Chapter II. Oligonucleotide immobilisation, characterisation and hybridisation detection on gold surfaces

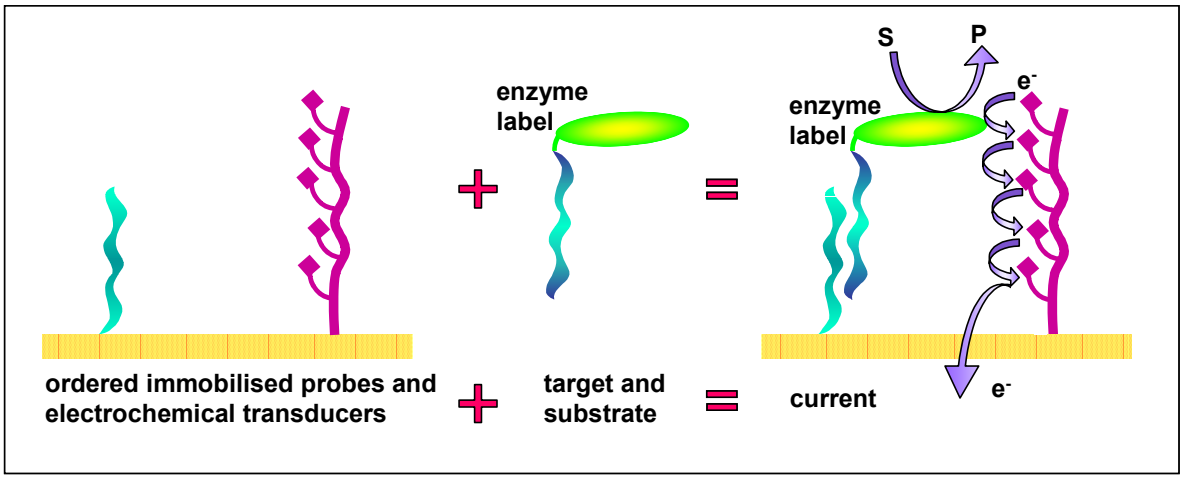
Introduction

The goal of this thesis is to develop novel DNA analytical instruments in the form of DNA sensors and arrays. As explained, the development of such devices passes through the following prerequisite steps:

a) Immobilisation of probes.
b) Arraying.
c) Transduction and hybridisation detection.

This chapter of the thesis describes the preliminary work that led to the choice of solutions for items a) and c). To choose the immobilisation techniques used for arraying, item c) described in Chapters III and IV, it was necessary to examine the different possible routes.

The initial strategy examined for probe immobilisation and hybridisation detection is summarised in Scheme II.1.



Scheme II.1. Immobilisation and hybridisation strategy.

The configuration described in Scheme II.1 consists of orderly immobilised probes in a mixed monolayer with redox polymers of controlled size. Upon hybridisation of the redox enzyme-labelled target, and in the presence of substrate, the electrons produced are transferred to the electrode surface thorough an electron hopping mechanism that is much more efficient than diffusional electron transfer. This improved electron collection efficiency combined with the reagentless nature of this configuration constitute a definitive advantage of these sensors compared to other available technologies.

Key to the success of this configuration is the ability to co-immobilise the recognition and transduction partners in such a way as to not impair their ability to hybridise whilst facilitating the transfer of electrons from relatively remote distances from the electrode surface. Thus, ordered monolayers on gold surfaces were chosen as the most appropriate strategy to fulfill these requirements.

The dative bonding of thiol-derivatised molecules on Au gives a significant flexibility for immobilisation and transduction. For example, mixed monolayers can achieve desirable spacing and orientation effects. The choice of gold as the immobilisation substrate has the additional advantage that it provides at the same time a transduction surface and the basis of the nanomodular arraying method described in subsequent chapters.

Related to the above is the ability to characterise the efficiency of immobilisation, functionality after immobilisation, and transduction. In the present chapter, these aspects are examined preliminarily. At first, the possibility to construct the SAMs described in Scheme II.1 is examined. Secondly, a survey of methods of quick and functional characterisation of the efficiency and functionality of immobilised biorecognition and transduction partners is made. Thirdly, optical and electrochemical strategies for hybridisation detection are surveyed.

Materials and methods

Materials. Gold (foils, wires, chips and quartz crystals) and glassy carbon were used as immobilisation substrates. Gold foil (0.05mm thickness, 99.99%) was purchased from Sigma. Gold wire (0.5mm diameter, 99.99%) was obtained from Incometal S.A. The National Microelectronics Research Centre (NMRC) of Ireland was the supplier of the 4 x 4mm gold chips on silicon substrate. Gold-covered quartz 5-MHz crystals were obtained from Universal Sensors Inc. 2-mercaptoethanol, 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), *p*-nitrophenyl phosphate (*p*NPP), 3,3',5,5'-tetramethyl-benzidine (TMB), polyoxyethylenesorbitan monolaurate (Tween 20), soybean peroxidase (SBP) and salmon testes DNA were purchased from Sigma. Thioctic acid, 4-mercaptopyridine, 3-mercapto-1-propanesulfonic acid sodium salt (MPS), glutaraldehyde and sodium borohydride ($NaBH_4$) were obtained from Aldrich. 2,2'-Azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS), cystamine, 3,3'-dithiodipropionic acid di(N-succinimidyl ester) (DPS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxisuccinimide (NHS) were purchased from Fluka. Antidigoxigenin-fluorescein-isothiocyanate Fab fragments (antidig-FITC) and antidigoxigenin-peroxidase Fab fragments (antidig-HRP) were obtained from Boehringer Mannheim. Antidigoxigenin-alkaline phosphatase Fab fragments (antidig-ALP) was purchased from Roche. 3' GTGCCAAGTCCCAATCGGTGTGAAACGCCC-SH 5' or 3' CTTTGTGGTTACTATAAATTTTTTTTTTTT-SH 5' (oligonucleotide-thiol), 3' FITC-GTGCCAAGTCC CAATCGGTGTGAAACGCCC-SH 5' (FITC-oligonucleotide-thiol), 3' GTGCCAAGTCCCAATCG

