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# DNA single-base mismatch study with an electrochemical enzymatic genosensor

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## Abstract

A thorough selectivity study of DNA hybridization employing an electrochemical enzymatic genosensor is discussed here. After immobilizing on a gold film a 30-mer 3'-thiolated DNA strand, hybridization with a biotinylated complementary one takes place. Then, alkaline phosphatase is incorporated to the duplex through the interaction streptavidin–biotin. Enzymatic generation of indigo blue from 3-indoxyl phosphate and subsequent electrochemical detection was made. The influence of hybridization conditions was studied in order to better discern between fully complementary and mismatched strands. Detection of 3, 2 and 1 mismatch was possible. The type and location of the single-base mismatch, as well as the influence of the length of the strands was studied too. Mutations that suppose displacement of the reading frame were also considered. The effect of the concentration on the selectivity was tested, resulting a highly selective genosensor with an adequate sensitivity and stability. © 2006 Elsevier B.V. All rights reserved.

**Keywords:** DNA mismatch; Genosensor; Hybridization; Electrochemical detection; SARS

## 1. Introduction

The development of DNA biosensors (genosensors) has become a field of great interest and application in different areas such as environment, food industry, pharmaceuticals, forensic medicine and clinical diagnosis. Genosensors are constituted by a recognition element that consists of single-stranded DNA (ss-DNA) immobilized on the sensor surface and a transduction system.

Electrochemical transducers have received considerable attention since they offer great advantages such as rapid and sensitive measurements. In addition, they are simple and low-cost devices with possibility of miniaturization. Strategies for electrochemical detection of DNA with solid electrodes have recently been reviewed (de-los-Santos-Álvarez et al., 2004) as well as electrochemical nucleic acid biosensors (Wang, 2002; Lucarelli et al., 2004). Sensitive methods are always required for DNA quantitation, specially when PCR preamplification is avoided (Patolsky et al., 2001). Moreover, selectivity is an important requirement for the detection of single nucleotide

polymorphism (SNP) in genes, the target of tailor-made medications (McCarthy and Hilfiker, 2000) and the key for obtaining medical information about important diseases (Brookes, 1999).

Basically, there are three different strategies for the electrochemical detection of DNA point mutations. Apart from indicator free approaches, charge transport through double-stranded DNA can be monitored. Single-base mismatches appear to induce significant perturbations in the electronic structure of the base-pair stack. Therefore, differences in the behaviour of redox-active intercalators (Yamashita et al., 2002; Wong and Gooding, 2003; Wakai et al., 2004) can be observed. Differences in hybridization of mismatched and complementary DNA strands with immobilized probes have been shown by surface plasmon resonance (Peterson et al., 2002) and surface plasmon fluorescence spectroscopy (Tawa and Knoll, 2004) measurements, deflection of atomic force microscopy cantilevers (Hansen et al., 2001) and frequency shifts of a piezoelectric biosensor (Minunni et al., 2003). Several practical physical aspects of interfacial nucleic acid oligomer hybridization for selective biosensor design have been recently reviewed (Watterson et al., 2002). Since a mismatched duplex is not as stable as that formed by a fully paired one, differentiation between both is possible. Thus, the third strategy relies on controlling experimental variables affecting the hybridization event (stringency) at the

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transducer-solution interface. As they present different thermal stability, control of temperature may help on differentiation (Caruana and Heller, 1999), even though markers that interact electrostatically with DNA are used (Lapierre et al., 2003). It is also reported in the bibliography that a repulsive potential preferentially denatures mismatched DNA hybrids (Heaton et al., 2001). Initial work from our laboratory showed that the presence of a 25% of formamide in the hybridization buffer allowed a single-base mismatch detection (Hernández-Santos et al., 2004, 2005). The use of a concentrated hybridization buffer containing a 45% of formamide and a 5% of dextran sulfate allowed the discrimination of a single-base mismatch (Xu et al., 2001).

The use of enzymes as labels permits to increase assay sensitivity due to their inherent amplification. In the detection of point mutations, a soybean peroxidase label was used for detecting a single-base mismatch in an 18-base oligonucleotide (Caruana and Heller, 1999). Discrimination of three (Abad-Valle et al., 2005) and single-base (Hernández-Santos et al., 2004) mismatch employing alkaline phosphatase (AP) as label and 3-indoxyl phosphate as substrate in conjunction with an electrochemical genosensor has been performed in our laboratory. A decrease of a 33.5% for a single-base mismatched strand has also been reported when  $\alpha$ -naphthyl phosphate is employed as substrate (Xu et al., 2001).

In this paper, we carry out a systematic study about the selectivity of DNA hybridization using an enzymatic electrochemical genosensor on gold films developed in a previous work (Abad-Valle et al., 2005). A sequence of the SARS (severe acute respiratory syndrome) coronavirus (CoV), the causative agent of an outbreak of atypical pneumonia, has been chosen as target. Because of the rate of mortality in patients, it is very important to identify SARS-CoV quickly and accurately. Sequence variations can cause viral transmission from animal to man (Ruan et al., 2003). Moreover, this virus is characterized by

the rapid mutation (Li et al., 2003), including point mutations and few short deletions or insertions that have been detected in different infected individuals (He et al., 2004). Here, we describe results of a rigorous study aimed at the better comprehension of the hybridization interaction from the point of view of selectivity. Stringency conditions are carefully revised. Mismatched strands with different location and type of mutation have been tested. Influence of the length has also been studied using oligonucleotide strands longer than those usually reported in the bibliography.

