatures, no interferences from common components of blood, serum, saliva, plasma and urine, compatibility with PCR amplification, no washing, real-time detection, reusability, low manufacturing costs and

possibility to miniaturise. Although fluorescence techniques are very difficult to compete with, Clinical Micro Sensors possesses at the time of writing the most competitive DNA sensor technology based on electrochemistry, although it might only be applicable to a small number of sensors, mainly for diagnostic applications.

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Abbreviations

A: adenine

AC: alternating current

AO: acridine orange

bDNA: branched DNA

bpy: 2,2'-bipyridine

C: cytosine

CCD: charge-coupled device

cDNA: complementary DNA

CV: cyclic voltammetry

DNA: deoxyribonucleic acid

DNP: dinitrophenol

dsDNA: double-stranded DNA

EB: ethidium bromide

ECL: electrogenerated chemiluminescence

EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

FITC: fluorescein-isothiocyanate

G: guanine

HCMV: human cytomegalovirus

HRP: horseradish peroxidase

IR: infra-red

ITO: indium-tin oxide

LSV: linear sweep voltammetry

mRNA: messenger RNA

NHS: N-hydroxysuccinimide

PCR: polymerase chain reaction

phen: phenanthroline
PNA: peptide nucleic acid
QCM: quartz crystal microbalance
RCA: rolling circle amplification
RNA: ribonucleic acid
rRNA: ribosomal RNA
SAM: self-assembled monolayer
SBH: sequencing by hybridisation
SBP: soybean peroxidase
SERS: surface-enhanced Raman scattering
SNP: single nucleotide polymorphism
SPR: surface plasmon resonance
ssDNA: single-stranded DNA
STM: Scanning Tunnelling Microscopy
T: thymine
TBR: tris(2,2'-bipyridine) ruthenium (II)
T_m: melting temperature
TSA: Tyramide signal amplification
UV: Ultra-Violet
vis: visible
XPS: X-ray photoelectron spectroscopy

1.2. Colloidal gold conjugations and deposition

One of the first studies of colloidal gold systems was reported by Faraday in 1857. He described the stability of colloids in different electrolytes. He observed that the addition of salt changed the solution colour from ruby towards blue and that solid tended to precipitate from the blue liquid. Neither the blue liquid nor the deposits could be changed back to ruby. However, he also demonstrated the protective effect of gelatine and other macromolecules, suggesting that the change induced by changing conditions was “not a change of the gold as gold, but rather a change in the relations of the surface of the particle to the surrounding medium”. His observations were the beginning of colloidal research.

1.2.1. Colloidal gold suspensions characteristics

A colloidal suspension is a dispersed phase (or discontinuous phase) distributed uniformly in a finely divided state in a dispersion medium (or continuous phase). An important characteristic of this kind of suspension is the high interfacial area between the discontinuous and the continuous phase. Additionally, each colloidal particle is made up of a limited number of molecules, and most of them lie at or close to the surface of the particle.

Of interest here are colloidal gold suspensions, where the discontinuous phase is formed by the colloidal gold particles and the continuous phase is the aqueous solution. A colloidal gold particle consists of an elemental gold core with adsorbed ions on its surface, such as Cl^-, $AuCl_2^-$ or citrate ions produced from its synthesis. These ions confer a negative charge on the particle and stabilise the dispersion in an aqueous suspension, preventing aggregation by electrostatic repulsion. Their detectability under light and electron microscopy, as well as the small size, the high stability, the non-toxicity and the possibility to conjugate them to a great variety of biomolecules, make colloidal gold particles suitable as markers (Roth and Binder, 1978). Furthermore, their ability to act as nucleation sites for the electroless deposition of silver enhances their detectability under light microscopy (Danscher and Rytter-Norgaard, 1983; Brada and Roth, 1984; De Waele *et al.*, 1989; Van den Brink *et al.*, 1990; Medalia *et al.*, 1999; and Hainfeld and Powell, 2000).

Most colloidal gold particles have a diameter between 2 and 150nm, depending on the synthesis method. All the synthesis methods are based on the reduction of Au^{3+}, usually in the form of gold trichloride, using different reducing agents and under varying conditions. This reduction produces a supersaturated molecular Au^0 solution and as the Au^0 concentration increases the gold atoms cluster and form seed of nuclei. Particle growth occurs by further deposition of metallic gold upon the nuclei (Slot and Geuze, 1985). The size of the particle depends on the concentration of Au^{3+}, the reducing agent used, its concentration, the reaction time and the hydrodynamics of the reacting mixture. In general terms, strong reductants produce a great number of nuclei and hence rather small particles, and vice versa. For example, small particle sizes (<5nm) are obtained with high

power reductants, such as sodium borohydride (Bonnard *et al.*, 1984) or yellow or white phosphorous (Faulk and Taylor, 1971; Horisberger and Rosset, 1977; and Pawley and Albrecht, 1989), and drastic conditions; medium-sized particles (6-15nm) are formed by treatment with sodium ascorbate (Horisberger and Tacchini-Vonlanthen, 1983 and Albrecht *et al.*, 1988); and larger particles are obtained with sodium citrate and milder conditions (Horisberger and Rosset, 1977; Horisberger, 1979; and Pow and Morris, 1991). Colloidal suspensions are not of uniform size, but instead a Gaussian distribution of sizes is produced (Weisbecker *et al.*, 1996; Mayya *et al.*, 1997; and Goia et Matijević, 1999). The colour of the suspension depends on the particle size, yellow-orange being the suspension of the smallest particles, red the suspension of the midrange particle size, and blue-green the suspension of the largest particles. This observed effect could be explained by Mie's theory (1908), which illustrates the size dependence of extinction by colloidal gold, relating it with the absorption and the scattering of light. On one hand, particles with diameter between 5.2 and 20.0nm absorb at around λ = 520nm, and as the diameter increases beyond 20.0nm, the absorption peak broadens and shifts towards longer wavelengths. On the other hand, scattering of white light by small particles is weak but starts to be important when the diameter is larger than 50nm. The colour of the suspension is the result of both preferential scattering and absorption at longer wavelengths for larger particles.

1.2.2. Stability of colloidal gold suspensions

The DLVO theory, developed independently by Derjaguin and Landau (1941) and Verwey and Overbeek (1948), established the basis of behaviour of colloidal systems in suspension, quantifying the combined effect of short- and long-range forces on the behaviour of this metastable system.

