

Direct Electrochemical Sensor for Fast Reagent-Free DNA Detection

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Abstract

A novel reagentless direct electrochemical DNA sensor has been developed using ultrathin films of the conducting polymer polypyrrole doped with an oligonucleotide probe. Our goal was to develop a prototype electrochemical DNA sensor for detection of a biowarfare pathogen, *variola major* virus. The sensor has been optimized for higher specificity and sensitivity. It was possible to detect 1.6 fmol of complementary oligonucleotide target in 0.1ml in seconds by using chronoamperometry. The sensitivity of the developed sensor is comparable to indirect electrochemical DNA sensors, which use electrochemical labels and reagent-intensive amplification. The developed sensing electrode is reusable, highly stable and suitable for storage in solution or in dry state.

Background

One of the fastest growing areas in DNA/RNA analysis technology is the development of DNA biosensors. Typically, the biosensor employs immobilized oligonucleotides as the recognition element and measures specific binding processes of complementary DNA or RNA at the biosensor interface. **Figure 1** shows a schematic of a typical DNA biosensor with target DNA already bound to the probe/recognition DNA.

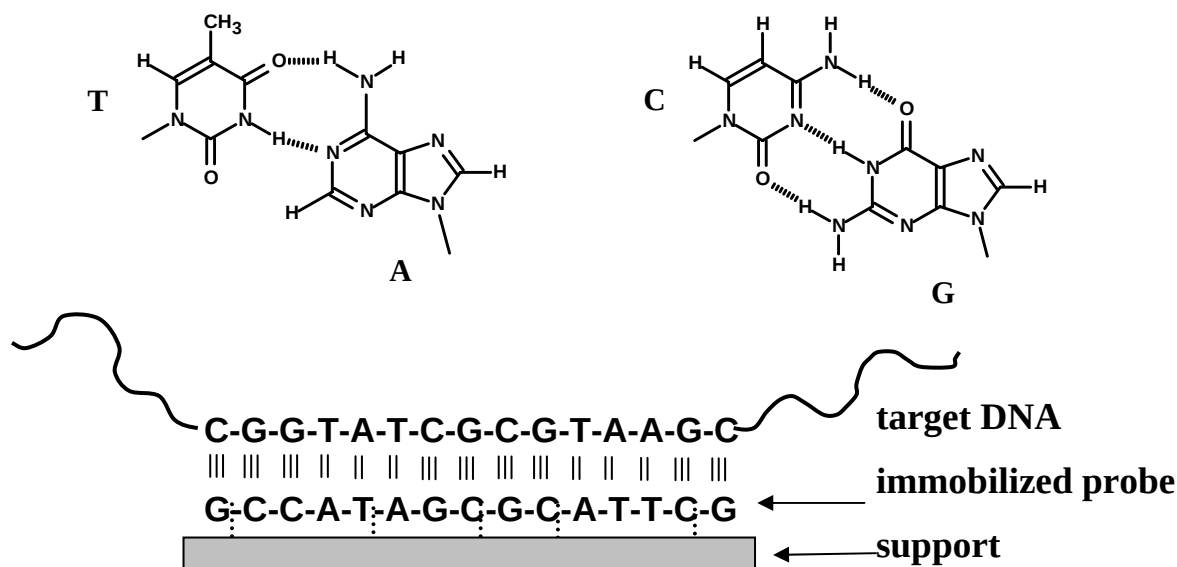


Figure 1. Hydrogen bonds forming between complementary DNA bases during the hybridization step (top). Probe DNA immobilized on the sensor's support with the recognized complementary target DNA attached to it (bottom).

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Existing electrochemical methods for DNA/RNA detection can be separated into two groups:

indirect methods - sensors that employ electrochemical labels and hybridization markers, and *direct methods* - sensors that use the ability of DNA to contribute to conductivity of a conductive polymer, which changes upon hybridization.

Indirect methods of electrochemical hybridization detection generally have better detection limit but take longer and require more steps, compared to direct methods. Detection of electrical current, generated by electroactive enzymes such as Horseradish Peroxidase (HRP) shown in **Figure 2** or Alkaline Phosphatase (AP) employed in some indirect electrochemical DNA sensors. For example, Zang et al could detect 3000 copies of 38-b.p. DNA oligonucleotide in 10 μ l droplet (0.5fM) with the HRP-labeled *detector probe* [Zhang].