## 2. Materials and methods

### 2.1. Reagents

Different synthetic oligonucleotides, whose sequences are listed in Table 1, were used. They were commercially prepared (Eurogentec) and supplied as liophilisates. The sequence of the complementary target corresponds to a portion (bases comprised between 29,218 and 29,247, both included) of the severe acute respiratory syndrome (SARS) coronavirus (Marra et al., 2003). Different number, position and chemical nature of base mismatches were tested. For the 30-mer oligonucleotide, three, two and one-base mismatches were considered. They were situated at the center and near the extremes for the three-base mismatch strand. When two bases are mismatched, point mutations situated at the center and the 3'-end, facing the solution, are maintained. In the case of 1-mismatch strands, this is located either at the center or at the 3'-end. For the 40-mer oligonucleotide, a complementary and a one-mismatch (central) strands were employed. Deletion and insertion of one base was also tested. Target strands were biotinylated at the 3'-end. All oligonucleotides were solved in 0.1 M TE buffer (Tris-HCl buffer with 1 mM EDTA) pH 8. Aliquots

Table 1  
Oligonucleotide sequences employed, nomenclature and  $T_m$

	Length	Sequence	$T_m$ (°C)
Probe	30-mer	5'-CTT TTT CTT TTT GTC CTT TTT AGG CTC TGT-3'-(CH <sub>2</sub> ) <sub>3</sub> -SH 5'-CTT TTT CTT TTT GTC CTT TTT AGG CTC TGT-3'-(CH <sub>2</sub> ) <sub>6</sub> -SH	53.1
Complementary target (c-DNA)		5'-ACA GAG CCT AAA AAG GAC AAA AAG AAA AAG-3'-biotin	53.1
Three-base mismatch target (3m-DNA)		5'-ACA <u>GCG</u> CCT AAA <u>AAC</u> GAC AAA AAG <u>AGA</u> AAG-3'-biotin	55.2
Two-base mismatch target (2m-DNA)		5'-ACA GAG CCT AAA <u>AAC</u> GAC AAA AAG <u>AGA</u> AAG-3'-biotin	54.3
One-base mismatch target 1 (1m-DNA, centre, C-C)		5'-ACA GAG CCT AAA <u>AAC</u> GAC AAA AAG AAA AAG-3'-biotin	53.1
One-base mismatch target 2 (1m-DNA, centre, T-T)		5'-ACA GAG CCT AAA <u>ATG</u> GAC AAA AAG AAA AAG-3'-biotin	53.1
One-base mismatch target 3 (1m-DNA, extrem, C-C)		5'-ACA GAG CCT AAA AAG GAC AAA AAG AAA <u>AAC</u> -3'-biotin	53.1
Non-complementary target		5'-GGT CTT <u>GCC</u> <u>CAA</u> <u>TCG</u> <u>GAA</u> CGT TTT TTT TTT-3'-biotin	56.3
Probe	40-mer	5'-TCA GTC TTT TTC TTT TTG TCC TTT TTA GGC TCT GTT GGT G-3'-(CH <sub>2</sub> ) <sub>3</sub> -SH	58.8
Complementary target		5'-CAC CAA CAG AGC CTA AAA AGG ACA AAA AGA AAA AGA CTG A-3'-biotin	58.8
One-base mismatch target		5'-CAC CAA CAG AGC CTA AAA <u>ACG</u> ACA AAA AGA AAA AGA CTG A-3'-biotin	58.8
Deleted base target	29-mer	5'-ACA GAG CCT AAA AAG <u>↑</u> ACA AAA AGA AAA AG-3'-biotin	51.8
Inserted base target	31-mer	5'-ACA GAG CCT AAA AAG <u>TGA</u> CAA AAA GAA AAA G-3'-biotin	53.4

Mismatches are indicated by bold and underlined characters. Complementary bases contained in the non-complementary target are noted with cursive and underlined characters.

were stored at  $-20^{\circ}\text{C}$ , whereas working solutions were kept at  $4^{\circ}\text{C}$ .

1-Hexanethiol was obtained from Sigma–Aldrich. It was solved in absolute ethanol (Merck). Hybridization was carried out in a  $2\times$  saline sodium citrate (SSC: 30 mM sodium citrate buffer with 300 mM sodium chloride) buffer pH 7. A 0.1 M phosphate buffer saline (PBS, 0.15 M in NaCl) was also prepared.

Alkaline phosphatase-labelled streptavidin (ST-AP) was purchased from Sigma. It was prepared in 0.1 M Tris-HCl buffer, 1 mM  $\text{MgCl}_2$  pH 7.2. Aliquots were maintained at  $-20^{\circ}\text{C}$  and working solutions at  $4^{\circ}\text{C}$ . 3-Indoxyl phosphate (3-IP), AP substrate, was purchased from Sigma. Solutions were daily prepared in 0.1 M Tris-HCl buffer, 10 mM  $\text{MgCl}_2$ , pH 9.8. They were kept at  $4^{\circ}\text{C}$  and protected from light.

Trizma base and NaCl were supplied by Sigma. Sodium citrate,  $\text{MgCl}_2$ , NaOH and HCl (25%) were purchased from Merck as well as phosphoric (85%) and sulphuric acids (95–97%). EDTA was obtained from Fluka. Formamide, formaldehyde and urea were obtained from Sigma.

Water was purified employing a Milli-Q plus 185 device from Millipore. Micropipettes obtained from Eppendorf and Rainin Instruments were employed as well as 1.5 mL tubes (Eppendorf). The rest of volumetric material was of analytical grade.

