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Label-Free DNA Detection Based on Modified Conducting Polypyrrole Films at Microelectrodes

Carla dos Santos Riccardi,[†] Hideko Yamanaka,[†] Mira Josowicz,*[,‡] Janusz Kowalik,[‡] Boris Mizaikoff,[‡] and Christine Kranz*[,‡]

Universidade Estadual Paulista, Instituto de Química de Araraquara, R. Prof. Francisco Degni, s/no C. P.: 355, CEP: 14801-970, Araraquara, SP, Brazil, and School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0400

A label-free electrochemical detection method for DNA hybridization based on electrostatic modulation of the ion-exchange kinetics of a polypyrrole film deposited at microelectrodes is reported. Synthetic single-stranded 27-mer oligonucleotides (probe) have been immobilized at 2,5-bis(2-thienyl)-*N*-(3-phosphorylpropyl)pyrrole film formed by electropolymerization on the previously formed polypyrrole layer. The 27- or 18-mer target oligonucleotides were monitored via the electrochemically driven anion exchange of the inner polypyrrole film. The performance of the miniaturized DNA biosensor system was studied in respect to selectivity, sensitivity, reproducibility, and regeneration of the sensor. Control experiments were performed with a noncomplementary target of 27-mer DNA and 12 base-pair mismatched 18-mer sequences, respectively, and did not show any unspecific binding. Under optimized experimental conditions, the label-free electrochemical biosensor enabled the detection limits of 0.16 and 3.5 fmol for the 18- and 27-mer DNA strand, respectively. Furthermore, we demonstrate reusability of the electrochemical DNA biosensor after successful recovery of up to 100% of the original signal by regenerating the DNA "label-free" electrode with 50 mM HCl at room temperature.

The development of electrochemical transduction schemes for DNA biosensors (so-called *genosensors*) has recently received increasing attention using photoelectrochemistry,[1] potentiometry,[2] and DNA-modified electrodes.[3,4] Electrochemical detection techniques may involve a variety of schemes such as metal complexes,[3,5] gold nanoparticles,[6,7] redox labels,[8,9] and redox-active hybrid indicators that bind more strongly to hybrids than to single DNA strands.[10−14] Besides these indirect schemes, direct electrochemical detection of hybridization events[15−19] including amplicon detection as the result of PCR amplification[20−22] is of particular interest.

The majority of approaches detecting hybridization events involve the covalent attachment of appropriate oligonucleotides at conductive electrode substrates[23] including inherently conducting polymers (ICPs).[24−26] For example, it has been shown that oligonucleotides can be covalently attached to pyrrole monomers

* Corresponding authors. E-mail: Christine.Kranz@chemistry.gatech.edu; Mira.Josowicz@chemistry.gatech.edu.

[†] Instituto de Química de Araraquara.

[‡] Georgia Institute of Technology.

(1) Qingwen, L.; Guoan, L.; Jun, F.; Dawen, C.; Qi, O. *Analyst* **2000**, *125*, 1908−1910.

(2) (a) Wang, J.; Bard, A. *J. Chem.* **2001**, *73*, 2207−2212. (b) Wang, J.; Xiaohua, C.; Jonsson, C.; Balakrishnan, M. *Electroanalysis* **1996,** *8*, 20−24. (c) Wang, J.; Xiaohua, C.; Fernandes, J. R.; Grant, D. H.; Ozsoz, M. *J. Electroanal. Chem.* **1998**, *441*, 167−172. (d) Wang, J.; Jiang, M.; Mukherjee, B. *Anal. Chem.* **1999**, *71*, 4095−4099. (e) Wang, J.; Zhang, X.; Parrado, C.; Rivas, G. *Electrochem. Commun.* **1999**, *1*, 197−202. (f) Wang, J.; Kawde, A. N.; Sahlin, E. *Analyst* **2000**, *125*, 5−7.

(3) Takenaka, S.; Yamashita, K.; Takagi, M.; Uto, Y.; Kondo, H. *Anal. Chem.* **2000**, *72*, 1334−1341.

(4) Zhao, Y.-D.; Pang, D. W.; Hu, S.; Wang, Z. L.; Cheng, J. K.; Dai, H. P. *Talanta* **1999**, *49*, 751−756.

(5) Erdem, A.; Meric, B.; Kerman, K.; Dalbasti, T.; Ozsoz, M. *Electroanalysis* **1999**, *11*, 1372−1376.

(6) Park, S. J.; Taton, T. A.; Mirkin, C. A. *Science* **2002**, *295*, 1503−1506.

(7) Taton, T. A.; Mirkin, C. A.; Letsinger, R. L. *Science* **2000**, 289, 1757−1760.

(8) Ihara, T.; Nakayama, M.; Murata, M.; Nakano, K.; Maeda, M. *Chem. Commun.* **1997**, *17*, 1609−1610.

(9) Lei, C.; Gheorghe, M.; Guiseppi-Elie, A. *Abstract of Papers*, Fall ACS National Meeting, Washington, DC, 2000; pp 552−553.

(10) Millan, K. M.; Mikkelsen, S. R. *Anal. Chem.* **1993**, *65*, 2317−2323.

(11) Hashimoto, K.; Ito, K.; Ishimori. Y. *Anal. Chem.* **1994**, *66*, 3830−3833.

(12) Carter, M. T.; Rodriguez, M.; Bard, A. J. *J. Am. Chem. Soc.* **1989**, *111*, 8901−8911.

(13) Zeman, S. M.; Phillips, D. R.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 11561−11565.

(14) Erkkila, K. E.; Odom, T. D.; Barton, J. K. *Chem. Rev.* **1999**, *99*, 2777−2795.

(15) (a) Wang, J.; Liu, G.; Merkoc, A. *J. Am. Chem. Soc.* **2003**, *125*, 3214−3215. (b) Wang, J.; Xu, D.; Kawde, A. N.; Polsky, R. *Anal. Chem.* **2001**, *73*, 5576−5581.

(16) Napier, M. E.; Loomis, C. R.; Sistare, M. F.; Kim, J.; Eckhardt, A. E.; Thorp, H. H. *Bioconjugate Chem.* **1997**, *8*, 906−913.

(17) Xi, C.; Cai, H.; He, P.; Fang, Y. *Fresenius J. Anal. Chem.* **2000**, *367*, 593−595.