A number of phenomena and physical forces are relevant to the study of colloidal systems and their electrostatic stabilisation. Particles move according to Brownian dynamics subject to inertial, viscous and gravitational force fields. Since the beginning of the century, Einstein and Langevin have set forth the theory describing these phenomena (Einstein, 1906 and Langevin, 1908). This movement is subject to dispersion and repulsive (mainly electrostatic) forces whose balance decides the stability of the suspension. Quantification of dispersive forces was developed by Hamaker in 1937. As for the electrostatic potential, it was not until Gouy (1910) and Chapman (1913) developed their theory relating the potential of the particles with the thickness of the diffuse layer depending on the ionic strength of the electrolyte, that a quantitative description of these phenomena could be made. As mentioned, DLVO theory integrates these effects in a quantitative description of the interparticle potential that in general presents two minima separated by a potential maximum at intermediate distances, as depicted in Figure I.1.

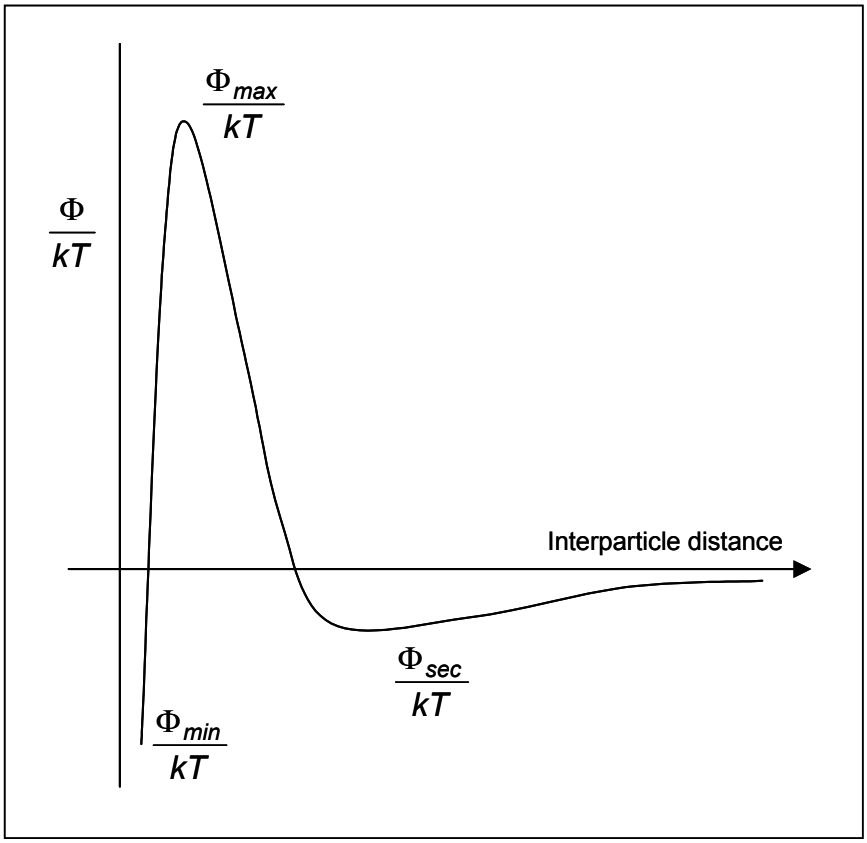


Figure I.1. General presentation of interaction potential of spheres in water.

At low ionic strength the $\frac{\Phi_{max}}{kT}$ is high enough so as to constitute a repulsive barrier that maintains the dispersion stable in the kinetic sense, although is not at thermodynamic equilibrium. An approximation of the interparticle potential expression can be reached for a symmetric electrolyte:

$$\frac{\Phi}{kT} = \frac{8\,a}{z^2 l_b} \tanh^2 \frac{ez\,\Psi_s}{4kT} \exp\left[-\frac{(r-2a)}{l_b}\right] - \frac{aA_{\text{eff}}(0)}{12kT(r-2a)} \qquad \text{(Eq. I.2)}$$

where $\frac{\Phi}{kT}$ is the dimensionless interparticle interaction potential, a is the particle radius, r is the centre-to-centre particle separation, l_b is the Debye length, z is the valence of the particle, e is the fundamental charge, Ψ_s is the particle surface potential, and A_{eff} / kT is the effective Hamaker constant. This expression indicates that Φ_{max} can be made to disappear at $\Phi = -\frac{d\Phi}{dr} = 0$ at decreasing Debye length (l_b). The Debye length can be minimised at increasing ionic strength and the critical salt concentration that brings about the irreversible flocculation can be calculated.

The above analysis leaves out of the picture chemical effects, such as the nature of the electrolyte (for example valence), although it can be accounted for implicitly. Despite this, it is a good starting

point for simple colloidal systems. Still, through the Hamaker constant (A_{eff}), the expression accounts for the properties of the dispersed phase.

Although Eq. I.2 gives a qualitative understanding of colloidal stability, the prediction of the same under particular conditions or the explanation of the instability of small particle colloids requires statistical thermodynamic considerations that lead to the definition of the stability ratio $W = J_0 / J$ where J_0 is the rate of flocculation calculated from the free diffusion of spheres, whereas J is the flux of particles under an arbitrary potential.

Substituting the linearised DLVO description of Φ allows the explicit calculation of W under specified conditions and thus the prediction of stability of a colloidal suspension. It should be noted that W is a dynamic measure of stability that also allows the calculation of the time scale of the formation of doublets that is considered the first event of flocculation. In the 1970s (Honig *et al.*, 1978) the effect of hydrodynamics on flocculation was included in the expressions for W predicting accurately shear effects on colloid stability.

The final modifications of the DLVO theory of relevance to this thesis have to do with the effect of osmotic forces and entropy on colloidal stability. Polymer addition to colloidal suspensions was known to stabilise them. Later observations rationalised the effects of polymers depending on their relation to the dispersed phase (Napper, 1983). Strongly adsorbing polymers change the nature of the surface interaction and depending on their extension in solution can stabilise or destabilise entropically the suspension. Non-adsorbing polymers can increase the osmotic drive for aggregation in the case they are excluded from the interparticle space. Quantitative descriptions of the interparticle potential as modified by the presence of polymers in colloidal systems have been developed (deGennes, 1980, 1982 and 1987).