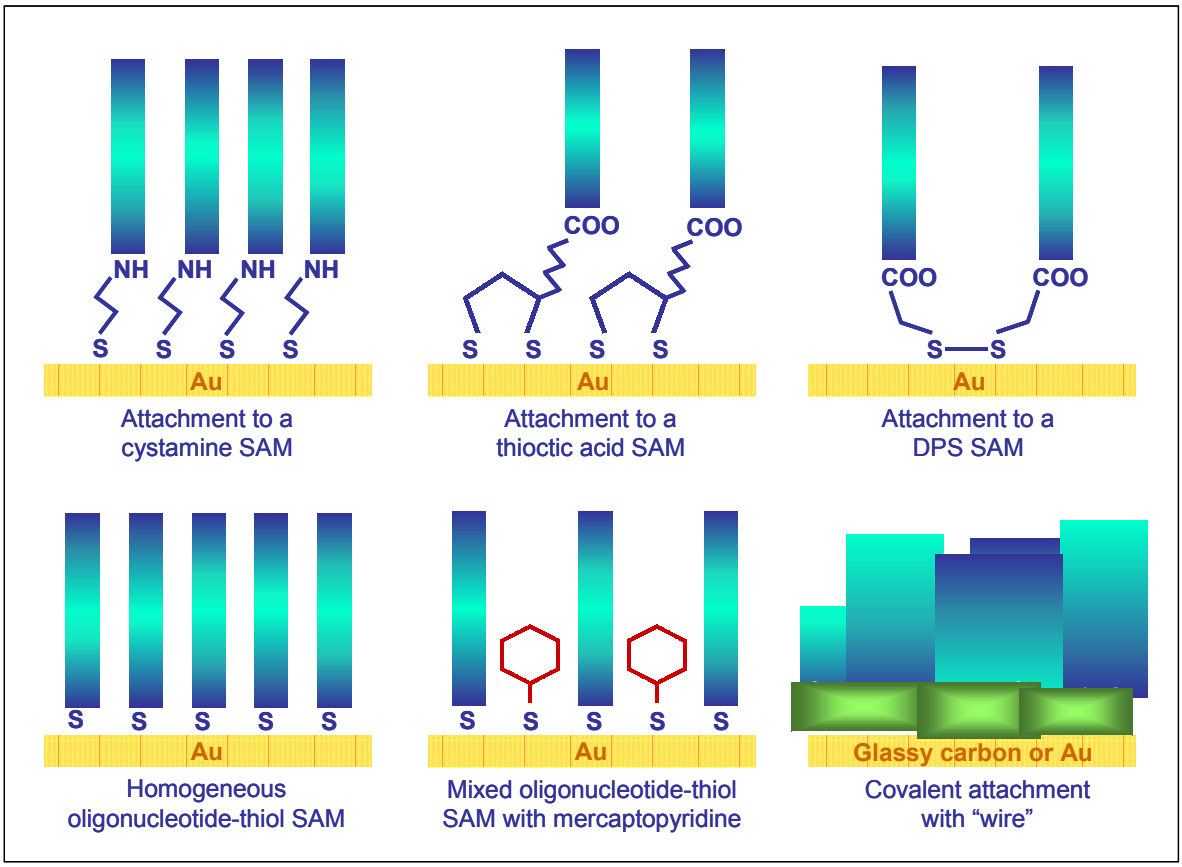
GTGTGAAACGCCC-amine 5' (amino-oligonucleotide), 3' GGGCGTTTCACACCGATTGGGACTTG GCAC 5' (complementary oligonucleotide), 3' digoxigenin-GGGCGTTTCACACCGATTGGGACTTG GCAC 5' (dig-labelled complementary oligonucleotide), 3' GGGCGTTTCACACCGATTGAGACTCG TAAC 5' (non-complementary oligonucleotide), 3' digoxigenin-GGGCGTTTCACACCGATTGAGA CTCGTAAC 5' (dig-labelled non-complementary oligonucleotide), 3' TTTATAGTAACCACAAAGCC CCCCCCCC CC-amine 5' (fully complementary amino-oligonucleotide), 3' ATAGTAGAAACCACAA AGCCCCCCCCCCCC-amine 5' (amino-oligonucleotide with 4-point mutations) were obtained from Isogen or Genosys. NAP^{TM}-10 columns (with Sephadex^{TM} G-25 DNA grade) were obtained from Amersham Pharmacia Biotech AB. [Os(2,2'-bipyridine)$_2$Cl(4-(aminomethyl)pyridine)]Cl, [Os(2,2'-bipyridine)$_2$Cl (pyridine-4-carboxylic acid)]Cl, [Os(2,2'-bipyridine)$_2$Cl(4-pyridylacetic acid)]Cl and Os(4,4'-dimethyl-2,2'-bipyridine)$_2$Cl$_2$ were synthesised as explained in Chapter V. The controlled size redox "wire" indicated in Scheme II.1 was synthesised by Peptide Technologies Corporation, and its structure was based on an oligopeptide (poly Lys) modified with isonicotinyl groups on the ε amino groups. This 15-mer was subsequently modified either on the electrode or in bulk with Os(4,4'-dimethyl-2,2'-bipyridine)$_2$Cl$_2$. The positive redox "wire" polymer used in the hybridisation detection was synthesised derivatising the PVP backbone with Os(2,2'-bipyridine)$_2$Cl$_2$ and 2-bromoethylamine hydrobromide, according to the procedure described by Katakis (1994).

Instrumentation. Cyclic voltammograms were obtained using either a BAS CV-1B or an AUTOLAB PGSTAT10 potentiostat in a conventional three-electrode cell, with Ag/AgCl as reference electrode and Pt as counter electrode. Frequency changes were measured with a Universal Sensors Inc. PZ-1000 quartz crystal microbalance (QCM). Colourimetric measurements were performed with a Molecular Devices 340PC 96-well plate reader. Fluorescence was measured with a Nikon E600FN fluorescence microscope equipped with a Sony CCD camera.