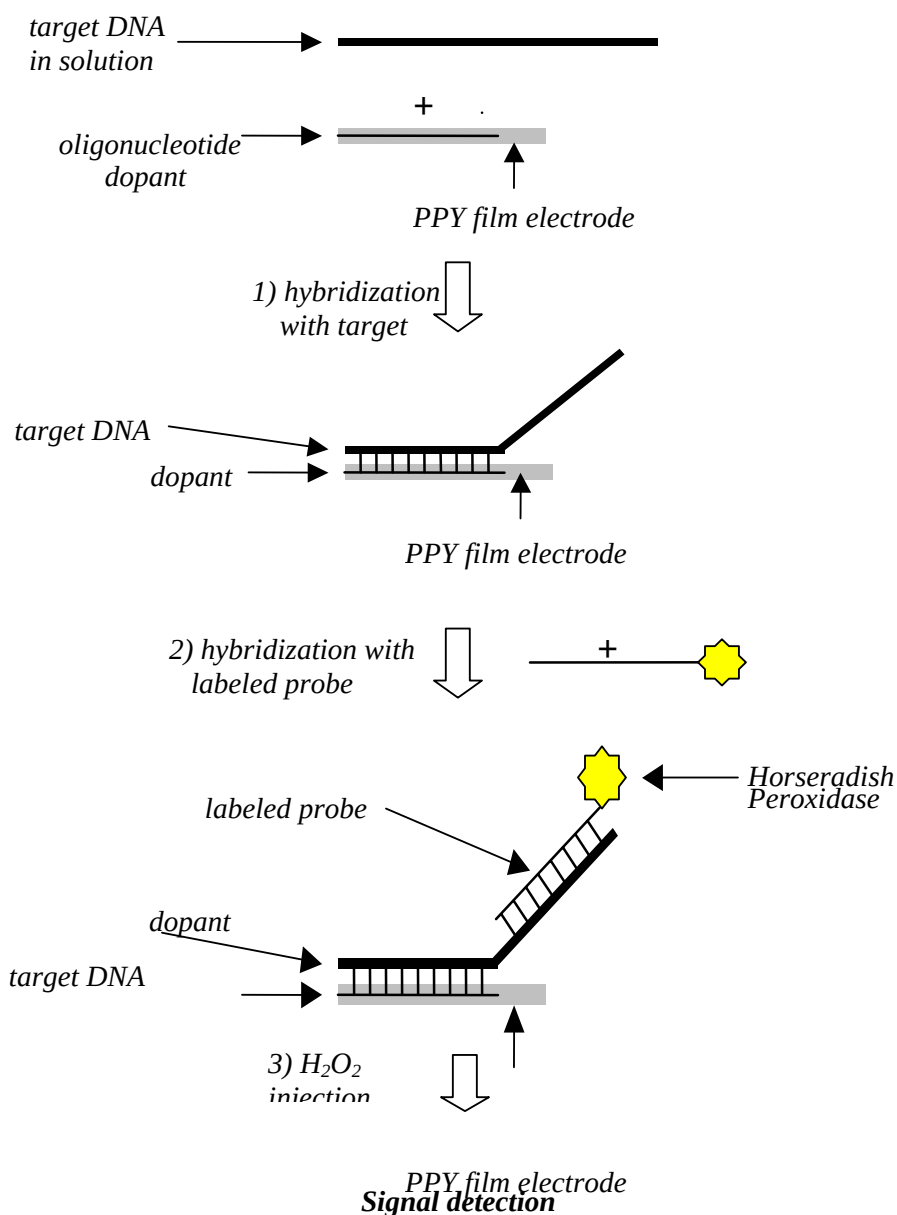


Figure 2. Schematic of a recognition event for an indirect electrochemical DNA sensor that employs a horseradish peroxidase electrochemical label.

Direct electrochemical methods of DNA hybridization detection (**Figure 3**) are simpler and faster, compared to indirect methods, utilizing electrochemical labels. However, until now reported indirect methods were more sensitive and more specific. Our goal was to optimize a direct method of electrochemical detection, which would be comparable in sensitivity and specificity to indirect (enzyme-enhanced) detection.

