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Two independent label-free detection methods in one electrochemical DNA sensor

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ABSTRACT

Two direct reagent-free detection methods were tested with Au/polypyrrole/oligonucleotide modified electrodes. Detection by monitoring guanine oxidation was realized amperometrically using an experimental setup which does not require any expensive electrochemical equipment and is therefore suitable for *in situ* detection. Target detection was also realized by monitoring the decrease in the amplitude of polypyrrole oxidation and reduction peaks in cyclic voltammetry experiments after incubation or injection of target into the electrochemical cell. Detection of 53 pM target within a 2000× excess of non-complementary sequences was possible. The possibility of a dual detection scheme in the same biosensor, with both detection schemes being totally independent from one another is very promising for genosensor design since it would result in a significant decrease in the number of false positive and false negative samples.

1. Introduction

DNA assays are currently standard methods for the identification of a large number of genetic and infectious diseases in clinical laboratories (Lee, 2008). Major drawbacks of current well-standardized molecular biology techniques are its time requirements for sample processing and amplification, false positives from sample or reagent contamination, and high instrumentation and labor costs (Gau et al., 2005). Fast, simple, low cost and trustworthy devices are needed for point-of-care diagnostics and self use, and will revolutionize the field of molecular diagnostics such as glucose amperometric biosensors did for the field of diabetes management (Soper et al., 2006).

Genosensors are analytical devices for the detection of specific DNA "target" sequences in solution, upon hybridization of the targets with complementary "probes" immobilized on a solid substrate. Among the different detection schemes, electrochemical DNA hybridization detection methods have attracted great attention due to their high sensitivity, low cost, ease of use, portability, and compatibility with micro fabrication (Hejazi et al., 2008). They are sub classified into either direct (label-free) or indirect methods, depending upon the need of introducing electrochemical labels which can discriminate between single stranded (ss) and double stranded (ds) DNA.

Electrochemical labels are mainly electro active intercalating agents which can be preconcentrated at the electrode surface through association with dsDNA (Millan and Mikkelssen, 1993). More recent approaches include the use of organic dyes such as methylene blue which interacts with accessible DNA bases (Pournaghi-Azar et al., 2006; Loaiza et al., 2005; Meric et al., 2002). Another interesting approaches are the use of redox enzymes such as horseradish peroxidase which can be preconcentrated on the electrode surface by sandwich assays involving probe/target/reporter hybrids (Zhang et al., 2003; Huang et al., 2002) or DNA modified Au nanoparticles which can be released and detected after hybridization (Authier et al., 2001).

In spite of the low detection limits which can be accomplished with indirect (label-based) detection methods, there exist a strong interest in developing alternative label-free detection schemes since labeling constitutes an additional costly and time-consuming preparatory step (Haring Bolívar et al., 2004) that can drastically change the binding properties of the labeled biomolecule (Daniels and Pourmand, 2007). For these reasons, we focused our attention in electrochemical, label-free (direct) and reusable DNA sensors which could render a single-step process since these attributes are considered useful for in field applications such as point-of-care diagnostics (Lai et al., 2006).

Direct methods rely on the intrinsic electrochemical properties of DNA (the oxidation of purine bases, particularly guanine) or in changes in some of the interfacial properties after hybridization. The first report in which guanine oxidation chemistry was used for target detection was described by Wang et al. (1997), who showed

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how the chronopotentiometric signal of guanine decreased after incubation of an oligo d[G] modified electrode with a guanine-free target (Wang et al., 1997). Lately, the use of inosine-substituted probes enabled the detection of guanine-containing targets by detecting an appearance of the guanine signal after hybridization (Wang et al., 1999). Besides chronopotentiometric methods, differential pulse voltammetry was also performed in other reports for DNA detection based on the guanine oxidation signal (Ozkan-Ariksoysal et al., 2008).

Polypyrrole (pPy) is a versatile conductive polymer which can be obtained by induction of pyrrole polymerization by either chemical oxidation, photo induced synthesis or electrochemical activation by anodic current (Ramanavicius et al., 2006). It has been used as a conductive matrix for probe immobilization in a number of protocols (Peng et al., 2005; Korri-Youssoufi et al., 1997; Livache et al., 1994). For instance, oligodeoxinucleotides (ODNs) can be directly incorporated into the pPy matrix as dopants during polymer synthesis when they are included in the polymerization solution as the only counter ions (Wang et al., 1999). ODNs are in this way attached to the polymer modified electrode by their phosphate backbone, and the nucleotidic bases remain accessible and exposed to the solvent. Direct target detection can be realized by monitoring changes in pPy interfacial properties before and after hybridization. This approach was followed in the two reports which inspired many of the experiments realized in the present study. Two opposite (anodic/cathodic) signals were registered by chronoamperometry upon injection of complementary and not complementary targets, respectively (Wang et al., 1999; Komarova et al., 2005). The nature of such an up/down response is not fully understood but is very attractive, though it implies a technological challenge since the non-specific signal can suppress the specific signal when a mixture of both complementary and non-complementary ODNs are used (Komarova et al., 2005).