Electrode preparation. Gold wires, foils and chips were cleaned with "piranha's solution" (70% H_2SO_4 : 30% H_2O_2, 60ºC) and rinsed with distilled water to remove any adventitious organic and inorganic material prior to adsorption of the SAM as a precaution. Extreme caution should be used with this solution since it reacts violently with organic material and all work should be carried out in a fumehood. This cleaning process was inappropriate for the gold-covered quartz crystals because the drastic conditions removed the gold form the corresponding surface. Consequently, they were cleaned by immersion in 1.2M NaOH for 5min, rinsing with water, immersion in 1.2M HCl for 5min, rinsing with water, placing a 12.2M HCl drop on the gold for 5min, rinsing with water and letting them dry. The cleanliness of the gold was checked by cyclic voltammetry in 0.1M H_2SO_4. Glassy carbon electrodes were polished with 5, 1 and 0.3μm alumina on polishing clothes (Buehler), rinsed with water and sonicated for 15min.

SAMs formation and characterisation. SAMs were formed by immersion of the gold surface into a solution with thiol-containing molecules for different time periods (Scheme II.2). Cystamine SAMs

were obtained by immersion into a 100mM aqueous cystamine solution for 2h with stirring. Thioctic acid SAMs were obtained by immersion into a 10 or 100mM thioctic acid in 50% ethanol : 50% water for 12h with stirring. DPS SAMs were obtained by immersion into a 10mM DPS solution in DMF for 30min with stirring. MPS SAMs were obtained by immersion into a 2mM MPS in 50% ethanol : 50% water for 12h with stirring. Mixed monolayers with thioctic acid and blocking agents like mercaptoethanol and mercaptopyridine were formed simultaneously, by immersion of the solid surface into a solution with both thiol-containing molecules (10 or 100mM thioctic acid and 1mM blocking agent in 50% ethanol : 50% water) for 2h with stirring, or sequentially, by immersion firstly into the thioctic acid solution (10 or 100nM in 50% ethanol : 50% water) for 12h with stirring and afterwards into the blocking agent solution (1mM in ethanol) for 2h with stirring.



Scheme II.2. DNA immobilisation techniques.

The effectiveness of cystamine, thioctic acid, mercaptoethanol and mercaptopyridine SAMs was characterised by cyclic voltammetry in 0.5M KOH or in 0.1M phosphate buffer, 0.15M NaCl, pH 6.0, at 100mV s^{-1} and under argon to avoid oxygen reduction, which would interfere in the detection of the thiol reduction that takes place at around -0.7V (*vs.* Ag/AgCl). Cystamine and thioctic acid SAMs were also characterised measuring the surface coverage due to the non-specific adsorption of [Os(2,2'-bipyridine)$_2$Cl(4-(aminomethyl)pyridine)]Cl and [Os(2,2'-bipyridine)$_2$Cl(pyridine-4-carboxylic acid)]Cl (dissolved in water or DMF) after immersion for 2h. The surface coverage of adsorbed

complex was determined in 0.1M phosphate buffer, 0.15M NaCl, pH 6.0, at 100mV s^{-1} after washing the electrodes. It was assumed that non-specific adsorption occurred on the pinholes.

To study the ability of cystamine and thioctic acid SAMs to react with oligonucleotides, the redox complexes [Os(2,2'-bipyridine)$_2$Cl(4-(aminomethyl)pyridine)]Cl and [Os(2,2'-bipyridine)$_2$Cl(4-pyridylacetic acid)]Cl were covalently attached to the reactive groups of the cystamine and thioctic acid SAMs, respectively, via EDC reaction in 0.1M acetate buffer, pH 5.1, at 100mV s^{-1}, and the surface coverage due to the complexes was measured electrochemically

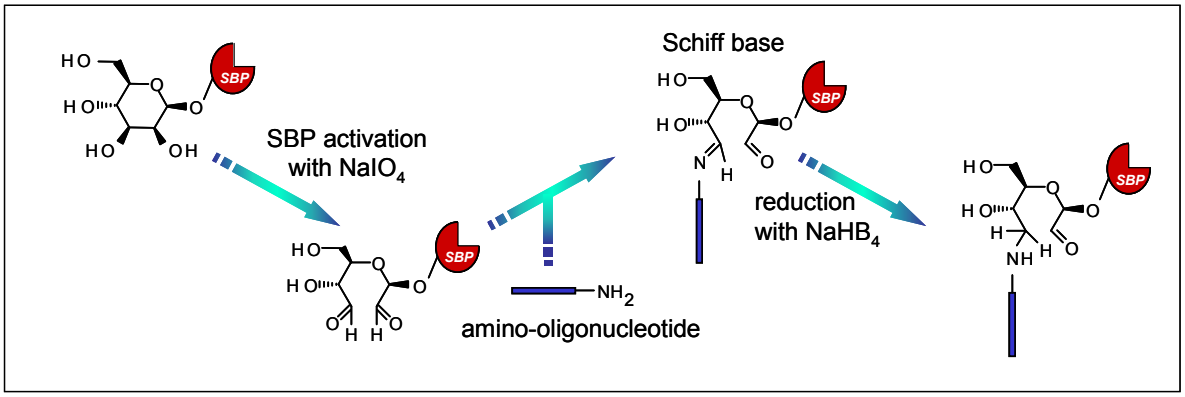
DNA immobilisation and characterisation. Amino-oligonucleotides were directly attached to the ester groups of the DPS SAM, simply by immersion of the DPS-modified surface into a 0.05, 0.5 or 50µg mL^{-1} amino-oligonucleotide solution for 2h with stirring. This binding was followed in real time by measuring the corresponding frequency change in the quartz crystal microbalance (QCM). In another characterisation technique, the amino-oligonucleotide was attached to a DPS SAM formed on a gold wire, hybridised with a dig-labelled complementary oligonucleotide (and with the dig-labelled non-complementary oligonucleotide as a control), and incubated with antidig-FITC.