(18) Pividori, M. I.; Merkoci, A.; Alegret, S. *Biosens. Bioelectron.* **2000**, *15*, 291−303.

(19) Peng, H.; Soeller, C.; Vigar, N.; Kilmartin, P. A.; Cannell, M. B.; Bowmaker, G. A.; Cooney, R. P.; Travas-Sejdic, J. *Biosens. Bioelectron.* **2005**, *9*, 1821−1828.

(20) Kuhr, W. G. *Nat. Biotechnol.* **2000**, *18*, 1042−1043.

(21) (a) Marrazza, G.; Chianella, I.; Mascini, M. *Anal. Chim. Acta* **1999**, *387*, 43−51. (b) Marrazza, G.; Chianella, I.; Mascini, M. *Biosens. Bioelectron.* **1999**, *14*, 43−51.

(22) Mikkelsen, S. R. *Electroanalysis***1996**, *8*, 15−19.

(23) Palecek, E. *Electroanalysis* **1996**, *8*, 7−14.

(24) Livache, T.; Fouque, B.; Roget, A.; Marchand, J.; Bidan, G.; Teoule, R.; Mathis, G. *Anal. Biochem.* **1998**, *255*, 188−194.

(25) Garnier, F.; Korri-Youssoufi, H.; Srivastava, P.; Mandrand, B.; Delair, T. *Synth. Met.* **1999**, *100*, 89−94.

(26) Wu, Y.; Moulton, S. E.; Too, C. O.; Wallace, G. G.; Zhou, D. *Analyst* **2004**, *129*, 585−588.

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forming electrochemically conductive electroactive copolymers.[24,25] Alternatively, oligonucleotides have been directly covalently attached to polythiophenes after polymer synthesis.[27] Using either of these polymer backbones, direct electrochemical detection of hybridization events is enabled.[25,28] Using similar copolymers, it was found that photocurrents generated at polypyrrole (PPy) electrodes are affected by hybridization events.[29] The specific hybridization of grafted oligonucleotides with the complementary nucleotide target induces a modification of the electrochemical behavior of the conductive polypyrrole backbone. This phenomenon has been exploited to establish sensitive electrochemical sensors for oligonucleotides with nano- to femtomolar detection limits of oligonucleotide in solution. It should be noted though that the electrochemical response of the polypyrrole−oligonucleotide electrode remains unchanged after incubation in buffered aqueous solutions containing noncomplementary oligonucleotides.[30]

An alternative approach to covalent binding is the immobilization of the oligonucleotide as a dopant into an ICP during electropolymerization. This approach was introduced by Wang and co-workers[31,32] utilizing an inverted electrochemical cell that enables electropolymerization in extremely small volumes. In these studies, both constant-potential amperometry and potentiometric stripping analyses were used to monitor hybridization.[31]

The electrochemical detection of nucleic acid targets at low concentrations based on electronic conducting polymer detection is of particular interest due to the inherent potential of sensor miniaturization. Millan et al. voltammetrically detected the intercalation of redox couples in double-stranded DNA and succeeded in observing the mutation associated with cystic fibrosis.[33] Xu et al. observed hybridization via electrogenerated chemiluminescence of a metal chelate tag after its intercalation in double-stranded DNA.[34] Korri-Youssoufi et al. measured the increase in the resistivity of electrochemically copolymerized pyrrole and oligonucleotide-substituted pyrrole upon hybridization detecting 10 pM oligonucleotide.[35] Furthermore, reproducible detection of the hybridization of 10^5 copies via reactive electrophoretic deposition of a chemically activated ss-oligonucleotide (electron-conducting redox polymer, PAA-PVI-Os) has been demonstrated. The melting of 10^5 copies of a simple 25−30-base hybrid could be amperometrically observed with a carbon microelectrode.[36] Enzyme-amplified sandwich-type amperometric assays of 38-base DNA strands at 0.5 fM concentration have been reported utilizing a 10-μm carbon microelectrode coated with an electrodeposited redox polymer containing a DNA capture sequence. With a probed volume of 10 μL, as few as 3000 copies of DNA were detected.[37] The hybridization of targets using electrocatalytic nucleobase oxidation has been evaluated on gold microelectrodes using $Os(bpy)_3^{3+/2+}$ as the mediator and modified nucleobases such as 8-oxoguanine or 5-aminouridine as the oxidized base. This system allowed for the sensitive (400 fM) and selective detection of target oligonucleotides at the gold monolayer platform.[38] A label-free detection of DNA hybridization events based on electrostatic modulation of the ion-exchange kinetics of a polypyrrole film was investigated by Thompson et al.[39]

In the present work, we report the label-free electrochemical detection of DNA hybridization based on electrostatic modulation of the electrochemically driven chloride ion-exchange of modified polypyrrole-bilayer films deposited at microelectrodes. The DNA-modified electrode surface was prepared following the procedure reported by Thompson et al. that requires only a minimal manipulation on the DNA. It was carried out only at macroelectrodes.[39,40] The layout of the modified electrode relies on the fact that DNA is a negatively charged polyelectrolyte owing to ionization of the phosphate group at physiological pH. The charge neutralization of nucleic acid is achieved through the interactions of the phosphate groups, sugar hydroxyls, and endocyclic nitrogen atoms of the nucleobases with positively charged metal ions.[41] These formed metal complexes may be held together by electrostatic forces or weaker van der Waals forces, including hydrophobic bonding. For example, electrostatic binding of Mg^{2+} to the phosphate groups provides significant stabilization of the double-helical structure.[41] To employ that electrostatic attraction between the phosphate anions of the nucleic acid and the magnesium cations, sequential electropolymerization of a self-doped pyrrole, 2,5-bis(2-thienyl)-*N*-(3-phosphorylpropyl)pyrrole (pTPTC3−PO_3H_2) on a polypyrrole-modified electrode was carried out. The oligonucleotide probe is linked to the conducting polymer by forming a bidentate complex between Mg^{2+} and an alkyl phosphonic acid group of the polymer. It is important to note, that the phosphate groups of the DNA probe are forming *a cloud of negative charge* at the solution/polymer interface. The negative charge represents an electrostatic barrier for the chloride anion moving in and out of the inner polypyrrole layer during electrochemical cycling of the modified electrode. Next to steric hindrances affecting the diffusion of counteranions into the polymeric coating resulting from the duplex formation, this increase of negative charge at that barrier due to formation of the double helix during hybridization is the core of the presented detection scheme for the DNA hybridization event.