In this work, two direct detection methods (based on guanine oxidation and pPy electro active properties) were tested in electrodes modified with pPy doped with the oligonucleotide probes. The aim of this work was to explore different possibilities for target detection within a same sensor system that could be compatible with miniaturization, automatization and *in situ* use. Since both methods described are independent from one another, we argue that this will benefit the specificity of the assay since false positives and false negatives arising from one method could be discarded by the other.

2. Materials and methods

2.1. Chemicals and solutions

Pyrrole (98% purity, reagent grade) was acquired form Sigma. After each use, it was bubbled for 2 min in a nitrogen atmosphere to avoid monomer oxidation. 0.5 M aliquots were prepared using MiliQ water and stored at 4 °C. Iron (III) chloride hexahydrate (97%) was purchased from Fluka. Phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), pH 7.4, was prepared with analytical grade reagents in MiliQ water.

DNA oligonucleotides were obtained from SBS Genetech (China). Oligonucleotide stock solutions were prepared using MiliQ water and stored at a temperature of −20 °C until use. The oligonucleotide sequences used in this study were:

Oligo d[AC]8: 5′ ACA CAC ACA CAC ACA C 3′
Oligo d[TG]8: 3′ TGT GTG TGT GTG TGT G 5′
Oligo d[C]10: 5′ CCC CCC CCC C 3′
Oligo d[G]10: 3′ GGG GGG GGG G 5′

2.2. Electrochemical equipment

Voltammetric characterization was realized using a VoltaLab PGZ 301 potentiostat (Radiometer Analytical) in a three-electrode electrochemical cell containing PBS as the electrolyte, an Ag/AgCl reference electrode and pencil graphite (Faber Castell, 2B) as the auxiliary electrode. Amperometric measurements were carried out using a custom-made potentiostat measuring 30 cm × 15 cm × 8 cm. The working electrode potential could be manually fixed at the desired value against the reference electrode by adjusting a screw. Current and voltage were measured with commercially available digital multimeters (Nippon America).

2.3. Pretreatment of electrodes

High purity (≥99.99%) gold wires of 1 mm diameter and 2 cm of length were used as the working electrodes for all experiments, and were purchased from Goodfellow (UK). Before use, electrodes were polished with 0.45 μm alumina until a mirror-like surface was obtained. Electrodes were then immersed in a cleaning solution (30% H_2O_2, 2% H_2SO_4) for 2 h, and rinsed with MiliQ water. Electrodes were then air-dried at room temperature and their lateral surface was covered with Teflon tape, exposing only the polished surface and 0.5 cm of the opposite end, for the establishment of electric contacts. Purity of the electrodes and effectiveness of the cleaning protocol were tested by voltammetric characterization in 0.5 M H_2SO_4 and in PBS containing the redox couple $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$.

2.4. Pyrrole electropolymerization and probe immobilization

For pPy electrosynthesis a modification of the method of the electro-spot was used (Livache et al., 2003). The narrow end of a 1000 μL micropipette tip was cut in order that the polished section of the gold electrode could be introduced into the tip (supplementary Figure 1). From the opposite end of the tip a platinum wire was introduced and placed near the surface of the working electrode. The tip was then filled with 20 μL of the polymerization solution, containing pyrrole (0.1 M) and the desired oligonucleotides (0.8 μg/μL). Electropolymerization was carried out at constant potential of +0.9 V between the working electrode and the platinum wire for 3 min. For pPy doped with an inorganic anion, 8 mM $FeCl_3$ was used.