## 2.2. Hybridization assay

An amount of  $5\ \mu\text{L}$  of the thiolated strand solution ( $1\ \mu\text{M}$ ) was deposited on the gold film for 12 h at  $4^{\circ}\text{C}$ . Non-immobilized strands were removed by cleaning with 0.1 M Tris-HCl buffer pH 7.2. Then,  $10\ \mu\text{L}$  of a 2% 1-hexanethiol solution was added and maintained for 10 min. After a new cleaning with  $2\times$  SSC buffer pH 7, hybridization took place by depositing on the film  $20\ \mu\text{L}$  of a biotinylated strand solution ( $2.5\ \text{nM}$ ) for 60 min at room temperature. Then, the film was washed with 0.1 M Tris-HCl buffer, 1 mM  $\text{MgCl}_2$ , pH 7.2 and a  $20\ \mu\text{L}$  drop of  $10^{-9}\ \text{M}$  ST-AP was deposited for 60 min. Afterwards, the film was washed again with 0.1 M Tris-HCl buffer, 10 mM  $\text{MgCl}_2$ , pH 9.8 and  $20\ \mu\text{L}$  of 3 mM 3-indoxyl phosphate solution was added. The enzymatic reaction took place for 10 min and then was stopped with  $5\ \mu\text{L}$  of concentrated  $\text{H}_2\text{SO}_4$ . An amount of  $5\ \mu\text{L}$  of Milli-Q water was immediately added and the measurement was made. Each measurement was performed twice.

Unspecific adsorptions were measured by following the same procedure but employing a  $2\times$  SSC buffer drop instead of a biotinylated strand solution drop in the hybridization step.

Unless otherwise noted, the analytical signal is considered the average between two measurements recorded in different areas of a gold film.

## 2.3. Electrochemical measurements

The details of the homemade miniaturized electrochemical system employed were reported in a previous paper (Abad-Valle et al., 2005). Briefly, a three-electrode potentiostatic system with a working electrode consisting of a 100 nm sputtered gold layer was employed. The working area was limited by self-adhesive

washers in such a manner that 23 assay sites *per* film can be employed. The Ag/AgCl (reference) and Pt (auxiliary) electrodes were external.

Measurements were performed with an Autolab PGSTAT 10 (Eco Chemie) potentiostat interfaced to an ADL Pentium 120 computer system and controlled by Autolab GPES software version 4.8 for Windows 98. A potential of  $-0.35\ \text{V}$  was applied for 30 s before scanning the potential between  $-0.15$  and  $+0.3\ \text{V}$  following a square wave format with 50 Hz of frequency and 50 mV of amplitude.

## 3. Results and discussion

In this work, a simple enzymatic genosensor fabricated on gold films is employed for the study of the hybridization selectivity. A thiolated probe is immobilized on a 100 nm gold film taking advantage of the strong gold-sulphur interaction. In this case, the oligonucleotides employed as probes are derivatized with a thiol-terminated aliphatic linker of three carbons. It permits higher mobility to DNA strand and therefore facilitates its interaction. Drying of the layer was necessary to obtain better results. Therefore it was maintained for 12 h at  $4^{\circ}\text{C}$ , although similar results are obtained for 30 min at  $37^{\circ}\text{C}$ . This layer is stable with time as demonstrated from assays performed on gold films with immobilized strands that were maintained for a period of time at  $4^{\circ}\text{C}$ . Signals were recorded after one, three and six months and compared to that obtained in gold films where the assay was carried out after twelve hours of immobilization. The value of these signals (after background subtracting) obtained after twelve hours (considering for one, three and six months) was  $50 \pm 7\ \mu\text{A}$  ( $n = 6$ ). The average difference between the signal recorded for one, three and six months and the corresponding after 12 h was  $14\ \mu\text{A}$ .

With the aim of minimizing non-specific adsorption at the gold surface, the alkane 1-hexanethiol was immobilized on the surface. The adsorption kinetics of alkanethiols on a gold surface are often described as a two-step process (Bain et al., 1989). Initially, there is a fast (few minutes) growth of the film thickness to 80–90% of the final value, followed by a slower process in which both the thickness and wettability approach an equilibrium value in approximately 10–20 h. As it depends on the composition and concentration, lower time (3 h) has been reported for a mercaptoundecanoic acid SAM (40 mM) (Campuzano et al., 2002). Self-assembled monolayers (SAMs) are conventionally immobilized on the surface by immersing a biosensor in the corresponding solution for over 24 h. In this work, the solution is directly applied on the gold surface and then allowed to air dry at room temperature (10 min), in order to decrease analysis time and enable a more efficient immobilization. The gravity would facilitate the formation of the Au-S covalent bond and an upright alignment of carbon chains would occur due to Van der Waals forces between the carbon chains in the standing-up phase (Akram et al., 2004). This alkanethiol immobilization leads to a decrease in the charging current, noted also by other authors (Akram et al., 2004), obtaining well-defined signals when compared with other common blocking agents such as albumin (Abad-Valle et al., 2005).

In SAMs generation, alkanethiols with acid or alcohol functionalities and different lengths are usually employed (Pan et al., 1996; Duan and Meyerhoff, 1994). However, the use of 1-hexanethiol is not common, although it gives better results than the corresponding alcohol. Its hydrophobicity would avoid electrostatic interaction with surface-charged molecules. Higher lengths could difficult electronic transfer (Akram et al., 2004) and the smaller ones result to be extremely volatile. This layer is also useful for removal of non-specifically bound DNA (Herne and Tarlov, 1997) and extending the thiolated DNA strand farther into the solvent phase (Levicky et al., 1998).