FITC-oligonucleotide-thiol and oligonucleotide-thiol SAMs were obtained after immersion into 0.5 or 1ng mL^{-1} (for the FITC-oligonucleotide-thiol), and 0.2 or 90ng mL^{-1} (for the oligonucleotide-thiol) solutions in 0.1M phosphate buffer, pH 8.0, for 1h with stirring. Mixed monolayers with oligonucleotide-thiol and blocking agents like mercaptoethanol and mercaptopyridine were formed simultaneously, by immersion of the solid surface into a solution with both thiol-containing molecules (0.1ng mL^{-1} oligonucleotide-thiol and 1.1, 100, 110nM, 1, 10 or 11mM blocking agent) for 2h with stirring, or sequentially, by immersion firstly into the oligonucleotide-thiol solution and afterwards into the blocking agent solution. When sequential mixed monolayers were formed, the blocking agents were used at 2.2, 220nM or 22mM concentration for different times (15, 30, 45min, 1 or 2h) with stirring. FITC-oligonucleotide-thiol SAMs were characterised by measuring the fluorescence emitted by the FITC moiety. The surface coverage of oligonucleotide-thiol SAMs was characterised by the QCM technique, by cyclic voltammetry in 0.5M KOH or in 0.1M phosphate buffer, 0.15M NaCl, pH 6.0, at 100mV s^{-1} and under argon, and also by the electrochemical detection of guanine at +0.8V (*vs.* Ag/AgCl) in 10mM acetate buffer, 0.1M NaCl, pH 4.7.

Covalent attachment immobilisation technique was also attempted. Bare or MPS-modified electrodes were immersed into a solution containing 30µg mL^{-1} of the positive redox "wire" polymer for 6h with stirring. The electrode was then introduced into a 5µg mL^{-1} amino-oligonucleotide or 0.2ng mL^{-1} oligonucleotide-thiol solution, for 3h with stirring. Some electrodes were immersed in 1.5µg mL^{-1} SPDP (in 0.1M phosphate buffer, pH 8.0) for 30min with stirring, between the "wire" and the oligonucleotide steps in order to favour the cross-linking. This last procedure was also used with glassy carbon. The amino-oligonucleotide or oligonucleotide-thiol immobilisation efficiency and

functionality were characterised by hybridisation with SBP-labelled complementary oligonucleotide and electrochemical detection.

Amino-oligonucleotide labelling with SBP. The reaction procedure is depicted in Scheme II.3. 5mg of SBP were dissolved in 1mL of 0.1M phosphate buffer, pH 7.0, and 0.1mL of freshly dissolved sodium periodate in water (110mM) was added to a final concentration of 10mM. The mixture was left to react for 30min with stirring protected from light. Afterwards, the reaction was stopped by adding 0.11mL of ethylene glycol for 20min at 4ºC. The activated enzyme was purified by filtration with a G-25 Sephadex separation column. 1mL of the activated enzyme (7-fold molar excess) was added to 0.25mL of a 70.84µM amino-oligonucleotide solution (fully complementary or with 4 mutations, in 0.1M phosphate buffer, pH 7.8) and allowed to react for 3h at 4ºC with stirring protected from light. 0.5mL of 0.4M $NaBH_4$ were added to a final concentration 0.1M and the solution was allowed to react overnight at 4ºC with stirring protected from light. The final concentration of oligonucleotide was 10.12µM. The activity of the conjugated SBP was measured spectrophotometrically with the ABTS enzymatic assay, recording the absorbance change at 405nm. The activity of conjugated SBP was 33.58U mg^{-1}, 58.62% of native SBP. The loss of activity was presumably due to the periodate oxidation step.

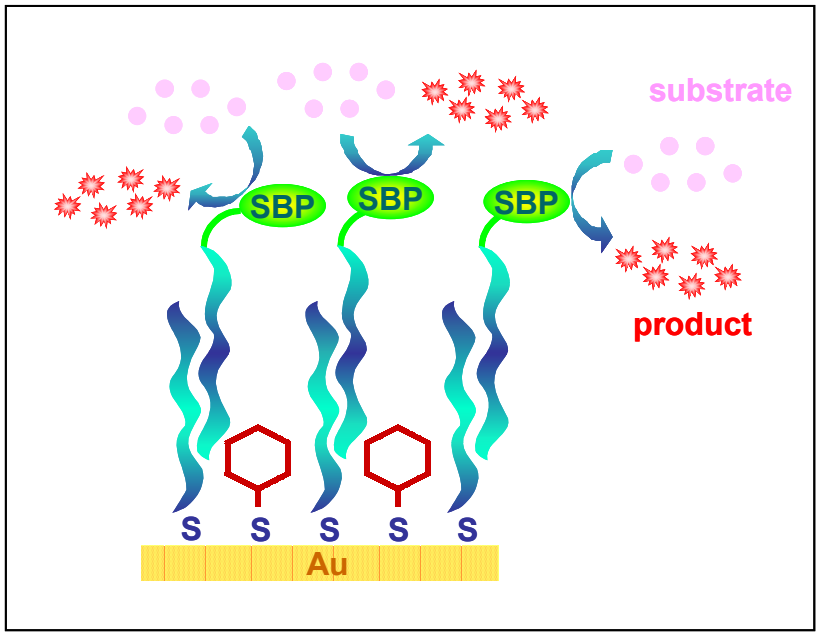


Scheme II.3. SBP-amino-oligonucleotide conjugation.

DNA hybridisation. Hybridisation was carried out with 0.04, 0.34, 0.4, 3.4 and 34ng mL^{-1} complementary oligonucleotide in 1 x SSC, 2.5 x Denhardt, 10mM tris-HCl, 1mM EDTA, pH 7.5, or 50mM tris-HCl buffer, 1M NaCl, pH 7.0, usually for 1h at 48 or 58ºC with stirring. Different blocking agents were used before (1% baby milk in 0.1M phosphate buffer, pH 7.0 for 1h at 37ºC with stirring) and/or during the hybridisation step (hybridisation buffer containing 1% baby milk, 0.2% Tween 20, 10ng mL^{-1} or 1µg mL^{-1} salmon testes DNA). Oligonucleotides were labelled with digoxigenin or SBP. Blanks were performed with non-complementary oligonucleotides or oligonucleotides with 4-point mutations depending on the needs.