2.5. Target detection by chronoamperometry

For detection of target sequences by the oxidation signal of guanine, a guanine-free probe (oligo $d[C]_{10}$) and a guanine-containing complementary target (oligo $d[G]_{10}$) were used, as well as other combinations of probes and targets. A three-electrode electrochemical cell was used, containing 12 mL of PBS as the electrolyte. The working electrode potential was fixed at a value of +1.0 V and the system was allowed to stabilize for 10–15 min. After that time, 2 μL of a 0.5 μg/mL solution of ODNs (complementary or not complementary to the probe, depending on the experiment) were injected into the cell and the electric current was registered at 5, 10, 15, 30, 45, 60, 120 and 180 s post-injection. At t = 180 s (3 min post-injection), 4 μL of the same ODN solution were injected. The procedure was repeated and increasing amounts of targets were injected every 3 min. The amounts of targets subsequently introduced in each experiment were: 1, 2, 4, 6, and 10 ng. Independent duplicates were realized for every combination of probe and target tested. Between duplicates, different electrodes were used.

The values of the electric current registered every 3 min post-injection of target were normalized to the initial value (before the

first introduction of ODNs) and plotted against the total amount of target which had been introduced at that point of the experiment.

2.6. Voltammetric characterization of the electrodes and target detection by monitoring changes in pPy oxidation and reduction peaks

Unless otherwise stated, cyclic voltammetry experiments were realized at room temperature in a three-electrode electrochemical cell, containing PBS pH 7.4 as the electrolyte. Potential sweep was realized at a speed of 100 mV/s and was set to start at −0.6 V. The potential was inverted at +0.6 V and 3–4 scans were registered in order to study if electrodes were stable in such range of potentials. Since no significant differences were observed between scans, the last one was taken into consideration for analysis.

Two different experimental approaches were tested for target detection. The first approach was to characterize the working electrode voltammetrically as described above prior to the introduction of a known amount of target sequence into the electrochemical cell. Cyclic voltammetry was realized again and the procedure was repeated with subsequent additions of targets followed by voltammetric characterization. A working electrode modified with pPy/oligo d$[TG]_8$ was used and oligo d$[AC]_8$ and oligo d$[TG]_8$ ODNs were used as complementary and non-complementary targets, respectively. The amounts of target subsequently introduced into the cell were: 1, 2, 3, 4, 5, 7.5, 10 and 20 ng. The amplitude of pPy oxidation peak was measured and normalized to the initial value (before the first addition of target).

Between experiments, the working electrode was immersed in 0.5 M NaOH for 15 s and three washes with MiliQ water (10 s per wash) were realized in order to ensure target dissociation and electrode regeneration. The cell was filled with new target-free PBS and counter and reference electrodes were carefully rinsed with MiliQ water.

In order to avoid the high degree of unspecific adsorption to the electrode surface during the voltammetric scan at positive potentials, a different approach was followed. Targets were not further introduced into the electrochemical cell. Instead, electrodes were incubated for 1 h at room temperature with 0.5 μg/mL complementary sequence in PBS (4 mL) prior to characterization by cyclic voltammetry. Electrodes modified with pPy/oligo d$[AC]_8$ and pPy/oligo d$[TG]_8$ were used. A control of specificity was realized by incubating a pPy/oligo d$[AC]_8$ electrode with 0.5 μg/mL of non-complementary ODNs (oligo d$[AC]_8$).

To test the performance of the sensor in samples where the target sequence is in the presence of a great excess of non-complementary sequences (as an approximation to real samples), pPy/oligo d$[TG]_8$ modified electrode was incubated under the same experimental conditions with 0.5 μg/mL complementary target in PBS for 1 h. A second incubation step was realized in 4 mL of PBS containing 0.25 ng/mL complementary target and 0.5 μg/mL (2000× excess) of non-complementary ODNs (oligo d$[TG]_8$). Cyclic voltammetry was performed after each incubation step and the voltammograms were superimposed in order to detect changes in pPy oxidation and reduction peaks.

3. Results and discussion

3.1. Electrode preparation

The desired DNA probes were immobilized on surface-polished high-purity gold electrodes by an adaptation of the method described by Wang et al. (1999), taking the optimizations made by Komarova et al. (2005) into consideration. Sequence-specific ssDNA probes are incorporated into polypyrrole films when incorporated into the polymerization solution as the sole dopant during electrochemical polymerization of pyrrole. Since the attraction forces between pPy and DNA are established by the sugar-phosphate backbone, the nucleotide bases are exposed to the surface and are thus accessible for hybridization.

Polymerization was carried out using a blue (1000 μL) pipette tip as the electrochemical cell, in a modification of the "electrospotting methodology" described by Livache et al. (2003). This approach (shown in supplementary Figure 1) allowed the use of low volumes of polymerization solution (20 μL), and so reagents (especially ODNs, which are used in high concentrations) were not excessively wasted in every experiment.