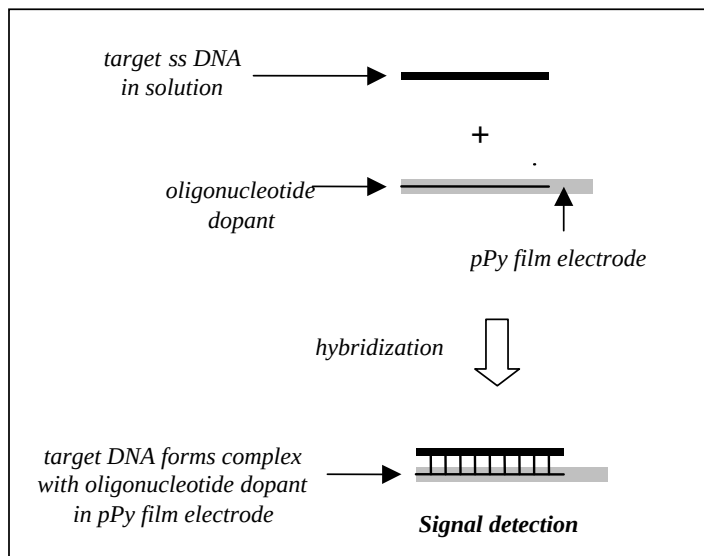


Figure 3. Schematic of a recognition event for a direct electrochemical DNA sensor.

Incorporation of Oligonucleotides in Conducting Polymer Films

Direct registration of DNA hybridization advanced significantly with the use of electroactive films of conductive polymers as electrodes. Conductive polymers consist of conjugated backbones that are easily oxidized or reduced (doped/undoped) with a concomitant increase or decrease in conductivity, with each polymer having its own redox characteristics.

DNA is electroactive and can serve as a dopant due to the negative charge of phosphates and, therefore can contribute to the polymer conductivity. Oligonucleotide probes can be incorporated into growing films of a conducting polymer [Wang 99, Lassalle], or the surface of the polymer electrode can be derivatized with covalent attachment of oligonucleotides [Lee, Cha, Pham]. Hybridization with a complementary DNA target causes changes in conductivity of the electrode, which can be measured by amperometry [Wang 99], impedance spectroscopy [Lee], cyclic voltammetry [Cha], differential pulse voltammetry [Pham] or photocurrent spectroscopy [Lassalle]. The detection limits of most direct DNA-sensing approaches are in micromolar range, however, the latest publications on direct DNA sensing report lower detection limits. Our choice is to use DNA as sole dopant during electrochemical polymerization of pyrrole (**Figure 4**)

Cyclic voltammetry was one of the methods for electrochemical polymerization of pyrrole in the presence of single stranded probe DNA. Electropolymerization was carried out in a custom-made electrochemical minicell (internal volume 100 μ l), with Indium Tin Oxide (ITO)-coated glass (Delta Technologies Ltd.) used as a working electrode and a platinum wire as an auxiliary electrode (**Figure 5**). Mini-reference electrode (Ag/AgCl /3M KCl, Abtech Scientific) was used in all experiments. Polymerization was performed using CHI-660 potentiostat (CHI Instruments), in 10 voltammetric cycles between 0 and 0.7V, using solution of 0.05M of pyrrole and 0.1-1 mg/ml of oligonucleotide (15, 20 or 30-mer) dopant in DNase/RNase-free water. The resulting film diameter was 3 mm.

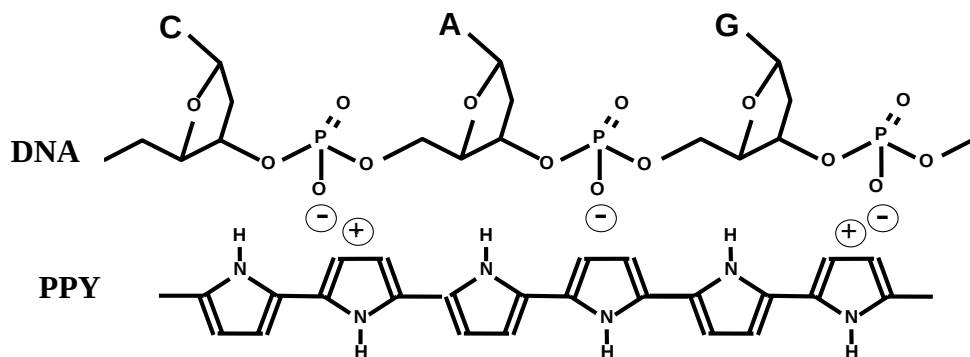


Figure 4. DNA attached to the backbone of polypyrrole as a sole dopant during electrochemical polymerization.