This approach takes advantage of the reversible ion-exchange kinetics of PPy after its surface has been modified with a layer of electrochemically grafted poly(2,5-dithienylpyrrole) modified with a phosphonic acid group (pTPTC3−PO_3H_2). The binding of the DNA probe via its phosphoric acid residues is mediated by the magnesium cations serving as a linker between the phosphonic acid group of the grafted polymer and the phosphate group of the oligonucleotide probe.[42] We have adapted this concept for the

(27) Nilsson, K. P. R.; Inganaes, O. *Nat. Mater.* **2003**, *2*, 419−424.
(28) Lee, T. Y.; Shim, Y. B. *Anal. Chem.* **2001**, *73*, 5629−5632.
(29) Lassalle, N.; Mailley, P.; Vieil, E.; Livache, T.; Roget, A.; Correia, J. P.; Abrantes, L. M. *J. Electroanal. Chem.* **2001**, 509, 48−57.
(30) Cosnier, S.; Gondran, Ch. *Analusis* **1999**, *27*, 558−564.
(31) Jiang, M.; Wang, J. *J. Electroanal. Chem.* **2001**, *500*, 584−589.
(32) Wang, J.; Jiang, M.; Fortes, A.; Mukherjee, B. *Anal. Chim. Acta* **1999**, *402*, 7−12.
(33) Millan, K. M.; Saraullo, A.; Mikkelsen, S. R. *Anal. Chem.* **1994**, *66*, 2943−2948.
(34) (a) Xu, X. H.; Yang, H. C.; Mallouk, T. E.; Bard, A. J. *J. Am. Chem. Soc.* **1994**, *116*, 8386-8387. (b) Xu, X. H.; Bard, A. J. *J. Am. Chem. Soc.* **1995**, *117*, 2627−2631.
(35) Korri-Youssoufi, H.; Garnier, F.; Srivastava, P.; Godillot, P.; Yassar, A. *J. Am. Chem. Soc.* **1997**, *119*, 7388−7389.
(36) Lumley-Woodyear, T.; Caruana, D. J.; Campbell, C. N.; Heller, A. *Anal. Chem.* **1999**, *71*, 394−398.
(37) Zhang, Y.; Kim, H.-H.; Heller, A. *Anal. Chem.* **2003**, *75*, 3267−3269.
(38) Gore, M. R.; Szalai, V. A.; Ropp, P. A.; Yang, I. V.; Silverman, J. S.; Thorp, H. H. *Anal. Chem.* **2003**, *75*, 6586−6592.
(39) Thompson, L. A.; Kowalik, J.; Josowicz, M.; Janata, J. *J. Am. Chem. Soc.* **2003**, *125*, 324−325.
(40) Aiyejorun, T., Kowalik, J., Janata, J., Josowicz, M. *J. Chem. Educ.* In press.
(41) Cowan, J. A., *Nucleic Acids Mol. Biol.* **2004,** *14*, 339−360.

Table 1. Investigated Oligonucleotide Sequences

description	sequence
	18-Mer Oligonucleotide
probe	5′-CGC TCA ATG CCT GGA GAT-3′
target	5′-ATC TCC AGG CAT TGA GCG-3′
mismatched DNA	5′-CAC TCT ATG TCC GGT CAT-3′
	27-Mer Oligonucleotide
probe	5′- GCT TTT ACT TAT TTG ATC ATT CCT TCA-3′
target	5′- TGA AGG AAT GAT CAA ATA AGT AAA AGC-3′
noncomplementary	5′- ATC CCC GTC CGC CCA GCT GCC TTC CTG-3′
	24-Mer AQ-DNA
DNA tagged with AQ	5′-AQ TTC CTT CCT TAT ATT TCC TTC CTT-3′
complementary sequence	5′- AAG GAA GGA AAT ATA AGG AAG GAA-3′

development of a miniaturized genosensor based on microelectrodes for studying the detection of short target DNA sequences (18- and 27-mer) in respect to selectivity, sensibility, and reproducibility of the biosensor. Additionally, the effect of HCl (50 mM) on the regeneration of the electrode has been studied. The main advantages of this strategy are low-cost, simple design, and miniaturization.

EXPERIMENTAL SECTION

Chemicals. Pyrrole monomer (Py, 98%) was purchased from Sigma-Aldrich (St. Louis, MO). Before use, pyrrole was purified by passing it through a neutral alumina column. pTPTC3−PO_3H_2) is not commercially available and was synthesized according to Hartung et al.[43] Magnesium chloride was obtained from Fluka. Tetra-*n*-buthylammonium perchlorate was obtained from Alfa Aesar (Ward Hill, MA). All other reagents were obtained from Sigma-Aldrich and used without further purification. A Tris-HCl buffer (0.1 mol L^{-1}, pH 7.2) was used as the supporting electrolyte. The sequences of the anthraquinone-labeled DNA (24-mer AQ-DNA) and probe oligonucleotides (18- and 27-mer), and its complementary, noncomplementary (27-mer), and mismatched (18-mer) strands were custom-made by Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). All chemicals were free of RNase and DNase and were used as received.

Apparatus. Cyclic voltammetry (CV) and constant-potential amperometry were carried out using an electrochemical workstation (660A, CH Instruments Electrochemical, Austin, TX). All experiments were performed at room temperature (25 ± 1 °C). A three-electrode cell with a volume of 3 mL, comprising a 25-μm-diameter platinum or gold disk working electrode (electroactive area, 4.90×10^{-6} cm^2) encased in glass, a Ag/AgCl reference electrode, and a platinum wire counter electrode was used for all electrochemical experiments. A detailed description of the fabrication process of the used microelectrodes is given elsewhere.[44] Following polishing, the microelectrodes were ultrasonically cleaned for 15 min in deionized water. Further cleaning of the platinum electrodes was performed by cycling in 0.5 mol L^{-1} H_2SO_4 between −0.80 and +2.0 V (1 cycle) and −0.61 to +1.0 V (10 cycles) followed by 2-min polarization at 0.21 V versus Hg/Hg_2SO_4 as reference electrode. CV was applied to characterize the microelectrodes and to obtain the actual radius of the embedded microwire. Ferrocenemethanol (C_0 = 2.0 mmol L^{-1}; $D = 7.8 \times 10^{-6}$ cm^2 s^{-1})[45] was used in these characterization experiments. The actual radius of the Au microelectrodes was $r = 11.2 \pm 0.2$ μm ($n = 3$) and the actual radius of the Pt microelectrodes, 11.2 ± 0.23 μm ($n = 3$).