The effectiveness of pPy electropolymerization (judged by the time in which a black film was first detectable on the electrode surface and by the opacity of the films obtained) was not the same when ODNs of different sequences were used. For instance, oligo d$[AC]_8$ and oligo d$[TG]_8$ are both 16nt long, but polymerization was always faster when using oligo d$[TG]_8$ as dopant. This observation was confirmed by the amplitude of the oxidation peaks of pPy in cyclic voltammetry experiments (see Section 3.3).

3.2. Direct electrochemical detection of complementary targets by monitoring the chronoamperometric signal of guanine

Following changes of the electrical current over time has a major advantage in terms of response speed (the detection is done in real time, without the need of incubating the electrode with the sample before the detection) and simplicity of the electrochemical setup needed. The following results were obtained using only a simple custom-made portable potentiostat, two commercially available non-expensive multimeters, a reference electrode and a platinum wire as the counter electrode. Thus, detection can be made outside the laboratory since no big or fragile and expensive equipment is needed, what is a must towards achieving a device suitable for point-of-care diagnostics or *in situ* testing.

Wang et al. (1999) and Komarova et al. (2005) reported the appearance of a transient anodic current peak upon addition of the complementary targets when using chronoamperometry as the detection technique, and a constant potential of +0.15 V vs. Ag/AgCl. However, opposite signals were registered when non-complementary targets were added to the cell. There is still no answer to this particular behavior of the electrodes, and the drawback of this approach is that the specific signal is suppressed by the non-specific signal when a mix of target oligonucleotide and 10× excess non-specific DNA is used (Komarova et al., 2005).

In order to overcome this intriguing, yet inconvenient upward/downward response, we carried out chronoamperometric experiments at a different potential. Our hypothesis was that the previously described appearance of such specific and non-specific signals was a consequence of pPy electroactive behavior since the working potential (+0.15 V vs. Ag/AgCl) is close to the oxidation peak of pPy, observed in cyclic voltammetry experiments (see Section 3.3). Instead, we carried out detection at a potential of +1.0 V vs. Ag/AgCl, hypothesising that a specific signal would be obtained as a consequence of guanine oxidation on the electrode surface upon hybridization with complementary targets. Honeychurch et al. (2007) determined the effect of pH on the oxidation peak potential ($E_{p,a}$) of guanine in voltammetric experiments using screen-printed carbon electrodes. Using this data, we determined by interpolation that the $E_{p,a}$ of guanine at pH 7.4 would be +0.87 V vs. Ag/AgCl, KCl (3 M). Ozkan-Ariksoysal et al. (2008) also reported an oxidation signal of guanine below +1.0 V vs. Ag/AgCl when performing differential pulse voltammetry at pH 7.4. Despite the fact that our working electrode is different to those used in both reports, performing detection at +1.0 V should be suitable for observing electrical currents associated to the oxidation of guanine.