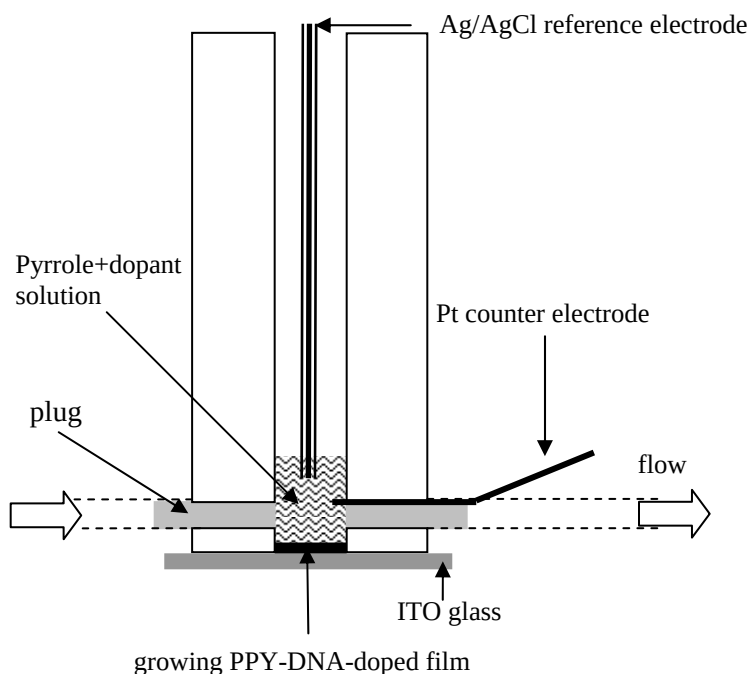


Figure 5. A custom-made electrochemical minicell (internal volume 100 μl)

The use of the oligonucleotide dopant shows a normal polymer growth with increasing current, which corresponds to incorporation of an oligonucleotide into the pPy film (**Figure 6**). The obtained PPY/oligonucleotide thin films had semitransparent, slightly colored appearance. The films were rinsed in the working buffer (0.1M.NaCl/0.1M glycine) and used for electrochemical detection of the target oligonucleotide.

Direct Detection of oligonucleotide target by chronoamperometric technique

Among *direct* electrochemical methods, chronoamperometry allowed us to register fast and reproducible signals immediately upon addition of the target oligonucleotide (**Figure 7A**). Other techniques (chronopotentiometry, cyclic voltammetry, impedance, differential pulse voltammetry) did not reveal any considerable changes even after prolonged incubation (up to 1 hour) of PPY/oligonucleotide film with the target oligonucleotide.

Chronoamperometry was performed at a potential of 0.15 V using CHI-660 potentiostat in the detection buffer (0.1M NaCl/0.1M glycine solution in water). PPY/oligonucleotide film on ITO-coated glass electrode was used as the working electrode. The system was allowed to stabilize for few seconds to achieve a flat baseline. The injection of the target DNA (5 μ l) was performed and the change in current was recorded.

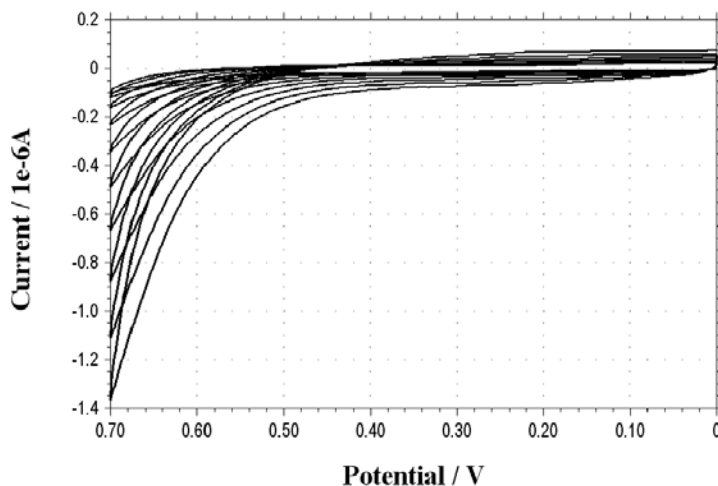


Figure 6. Cyclic voltammogram (10 cycles) of electropolymerization of pyrrole (0.05 M) on ITO glass in the presence of dopant-30 (100 μ g/ml).