Then, hybridization with biotinylated target DNA is followed by interaction with alkaline phosphatase-labelled streptavidin. Further incubation with 3-IP produces indigo blue that is solubilised. Reversibility of the electrochemical process permits signal enhancement when square wave voltammetry is employed.

### 3.1. Selectivity of hybridization

The investigation of the selectivity of hybridization and the possibility of electrochemically detecting mutation points could be carried out from three different points, as was commented in the introduction. In one of the approaches, DNA hybridization is carried out under low stringent conditions. On the other hand, mismatches appear to induce perturbations in the electronic structure of the base-pair stack and therefore the behaviour of a redox-active intercalating agent could change. As in this work an enzymatic detection is used, differences in the electron transfer are not seen. Indeed, when hybridization between the 30-mer probe (1.02  $\mu\text{M}$ ) and both, complementary and 3-mismatched strands (4.04 nM), was performed in a  $2\times$  SSC buffer for 15 min at  $37^\circ\text{C}$ , no discrimination was observed. However, when hybridization took place between the probe and a 30-mer strand with only six common bases, a differentiation was seen. The signal for the non-complementary strand (with six separate complementary points) was a  $25 \pm 1.2\%$  of the corresponding to the fully complementary. Although there are studies presenting evidence of stable and measurable hybridization between four-base pair complementary sequences (Hansen et al., 2001), the hybridization is generally considered thermodynamically unstable when complementarity exists over only a very short stretch (less than six nucleotides).

Applying stringency conditions is a simple way to differentiate between fully complementary and mismatched strands. Therefore, hybridization was carried out under different conditions. As the DNA strand is polyanionic, the electric field could control electrostatic interactions and would suppose stringency to the biomolecular interaction (Heaton et al., 2001). A potential of  $-0.35\text{ V}$  was applied after hybridization for 30 s with the aim of destabilizing and removing unpaired strands. However, similar signals were observed,  $41 \pm 4.3$  and  $40 \pm 4.7\ \mu\text{A}$  for the complementary and unpaired (three-base mismatched) strands, respectively. The employment of different ionic strength (1, 2, 5 and  $10\times$  SSC), buffer composition (TE, Tris-HCl or PBS), formamide percentage (5, 25 and 50%) and temperature (4 and  $45^\circ\text{C}$ ) for the 15-min hybridization step did not produce a clear discrimination.

Strands interact rapidly as can be seen in SPR (Peterson et al., 2002) or cantilever deflection (Hansen et al., 2001) experiments. However, hybridization time is an important variable in obtaining selectivity. When hybridization step was performed placing  $20\ \mu\text{L}$  of the target solution (containing complementary or three-base mismatched strand) on the sensor surface for 15, 30, 45 and 60 min in  $2\times$  SSC buffer pH 7 with 25% formamide, an increase in the signals with time was seen as observed in Fig. 1. However, slower hybridization kinetic is presented by the sequence that is mismatched with respect to the probe. As the signal enhancement is much higher for the complementary strand, discrimination between both strands could be achieved with time. For 1 h, a  $38 \pm 1.3\%$  of decrease (comparing the signal of the mismatched strand to that of the complementary one) was obtained. Meanwhile no differentiation is seen for 15 min. Higher times were not tested for avoiding drop evaporation that would cause a decrease in the hybridization signal (Abad-Valle et al., 2005). Furthermore, a longer hybridization time does not have to suppose an increase in selectivity. A time of 30 min is employed by other authors as optimum for the best discrimination between complementary and four-base mismatched targets (Kara et al., 2004), decreasing notoriously the selectivity for higher times. In the discrimination of *Helicobacter pylori* sequences, when hybridization was permitted to proceed longer than 12 h at  $40^\circ\text{C}$ , comparable results were obtained for the complementary and mutated sequences (Lapierre et al., 2003). Therefore, 1 h was used as hybridization time for the rest of the assays. Lower hybridization time produced less effect on the discrimination and longer time, apart from producing a decrease in both signals due to evaporation, seemed to act increasing the signal corresponding to the mismatched strand.

### 3.2. Stringency conditions influence on mismatched strands discrimination

Once discrimination has been achieved, the effect of different stringency conditions, which destabilized the unpaired duplex, was studied. Ionic strength, pH, addition of chemicals and potentials application were tested for the discrimination of the 3-mismatched strand. High temperature destabilizes DNA duplex and therefore it is an important factor in selectivity (Caruana and Heller, 1999; Millan et al., 1994), but the employment of

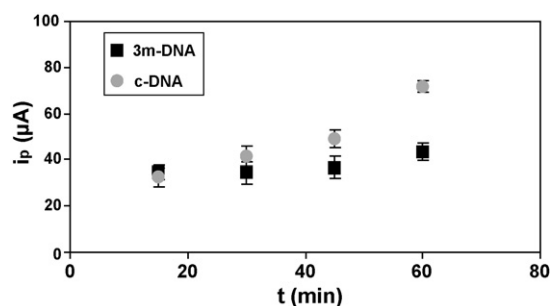


Fig. 1. Influence of the hybridization time on selectivity. Signals correspond to the complementary strand (c-DNA) and the three-base mismatched one (3m-DNA).  $V_{\text{target}} = 20\ \mu\text{L}$ ,  $2\times$  SSC buffer pH 7, 25% formamide.

high temperatures during the hybridization step complicates the assay and therefore it was not studied.