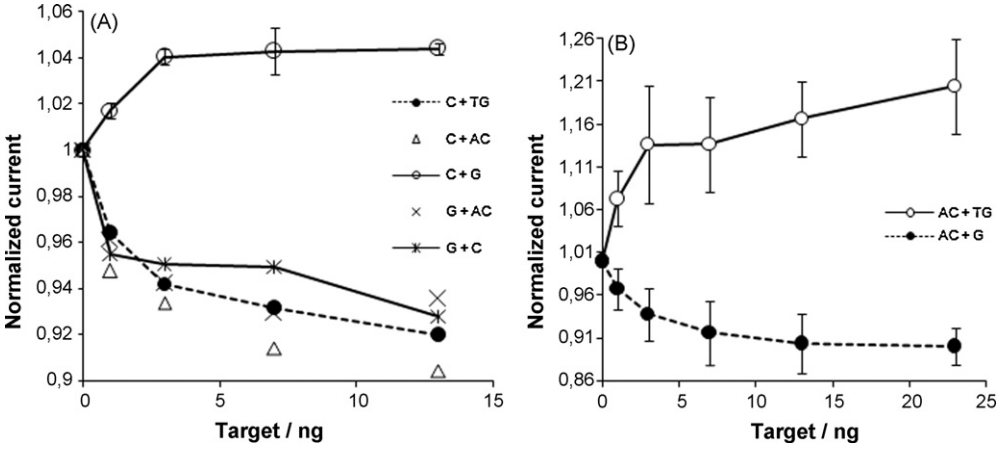

Fig. 1. Variation of the normalized electric current measured 180 s after ODN injection vs. the total amount of complementary or non-complementary ODNs injected into the electrochemical cell. Solid lines represent injection of complementary targets. Dotted lines represent cases in which guanine-rich non-complementary target were injected. Error bars represent the maximum and minimum value of two independent experiments. The experiments were realized in a non-stirred three-electrode electrochemical cell containing 12 mL of PBS pH 7.4 at a constant potential of aproximately +1.0 V vs. Ag/AgCl after 10 min of system stabilization. Labels indicate the sequence of the ODNs used in each experiment (probe + target). C: oligo $d[C]_{10}$; G: oligo $d[G]_{10}$; AC: oligo $d[AC]_8$; TG: oligo $d[TG]_8$.

Since a seminal work described by Wang's group (Wang et al., 1997), the oxidation signal of guanine has been used as the basis of many electrochemical label-free detection schemes (Ozkan-Ariksoysal et al., 2008; Hejazi et al., 2008; Kerman et al., 2003). Detection of complementary targets can be realized by monitoring the appearance of the guanine signal after hybridization of guanine-free (inosine-substituted) probes with guanine-containing targets (Wang et al., 1998). In our study, ODNs were designed in order to be either guanine-free or guanine-rich, so there was no need of utilizing inosine-substituted oligos.

The electroanalytical techniques used in previously published reports on the basis of guanine oxidation electrochemistry include chronopotentiometry (Wang et al., 1998), differential pulse voltammetry (Hejazi et al., 2008; Ozkan-Ariksoysal et al., 2008) and square wave voltammetry (Kerman et al., 2003) principally. To our concern, genosensor design on the basis of guanine oxidation chemistry monitored by constant potential techniques such as chronoamperometry has not been reported elsewhere or has not been given enough attention. The benefit of our approach is that results are obtained in real time with portable and non-expensive equipment, what is desirable for the development of a future genosensor which can be used outside laboratory facilities.

Once the potential is fixed at the desired value and the circuit is closed, there is an exponential current decrease with time. Before each experiment, the system was enabled to stabilize for about 10–15 min until the current reached a plateau. Different amounts of oligonucleotides were injected in the cell every 3 min in a progressively growing fashion, and the electric current was recorded against time. Supplementary Figure 2 shows the electric current registered against time when a guanine-free probe (oligo $d[C]_{10}$) is incubated with a guanine containing complementary target (oligo $d[G]_{10}$, A) and a guanine-containing non-complementary target (oligo $d[TG]_{10}$, B). Immediately upon addition of the target (in the figures, indicated by asterisks), a cathodic (A) or anodic (B) transient current is registered. The nature of such a current does not depend on whether the target is complementary or not complementary to the probe, since it was found that its sign could change even between repetitions of the same experiment, thus indicating that at least two distinct processes are involved, with the net current reflecting the relative contributions of both.

Many combinations of different probes and targets were tested. In the majority of cases, the electric current quickly adopted a decreasing fashion (as seen in supplementary Figure 2B). Interestingly, there were only two exceptions in which the electric current grew with time upon addition of the targets: oligo $d[C]_{10}$ (probe) + oligo $d[G]_{10}$ (target) and oligo $d[AC]_8$ (probe) + oligo $d[TG]_8$ (target). Fig. 1 shows the values of the electric current 3 min after the injection of target (normalized to the initial value, before the first injection of target) against the total amount of target injected in the cell.

Taken together, these results clearly show that the growth of the electrical current over time is a consequence of the oxidation of the guanine nucleotides present in the target sequence, and not of the hybridization event by itself. For instance, such behavior is not observed when testing the electrodes with guanine-free complementary targets (as indicated in Fig. 1, by a solid line labeled "G + C"). However, this signal is specific since it is not observed when injecting guanine-rich non-complementary targets. The electrical current growth over time as a consequence of guanine oxidation in complementary targets shows great promise for future genosensor design, since the detection is done in real time and no expensive equipment is needed.

3.3. *Cyclic voltammetry of the modified electrodes and target detection by monitoring changes in polypyrrole oxidation and reduction peaks*

Cyclic voltammetry of the electrodes modified with pPy doped with any of the ODNs used in this study shows two well-defined peaks corresponding to pPy oxidation and reduction (Fig. 4). In order to demonstrate that those peaks appear as a consequence of pPy redox activity and not because of the immobilized ODNs, we obtained a pPy film-free of DNA by using inorganic ions (Cl^-) as dopants during polymerization. The cyclic voltammograms of these electrodes have the same overall shape as those shown in Fig. 4 with an anodic (oxidation) peak at +0.15 V and a cathodic (reduction) peak registered at −0.11 V.