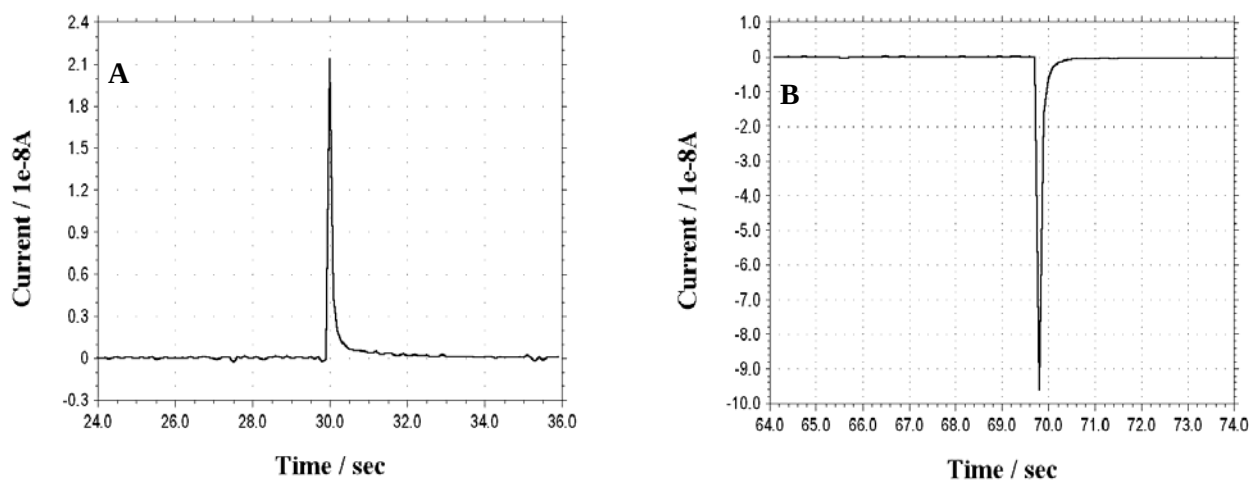


Figure 7. Direct chronoamperometric DNA sensing at 0.15V: Response of PPY/dopant-20 film, prepared by constant potential electropolymerization, to injection of (A) specific oligonucleotide (50ng of “target-41” in 0.1M NaCl/0.1M glycine); and (B) non specific DNA (500ng of linearized, denatured pUC19 in 0.1M NaCl/0.1M glycine), sensing volume 0.1ml.

The speed of chronoamperometric response is the major advantage of the technique. The steep increase in current happens immediately after the injection of the target oligonucleotide in a 0.1M NaCl /0.1M glycine buffer into the electrochemical cell, with the PPY/oligonucleotide film serving as a working electrode (**Figure 7A**). The drawback of chronoamperometry is the appearance of the strong signal of the opposite polarity upon addition of non-complementary DNA (**Figure 7B**). The non-specific signal suppresses the specific signal produced by target DNA when a mix of target oligonucleotide and excess of

non-specific DNA were used. It is worth mentioning, however, that no problems arose while detecting hybridization of 15 nucleotides (using PPY films with dopant-15) in a 41-oligonucleotide target.

Further optimization of direct detection was targeted at eliminating the non-specific signal, along with increasing the sensitivity of direct detection. We have tested a number of approaches to optimize specificity and sensitivity of the PPY/oligonucleotide sensing films, which are discussed below. They can be separated into (1) optimization of film preparation parameters (2) blocking the surface of the film and (3) altering conditions of target detection.

Sensitivity optimization of direct electrochemical DNA detection

1) *Altering the length of the dopant in PPY film:* Oligonucleotide dopants of different lengths (**Figure 8**) were used in PPY film preparation by CV electropolymerization. Films obtained with shorter dopants (dopant-15 and -20) produced stronger amperometric signals compared to films containing dopant-30.

(5') CGGAAGATGCAATAGTAATCAGGTAGAGAC (3')	Dopant-30	$T_m = 73.6^\circ\text{C}$
(5') GCAATAGTAATCAGGTAGAG (3')	Dopant-20	$T_m = 55.1^\circ\text{C}$
(5') GCAATAGTAATCAGG (3')	Dopant-15	$T_m = 45.6^\circ\text{C}$

Figure 8. Different length dopants.