DNA hybridisation measurement. For the hybridisation detection with dig-labelled oligonucleotides, after the hybridisation and a blocking step with BSA (1% in 50mM tris-HCl buffer,

150mM NaCl, pH 7.6, for 1h at 37ºC), the electrode was incubated in 50mM tris-HCl buffer, 150mM NaCl, pH 7.6, 1% BSA, containing antidig-ALP (1:2500 dilution) or antidig-FITC (1:5 dilution) conjugate for 1h at 37ºC with stirring. The fluorescence emitted by the antidig-FITC was detected by fluorescence microscopy. The electrodes with antidig-ALP were incubated with *p*NPP (1mg mL^{-1} in 0.1M DEA buffer, 50mM KCl, 1mM $MgCl_2$, pH 9.5) for 1h at 37ºC with stirring, and the reaction was stopped with H_2SO_4. Afterwards, the absorbance change was measured using a 96-well plate reader. Hybridisation with SBP-amino-oligonucleotide conjugate was detected by colourimetry, after incubation in TMB / H_2O_2 (100µL of TMB (10mg mL^{-1} in DMSO) + 2µL H_2O_2 + 10mL of 0.1M acetate buffer, pH 6.0) for 1h at 37ºC with stirring, or electrochemically, measuring the response to a saturated concentration of H_2O_2 in the presence of a positive redox polymer adsorbed *a posteriori* (in oligonucleotide-thiol SAMs) or cross-linked (in covalent oligonucleotide immobilisation) in 0.1M phosphate-acetate buffer, pH 5.0, and the working potential being at +0.1V (*vs.* Ag/AgCl). Scheme II.4 shows the global colorimetric system.



Scheme II.4. Colorimetric detection of hybridisation: mixed SAM / SBP-labelled complementary sequence / TMB + H_2O_2 / H_2SO_4.

Results and discussion

Electrode preparation. Gold electrode cleanliness was checked by cyclic voltammetry in 0.1M H_2SO_4, as the cyclic voltammogram of clean gold shows an oxidation peak at +1.3V (*vs.* Ag/AgCl) and a reduction peak at +0.9V (*vs.* Ag/AgCl) corresponding to metal oxide formation and reduction (Figure II.1). Furthermore, any residue of organic contaminant left by preceding cleaning treatments is removed during repeated oxidation and reduction of the gold. By finishing the scan at a negative potential an oxide-free gold surface was ensured. Although cleaning with “piranha’s solution” is a

very drastic treatment, it is necessary to mention that even under these extreme cleaning conditions, on some occasion adsorbed osmium complexes were difficult to remove and the repetition of the cleaning procedure was needed.

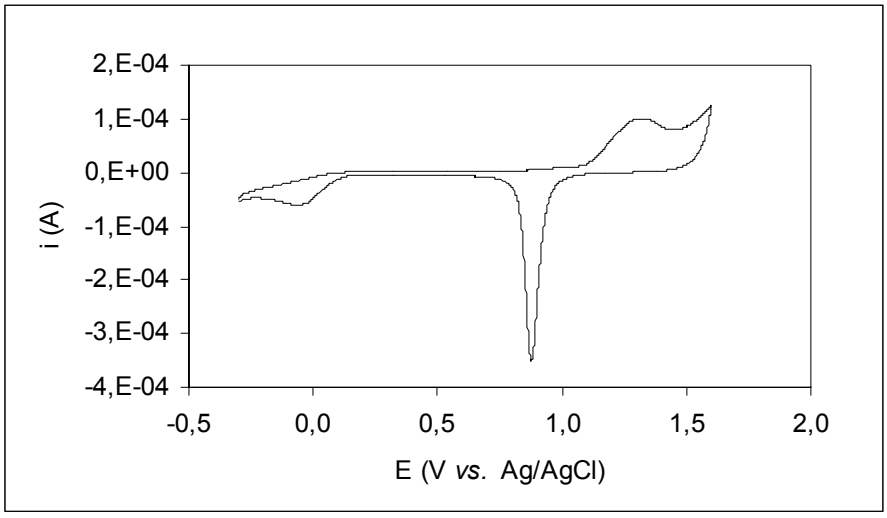


Figure II.1. Cyclic voltammogram of a gold electrode in 0.1M H_2SO_4 at 100 mV s^{-1}.

SAMs formation and characterisation. At sufficiently negative potentials the dative bond of thiol groups to Au can be reduced with consequent thiol desorption. Cystamine, thioctic acid and mercaptopyridine SAMs were characterised making use of this property. Cyclic voltammograms showed a reduction peak at -0.7V (*vs.* Ag/AgCl), indicating the SAM presence. By repetitive scans, the reduction peak intensity decreased, reflecting the desorption of thiols bound to the gold and, consequently, the loss of material assembled to the surface (Figure II.2).