The presence of ions in DNA solutions stabilizes DNA negative charges and therefore ionic strength will affect to hybridization step. This means that selectivity could be affected, although this effect depends on oligonucleotide packing density (Ikuta et al., 1987). In this study, the signal obtained after hybridization of the probe (1.01  $\mu\text{M}$ ) with c-DNA and 3m-DNA (3.03 nM) employing 1, 2, 5 and 10 $\times$  SSC pH 7 buffers (without formamide) for the hybridization step was recorded. Values of 1.02, 1.24, 1.24, and 1.15 for the c-DNA/3m-DNA ratio were obtained, respectively. This indicates that ionic strength does not affect significantly to the selectivity, so a 2 $\times$  SSC buffer was employed for further studies.

For oligonucleotide strands of short lengths, a commonly accepted model for duplex formation is that of nucleation followed by helix zipping (Craig et al., 1971). Acid and basic pH values diminish nucleation of DNA bases and therefore the capacity of discerning complementary and mismatched strands will be modified. The hybridization step was performed in 2 $\times$  SSC buffer at pH 6, 7 and 8 for 60 min with the aim of studying its influence. Extreme pH values were not tested, since it would cause DNA denaturation. The values of c-DNA/3m-DNA ratio obtained were 1.17, 1.16 and 1.02 for pH 6, 7 and 8, respectively. As this parameter neither affects greatly the selectivity, the pH 7 was maintained.

Compounds with amino and carbonyl groups in their structure present in the hybridization step will compete with nucleotide bases for hydrogen bonds formation. Therefore, they facilitate DNA duplex destabilization and strands separation. Formaldehyde (H–CO–H), formamide (H–CO–NH<sub>2</sub>) and urea (H<sub>2</sub>N–CO–NH<sub>2</sub>) are some examples of these compounds. Each one of them was added in a 25% proportion to the 2 $\times$  SSC buffer pH 7 and under these conditions hybridization with c-DNA and 3m-DNA took place. Results obtained are shown in Fig. 2, where the signals recorded without added modifiers were also included. Formaldehyde produces a sharp decrease of both signals (c-DNA and 3m-DNA). The presence of 3 mismatches signifies a decrease of a 22.2% when urea is employed. The best discrimination capacity was obtained with formamide (a 58.9% decrease) and therefore, this chem-

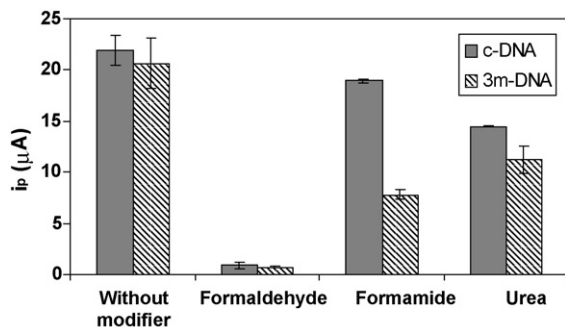


Fig. 2. Effect of addition of modifiers to the hybridization buffer (2 $\times$  SSC pH 7) in a 25% proportion on the genosensor response for the complementary target (c-DNA) and the three-base mismatched one (3m-DNA).  $V_{\text{target}} = 20 \mu\text{L}$ ,  $t_{\text{hybr}} = 60 \text{ min}$ .

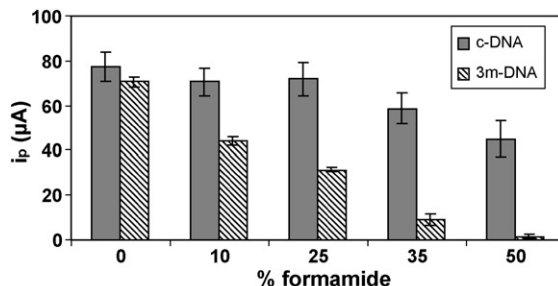


Fig. 3. Influence of formamide concentration in the hybridization buffer on the genosensor response for the complementary target (c-DNA) and the three-base mismatched one (3m-DNA).  $V_{\text{target}} = 20 \mu\text{L}$ ,  $t_{\text{hybr}} = 60 \text{ min}$ .

ical was added to the hybridization buffer for the rest of the assays.

Formamide concentration in the hybridization buffer was varied between 10 and 50% in order to study its influence and the optimum proportion for attaining the highest selectivity. As it is showed in Fig. 3, the discrimination between strands increases with formamide concentration. The signal diminishes from a 37% in the case of using a 10% of concentration to a 92% for the 50%. Since with this last percentage the discrimination attains almost the 100%, with the lowest 3m-DNA signal and the highest c-DNA/3m-DNA ratio, this concentration was employed when clear differentiation wanted to be observed.

DNA duplex is negatively charged because of DNA backbone phosphate groups. Applying a negative potential after the hybridization step would cause DNA destabilization and denaturation of the wrong hybridized strands. To study this effect, once that hybridization took place in 2 $\times$  SSC buffer pH 7 with 25% formamide (a 50% was not employed in order to better observe some change in the 3m-DNA signal) a  $-0.30 \text{ V}$  potential was applied for 2 min in a 0.1 M Tris–HCl buffer pH 7.2. When c-DNA/3m-DNA ratios are compared, values of 1.48 and 1.54 are obtained with and without potential application, respectively. No improvement was either observed when the experience was repeated with a buffer that did not contain formamide. As selectivity is not enhanced, this step was not considered for the rest of the studies.