1.2.3. Colloidal gold conjugation: stabilisation or aggregation?

As mentioned, addition of macromolecules or biomolecules, especially proteins, can protect colloidal gold from aggregation by conjugation to it, preventing cohesion of one particle to another and at the same time converting the lyophobic colloidal gold suspension to a lyophilic one that assumes many of the surface properties characteristics of the protein. However, the eventual stabilisation or destabilisation of the suspension will depend on the properties of the conjugating biomolecule, the extent of conjugation and the characteristics of the solution. The conjugation of biomolecules to colloidal gold depends on three parameters: 1) the electrostatic attraction between the negatively charged particle and the positively charged sites on the biomolecules, 2) the adsorption forces between the metal surface and the hydrophobic pockets on the biomolecule, and 3) the dative binding between gold and sulfhydryl groups, if present in the biomolecule. The temperature, the concentrations of the reagents, the pH and the ionic strength of the media have to be considered during and after conjugation. The optimum pH for conjugation is close to or slightly

above the isoelectric point (pI) of the biomolecule because at this pH the maximum adsorption efficiency and the highest stability are obtained. This point corresponds to the maximal interfacial tension and lowest solubility for the protein, favouring adsorption. If conjugation is attempted at lower pH, aggregation of colloidal gold occurs immediately upon addition of protein, due to the strong electrostatic attraction forces that cause the bonding of one protein to several gold particles. At higher pH, the adsorption is lower due to the higher charge repulsion between the protein and the colloid, and the increased hydration of the biomolecule.

Once the conjugation is concluded, blocking agents are used to block any remaining free sites, usually by adsorption, and to further stabilise the colloidal gold suspension. These blocking agents are also called protective agents. If they are negatively charged, they provide an extra repulsive force to the system, preventing aggregation. If they are positively charged, they can have a detrimental effect if not used in the appropriate concentration.

Usually, the formation of biomolecule-gold conjugates does not affect the biological activity of the biomolecule (Crumbliss *et al.*, 1992). However, alteration of biological activity may occur due to steric effects. In fact, depending on the number of contacts per molecule with the colloidal gold particle and its orientation, the adsorbed biomolecule can partially lose its specific activity or biorecognition capacity.

A variety of biomolecules can be conjugated to gold particles. Several types of proteins and small molecules have been conjugated, such as antibodies (Geoghegan *et al.*, 1977; Slot and Geuze, 1985; De Waele *et al.*, 1989; Lyon *et al.*, 1998; and Seelenbinder *et al.*, 1999), immunoglobulin binding proteins such as protein A (Brada and Roth, 1984 and Slot and Geuze, 1985), avidin (Morris and Saelinger, 1984 and González-García *et al.*, 2000) and streptavidin (Bonnard *et al.*, 1984 and González-García *et al.*, 2000), enzymes (Geoghegan *et al.*, 1977; Crumbliss *et al.*, 1992; and O'Daly *et al.*, 1995), lectins (Benhamou *et al.*, 1988) and alkanethiolates (Weisbecker *et al.*, 1996), as well as DNA (Elghanian *et al.*, 1997; Mucic *et al.*, 1998; and Storhoff *et al.*, 1998), RNA (Medalia *et al.*, 1999) and polymers.

1.2.4. Colloidal gold electrodeposition: the theory

When colloidal particles are charged, externally applied electric fields can cause them to migrate by electrophoresis. This discovery dates from 1809, when Reuss saw that colloidal particles were charged and noted their motion under an electric field. After him, Linder and Picton found that synthetic sols of sulphur, ferrocyanide, gold, silver and platinum were negatively charged, while oxide sols of iron, chromium, aluminium and cerium were positive. In 1940, Hamaker and Verwey postulated that the chief action of the electric field is to move the particles towards the electrode and produce a force that presses the particles together on the surface of the electrode in the same

way as the force of gravity presses them on the bottom of a container. In a more quantitative way, Yang *et al.* (1998) divided the deposition process in four stages:

1. At large distances from the electrode, particle transport is due to convection and migration due to external forces.
2. As a particle approaches the collector within a distance comparable to the particle size, the resulting reduction in particle mobility is due to the particle-wall hydrodynamic interaction.
3. At closer distances to the electrode, apart from hydrodynamic interaction, colloidal forces (Van der Waals and electrostatic interactions) appear. In addition, hydration, steric and hydrophobic forces may also be present and the whole process may be controlled by solution hydrodynamics.
4. When the particle is close to physical contact with the electrode, stochastic effects such as flux due to discrete charges at the collector and particles surfaces, surface heterogeneity, roughness and polymer bridging, may play significant roles. Again, hydrodynamics and particle-to-particle repulsion may play a role in the establishment of the eventual equilibrium.

Several models, like the random sequential adsorption (RSA) model, have been developed and are being refined to account for adsorbed particle interactions (Adamczyk *et al.*, 1994 and 1997, and Adamczyk and Weroński, 1997) and transport mechanisms, such as diffusion, convection, etc., in an effort to describe irreversible colloidal adsorption. Lavalle *et al.* (1999) also modified the RSA model including the diffusion of the particles during deposition, allowing an accurate description of the irreversible deposition process. In this direction, Wojtaszczyk *et al.* (1997) and Faraudo and Bafaluy (1999) modelled the same system taking into account both the transport from the bulk and the interaction with pre-adsorbed particles, which was also useful in describing irreversible colloidal adsorption.

Although the RSA model or modifications can successfully predict the adsorption of colloidal gold particles, during electrodeposition, in the presence of an electrical field, the situation changes. The general belief is that colloidal particles are electrodeposited by an irreversible process, and that mobility on the electrode is unexpected due to both the strong attraction between the positive electrode and the negatively charged colloids. However, Trau *et al.* (1996 and 1997) observed migration and postulated the appearance of an attractive force between deposited like-charged colloidal particles that overcomes the effect of the repulsive forces. This attractive force probably occurs due to electrohydrodynamic effects arising from charge accumulation near the electrodes due to the passage of ionic current through the solution and can, not only lead to particle approach, but also to monolayer or aggregation formation. Böhmer (1996) and Solomentsev *et al.* (1997) observed that electroosmosis effects could also explain particle motion.