Preparation of DNA Microelectrodes Based on a Layered Polypyrrole/Conducting 2,5-Bis(2-thienyl)-*N*-(3-phosphorylpropyl)pyrrole Layer. Pyrrole solution (0.1 mol L^{-1}) was prepared in 0.1 mol L^{-1} tetra-*n*-butylammonium perchlorate in acetonitrile (AcCN) and stored in the absence of light. Polypyrrole films were electrochemically formed at 0.7 V (vs Ag/0.1 mol L^{-1} $AgNO_3$ in AcCN/0.1 mol L^{-1} TBAP in AcCN) at the Pt or Au microelectrode surface. The pyrrole polymerization was controlled by chronocoulometry. It was terminated when the total charge supplied by the electrode reached 0.03×10^{-6}, 0.15×10^{-6}, 0.75×10^{-6}, 3.0×10^{-6}, or 15.0×10^{-6} C, respectively. Sequentially, the pTPTC3−PO_3H_2 layer was grafted following the procedure described in the literature.[39] The pTPTC3−PO_3H_2 polymerization was always stopped at 5.5×10^{-7} C. The electrodes were rinsed several times with AcCN after each polymerization step, removing excessive or unbound pyrrole and oligomers from polymerized or unbound TPTC3−PO_3H_2. The chemical stability of the PPy−pTPTC3−PO_3H_2 bilayer deposited at the electrode surface was improved by application of a potential step from the open cell potential to 1.2 V for 1 min in Tris-HCl buffer solution (pH 7.2). Following, the electrode was immersed in 5×10^{-3} mol L^{-1} $MgCl_2$ solution and kept under stirring for at least 15 min to complete the preparation of a generically "activated" electrode (PPy−ppTPTC3−PO_3H_2−Mg^{2+}) ready for binding the DNA probe. Subsequently, the electrode was washed under stirring in 0.1 mol L^{-1} Tris-HCl buffer (pH 7.2) to remove Mg^{2+} ions not involved in the formation of the bidentate complex. In the following, CVs were recorded within a potential range of +0.3 to −0.3 V (vs Ag/AgCl at 0.05 V s^{-1}) in 0.1 mol L^{-1} Tris-HCl buffer (pH 7.2). The DNA probe immobilization (27-mer, 1.0×10^{-6} mol L^{-1}, or 18-mer, 1.0×10^{-5} mol L^{-1}) was allowed to proceed for 5, 10, 20, 30, and 40 min, respectively. Thereafter, the electrode was thoroughly washed using 0.1 mol L^{-1} Tris-HCl buffer solution for 5 min to ensure that removing the DNA probes that were not involved in the complex formation with the Mg^{2+} attached to the bilayer of the electrode took place.

Hybridization and Detection. The PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA (27-mer)-modified electrodes were exposed to a solution of the noncomplementary oligonucleotide (1.0×10^{-6} mol L^{-1}) for 10 min at room temperature while gently mixing. After washing, CVs were recorded evaluating any nonspecific interactions. In the following, the complementary interaction with 27-mer, 1.0×10^{-18}−1.0×10^{-7} mol L^{-1}, and 18-mer, 1.0×10^{-18}−1.0×10^{-9} mol L^{-1} (prepared by serial dilution), was performed at identically processed electrodes, respectively. After hybridization, the microelectrodes were thoroughly rinsed under stirring (5 min) in an excess of Tris-HCl buffer (pH 7.2) and again CVs were recorded. Likewise, the hybridization of immobilized 18-mer

(42) Koutsodimou, A.; Kovala-Demertzi, D.; Katsaros, N. *J. Coord. Chem.* **1998**, *43*, 1, 1−12.
(43) Hartung, J., Kowalik, J., Kranz, C., Janata, J., Josowicz, M., Sinha, A., McCoy, K. *J. Electrochem. Soc.* **2005**, *152*, E345−E350.
(44) Kranz, C.; Ludwig, M.; Gaub, H. E.; Schuhmann, W. *Adv. Mater.* **1995**, *7*, 38−40.
(45) Miao, W. J.; Ding, Z. F.; Bard, A. J. *J. Phys. Chem. B* **2002**, *106*, 1392−1398.

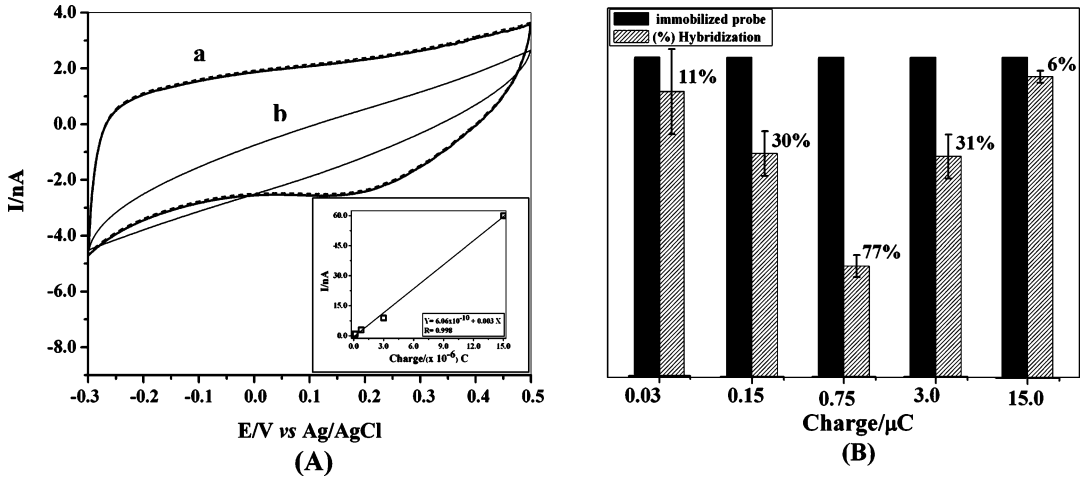

Figure 1. (A) Cyclic voltammograms recorded in 0.1 mol L^{-1} Tris-HCl buffer (pH 7.2) after electropolymerization of PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA (27 mer) at the electrode surface at 0.75 × 10^{-6} C: (dark solid line) PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA (27-mer)-modified electrode (dashed line) after incubation with noncomplementary DNA and (light solid line) after hybridization with target DNA, Scan rate, 0.050 V s^{-1}. (B) Histograms derived from the difference between the initial signal provided by the PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA layer and the current value obtained after DNA hybridization corresponding to the percentage of hybridization for different thickness conditions. The error bars are derived from two experiments.

probes (0.1 × 10^{-6} mol L^{-1}) with complementary and mismatched DNA (1.0 × 10^{-9} mol L^{-1}) strands was investigated. At low DNA concentrations, a smoothing algorithm was applied after each voltammetric measurement to remove random noise and electromagnetic interference.