Interestingly, the amplitude of the oxidation and reduction peaks of pPy/ODN modified electrodes increases upon addition of non-complementary ODNs and decreases when injecting complementary targets (Figs. 2 and 3, and supplementary Figure 3). This phenomenon resembles previous observations made in chronoamperometic experiments (at a constant potential of +0.15 V) in which currents of opposite signs were registered upon injection of complementary and non-complementary targets (Wang et al., 1999 and Komarova et al., 2005).

A direct application of this phenomenon for target detection is not possible since the decrease in the oxidation peak of polypyrrole

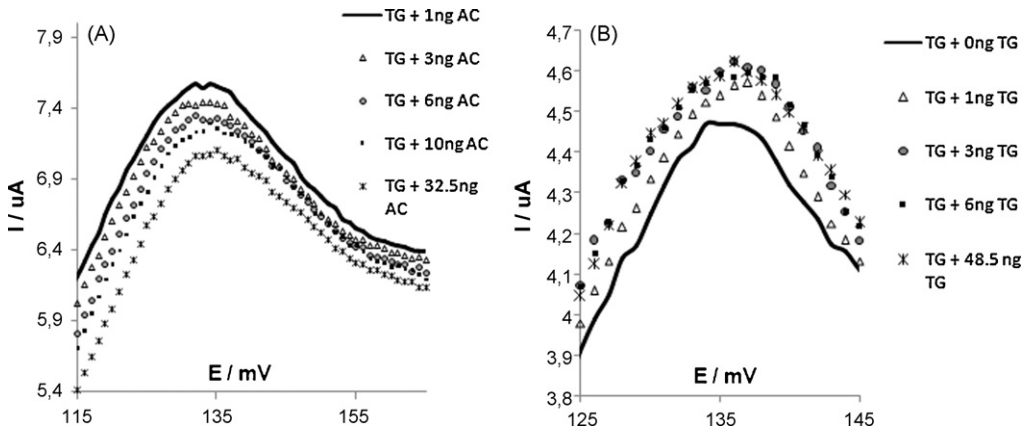

Fig. 2. Windows of the cyclic voltammograms of pPy/oligo d[TG]$_8$ modified electrode, showing pPy oxidation peaks. Increasing amounts of complementary target (A) and non-complementary oligo d[TG]$_8$ (B) were introduced into the cell (containing 12 mL of PBS pH 7.4) and cyclic voltammetry was realized immediately upon each introduction step. Third cycles of some of the voltammograms obtained are shown and superimposed in the figures. The legend shows the total amount of target (in ng) which had been introduced by the corresponding point of the experiment. Windows of the same voltammograms showing pPy reduction peaks are shown in supplementary Figure 3.

cannot be seen when injecting a mixture of complementary and non-complementary targets in similar proportions. A similar situation was described in the studies made by chronoamperometry (Komarova et al., 2005). The challenge is to minimize non-specific adsorption to the electrode surface, in an effort to suppress the non-specific signal which can hide the specific signal in real samples. This was achieved in the previously cited report by blocking the electrode with calf-thymus DNA.

One problem which arises form the design of our experiment is that the targets are driven to the electrode' surface by migration forces while the electrode potential is getting increasingly positive during the potential sweep. The consequence of this is that non-specific adsorption becomes maximized. In order to bypass this situation, we tried a different experimental setup in which the electrodes were incubated with the target ODNs prior to cyclic voltammetry. In this way, non-specific targets could be carried from the bulk of the solution to the interface only by diffusion gradients and thermal motion.