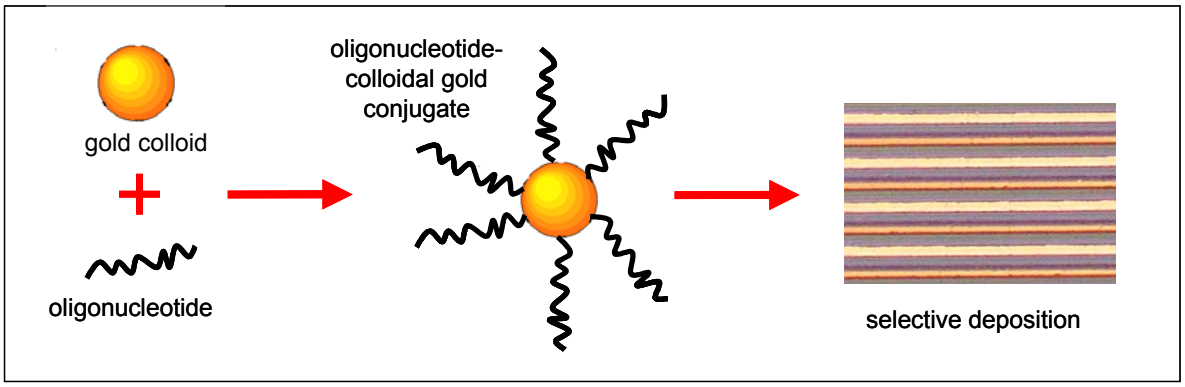
1.2.5. Colloidal gold electrodeposition: the practice

Colloidal particle deposition in general is both a well-studied and long-applied technique, for protection of metal surfaces, painting, etc. (Narayan and Narayana, 1979; Celis and Roos, 1982; and Hovestad and Janssen, 1995). Despić and Pavlović (1984) were the pioneers in colloidal gold electrodeposition on electrodes. They took advantage of the electrophoretic mobility of colloidal gold to deposit it on a carbon electrode under an applied potential. They suggested a discharge-controlled process resulting from the oxidation of the oxygen-containing species that had adsorbed on the gold particles and the formation of oxide or oxygen, which could react with the carbon electrode and thus stabilise the deposition. In the same direction, Bailey *et al.* (2000) deposited citrate-stabilised colloidal gold on micro-patterned conductive indium-tin oxide (ITO) substrates by electrophoresis, and examined the optical properties of the patterned films. Whereas in the assembly on thiol-activated surfaces the colloidal gold particles are immobilised in a randomly and low-packed distribution (Grabar *et al.*, 1996), the structure of electrodeposited colloidal gold particles is ordered and close-packed (Giersig and Mulvaney, 1993). As mentioned above, the attractive interaction that overcomes the repulsion between colloids probably comes from electrohydrodynamic and electroosmic effects. This force of attraction can not only make colloidal particles move toward each other, but also form multilayers or agglomerates, as Despić and Pavlović (1984) and Trau *et al.* (1996) observed experimentally. In fact, Zhao *et al.* (2000) observed that higher field strength resulted in more orderly packed structures and higher coverage, probably due to the stronger attractive forces between particles.

Colloidal gold can be used as probe immobilisation tool, when conjugated to a biomolecule, such as GOx (Yabuki and Mizutani, 1995), HRP (O'Daly *et al.*, 1995), xanthine oxidase and bovine carbonic anhydrase (Crumbliss *et al.*, 1992). Combining the electrodeposition with the electrochemical determination of gold by cyclic voltammetry, differential pulse voltammetry or linear sweep voltammetry (Alexander *et al.*, 1978; Despić and Pavlović, 1984; González-García and Costa-García, 1995; Trancoso and Barros, 1989; Dequaire *et al.*, 2000; and González-García *et al.*, 2000), it is possible to determine the conjugate immobilisation on an electrode surface. Furthermore, steady-state amperometry monitoring of the enzymatic substrate consumption or enzymatic activity assay of the conjugates lead to determine if the biomolecule has retained its biological activity after conjugation and deposition under an applied potential, which makes this strategy attractive for the development of amperometric sensors (Crumbliss *et al.*, 1992; O'Daly *et al.*, 1995; and Yabuki and Mizutani, 1995).

The modular approach for DNA chip arrays proposed in the present work, depicted in Scheme I.1, is based on the dual ability of colloidal gold to be electrodeposited on the electrode surface and to be conjugated to biomolecules. Thiol-modified oligonucleotides were conjugated to colloidal gold, and the conjugations resulted in stable and functional DNA-gold nanomodules. In continuation, the

conjugates were selectively electrodeposited on different electrode surfaces, and the depositions were examined by different characterisation techniques. Moreover, the electrodeposited biorecognition modules were functional, i.e. hybridisable. The ability to selectively deposit these nanomodules and the observed functionality after immobilisation make the strategy suitable as a probe arraying method for DNA chips.



Scheme I.1. Strategy based on the conjugation of oligonucleotides on colloidal gold and the subsequent selective electrodeposition on arrays.

1.2.6. Oligonucleotide-colloidal gold hybridisation

Until now, the gold particle in the oligonucleotide-colloidal gold conjugates has been mainly used as a label in *in situ* hybridisation detection using microscopy (Van den Brink *et al.*, 1990). However, some authors have taken advantage of other colloidal gold properties in conjunction with DNA hybridisation. For example, Mirkin *et al.* (1996) constructed a nanocrystal assembly, forming dimers or trimers by hybridisation. Firstly, they tagged 13nm gold particles with non-complementary oligonucleotides. Afterwards, a duplex DNA with sticky ends complementary to the oligonucleotides on the particles was added. In that moment, aggregation occurred and as consequence a macroscopic network was created. The process could be reversed by changing the temperature above the T_m of the hybridised complementary strands, and the phenomenon was monitored by spectrophotometry. Mucic *et al.* (1998) used colloidal gold particles of different sizes simultaneously to create mixed nanostructures. Similarly, Alivisatos *et al.* (1996) demonstrated that individual 1.4nm gold particles functionalised with oligonucleotides were aligned upon a single strand of DNA in a “head-to-head” of “head-to-tail” fashion, based on the hybridisation event. These works are examples on how oligonucleotide-conjugated colloids are being used as nanomodules to construct nanoarchitectures. Modified colloids as building microblocks or nanomodules are thus becoming a powerful tool to form bottom-up superstructures with many possible applications.

Additionally, oligonucleotide-colloidal gold conjugates have been used to develop highly sensitive DNA detection methods. Reynolds *et al.* (2000) functionalised latex microspheres and gold particles with different oligonucleotide sequences and then introduced the target oligonucleotide into the

sample. When the target was complementary to both probes, the linking event between the gold particles and the latex microspheres resulted in a white-to-red colour change. Their detection limit was 500pM for a 24-base target. Elghanian *et al.* (1997) and Storhoff *et al.* (1998) used the same colloidal gold property but they observed red-to-purple/blue colour change associated to the hybridisation and consequent aggregate and polymeric net formation, which enabled to distinguish target sequences with one-base mismatches, deletions or insertions from the fully complementary ones. They detected about 10 femtomoles of a 30-base oligonucleotide.