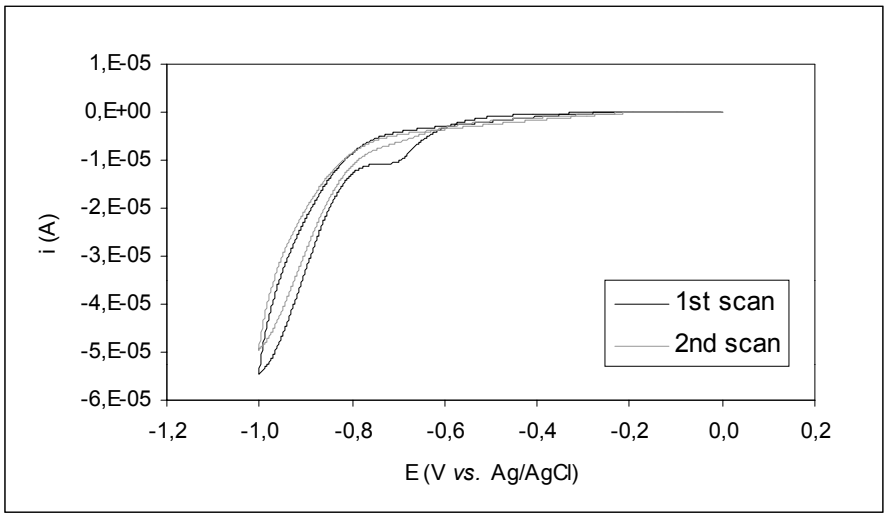


Figure II.2. 1st and 2nd cyclic voltammograms for the reduction of the Au-S bond in the mercaptopyridine SAM in 0.1M phosphate buffer, 0.15M NaCl, pH 6.0 at 100mV s^{-1}.

This technique, although useful to verify and quantify SAM formation, is destructive and does not allow the further use of the electrodes, but it was used to optimise the SAM formation assuming in subsequent experiments that equivalent SAMs were formed when the same conditions were used. This reduction peak, although prominent with mercaptopyridine SAMs, was not as clear or reproducible with cystamine or thioctic acid monolayers. For this reason, these compounds' self-assembly was characterised by the adsorption of osmium redox compounds on the pinholes or the SAM.

Ordered immobilisation of controlled size transduction oligomers. When the oligopeptide described in the experimental section was modified in bulk or on the surface of electrodes with Os redox compounds, identical surface coverage and low transduction efficiency even from redox enzymes led to the suspicion that the majority of the electrochemical signal was produced by a non-specific adsorption event of the Os complex directly on the gold surface. This phenomenon (if true) would prohibit exploitation of the ordered architecture for efficient electron transduction from the redox enzyme label. For this reason, two redox couples [Os(2,2'-bipyridine)$_2$Cl(4-(aminomethyl) pyridine)]Cl and [Os(2,2'-bipyridine)$_2$Cl(pyridine-4-carboxylic acid)]Cl were left to adsorb on differently modified Au surfaces. The results are summarised in Table II.1.

Table II.1. Adsorption of Os redox couples on Au-SAMs*.

Type of SAM	Adsorption solution	Redox couple	Os surface coverage (x 10^{11} mol cm^{-2})	Standard deviation
None	water	[Os(bipy)$_2$Cl(py-NH$_3^+$)]Cl	6.18	0.08
Cystamine	"	"	2.16	1.14
Thioctic acid	"	"	30.30	2.50
Mixed thioctic acid / mercaptoethanol	"	[Os(bipy)$_2$Cl(py-NH$_3^+$)]Cl	14.3	1.20
Mixed thioctic acid / mercaptopyridine	"	"	9.57	0.78
None	DMF	[Os(bipy)$_2$Cl(py-NH$_3^+$)]Cl	none	none
Cystamine	"	"	none	none
Thioctic acid	"	"	2.38	0.40
None	"	[Os(bipy)$_2$Cl(py-COOH)]Cl	none	none
Cystamine	"	"	none	none
Thioctic acid	"	"	0.32	0.08
Mixed thioctic acid / mercaptopyridine	"	"	0.15	0.004

* Surface coverage was determined by the integration of charge passed during CVs (200mV s^{-1}) in fresh buffer after washing the electrodes subsequent to modification as described in the experimental part.

Further to these results should be added the following observations: a) that sequential *vs.* simultaneous formation of the mixed monolayers did not result in any difference in the results, although sequential formation seemed to lead to more reproducibility and b) that protonation of the

thioctic acid SAM yielded one order of magnitude lower non-specific adsorption of [Os(2,2'-bipyridine)$_2$Cl(4-(aminomethyl)pyridine)]Cl.

The aforementioned demonstrates that a very high non-specific interaction exists between the osmium complexes and the gold surface, regardless of the existence of the SAM. This fact was underlined by the fact that Os-treated gold surfaces were almost impossible to clean even after repeated treatments in "piranha's solution". It was therefore determined that only the use of DMF solution for further SAM modification could lead to the type of architectures stipulated in Scheme II.1. Apparently, due to the low dielectric constant of DMF and the fact that it is a better solvent for the Os complexes (except for [Os(2,2'-bipyridine)$_2$Cl(4-pyridylacetic acid)]Cl), it is possible to achieve this ordered immobilisation procedure. A similar effect was observed if the thioctic acid-modified electrodes were immersed in a buffer of a lower pH before the mediator adsorption step, apparently due to the protonation of thioctic acid and the reduction of the electrostatic attraction of the SAM with the modifying Os moieties. For this reason, subsequent experiments to determine the capacity of selective (as opposed to non-specific) attachment of Os centres to SAMs were carried out under these conditions.

The reactivity of cystamine and thioctic acid SAMs was studied reacting [Os(2,2'-bipyridine)$_2$Cl(4-(aminomethyl)pyridine)]Cl and [Os(2,2'-bipyridine)$_2$Cl(4-pyridylacetic acid)]Cl with thioctic acid and cystamine SAMs, respectively, via EDC, the surface coverage providing an indication of the reaction yields. The surface coverage from the non-specific adsorption of these osmium complexes on the SAMs was compared with that after EDC. [Os(2,2'-bipyridine)$_2$Cl(4-pyridylacetic acid)]Cl was used instead of [Os(2,2'-bipyridine)$_2$Cl(pyridine-4-carboxylic acid)]Cl because the carboxylic group was more reactive towards activation with EDC. The conclusions were:

1) There were no differences between the EDC reaction in 0.1M acetate buffer, pH 5.1, between the cystamine SAM and the [Os(2,2'-bipyridine)$_2$Cl(4-pyridylacetic acid)]Cl complex ($\Gamma = 2.11 \times 10^{-11} \pm 0.21 \times 10^{-11}$mol cm^{-2}) and the non-specific adsorption in DMF ($\Gamma = 1.83 \times 10^{-11} \pm 0.82 \times 10^{-11}$mol cm^{-2}), suggesting that this could not be a suitable strategy for the ordered and selective modification of the SAM.