Evaluation of the Immobilized Probe DNA at the Layered Polypyrrole/Conducting 2,5-Bis(2-thienyl)-*N*-(3-phosphorylpropyl)pyrrole. An estimation of the amount of probe DNA immobilized at the electrode surface followed the procedure as described in the literature using 24-mer AQ-DNA.[46] The PPy−pTPTC3−PO_3H_2−Mg^{2+}-modified electrode was immersed in buffer solution of the appropriate anthraquinone DNA probe (0.5 × 10^{-6} mol L^{-1}) for 30 min. After washing, the efficiency of the probe DNA immobilization (AQ-DNA, 0.5 × 10^{-6} mol L^{-1}) at the layered PPy/conducting pTPTC3−PO_3H_2−Mg^{2+} layer was evaluated by square wave voltammetry in 0.1 mol L^{-1} Tris-HCl buffer solution. The behavior of the system was also studied using an oligonucleotide sequence without anthraquinone.

RESULTS AND DISCUSSION

Influence of the Inner Polypyrrole Thickness of the Pt/PPy−pTPTC3−PO_3H_2−Mg^{2+} ssDNA (Probe) to DNA Hybridization Event. The influence of the thickness of the electropolymerized polypyrrole on detection of the DNA (27-mer) hybridization was studied by terminating the electric charge used for its polymerization at 0.03 × 10^{-6}, 0.15 × 10^{-6}, 0.75 × 10^{-6}, 3.0 × 10^{-6}, and 15.0 × 10^{-6} C. The thickness of the outer layer, pTPTC3−PO_3H_2−, was always kept constant. An increase in peak current was observed, which was directly proportional to the increase of the charge monitored during the PPy polymerization step (inset, Figure 1A). A linear regression results in $Y = 6.06 \times 10^{-6} + 0.003X$ (X, applied charge; Y, current in nA) with a correlation coefficient of 0.998. The reproducibility of the film deposition was estimated using the same microelectrode for 10 individual electrodeposition cycles at different days resulting in an average current of 2.76 × 10^{-9} A with SD = 0.435 × 10^{-9} and RSD = 16%. The reproducibility of the measurement was also estimated using different electrodes (n = 5) within 1 day resulting in an average current of 1.72 × 10^{-9} A with SD = 0.352 × 10^{-9} and RSD = 20%. Figure 1A shows the CV (a) obtained in 0.1 mol L^{-1} tris-HCl buffer solution (pH 7.2) immediately after the polymerization of PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA (27-mer) at the electrode surface at 0.75 × 10^{-6} C. The DNA probe (27-mer, 1.0 × 10^{-6} mol L^{-1}) was immobilized at the PPy−pTPTC3−PO_3H_2−Mg^{2+}-modified electrode for 30 min. Following, the PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA electrodes were exposed to target DNA (27-mer, 1.0 × 10^{-7} mol L^{-1}) for 10 min. After washing, CVs (b) were again recorded in 0.1 mol L^{-1} Tris-HCl buffer (pH 7.2). A significant decrease in the current was observed. Figure 1B shows the histograms derived from the difference between the initial signal (PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA response) and the current value obtained after DNA hybridization, which corresponds to the percentage of hybridization. The decrease in percent area was calculated comparing the signal observed with the probe DNA immobilized (this value is set as 100%) and the ones obtained after the subtraction of the complementary interaction (curve b, Figure 1A) from PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA (curve a, Figure 1A) cyclic voltammogram. The addition of negative charges to the modified electrode surface due to phosphate groups of the complementary strand decreases the chloride ion exchange providing the decrease in the observed CV current. For thin films, only a small decrease of the signal was observed (11%, 0.03 × 10^{-6} C), which becomes more pronounced at 0.15 × 10^{-6} C. The optimum response for the DNA hybridization has been observed at 0. 75 × 10^{-6} C, which corresponds to a decrease of an area of 77% in contrast to the signal obtained from the PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA layer. However, if the PPy films reach a certain thickness (at ∼0.75 × 10^{-6} C), the response of DNA hybridization may be affected by the increase of the resistivity

(46) Kertesz, V.; Whittemore, N. A.; Inamati, G. B.; Manoharan, M.; Cook, P. D.; Baker, D. C.; Chambers, J. Q. *Electroanalysis* **2000**, *12*, 889−894.