Patolsky *et al.* (2000) used colloidal gold as an amplification tool. They labelled oligonucleotides with 12nm colloidal gold particles and used them as amplification probes in the detection of hybridisation on a QCM crystal. Their limit of detection was 100pM, but they could go to lower limits of detection by using a second amplification with dendritic oligonucleotide-colloidal gold structures, which amplified the response by 3 times. Zhao *et al.* (2001) used the same system but with 50nm colloidal gold particles and they obtained a lower detection limit of detection of 10fM.

In the present work, the colloidal gold-DNA conjugates have been used as nanomodules to develop a new and promising DNA arraying method. As it has been mentioned before, the biorecognition modules formed by thiol-modified oligonucleotides conjugated to colloidal gold were functional. In other words, the oligonucleotide sequence was able to recognise its complementary sequence when it was present into the sample. The hybridisation event was examined both colourimetrically and electrochemically, and both techniques reported satisfactory results. Moreover, the DNA sensor was able to discriminate a sequence with 4-point mutations. It is also necessary to mention that this strategy is not useful only to construct DNA arrays, but also to create any multi-sensoric platform, as colloidal gold can be conjugated to any biomolecule, and the selective electrodeposition of the nanomodules does not depend of the kind of biorecognition element.

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Abbreviations

att: attraction

DLVO: Derjaguin - Landau - Verwey – Overbeek

DNA: deoxyribonucleic acid

GOx: glucose oxidase

HRP: horseradish peroxidase

ITO: indium-tin oxide

rep: repulsion

QCM: quartz crystal microbalance

RNA: ribonucleic acid

RSA: random sequential adsorption

T_m: melting temperature

1.3. Signal amplification: electron transfer kinetics

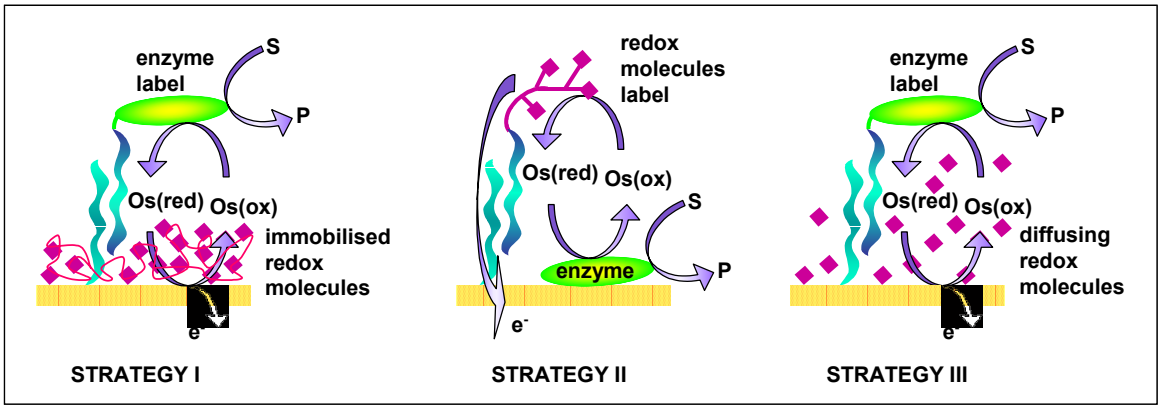
1.3.1. Hybridisation detection and amplification in DNA sensors

Detection of DNA hybridisation depends on the signal generated by the biorecognition event. One of the problems is that the amount of DNA that has to be detected is at the femtomolar (10^{-15}M) or attomolar (10^{-18}M) level. To solve this problem, samples are subjected to PCR, a technique that increases the amount of DNA in the sample. Different methods can be used to detect hybridisation. In this work, an electrochemical method of transduction was employed. Among the different signal transducers, the electrochemical ones (Millan and Mikkelsen, 1993; Millan *et al.*, 1994; Hashimoto *et al.*, 1994; Kolakowski *et al.*, 1996; Palanti *et al.*, 1996; Wang *et al.*, 1996, 1997a, b, c; Napier and Thorp, 1997; Napier *et al.*, 1997; Marrazza *et al.*, 1999; Caruana and Heller, 1999; Ontko *et al.*, 1999; and Azek *et al.*, 2000) have certain advantages, such as high sensitivity, fast response, robustness, potential for cost savings, miniaturisation, mass production, portability and automatisation of measurement and data processing.

The goal in this work is to develop integrated DNA amperometric array devices. The strategy is based on the integration of probe arraying and target sensing by the same method. It consists of the arraying of biorecognition nanomodules on biochips, by selective electrodeposition of the oligonucleotide-thiol-colloidal gold conjugates on determined sites of the array, and of the measurement of the biorecognition event, using the appropriate electrochemical enzymatic label, whose electron communication with the transducer is usually mediated by a redox compound.

In the amperometric DNA sensors based on redox enzyme-labelled targets, the oxidation current obtained at the electrode is proportional to the amount of enzyme in proximity to the electrode. As the enzyme is the label, this oxidation current will be proportional to the hybridisation yield. An essential prerequisite for the development of such sensors with high sensitivity and fast response is to establish a fast electron transfer from the redox enzyme to the electrode. As it is known, most redox enzymes cannot transfer electrons to the electrode and it is here where mediators play their role. Mediators catalyse the electron transfer between enzymes and electrodes by shuttling electrons between the enzyme active site and the electrode.

For the purposes of the present work, the state-of-the-art on redox electron exchange and the study of electron transfer between biomolecules and mediators are relevant because in a label-dependent detection of hybridisation, one of three possible strategies can be envisioned as shown in Scheme I.2. Any one of these strategies can be combined with one or more enzyme cascades for signal amplification and reagentless operation (as for example when the sample contains the substrate of the immobilised enzyme whose product is substrate for the label enzyme, having the additional advantage that an inverted diffusion layer is created for the label‘s substrate starting at the electrode surface and thus contributing to the possibility of separation-less assays).



Scheme I.2. Strategies for the electrochemical detection of hybridisation.

In all these strategies, two phenomena involved in electron transport are relevant for the optimum operation of the detection scheme:

a. **Electron tunnelling**: whenever electrons have to "jump" through a distance (be it from the label's position to the electrode surface or from the enzyme active site to the mediator), it is important to take into consideration the Marcus theory of biological electron transfer to predict the electron tunnelling.

b. **Electron exchange kinetics**: when electrons are shuttled through a mediator, it is important to achieve optimum electron exchange rate constants.