### 3.3. Single-base mismatch differentiation

As the presence of base mismatches is frequently associated with human diseases, detecting a single-base mismatch is of increasing interest. The SARS-CoV is characterized by rapid mutation (Li et al., 2003). Six positions with high-mutation rate were identified closely with the three phases of the SARS epidemic (Long et al., 2004) and deletions or insertions were detected in different infected individuals (He et al., 2004). Then, the detection of the mutations results helpful for the development of other studies, i.e., specific pharmaceuticals design or study of infectious mechanisms.

In this case, base substitutions were located at 5, 15 and 26 positions, where T–A, C–G and T–A bonds were changed by T–C, C–C and T–G, respectively. Therefore, transversions (purinic by pyrimidinic bases, i.e., A  $\rightarrow$  C and G  $\rightarrow$  C) and

transitions (purinic by other purinic base, i.e., A → G) are involved. A total of seven hydrogen bonds disappeared. As the possibility of detecting a single-base mismatch is relevant, the hybridization was performed with two and one-base mismatch strands. The mismatch at position 15 is maintained for both, as well as the 26th for the 2m-strand. In Fig. 4, the signals corresponding to the complementary and 3, 2 and one-base mismatched strands are represented. Values of the signals after subtracting background were,  $0.064 \pm 0.0071$ ,  $2.4 \pm 0.28$  and  $3.5 \pm 0.36 \mu\text{A}$  for 3, 2 and 1m-DNA, respectively. Since the signal for the complementary strand is  $26.7 \pm 3.0 \mu\text{A}$ , a 99.8, 91.0 and 86.9% of decrease is, respectively, observed. Although the one-base mismatch strand provides a higher signal than that with three-base mismatches, where 100% of discrimination is almost achieved, c-DNA and 1m-DNA can be discerned perfectly. In this case, only three hydrogen bonds are suppressed.

Selectivity seems to depend on the chemical nature of the mismatching base pair and localization along DNA strand (Ikuta et al., 1987). In the assay commented in the previous paragraph, a 1m-DNA strand with a C–C mismatch located at the centre of the strand was employed. A transversion, with change of a purinic (G) by a pyrimidinic (C) base occurs. This is one of the more favourable discerning cases, taking into account that the stability for base pairs obtained from statistical simulation results. (Allawi and SantaLucia, 1997, 1998a,b; Peyret et al., 1999) follows the sequence: G–C > A–T > G–G > G–T = G–A > T–T = A–A > T–C ≥ A–C ≥ C–C. In this case, G–C, which is the more stable interaction, with three hydrogen bonds implied is substituted by a C–C interaction, which is situated at the end of the classification. Hybridizations with other one-base mismatched strands including other type of interaction and different localization were tested. Employing the same experimental conditions as before, signals for two one-base mismatch strands, one with a T–T mismatch in the centre of the strand (position 14) and the other with a C–C mismatch in the 3'-extreme (sequences indicated in Table 1) were recorded. They are compared in Fig. 5 with signals obtained for the 1m-DNA with the mismatch in position 15 (C–C type) and the complementary strand. In all the cases one-base mismatch strands can be distinguished from the complementary one. Signals corresponding to the two strands with the mismatch in the centre are similar, although the T–T mismatch strand gives a slightly higher signal. An A–T interaction (two hydrogen bonds) is changed in

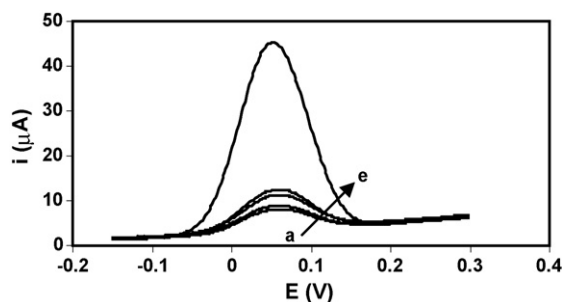


Fig. 4. Square wave voltammograms corresponding to the signals of (a) background (b) 3, (c) 2, (d) 1-mismatched and (e) complementary strands.  $V_{\text{target}} = 20 \mu\text{L}$ ,  $2 \times \text{SSC pH 7 buffer with 50\% formamide}$ ,  $t_{\text{hybr}} = 60 \text{ min}$ .

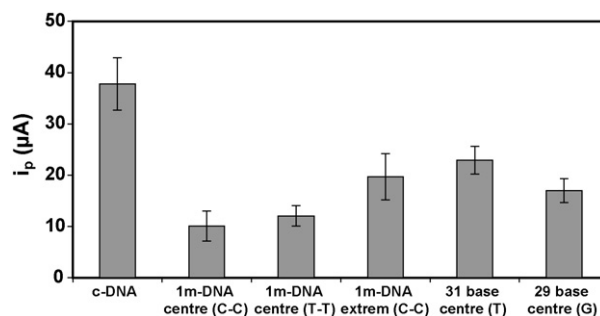


Fig. 5. Comparison between signals for the complementary and point mutated strands: substitutions at the centre or 3'-extreme, insertion and deletion.  $V_{\text{target}} = 20 \mu\text{L}$ ,  $2 \times \text{SSC pH 7 buffer with 50\% formamide}$ ,  $t_{\text{hybr}} = 60 \text{ min}$ .

this case by a T–T one (transversion with change of purinic (A) by pyrimidinic (T)), with less difference in stability. Therefore, the destabilization in DNA duplex will be smaller.