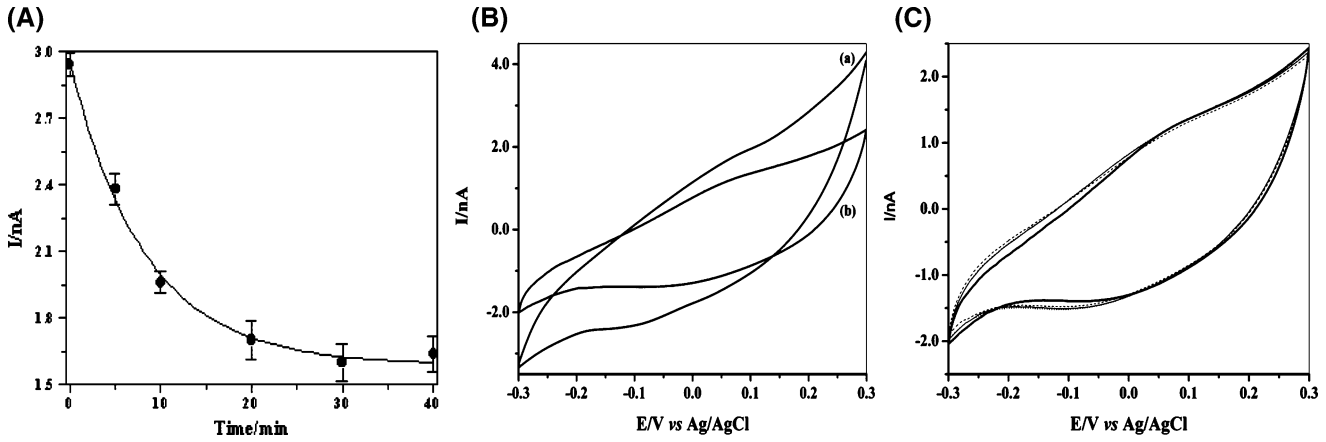

Figure 2. (A) Effect of the incubation time of probe DNA (27-mer) immobilization at the modified electrode surface. (B) Cyclic voltammograms obtained in 0.1 mol L^{-1} Tris-HCl buffer (pH 7.2) at a PPy−pTPTC3−PO_3H_2−Mg^{2+} layer (a) and after exposure to 1.0×10^{-6} mol L^{-1} probe DNA for 30 min (b). (C) Cyclic voltammograms recorded in Tris-HCl buffer (pH 7.2) at a PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA layer (dark solid line) and the same electrode after storage in deionized water for 15 (solid line), 30 (dashed line), and 90 (dotted line) min; scan rate, 0.05 V s^{-1}.

due to the conducting properties of PPy. Therefore, the PPy polymerization was terminated as soon as the charge exceeded 0.75×10^{-6} C by recording the current as a function time at constant potential (0.7 V).

Effect of the Incubation Time of Immobilized Probe DNA on the Response. As shown in Figure 2A, a significant decrease in response for the PPy−pTPTC3−PO_3H_2−Mg^{2+}-modified electrode (at 0.3 V) after successive immersion in 1.0×10^{-6} mol L^{-1} probe DNA (27-mer) solution for 5, 10, 20, 30, and 40 min was observed. After 20 min, the response of PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA has stabilized, indicating an effective immobilization of the 27-mer probe DNA at the PPy−pTPTC3−PO_3H_2−Mg^{2+} layer. Thus, a period of 30 min has been established as optimum for probing DNA immobilization at the modified electrode surface. Figure 2B shows the change in profile of the cyclic voltammogram after exposure of the PPy−pTPTC3−PO_3H_2−Mg^{2+} in probe DNA solution for 30 min. In the following, the stability of the immobilized probe DNA at the same electrode was evaluated. The PPy−pTPTC$_3$−PO_3H_2−Mg^{2+}/probe DNA film exhibited sufficient stability when stored in deionized water for 15, 30, and 90 min (Figure 2C) after the DNA incubation period.

Target Detection. The hybridization of complementary (27- or 18-mer), noncomplementary (27-mer) sequences, or 12-mismatched DNA (18-mer) to the PPy-pTPTC3−PO_3H_2−Mg^{2+}/probe DNA layer was investigated. In a first study, the effect of noncomplementary strands (27-mer, 1.0×10^{-6} mol L^{-1}) and mismatched strands (18-mer) on the response of the sensor was evaluated. No significant change of the cyclic voltammogram in the presence of 27-mer target DNA was observed as shown in Figure 3A. However, in the presence of the complementary oligonucleotide (27-mer, 1.0×10^{-12} mol L^{-1}), a significant change of the shape of the cyclic voltammogram was observed corresponding to a 14.5% decrease in response when compared to the signal derived from the cyclic voltammogram of the PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA before hybridization. The hybridization process was completed within 10 min. The same behavior could be observed for 18-mer target DNA (inset in Figure 3A). However, the 18-mer DNA hybridization process was substantially slower and completed only after 30 min of target DNA incubation time. This difference may be explained with the different ratio of base pairs (8 AT and 10 CG for the 18-mer and 19 AT AND 8 CG for the 27-mer). As hypothesis, a stronger base-pair interaction for AT base pairing (AT base pairs are more then doubled for the 27-mer) may lead to the faster hybridization process. This effect is subject of further investigations. The hybridization events can be represented by subtraction of the complementary and noncomplementary interactions from the PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA CV, as shown in Figure 3B. As explained above (Figure 1), the addition of negative charges to the modified electrode surface due to phosphate groups of the complementary strand decreases the chloride ion exchange. Thereby, the hybridization of the specific DNA sequence (target DNA) could be confirmed. The reproducibility of the measurements for the same DNA detector was investigated by comparing the hybridization signals obtained with the same concentration of complementary target oligonucleotide with different film depositions. For each surface, at least three replicate measurements were performed. The average hybridization value, standard deviation, and relative standard deviation obtained with the complementary target oligonucleotide were as follows: 1.0×10^{-16} mol L^{-1} (0.140 ± 0.005 nA, RSD = 3.6%), 1.0×10^{-14} mol L^{-1} (0.250 ± 0.01 nA, RSD = 4%), 1.0×10^{-12} mol L^{-1} (0.300 ± 0.007 nA, RSD = 2.3%), and 1.0×10^{-9} mol L^{-1} (0.445 ± 0.001 nA, RSD = 3%).

Figure 4 displays the effect of target DNA (27-mer) concentrations on the modified electrode response. Each concentration was tested in three replicates ($n = 3$). The curve results from the averaged surface areas obtained after the subtraction of the voltammogram before and after hybridization. The obtained response increased with the target concentration (ranging from 1.0×10^{-18} to 1.0×10^{-6} mol L^{-1}) up to 0.1×10^{-6} mol L^{-1} and leveled off above 0.1×10^{-6} mol L^{-1}. Such behavior reflects the probe hybridization sites available at the electrode surface above 0.1×10^{-6} mol L^{-1}. Desorption of the hybrid from the modified electrode surface was not observed, as the CV signal remained constant after successive measurements. For comparison, Figure 5 shows the logarithmic response function in dependence of 18- or 27-mer target DNA concentration; data for the 27-mer are the