In the following, the parameters influencing both phenomena are briefly discussed.

1.3.2. Marcus' theory and mediators' role

For an electron transfer reaction to occur, reactants must approach each other to facilitate electron coupling. The Marcus semiclassical theory (Marcus, 1965 and Marcus and Sutin, 1985) predicts and experiments show that electron transfer rates decay exponentially with distance, when distance exceeds atomic dimensions (>3Å), and that they also depend on the nature of the intervening medium. This dependence can be expressed as:

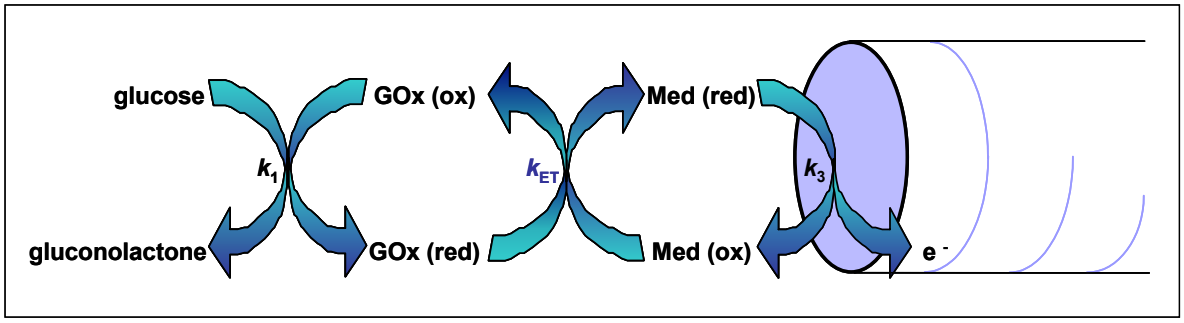
$$k_{ET} = 10^{13}\, exp\,(-ß(d - 3))\, exp\,(-(\Delta G^{0} + \lambda)^{2} / 4RT\lambda) \qquad \text{(Eq. I.3)}$$

where k_{ET} is the electron transfer rate constant, ß is a constant reflecting the effect of the medium, $-\Delta G^{0}$ is the reaction free energy, related to the driving force, i.e. the formal redox potential difference between the acceptor and the donor, and λ is the Marcus reorganisation energy.

The first application of Marcus' theory in the design of DNA hybridisation detection schemes has to do with the calculation of the possible distance of immobilisation of the label through which electron transfer can still occur at meaningful rates. Numerous works (Closs *et al.*, 1986; Mayo *et al.*, 1986; Lawson *et al.*, 1989; Wuttke *et al.*, 1992; Bjerrum *et al.*, 1995; Langen *et al.*, 1995; Smaley *et al.*,

1995; Gray and Winkler, 1997; and Winkler *et al.*, 1999) have shown the determined values of β to be 0.7-1.6Å^{-1} in biological layers and 0.8-1.1Å^{-1} in saturated alkane bridges. Furthermore, Bretz and Abruña (1995 and 1996) have shown that efficient electron transfer can occur even in 6-bonded monolayers through 6 bonds or through electron jumping in layer-by-layer architectures. The significance of these findings is that Strategy II of Scheme I.2 would only be feasible if the redox moiety could approach the electrode surface at short distances. Since this would require labelling of the proximal end of the target, it is likely that the specificity of the hybridisation event would be affected due to steric and electrostatic interferences of the label. Therefore, work should centre on strategies I and III, each one having relative advantages.

The second application of Eq. I.3 is relevant when studying electron transfer itself: usually enzymes are not able to transfer directly electrons to the electrode surface, due to the poor accessibility of their active site, which is imbedded in the insulating protein shell. One example was reported by Hecht *et al.* (1993), who observed a long funnel in glucose oxidase (GOx), allowing little accessibility to the solvent and with the flavin adenine dinucleotide (FAD) at the bottom. Mediators, immobilised or freely diffusing, somehow establish electrical contact between the enzyme active site and the electrode surface. In the example of GOx, mediators accept electrons from the $FADH_2$, competing with O_2, and afterwards, transfer these electrons to an electrode at a fixed potential where they are regenerated. Scheme I.3 shows these events.



Scheme I.3. Electrochemical transduction of mediated GOx.

In some cases electron transfer between proteins and redox mediators has been observed to occur at very high rates, even at long distances (it is accepted that the mediator cannot reach the enzymatic active site). These high rates may be due to the presence of electron-conducting "pathways" where an electron tunnelling effect is produced. The existence of these "pathways" has been experimentally verified in proteins like cytochrome *c* and myoglobin using semi-synthesised proteins with incorporated redox complexes (Mayo *et al.*, 1986; Wuttke *et al.*, 1992; Casimiro *et al.*, 1993; and Gray and Winkler, 1997). These electron-conducting "pathways" are especially interesting in the DNA hybridisation detection schemes because their presence in the enzyme label can contribute to an increase in the rate constant for the electron transfer reaction with the redox mediator, permitting to obtain higher catalytic currents and lower limits of detection.

1.3.3. Electron transfer rate constant determination

The homogeneous electron transfer rate constant of the reaction between a redox couple and a redox enzyme is thus of interest in the design and improvement of biosensors, be them catalytic or affinity using redox enzyme labels, especially for the implementation of Strategy III in Scheme I.2.

These constants can be determined by cyclic voltammetry, based on the analysis of electrocatalytic reactions described by Nicholson and Shain in 1964. Two criteria must be fulfilled in order to apply their analysis: the electrode reaction of the mediator has to be fast compared to the rate between the mediator and the enzyme, and the substrate (in the case of GOx, glucose) has to be in a saturating concentration to ensure that all the enzyme is in the reduced form. If these hypotheses are fulfilled, the limiting step of the overall mechanism is the reaction between the reduced enzyme and the oxidised mediator.