On the other hand, and referring to the location of the mismatch, differences have been found in the bibliography. Kelley et al. (1999) reported that films containing mismatches closest to the electrode surface showed the largest attenuation in signal, that consisted on the cathodic charge of the intercalator daunomycin. However, Tawa and Knoll (2004) studied the kinetics of DNA–DNA hybridization by surface plasmon fluorescence spectroscopy and stated that a double strand is found to be more destabilized if a mismatched base pair between the target DNA and the probe DNA is located farther away from the solid sensor surface, facing the solution. In our case, the 3'-extreme-mismatch (C–C type) strand signal is clearly higher than centre-mismatch (C–C type) strand one (Fig. 5), which means that although is also distinguished from the fully complementary, it destabilizes less the DNA duplex.

It has been reported that the mechanism of interfacial oligonucleotide hybridization may consist of two separate pathways: by direct diffusion of target DNA in bulk solution directly to immobilized oligonucleotides for selective binding or by non-selective adsorption of the target DNA onto the surface, followed by “two-dimensional diffusion” of adsorbed target oligonucleotides to immobilized probes for selective binding (Watterson et al., 2002). In both cases, adsorption and hybridization of smaller targets occur more rapidly than with larger targets. However, as the number of interactions increases with the length of the strands (number of nucleotides), stability is supposedly higher for longer strands. In order to study the influence of the length of the oligonucleotides on the hybridization and on the one-base mismatch differentiation, 40-mer complementary and one-base mismatch (C–C centre) strands were employed as well as the 30-mer ones. The strand sequences used are reported in Table 1. The signal for complementary 40-mer is higher than that of 30-mer, as well as the corresponding mismatched 40-mer and 30-mer strands (data not shown), due to the higher number of interactions involved in the longer strand. On the other hand, the c-DNA/1m-DNA ratio is lower when 40-mer strands are employed. It seems therefore that selectivity depends on the nucleotide bases number. One-base mismatch supposes a 1/30 (3.3%) of the total bases of a 30-mer strand, meanwhile in the

case of 40-mer strands it is 1/40 (2.5%). As the influence of the mismatch in the whole molecule is higher the discrimination increases.

Another important related study is the comparison between hybridization that takes place with crossed strands, that is to say a 40-mer immobilized strand with a 30-mer one (40–30) and vice versa, 30-mer immobilized strand with a 40-mer one (30–40). Signal for the first case (40–30) is higher than for 30–40 (data not shown). In the last case there is a rest of five oligonucleotides that can hinder the hybridization, which anyway takes place very close to the electrode. Meanwhile, in the 40–30 hybridization the interaction takes place at the centre of the 40-mer strand. 1-mismatched strands are in both cases discriminated, obtaining for similar reasons a higher signal in the case of hybridization between immobilized 40-mer and a 30-mer strand. The ratio c-DNA/1m-DNA is also greater for the 40–30 case.

When the basic 30–30 hybridization is compared with crossed interactions, always between complementary strands, the following order is established: 40–30 > 30–30 > 30–40. Although the same number of interactions are involved in 40–30 than in 30–30 hybridization, the presence of a five nucleotide spacer confers a higher mobility to the immobilized strand and favours the hybridization. This result is in agreement with that obtained for a 30–30 hybridization experience employing for the immobilized strand either a  $-(\text{CH}_2)_3$  or a  $-(\text{CH}_2)_6$  spacer. The extension of hybridization is slightly higher when a higher spacer  $-(\text{CH}_2)_6$  instead of  $-(\text{CH}_2)_3$  is employed (data not shown). This is the reason why some genosensors employ in the immobilized strand a nucleotide tail that does not hybridize, with the aim of permitting mobility (Hernández-Santos et al., 2004), although studies performed with different linker lengths demonstrated that kinetic of electron-transfer process was slower for longer linkers (Taft et al., 2003). In this case, however, the difference in the signal is not so big to justify the difference in price (the strand with a  $-(\text{CH}_2)_6$  spacer almost doubles the price of that with a  $-(\text{CH}_2)_3$  spacer). Moreover, the discrimination of 1 m (C–C type in the centre) DNA is similar for both cases.

Mutations not only consist on the change of bases (base substitution mutations) but also deletions and insertions can occur. Deletion of a guanine base in the exon 5 of the lipoprotein lipase gene has been detected by enzymatic recognition (Wakai et al., 2004). Different phases of the SARS epidemic were related closely with genotypes at different positions with high-mutation rate (Long et al., 2004) and also few short dele-

tions or insertions were detected (He et al., 2004). In this case, when a number of bases different from three (or a multiple) are involved, a change in the reading frame occurs. With the aim of detecting this type of mutation, biotinylated targets with deletion (29-mer) and insertion (31-mer) of one base at the central position (16th) were tested for hybridization. An illustrative scheme is depicted in Fig. 6. Guanine is deleted in the first strand and a thymine was introduced in the last one. Results are represented in Fig. 5, from which interesting conclusions can be obtained. Discrimination from complementary strand was always possible but the signal was higher for mutations implying an insertion (30–31 hybridization (Fig. 6c) or deletion (30–29 hybridization (Fig. 6d)) than those with a base substitution mutation in the centre (30–30 hybridization (Fig. 6b)). This means that when a base substitution occurs, interactions due to 29 bases can be produced but the mismatched pair C–C introduces a higher destabilization. However, when a deletion is present, a probable interaction between 29 bases occurs and the non-complementary base of the 30-mer immobilized strand (cytosine) remains unpaired. For the case of the insertion, the probable interaction occurs between 30 bases and the non-complementary base corresponding to the biotinylated 31-mer strand (thymine) is supposedly unpaired. This also explains why the signal for the insertion is higher than the deletion; a higher number of interactions are involved.