Fig. 4A shows the cyclic voltammogram of an electrode modified with pPy/oligo d[AC]8 before (grey line) and after (dotted line) 1 h incubation at room temperature in a solution of 4 mL containing 2 μg of non-complementary target oligo d[AC]$_8$. In contrast with our previous results, prolonged incubation with a high concentration of non-complementary target did not affect the amplitude of the reduction peak of pPy (a detailed view is shown in supplementary Figure 4B). Superimposed in the figure is the cyclic voltammogram of an electrode modified with pPy/oligo d[AC]$_8$ after incubation of 1 h at room temperature in a solution of 4 mL containing complementary target oligo d[TG]$_8$ (black line). The obvious observation is the complete disappearance of both the oxidation and reduction peaks of pPy. This was further confirmed in an independent experiment realized in the same conditions

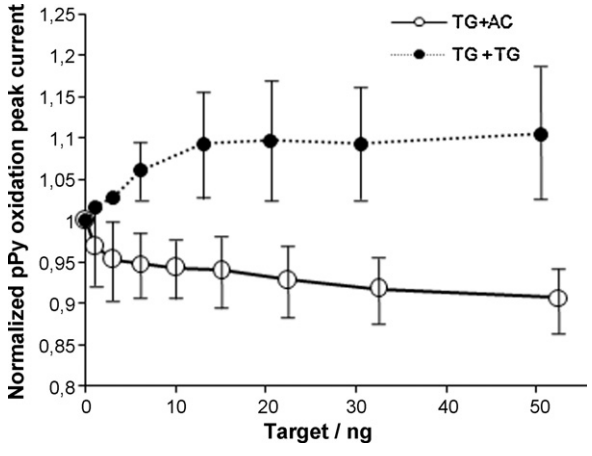

Fig. 3. Plot of the normalized pPy oxidation peak current vs. the total amount of complementary or non-complementary ODNs injected into the electrochemical cell. The electrochemical setup and the experimental procedure are summarized in Fig. 2. The amplitude of pPy oxidation peak was normalized to the value before the first introduction of ODNs into the cell. A similar behavior was found when analyzing pPy reduction peaks (supplementary Figure 3). Error bars indicate the maximum and minimum value of two experiments.

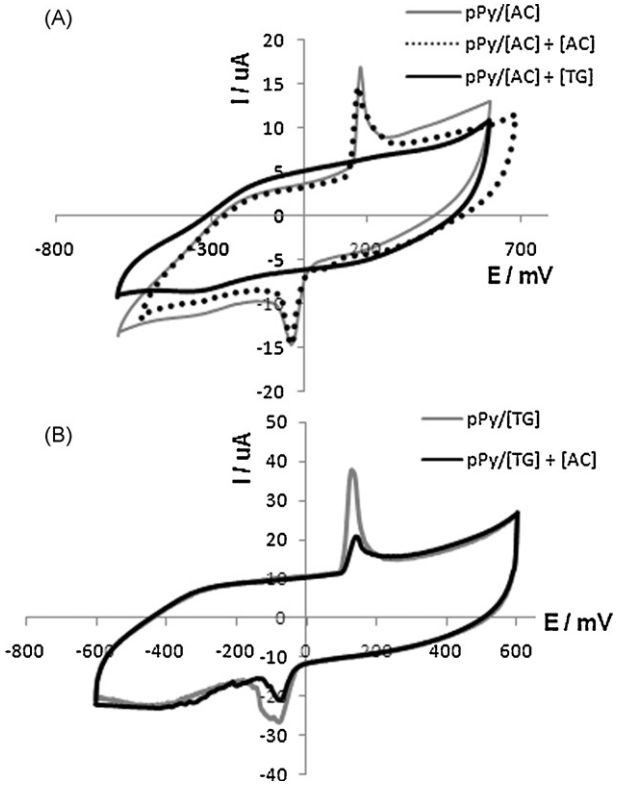

Fig. 4. Cyclic voltammograms of pPy/oligo d[AC]$_8$ (A) and pPy/oligo d[TG]$_8$ (B) modified electrodes before (grey line) and after 1 h incubation with 0.5 μg/mL of complementary target (solid line) or non-complementary ODNs (dotted line). There is no decrease of the reduction peak when incubating with non-complementary ODNs.

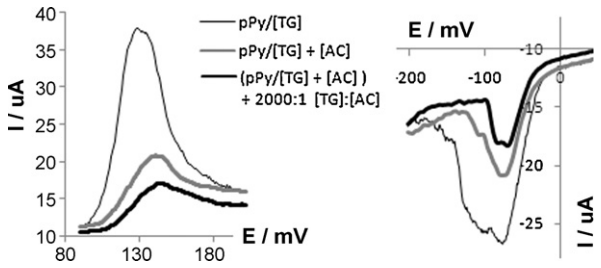

Fig. 5. Windows of the cyclic voltammograms of a pPy/oligo d[TG]8 modified electrode (thin line), after incubation with 0.5 μg/mL of complementary target (grey line), and after a second incubation step with 0.25 ng/mL of complementary target and a 2000× excess of non-complementary target oligo d[TG]8 (black thick line). pPy oxidation (left) and reduction (right) peaks are shown.