2) In the EDC reaction between [Os(2,2'-bipyridine)$_2$Cl(4-(aminomethyl)pyridine)]Cl in 0.1M acetate buffer, pH 5.1, and the carboxylic acid of thioctic acid / mercaptopyridine mixed SAMs, the non-specific adsorption in DMF was 53% ($\Gamma = 8.00 \times 10^{-11} \pm 1.46 \times 10^{-11}$mol cm^{-2} *vs.* $\Gamma = 4.2 \times 10^{-11} \pm 0.29 \times 10^{-11}$mol cm^{-2}). Although there results showed some promise, they did not guarantee a background-free measurement of hybridisation.

This high degree of Os adsorption combined with the low degree of reactivity of the cystamine and thioctic acid SAMs, also demonstrated for DPS SAMs by other techniques explained below, obliged a change of immobilisation strategy for the transduction chemistry. Additionally, it indicated that

direct oligonucleotide-thiol SAMs should be attempted, as they eliminate the low-yield EDC reaction step. Parallel experiments carried out by Narváez (1999) demonstrated that the surface coverage from osmium polymers covalently linked to previously formed SAMs was not higher than the surface coverage from covalently linked osmium monomers, indicating that these polymer redox sites had a low electrochemical transduction capability. Consequently, the initial strategy of having an ordered transduction monolayer of controlled size was abandoned.

DNA immobilisation and characterisation. QCM measurements showed frequency changes neither after the amino-oligonucleotide addition on DPS SAMs nor after subsequent hybridisations. These data suggested that the oligonucleotide was not attached, probably due to the deactivation of the ester groups of the DPS after DPS assembling and before frequency baseline stabilisation. The fluorescence measurement corroborated this result, as no fluorescence was observed after hybridisation with dig-labelled complementary oligonucleotide and incubation with antidig-FITC. Although reports in the literature indicate that the distance between the fluorophore and the Au surface is sufficient to avoid it, the absence of fluorescence emission could be due to the quenching effect due to the fluorescence resonance energy transfer (FRET) with the gold surface.

Supporting the FRET effect, FITC-oligonucleotide-thiols immobilised on gold did not emit any fluorescence signal either. It was therefore decided that an alternative method should be used to quantify fluorescing moieties immobilised on gold.

Since EDC activation of pre-formed SAMs proved unreliable, it was decided to use direct oligonucleotide SAMs. With the QCM technique, frequency changes on exposure to oligonucleotide-thiol solutions were observed to be 27 $\pm$ 4Hz for 1ng mL^{-1} and 13 $\pm$ 3Hz for 0.25ng mL^{-1} (*see example in Figure II.3*).

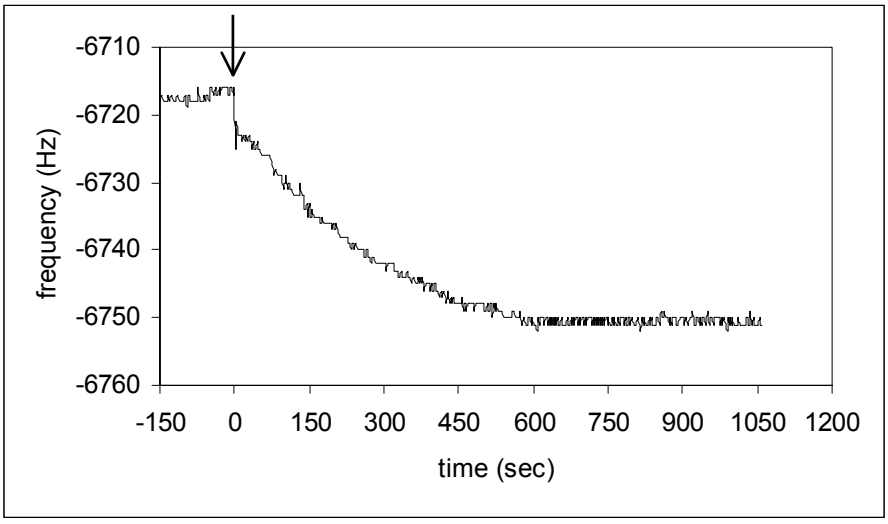


Figure II.3. Frequency change after oligonucleotide-thiol immobilisation (from a 1ng mL^{-1} solution) on a gold quartz crystal. Arrow indicates injection of oligonucleotide-thiol sample.

These results suggest successful oligonucleotide-thiol self-assembling. Moreover, the frequency stabilised 12min after the oligonucleotide-thiol solution injection, suggesting a quick monolayer formation. However, it was not possible to corroborate the surface coverage by the reduction of the thiol-Au bond or by the oxidation of guanine by cyclic voltammetry and thus, the gravimetric results were used to decide that the best strategy for probe immobilisation was the self-assembly of oligonucleotide-thiol, as it provides a direct, easy, simple, one-step probe immobilisation on gold surfaces.

DNA hybridisation measurement. A colorimetric assay technique was used to both verify and quantify the active functionality of immobilised oligonucleotides. In this technique, oligonucleotide-thiol homogeneous or mixed SAMs were immobilised on gold chips and hybridised with the dig-labelled complementary sequences. To detect hybridisation, chips were incubated with antidig-ALP and the absorbance change associated to the enzymatic reaction with the corresponding substrate was measured. Experiments showed a high non-specific adsorption of this conjugate on gold, on the oligonucleotide-thiol SAMs and on mercaptoethanol SAMs. Although a previous blocking step with BSA decreased this non-specific adsorption, no differences were observed in the hybridisation between complementary and non-complementary sequences. Consequently, the ALP label was changed by SBP, a thermostable peroxidase, in the hope to eliminate non-specific interactions. Furthermore, the modification according to Scheme II.3 allows the elimination of one step of the assay and ensures 1 SBP molecule per 1 complementary sequence, since the SBP was directly linked to an amino-oligonucleotide (fully complementary or with 4-point mutations). These experiments demonstrated that:

1) SBP-labelled oligonucleotides adsorbed non-specifically on the immobilised oligonucleotide SAMs. Nevertheless, the incorporation of baby milk (1%), Tween 20 (0.2%) and DNA salmon testes (1µg mL^{-1}) as blocking agents in the hybridisation step reduced this non-specific adsorption by 40%. The response from the non-complementary sequence was 65-82% of that of the complementary sequence, depending on the SBP-labelled oligonucleotide concentration, a fact that demonstrated the possibility to detect 4 mutations.