2) *Altering the concentration of the dopant in PPY film:* We investigated the effect of dopant concentration on film sensitivity towards target detection, comparing films prepared by CV electropolymerization with 100 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$, and 1000 $\mu\text{g/ml}$ of dopants, 0.05 M of pyrrole, 10 cycles from 0-0.7 V at a rate of 25 mV/s. The concentration of dopant dramatically influenced film sensitivity towards target DNA detection. Using higher dopant concentrations, amperometric hybridization signals were more pronounced, and smaller concentrations of the target oligonucleotide could be detected.

3) *Electropolymerization technique:* Conductive films polymerized at *constant potential* (CP) showed lower noise level in DNA-sensing chronoamperometry experiments (compared to films obtained using cyclic voltammetry), which allowed sensing of lower target DNA concentrations.

4) *Using ultra-thin PPY/DNA films:* Ultrathin films of PPY/oligonucleotide were prepared by modifying a preformed Langmuir-Blodgett (LB) film of stearic acid by exposure to pyrrole vapor and then electrochemically polymerizing it in the presence of the DNA dopant. Polymerization of pyrrole within stearic acid LB films allowed us to significantly increase sensitivity towards target oligonucleotide detection. Ultra-thin film preparation was performed as follows: For stearic acid Langmuir-Blodgett (LB) film deposition, the technique described elsewhere was used [Milella]. The only change from the published procedure was that 1 mg/ml solution of stearic acid in chloroform was spread onto a subphase of deionized water that contained no additives (FeCl_3 or HCl). After proper drying, the film was exposed to vapors of pyrrole for 48 hours at 4°C in the dark. The pyrrole-saturated LB film was electrochemically polymerized under a constant potential of 0.7 V for 1 min. in the presence of 100 μl of dopant-20 (1mg/ml) in deionized water.

Optimization of Specificity

In order to eliminate the non-specific signal we tried several approaches:

1. *Influence of hybridization potential on specificity of DNA recognition event:* We investigated whether changing the potential applied on the PPY/oligonucleotide film electrode during hybridization/sensing will allow us to increase specificity of DNA recognition. Routinely, we were performing chronoamperometry at 0.15V, with the potential applied prior to DNA injection. We hypothesized that applying negative potential will repel weaker-bound non-specific DNA. Both specific and non-specific signals remain unchanged in the 0.15-0.3 V range. More negative potentials caused reduced intensity of non-specific signals and increased intensity of specific signal starting at -0.4V working potential. Lowering the potential to -0.7 V resulted in disappearance of the non-specific signal and further increase of the specific one; however, the film lost stability and conductivity due to undoping.

2. *Altering ionic strength of hybridization buffer in the range 0.1M - 0.5M NaCl* did not affect the height of specific or non-specific peaks.

3. *Blocking the surface of the film with irrelevant DNA:* To eliminate the non-specific signal, we performed “blocking” of the PPY/DNA film with 1mg/ml of single stranded (freshly denatured) fragmented calf thymus DNA for 1h at room temperature with 0.15 V applied potential. Blocking resulted in complete disappearance of non-specific signal from oligo(dT₁₃₋₁₈), and in reduction of non-specific signals from oligo(dA₁₅) and from single-stranded fragmented pBR322 and pUC19 in the PPY/oligonucleotide films prepared by CV- or CP-electropolymerization. Additional incubation with 1mg/ml of calf thymus DNA with an applied potential of 0.15 V did not result in the complete disappearance of non-complementary peaks of poly(dA₁₅) and single-stranded fragmented pBR322. When blocking was performed on ultrathin LB films, it resulted in complete elimination of the non-specific signal from single-stranded fragmented pBR322 and pUC19 (**Figure 9A**).

Combining optimization approaches

Using blocked ultrathin PPY/oligonucleotide films, prepared by CP polymerization in preformed stearic acid LB film substrate, we were able to directly detect 10 pg or 1.6 fmol of the target oligonucleotide in 0.1ml in the presence of 250x weight excess of noncomplementary ss-DNA (**Figure 9B**).