(supplementary Figure 4A). In addition, Fig. 4B shows a dramatic decrease on pPy electroactivity after 1 h incubation of a pPy/oligo $d[TG]_8$ modified electrode with 4 mL of a solution containing 2 μg of the complementary target (a detailed view of the reduction peak is shown in supplementary Figure 4C). Thus, incubation with complementary targets provokes a dramatic decrease in both oxidation and reduction peaks of pPy regardless the sequence of the immobilized probe.

One possible explanation for the decrease in pPy peaks after injection or incubation with complementary targets is an increase in the resistance to electron transfer by anions as a consequence of the sugar phosphate backbone of target DNA, which is exposed to the solvent after hybridization. In a recent report, hybridization could be detected by impedance spectroscopy due to an increment in the electron transfer resistance when using the anionic redox couple ferri/ferrocyanide (Kafka et al., 2008). Since one of the mechanisms described for pPy electrochemical oxidation imply the incorporation of anions from the electrolyte to counter-balance oxidized pPy (Gandhi et al., 1995), it is possible that the electrostatic repulsion towards anion incorporation could reduce the extent of pPy oxidation. In contrast, non-specific adsorption to the electrode surface may probably orientate the adsorbed targets in a similar way as the immobilized probes are: with the nucleotide bases faced to the solvent while the negative charged phosphates remain close to the pPy film. This increases the polymer's conductivity by doping, which may be the cause of the pPy oxidation peak increase after injection of non-complementary targets.

Since the effect of non-complementary targets is to increase pPy electroactivity, and the effects of complementary targets is exactly the opposite, and since such an increase was not registered when incubating with non-complementary targets for up to 1 h (reflecting that the effects of non-specific adsorption are not as significant as when the electrode's potential is turned positive), we attempted to determine if it was possible to detect specific targets in the presence of a high excess of non-complementary ODNs. Thus, we tested the performance of our system in a mixture of non-complementary and complementary targets in a ratio of 2000:1 (4 mL of PBS solution containing 2 μg non-complementary target oligo $d[TG]_8$ and 1 ng oligo $d[AC]_8$). Fig. 5 shows windows of the cyclic voltammograms obtained, in the vicinity of pPy oxidation (left) and reduction (right) peaks, before (dark grey) and after (black) incubation with the mix of targets. Interestingly, a decrease of the oxidation and reduction peaks is clearly noticed (the value of the oxidation peak is 18.4% lower than the value registered before incubation, while the reduction peak is 12.7% lower).

Further investigation needs to confirm that the effect of non-complementary targets is always an increase in electroactivity of pPy (as it was shown in Fig. 3B and suggested later). Assuming that as true, the 12.7% decrease observed in pPy reduction peak is a direct consequence of the complementary targets present in the mixture. This establishes the detection limit of the method below 0.25 ng/mL or 53 pM. Furthermore, these values are obtained in a 2000× excess of non-complementary target, what makes the method suitable for real sample analysis.

False positives and negatives can have immense impact in biosensing for medical and biowarfare applications, and the efforts to enhance assay rapidity, sensitivity and simplicity can result in an increase in false positives or in false negatives (Satterfield et al., 2007). For this reason, the possibility of combining the two direct detection schemes described in this work is very promising.

4. Conclusions

We have studied two independent detection approaches which can in turn be combined in order to yield a more reliable analysis. A vast number of reports have used the oxidation of guanine for target detection, but our system, based on the monitoring of current increments over time, enables obtaining clear results without the need of sophisticated and expensive electrochemical equipment. This is a need towards the design of genosensors suitable for point-of-care testing. The detection limit and the sensor performance with real samples are being currently investigated. On the other hand, we have demonstrated that hybridization produces major changes in polypyrrole electrochemical properties, which can be easily detected by cyclic voltammetry. If future experiments confirm that the decrease of the oxidation and reduction peaks of polypyrrole is an exclusive consequence of hybridization as we have shown and suggested, we have achieved a new sensitive and direct method which can detect target sequences in a concentration of 53 pM, with a 2000× excess of non-complementary sequences. In a possible scenario, the first approach may be used for field analysis, while doubtful samples may be delivered to the laboratory for cyclic voltammetry experiments. This will reduce time, cost, as well as the number of false positives and false negatives since every sample can be tested by two independent methodologies within a single genosensor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.03.016.

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