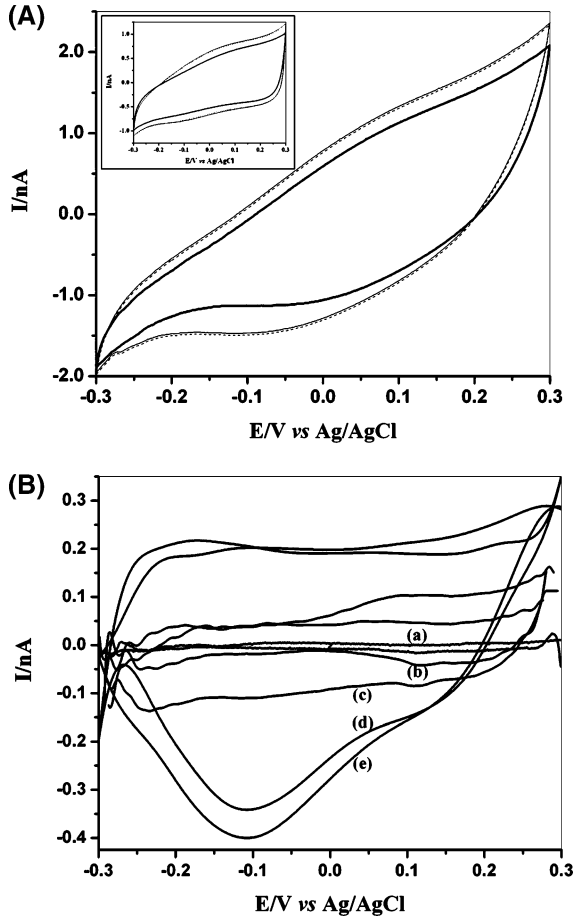

Figure 3. (A) Cyclic voltammograms recorded in 0.1 mol L^{-1} Tris-HCl buffer pH 7.2 for the detection of 27-mer DNA hybridization: (light solid line) PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA; (dashed line) after exposure to 0.1 × 10^{-6} mol L^{-1} noncomplementary DNA; (dark solid line) same electrode after hybridization with target DNA (0.1 × 10^{-12} mol L^{-1}) for 10 min. Inset: results obtained for 18-mer DNA sequences. Scan rate, 0.05 V s^{-1}. (B) Results of subtraction of the cyclic voltammograms for the noncomplementary (a) and complementary interactions (b, 1.0 × 10^{-16} mol L^{-1}; c, 1.0 × 10^{-14} mol L^{-1}; d. 1.0 × 10^{-12} mol L^{-1}; and e, 1.0 × 10^{-9} mol L^{-1}) from PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA cyclic voltammograms.

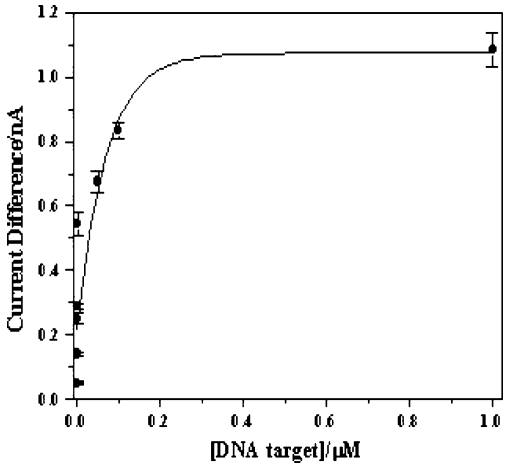

Figure 4. Concentration−response curve obtained for increasing concentration of 27-mer target DNA (ranging from 1.0 × 10^{-18} to 1.0 × 10^{-6} mol L^{-1}). The results correspond to the values of subtraction of the complementary interactions from PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA cyclic voltammograms (at 0.3 V). Each concentration was recorded in three replicates (n = 3).

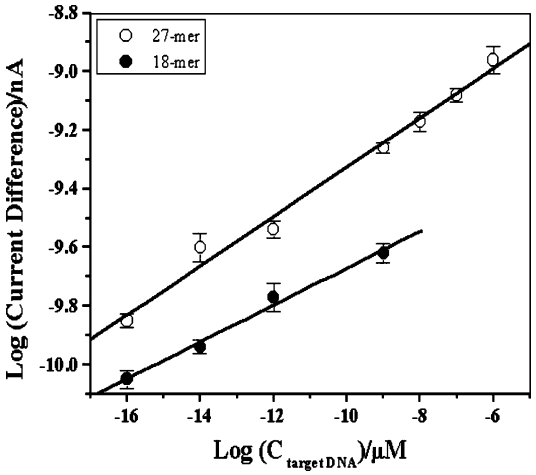

Figure 5. Double logarithmic calibration plot for the dependence of PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA-modified electrode response upon increasing the concentration of 27- and 18-mer target DNA. Each concentration was recorded in three replicates (n = 3).

same as shown in Figure 4. The sensitivity is defined by the slope of the fitted linear regression function and amounts 1.87 × 10^{-19} and 2.62 × 10^{-16} mol L^{-1} for 18-mer and 27-mer sequences, respectively. Therefore, the DNA biosensor shows exceptional sensitivity for the detection of the 18- and 27-mer DNA specific sequences. The background noise level was approximated by the average value of the signal at three points using noncomplementary (27-mer) or mismatched (18-mer) strands at the lowest concentration (1 × 10^{-18} mol L^{-1}). The background noise was determined at 0.050 ± 0.005 and 0.024 ± 0.005 nA, respectively. Consequently, the limit of detection was determined at 0.16 (18-mer) and 3.5 fmol (27-mer).

Chemical Regeneration of the DNA Biosensor. Repeated usability is a desired feature for biosensors in practical applications. Usually, the regeneration of a DNA sensor is achieved by either a thermal or a chemical regeneration procedure.[47] In the present study, chemical regeneration with 50 × 10^{-3} mol L^{-1} HCl solution was applied. The electrode response returned to its original signal after 30 min of treatment (Figure 6A), indicating that the double-stranded DNA hybrid was dissociated into single strands and that the signal of the immobilized probe DNA was not destroyed after the regeneration. The regenerated sensor (Figure 6B) produced a similar decrease of the CV when hybridized with the target strand, demonstrating the regeneration and reusability of the DNA biosensor. However, successive cycles of hybridization/regeneration using HCl 50 × 10^{-3} mol L^{-1} at the same electrode with the same probe were not successful. It is assumed that the contact with acid solution affects the binding between the probe and Mg^{2+} ions involved in the formation of the bidentate complex after repeated regeneration cycles. It is possible that repetitive dipping in magnesium chloride solution after the duplex hydrolysis can extend the usability of the "label-free" probe.

Evaluation of the Surface Concentration of the Immobilized Probe DNA To evaluate the surface concentration of the

(47) Abel, A. P.; Weller, M. G.; Duveneck, G. L.; Ehrat, M.; Wildmer, H. M. *Anal. Chem.* **1996**, *68*, 2905−2912.