Finally, the variation of c-DNA/1m-DNA ratio with target concentration was studied. This parameter is important in real sample analysis where DNA concentration is unknown and/or the more adequate DNA concentration can not be chosen. Following the optimized procedure, different c-DNA and 1m-DNA (C–C mismatch in the centre) concentrations were incubated in  $2 \times$  SSC buffer pH 7 with 50% formamide for 60 min. 30-mer target concentrations tested were 0.01, 0.1, 1 and 5 nM. The results obtained are showed in Fig. 7. Selectivity is achieved in all the tested concentrations. Discrimination varies slightly with target concentration with percentages always superior to 40%, the highest corresponding to 1 nM. It is derived from here that clear differences can be observed between complementary and mismatched strands at a concentration as low as 10 pM, demonstrating that the methodology is not only selective but sensitive to the hybridization. Linearity is obtained for both strands between 0.01 and 1 nM, with slopes of 21.2 and 9.6  $\mu\text{A}/\text{nM}$  for the complementary and mismatched strands, respectively. The detection limit, calculated as the concentration corresponding to a signal that is three times the standard deviation of the intercept,

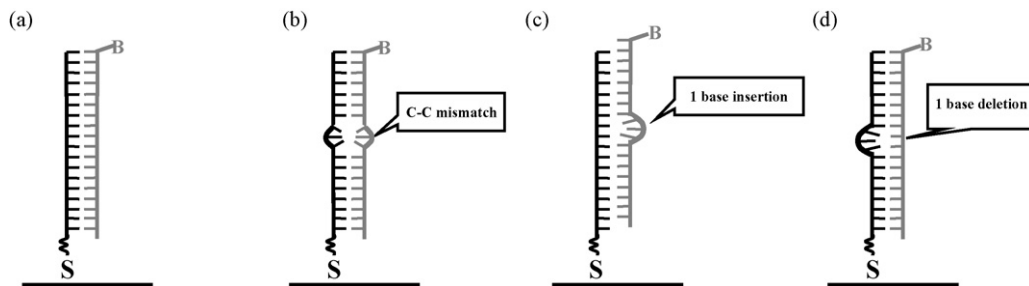


Fig. 6. Diagram of the hybridization of the immobilized strand with a fully complementary one (a) or with a strand with a substitution (b), insertion (c) or deletion (d).



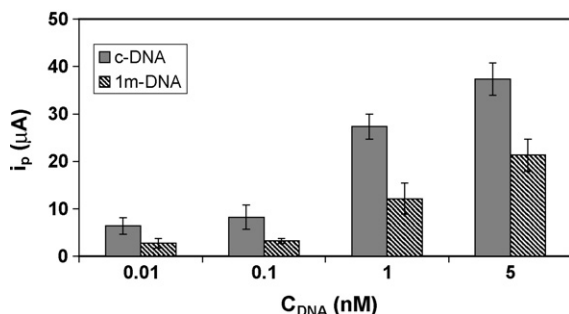


Fig. 7. Effect of the concentration on the selectivity. Signals for the complementary (c-DNA) and the one-mismatched strand (C–C at the centre, 1m-DNA).  $V_{\text{target}} = 20 \mu\text{L}$ ,  $2 \times \text{SSC pH } 7$  buffer with 50% formamide,  $t_{\text{hybr}} = 60$  min.

was found to be 5 and 70 pM, respectively, in the order of other found in the bibliography. Miyahara et al. (2002) reports 0.1 nM for the 20-meric mismatch detection system and Kara et al. (2004) estimated detection limits around 15 pM for both complementary and four-base mismatched 22-mer sequences. A very sensitive assay, 0.5 fM for a complementary strand and detection of a single-base strand at 10 fM level, was obtained by Zhang et al. (2003) by reducing dimensions: employment of a 10  $\mu\text{m}$ -diameter carbon microelectrode and a 10  $\mu\text{L}$ -droplet.

#### 4. Conclusions

A complete study on the selectivity of DNA hybridization has been carried out employing a sensitive, simple and stable electrochemical enzymatic genosensor. It is of relevant interest in the study of single nucleotide polymorphisms (SNP) as well as for the study of virus mutation. The methodology described here can easily discern hybrids, both fully complementary and unpaired at room temperature. This has been demonstrated employing a sequence of SARS virus as target. After testing many stringency conditions, it was observed that carrying out the interaction between strands in a medium containing a 50% of formamide during 1 h was enough for achieving a high degree of discrimination for all the tested mutated strands.

Studies with mutated (base substitution) 30-mer synthetic oligonucleotides revealed differences for 3, 2 and 1 mismatched strands. One mismatch is detected even if it is located at the extreme situated far from the electrode, being the attenuation of the signal lower than when the substitution occurs near the electrode surface. Hybridization studies carried out between 30-mer strands were compared with those with 40-mer oligonucleotides. The length of the strand influences the hybridization. Experiences with crossed strands revealed the importance of an oligonucleotide tail in the immobilized strand for favouring the mobility and therefore the hybridization. When an aliphatic spacer is used, the augmentation in three carbons implies an increase in the signal for both, complementary and mismatched strands. However, as the magnitude of the increase is similar, discrimination is not enhanced notoriously.

Mutations that involve deletions and insertions have also been proved. Both are differentiated from the fully complementary strand. However, although destabilization of the duplex

occurs, lower discrimination than with a substitution mutation is obtained. As a higher number of interactions is present, higher signals are obtained for the base insertion mutation.

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

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