2) Mixed monolayers with different mercaptoethanol spacer concentrations in the oligonucleotide SAMs were attempted. When using mercaptopyridine concentrations higher than 50mM, the absorbance values obtained after the hybridisation and incubation step were lower than when using pure SAMs, due to the displacement of the oligonucleotide-thiol SAM to an extent higher than desired. Unexpectedly, lower mercaptopyridine concentrations did not change significantly the absorbance values, compared to pure oligonucleotide SAMs. Although the purpose of the mixed monolayers was to separate the immobilised strands in order to favour the complementary sequence transport, it was not

possible to observe such an effect. Therefore, the idea to construct mixed monolayers was abandoned for the rest of this thesis.

3) The hybridisation time needed for optimum spectrophotometric detection was optimised. At 20min, the absorbance values were only around 0.4, indicating that hybridisation had not reached saturation. At 40min, the response due to the complementary sequence was at maximum and the non-complementary sequence (4-point mutations) showed a non-specific adsorption of 81%. At longer times, no further differentiation was achieved and it was assumed that for the model oligonucleotides hybridisation had completed with 40min.

The final goal of this thesis is not a colorimetric DNA sensor, but an electrochemical DNA sensor. Although the colorimetric results serve for characterisation, the electrochemical detection strategy is outlined in Scheme II.1. However, the initial strategy of self-assemble osmium-derivatised polymers next to immobilised oligonucleotides in an organised structure was proven impossible as described before.

In order to achieve the same electrochemical communication, osmium redox polymers of uncontrolled MW were not self-assembled but cross-linked. Firstly, an osmium redox polymer was adsorbed *a posteriori* on the SBP-amino-oligonucleotide/oligonucleotide-thiol/gold system, as described in the “Materials and methods” section. Cyclic voltammetry showed oxidation and reduction peaks at +0.355 and +0.335V (*vs.* Ag/AgCl), respectively, indicating the presence of the redox polymer on the electrodes. However, chronoamperometry showed very low intensity values and no differences between complementary and non-complementary sequences. As these low intensity values could be due to the desorption of the redox mediator from the system, the osmium polymer was then adsorbed directly on gold or cross-linked to MPS modified electrodes. Afterwards, amine- or thiol-modified oligonucleotides were immobilised by covalent attachment. Electrochemical detection was then attempted. The gold electrodes showed the characteristic surface-immobilised redox species wave by cyclic voltammetry but very low intensity currents and no differences between complementary and non-complementary sequences upon SBP-oligonucleotide hybridisation. In order to improve the cross-linking efficiency of the redox polymer, SPDP was also added in the cross-linking step, but the same electrochemical behaviour was observed. These results demonstrated that a reliable electrochemical detection of hybridisation with these transduction chemistries is not possible. In Chapters IV and V, the possible integrated solutions to this problem are examined.

Conclusions

This chapter describes the preliminary studies to evaluate several probe immobilisation strategies and hybridisation detection. Although the presence of SAMs was demonstrated by cyclic voltammetry, they were not very reactive, which made them inappropriate for subsequent

oligonucleotide attachment. Additionally, the experiments showed a lack of electrical communication between ordered immobilised redox polymers and electrodes and a high non-specific adsorption of Os complexes on gold. Consequently, the strategy of ordered and controlled size transduction chemistry immobilisation was abandoned. In parallel, and to avoid the low yield of the oligonucleotide attachment step, direct oligonucleotide-thiol SAMs were attempted. QCM frequency changes and several colorimetric experiments proved the presence and functionality of these oligonucleotide-thiol monolayers on gold electrodes. This strategy provided a rapid, uniform and homogeneous immobilisation with only one step. Once the immobilisation step was demonstrated and optimised, the possibility to detect hybridisation was studied. In order to avoid an extra step and to reduce the analysis time, SBP-labelled complementary oligonucleotides were used in the hybridisation step. The experimental parameters concerning the presence of blocking agents, the hybridisation time and the effect of mixed monolayers were optimised by colourimetry. As the final goal of this thesis is the development of an electrochemical DNA sensor, an osmium polymer was incorporated into the system to act as electron transfer mediator. However, despite the successful colorimetric results, electrochemistry did not provide significant intensity currents, suggesting that a signal amplification method was necessary for the electrochemical hybridisation detection.

References

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Abbreviations

A: adenine
ABTS: 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid)
antidig: antidigoxigenin
ALP: alkaline phosphatase
BSA: bovine serum albumin
C: cytosine
CCD: charge-coupled device
DNA: deoxyribonucleic acid
DMF: dimethylformamide
DPS: 3,3'-dithiodipropionic acid di(N-succinimidyl ester)
EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA: ethylenediaminetetraacetic acid
FITC: fluorescein-isothiocyanate
G: guanine

HRP: horseradish peroxidase

MPS: 3-mercapto-1-propanesulfonic acid sodium salt

MW: molecular weight

NHS: N-hydroxysuccinimide

NMRC: National Microelectronics Research Centre

*p*NPP: *p*-nitrophenyl phosphate

PVP: poly(4-vinyl pyridine)

QCM: quartz crystal microbalance

SAM: self-assembled monolayer

SBP: soybean peroxidase

SPDP: 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide

SSC: saline-sodium citrate

T: thymine

TMB: 3,3',5,5'-tetramethyl-benzidine

tris-HCl: tris[hydroxymethyl]aminomethane hydrochloride