The theoretical treatment of Nicholson and Shain (1964) is suitable for the EC_{cat} reactions:

$$Enz_{red} + Med_{ox} \longrightarrow Med_{red}$$

$$Med_{red} \longleftrightarrow Med_{ox} + ne^-$$

where Med_{ox} and Med_{red} are the respective redox forms of the mediator and Enz_{red} is the reduced enzyme. This theory provides an equation for the steady-state current, i_{SS}, which is an expression derived from the Savéant and Vianello (1965) equation:

$$i_{SS} = n\,F\,A\,C_{med}\,(D_{med}\,k_{ET}\,C_{enz})^{1/2} \qquad \text{(Eq. I.4)}$$

where i_{SS} is the steady-state current, A is the electrode area, C_{med} is the solution concentration of mediator, D_{med} is the diffusion coefficient of mediator, k_{ET} is the electron transfer rate constant between the enzyme and the mediator, and C_{enz} is the enzyme concentration in solution. This equation predicts a scan rate independent steady-state intensity, and a dependence of this intensity on the square root of the enzyme concentration. Plots of i_{SS} *vs.* $C_{enz}^{½}$ are linear and, according to this equation, provide the k_{ET} values if the other parameters are known.

Many works have been performed to study the “best” enzyme-mediator combination. Although ferrocene and its derivatives were the first mediators used for GOx, due to their high efficiency, stability and pH-independent redox potentials (Cass *et al.*, 1984 and 1985; Green and Hill, 1986; Jönsson *et al.*, 1989; Liaudet *et al.*, 1990; and Bourdillon *et al.*, 1993), other mediators, like derivatives of phenothiazines and phenoxazines, Wurster’s salts, benzoquinones, and cobalt, iron, ruthenium and osmium complexes have also been used (Kulys *et al.*, 1988 and 1994; Scheller *et al.*, 1989; Zakeeruddin *et al.*, 1992; and Fraser *et al.*, 1993). The apparent self-exchange rate constants of the cytochrome *c* heme site have also been studied (Coury *et al.*, 1990, 1991 and 1993). Apart from these electrochemical studies, it is necessary to mention the extensive work on

photo-induced electron transfer carried out by Gray and co-workers (Wherland and Gray, 1976; Crutchley *et al.*, 1984; Mayo *et al.*, 1986; Wuttke *et al.*, 1992; Casimiro *et al.*, 1993; Bjerrum *et al.*, 1995; Winkler and Gray, 1997; Gray and Winkler, 1997; and Winkler *et al.*, 1999), who studied the long-range electron transfer in biological systems, like myoglobin and cytochrome *c*, and helped to understand the electron tunnelling process in proteins. In recent studies, Savéant's group has developed a detailed theoretical model applicable to a large variety of experimental systems, which analysed the competition between substrate and cosubstrate on the kinetic control of the overall process, and its effect on the electrochemical response (Limoges *et al.*, 2002a, b).

In this thesis, a systematic study has been carried out independently varying the global charge and redox potential of several osmium redox complexes, and examining the effect of ionic strength and pH on the electron transfer rate constant to rationally design redox mediators for redox enzymes. These studies provide the information to elaborate enzymatic mechanisms and to rationally design electrochemical signal amplification schemes for affinity sensors.

1.3.4. Electrochemical amplification

As mentioned above, apart from intrinsically increasing the current from the redox system with more efficient mediators, it is possible to amplify the electrochemical signal using enzymatic cascades. Most electrochemical amplification schemes use dehydrogenase enzymes combined with diaphorase (Tang and Johansson, 1995) or tyrosinase (Bauer *et al.* 1996 and 1998) to detect alkaline phosphatase (ALP). The tyrosinase amplification schemes can also be used to detect phenols, although in this case it is also possible to directly oxidise the phenolic compound on the electrode surface and to reduce it using glucose dehydrogenase (GDH) (Rose *et al.*, 2001). ALP has also been detected using GOx as amplifying enzyme (Della Ciana *et al.*, 1995). In this system, *p*-hydroxyphenyl phosphate (HQP) is dephosphorilated and converted to hydroquinone (H_2Q) by ALP. H_2Q is then consumed at the electrode surface but is regenerated by GOx. In a similar way, GOx has been used to recycle and detect pentachlorophenol in contaminated soil (Male *et al.*, 1998). GOx has also been used in combination with GDH to increase the sensitivity for glucose determination in a fermentation bioreactor (Lapierre *et al.*, 1998). Such amplification schemes that include GOx suggest that the results obtained in Chapter V can be applied to rationally design hybridisation signal amplification.

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Abbreviations

ALP: alkaline phosphatase

DNA: deoxyribonucleic acid

Enz: enzyme

FAD: oxidised flavin adenine dinucleotide

$FADH_2$: reduced flavin adenine dinucleotide

GOx: glucose oxidase

GDH: glucose dehydrogenase

H_2Q: hydroquinone

HQP: *p*-hydroxyphenyl phosphate

Med: mediator

ox: oxidised

PCR: polymerase chain reaction

red: reduced

SAM: self-assembled monolayer

1.4. Presentation of the thesis

1.4.1. Objective

The objective of this thesis is to demonstrate a new concept of DNA chip arraying and establish an electrochemical method for hybridisation. This concept is based on the construction of biorecognition nanomodules: colloidal gold suspensions modified with quality-controlled probes that are stable for the time scale of manufacturing. These biorecognition nanomodules are subsequently electrodeposited in a directed way that in principle should allow arraying with photolithographic resolution. For the technique to have technological significance, an easy electrochemical hybridisation detection method should accompany the demonstration of arraying.

1.4.2. State-of-the-art

As examined in parts 1.1-1.3, the state-of-the-art in DNA chip arraying is characterised by two kinds of systems: *in situ* synthesis of oligonucleotide probes that allows photolithographic resolution of arraying but is subject to errors and therefore needs redundant probe locations and sophisticated compensation software, and ink-jetting or pin deposition of pre-fabricated probes with significantly lower site density but with controlled probe quality. Between these two extremes exist techniques such as polypyrrolisation or photolithographic patterning of pre-activated and pre-fabricated probes that combine the benefits of both techniques. As an indicator of the capacities of each method, assuming a 128-probe device of 16-nucleotide probes and state-of-the-art manufacturing facilities, and operating at the state-of-the-art resolution for each technique, 195 x 10^3 and 5 devices per cm^2 could be packed by the photolithographic and pin deposition techniques, respectively. The manufacturing capacity could reach 875 x 10^4 and 3.1 x 10^4 devices per hour, respectively. This means that for markets larger than about 80 million devices per year, the photolithographic technique presents a definite advantage despite the higher initial investment required for the manufacturing facilities. Therefore, the answer to the question to which method belongs the future of DNA chips should be that this depends on the product concept. For the high throughput applications (pharmacogenomics, toxicogenomics) probably the pin and ink-jet deposition methods will prove competitive if sample volume is not critical. For genome analysis and sequencing or gene discovery applications probably the photolithographic techniques will prevail. However, if the main driver for the future is diagnostics or decentralised, on site diagnostics at the physician's office, or even, home diagnostics, then the deciding parameters change radically. In that case, low-cost disposable amplification cartridges can be envisioned coupled with one-use chips that have to be produced at the 1-10 euro range. It is improbable that the existing methods could provide such a solution.