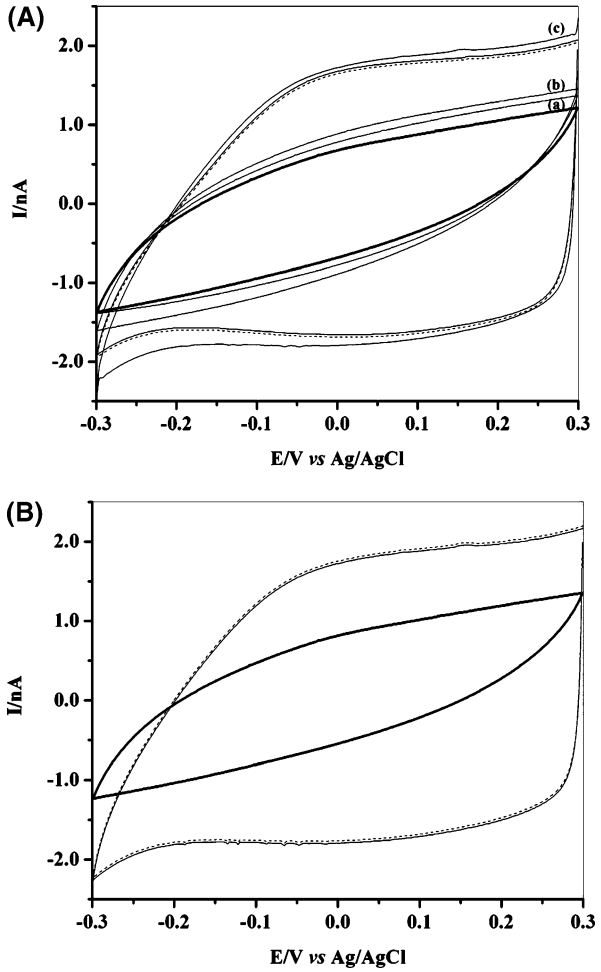

Figure 6. Cyclic voltammograms obtained from PPy−pTPTC3−PO_3H_2−Mg^{2+}/DNA1 (solid line) and after hybridization with 0.05 × 10^{-6} mol L^{-1} DNA-2 target (dotted line) on the electrode surface. The effect of HCl solution (50 × 10^{-3} mol L^{-1}) on the regeneration of the modified electrode surface is shown after 5 (a), 15 (b), and 30 (c) min.

immobilized probe DNA at the layered polypyrrole/conducting pTPTC3−PO_3H_2, an oligonucleotide modified with anthraquinone group was used as a probe. In Figure 7, square wave voltammograms (dark solid line, $Q = 8.5 \times 10^{-10}$ C) characteristic for the immobilized AQ DNA probe and the AQ-free DNA probe of the same sequence (see Table 1) are shown. The anodic peak at −148 mV ($I_{pa} = 6.81 \times 10^{-10}$ A) could be attributed to the chloride ions expelled from the inner PPy layer of the label-free DNA detector. The value for surface concentration was calculated from the integrated current under both the cathodic and anodic peaks corresponding to the immobilized probe molecule ($\Gamma = 4.48 \times 10^{-10}$ mol cm^{-2}). Figure 7 displays the oxidation wave. The experimental conditions are similar to the experiments described above. The PPy−pTPTC3−PO_3H_2−Mg^{2+}-modified electrode was immersed in oligonucleotide modified with anthraquinone probe for 10 min. The hybridization was finished after 10 min.

CONCLUSIONS

A label-free detection scheme for short-sequence (18- and 27-mer) DNAs based on conducting modified polypyrrole films deposited at a microelectrode surface is reported. The addition of negative charges to the PPy−pTPTC3−PO_3H_2−Mg^{2+}/probe DNA-modified electrode surface due to phosphonic acid groups of the complementary strand hinders the chloride ion exchange and causes a decrease in current of the recorded voltammogram. Thereby, the hybridization of short DNA sequences could be determined. The estimated amount of the probe DNA immobilized at the PPy−pTPTC3−PO_3H_2−Mg^{2+}-modified electrode using a 24-mer tagged AQ-DNA was found to be 4.48×10^{-10} mol cm^{-2}. The reported studies demonstrate exceptional sensitivity of the label-free DNA microgenosensor based on modified polypyrrole films deposited at the microelectrode surface with rapid response and sufficient reproducibility of the DNA hybridization events. The developed label-free electrochemical microbiosensor enabled the detection of short-sequence (18- and 27-mer) target DNAs with a limit of detection at 0.16 and 3.5 fmol, respectively. Furthermore, dissociation of the DNA duplex using HCl as regeneration solution was obtained. These sensors have potential applications for field experiments, as the described devices are already miniaturized, can be prepared in advance, and can be stored in dry state. Furthermore, we could demonstrate that the response time for hybridization detection could be as low as 10 min.

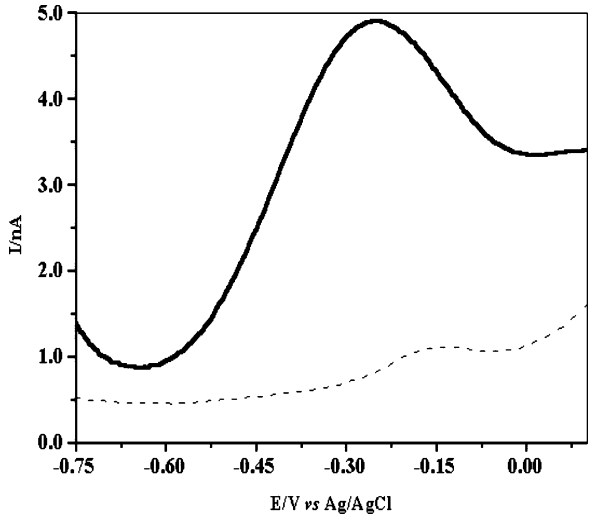

Figure 7. Square wave voltammograms in 0.1 mol L^{-1} Tris-HCl buffer of the (solid line) modified probe AQ-DNA or (dashed line) probe DNA without AQ marker at the layered PPy−pTPTC3−PO_3H_2−Mg^{2+}; a = 25 mV s^{-1}, ΔE_s = 2.0 mV, and f = 5.0 Hz.

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