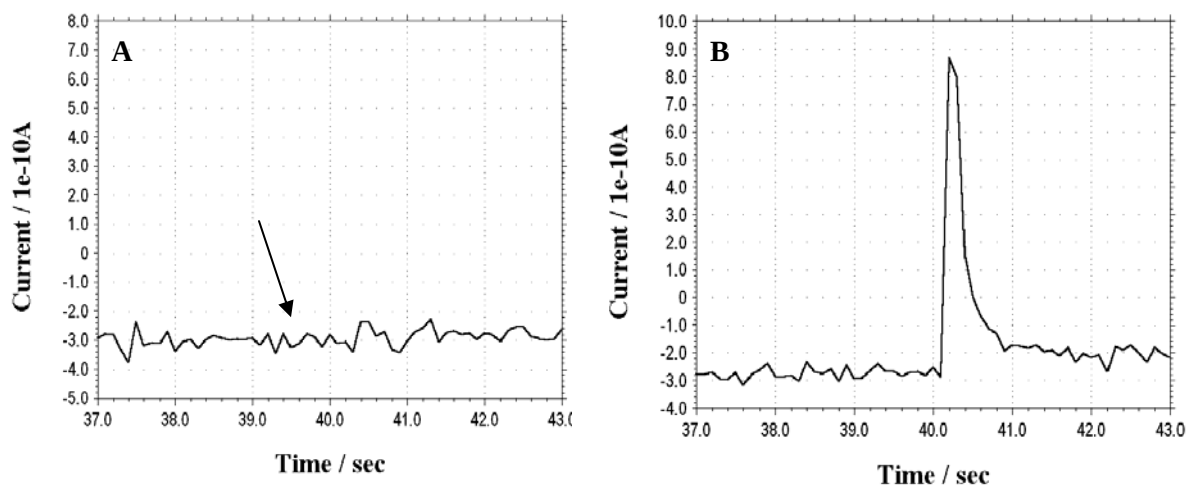


Figure 9. Specificity of direct chronoamperometry using ultra-thin films at the detection limit: (A) Absence of amperometric response to 2.5 ng of ss-pUC-19 in 0.1M NaCl/0.1 M glycine solution, injected at 40s. (B) Amperometric response to 10 pg of target-20 (concentration 16 pM) in the presence of 2.5 ng of ss-pUC-19 (both in 0.1M NaCl/0.1 M glycine solution, 0.1ml)

High sensitivity of the ultrathin PPY/oligonucleotide films results from considerable increase of signal-to-noise ratio compared to films obtained by CV or CP electrochemical polymerization. It was possible to

reliably register 1nA signal. The achieved detection limit of 10 pg or 1.6 fmol of target oligonucleotide in 0.1ml by direct electrochemical DNA sensing is orders of magnitude lower than reported for other direct DNA sensors. It is competitive with the indirect electrochemical DNA sensors reported lately, which use sandwich approaches, electrochemical labels and reagent-intensive amplification, and therefore employ longer detection times and are more difficult to make.

Stability

PPY/oligonucleotide films, prepared by CV or CP electropolymerization, were repeatedly tested during the course of 2 weeks for amperometric hybridization detection and were showing stable performance, with no decrease in detection speed or sensitivity. Films were regenerated by washing in deionized water (as reported by Park et al, 2002), which resulted in dissociation of hybridized DNA target from the complementary oligonucleotide in the film. No elevated temperatures or elevated salt concentrations were needed for film regeneration.

DNA-sensing ability remained unchanged upon storage in the working buffer, and films could be reused. The possibility of storing the PPY/oligonucleotide sensing films in a **dry** state a) at 4°C in the dark and b) at room temperature with light for prolonged periods of time, up to 2 months has been investigated as well. Stored in both conditions, the sensing films did not lose their sensing abilities and were 100% functional towards DNA detection when placed in the working buffer.

Conclusions

Compared to the published research on direct DNA sensing, we have achieved a significantly lower detection limit of 1.6 fmol in 0.1ml by using direct chronoamperometric hybridization detection on ultrathin films of DNA-doped polypyrrole. This makes our direct method competitive with indirect electrochemical DNA sensors, which use electrochemical labels and reagent-intensive amplification. Compared to that, our sensor is reagentless and detects DNA in one step. In addition, we can detect DNA in seconds, which is unsurpassed by any other detection method of comparable sensitivity. The sensing electrode is reusable, highly stable, and suitable for storage in solution or in the dry state.

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