Related to the product concept is the need for low detection limits. Fluorescence detection is at its limit with a monolayer of 50-100μm dots even if all the closely-packed monolayer is hybridised with

fluorescence tags. Electrochemical detection limits are in general 4 orders of magnitude higher. It is therefore questionable that the current state-of-the-art in resolution and miniaturisation can be met by the state-of-the-art in detection sensitivity. By enzyme cascades and multi-labelling, both techniques can gain in sensitivity 2 or 4 orders of magnitude. How to achieve this signal manipulation in a way that is reliable and does not interfere with biorecognition is a challenge that meeting it might define the shape of the DNA chips in the future.

Colloidal modification and use that has been chosen as arraying vehicle and immobilisation matrix presents a series of related challenges. Although the last century has been marked by an improved understanding of these metastable systems, they are still far from robust formulations that can be used in a manufacturing setting.

1.4.3. Hypothesis - significance

The basic hypothesis of this thesis is that biorecognition nanomodules can be synthesised in the form of colloidal gold particles modified with oligonucleotide probes. It is aimed that these nanomodules can form stable suspensions. It is further stipulated that they can be selectively arrayed under an electric potential field with photolithographic resolution. Additionally, it is proposed that the first steps be achieved towards proposing a hybridisation detection scheme based on electrochemistry that could yield currents of the order of 1nA from electrodes of diameters smaller than 50μm, assuming fully hybridised monolayers. This task in its entirety (not proposed to be resolved in this thesis) would mean a 5 to 7 order of magnitude signal amplification.

The significance of these propositions is that micrometric arraying will be achieved for DNA chips and an electrochemistry-based manufacturing method will be developed. If at the same time, a reliable and sensitive electrochemical detection method is reached, the technological basis will be in place to achieve low-cost, minimum-volume DNA analysis devices and microsystems. In the process, it is expected that interesting contributions will be made in the areas of colloidal science, bioorganic chemistry and redox bioelectrochemistry.

1.4.4. Methodology

The first task was to establish a reliable DNA immobilisation technique and characterisation methods. For this reason, monolayers were self-assembled simply by immersion of gold electrode surfaces into solutions containing thiol-modified oligonucleotides. The presence and reactivity of these SAMs were characterised by electrochemical, piezoelectric and colourimetric techniques. The hybridisation with enzyme-labelled sequences was verified by hybridisation ELONA assays. This activity is described in Chapter II.

The second task was to prepare functional bionanomodules. The stability of unmodified and modified colloidal gold suspensions was characterised by spectrophotometry. The conjugation of

oligonucleotides to colloidal gold particles was studied by sandwich ELONA assays and fluorescence. The functionality of the conjugated oligonucleotides was demonstrated by hybridisation ELONA assays. Finally, fluorescence was also used to study the thermal stability of the conjugation. This activity is described in Chapter III.

The third task was to achieve and study the selective electrodeposition of biorecognition nanomodules on different electrode surfaces and also to assure that they were functional through hybridisation. Several characterisation methods were used: light and electronic microscopy, spectrophotometry, colourimetry, electrochemistry and piezoelectric techniques, all described in Chapter IV.

Finally, electrochemistry was used to obtain the rate constants for the electron transfer between GOx and osmium complexes as a first step to design efficient amperometric detection techniques by molecular engineering of redox partners. This effort is described in Chapter V.

1.4.5. Most important conclusions

Regarding the preliminary “immobilisation and hybridisation” system, the absorbance from sequences with 4-point mutations was 74 $\pm$ 9% of complementary sequences, indicating the ability of the system to discriminate mutations.

It was found that 8.5mM carbonate, phosphate and citrate buffers gave the best results for the stabilisation of colloidal gold suspensions. High salt concentrations in the colloidal gold suspension produced aggregation, the effect being more important when using salts with divalent counterions. Nevertheless, this effect could be inhibited with 1% of BSA as blocking agent.

The conjugations of two model oligonucleotides to colloidal gold particles were successful and the biorecognition nanomodules were stable and functional under hybridisation temperatures, allowing to differentiate an oligonucleotide with 4-point mutations.

Selective electrodeposition (optimal conditions: +1.2V (*vs.* Ag/AgCl) for 2min) of oligonucleotide-colloidal gold conjugates was demonstrated with a 5μm resolution (limited by the resolution of the photolithographed electrodes), making the strategy suitable for array manufacturing. Additionally, after hybridisation, mutated sequences gave 32% of response compared to complementary ones, proving the viability of the strategy to differentiate 4 mutations in a 19-mer oligonucleotide.

More than 1 order of magnitude of amperometric signal amplification can be achieved by simply engaging in molecular engineering of redox partners.

1.4.6. Limitations and future work

Despite the successful results for the selective deposition, the current intensities from the hybridised sequences were always very close to current intensities from the blanks. This problem has hindered

the evaluation of the percentage of non-specific adsorption compared to selective deposition. This limitation of the electrochemical technique suggests that the effect of the non-specific adsorption could be lower. Consequently, the amplification of the electrochemical signal is required to detect not only the electrodeposition but also the biorecognition event in which a redox enzymatic label is involved. This methodology however did not allow a rational optimisation of the deposition conditions for lack of discrimination. An additional effort has to be made to eliminate any possible non-specific adsorption. Since the non-specific events are a function of shear stress and osmotic phenomena, manipulating the Reynolds number of the mobile phase in contact with the electrode and the solution osmotic parameters is warranted.

The electrochemical quartz crystal microbalance (EQCM), a device that permits label-free monitoring, has been used in preliminary experiments to evaluate the electrodeposition of colloidal gold particles. However, limitations associated with the flaw-less function of the device in the electrochemical mode appeared and this work has not advanced as expected. Once the system is fully set up, this piezoelectric technique will provide useful information, since it is highly sensitive. Additionally, real-time hybridisation measurements will be possible. This technique combined with other concurrent surface analysis techniques (electrochemical SPR) and colloidal phase characterisation (zeta potentials, light scattering) will eventually facilitate rationalised selective deposition and correlate it with suspension properties.