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New acridone derivatives for the electrochemical DNA-hybridisation labelling

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Abstract

In the field of DNA sensing, DNA hybridisation detection is generally performed by fluorescence microscopy. However, fluorescence instrumentation is difficult to miniaturise in order to produce fully integrated DNA chips. In this context, electrochemical detection of DNA hybridisation may avoid this limitation. Therefore, the use of DNA intercalators is particularly attractive due to their selectivity toward DNA double strand enabling DNA labelling without target chemical modification and, for most of them, to their electroactivity. We have synthesized a pyridoacridone derivative dedicated to DNA hybridisation electrochemical-sensing which presents good electrochemical reversibility, electroactivity at mild potentials and specificity toward DNA double strand. The electrochemical behaviour of this molecule has been assessed using cyclic voltammetry (CV). DNA/intercalator interactions were studied by differential pulse voltammetry (DPV) before application to hybridisation detection onto DNA sensors based on polypyrrole modified electrodes.
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Keywords: DNA hybridisation detection; Redox intercalator; Pyridoacridone

1. Introduction

In recent years, there has been a considerable interest in the development of DNA biosensor for the characterization of DNA hybridisation. Fluorescence microscopy is the most commonly used detection technique due to its high sensitivity and spatial resolution. However, this method requires relatively cumbersome monitoring instruments which limits its application in fully integrated biochips. Electrochemistry is a simple and promising method suitable for rapid detection of specific DNA sequence, combining high sensitivity, low cost and compatibility with microfabrication technology of transducers. Recently, several electrochemical DNA biosensors have been reported. Thus, the electrochemical signal of these DNA biosensors are based on label-free detection using the intrinsic electroactivity of DNA [1], modulation of the electrochemical comportment of conducting polymers [2], electrocatalytical oxidation of guanosine [3], redox-enzymes labelling [4] or utilization of colloidal gold labels [5] for example. Another route of DNA hybridisation detection dealing with the use of small molecules interacting directly and specifically with the DNA duplex, such as intercalators, DNA groove binders, metal complexes and threading agents, has been recently reviewed by Takenaka [6]. However, most of these molecules, designed for therapeutic applications, present poor electrochemical performances in terms of reversibility, overpotential and/or stability. Acridine and acridone derivatives are well-known antitumor drugs and their planar structure confers on these molecules the ability to bind DNA by intercalation. Therefore, a new acridone derivative (molecule **1**) designed for the electrochemical labelling of DNA hybridisation by intercalation has been synthesized [7].

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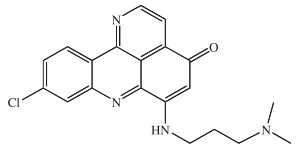
Molecule **1**



1 has been characterized electrochemically by cyclic voltammetry and its interaction properties with ss-DNA and ds-DNA have been assessed by differential pulse voltammetry (DPV) and UV–Visible spectroscopy. The use of **1** as an electrochemical indicator for the fabrication of DNA electrochemical sensor has been effected using DNA immobilization method based on the copolymerization of pyrrole and ODN-modified pyrrole monomer [8]. The intercalation process was recorded by differential pulse voltammetry (DPV) and the effectiveness of the obtained results was assessed using fluorescence microscopy as reference method.

2. Experimental section

All the electrochemical measurements were made in 0.1 M phosphate buffer (PB) solutions prepared with Na_2HPO_4 and NaH_2PO_4 (Aldrich) excepted electropolymerization procedures which are carried out in a 0.1 M $LiClO_4$ (Fluka) solution. Pyrrole was provided by Acros. Double strand (ds-) salmon sperm DNA and single strand (ss-) calf thymus DNA were purchased from Sigma. All chemicals were of the highest purity available and were used without further purification. The synthesis of compound **1** has been described elsewhere [7]. Oligonucleotides (ODN) have been synthesized using specific reagents (Perkin Elmer) on a 381A DNA synthesizer from Applied Biosystems. The 40 mer ODN probe (5′-Pyr(T5)AGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGT-3′) corresponds to a point mutation of k-ras protooncogene. Complementary ODN target is 40 mer long and presents the following sequences: 40 c 5′-biotin-ACAAAATGATTCTGAATTAGCTGTATCGTCAAGGCACTCT-3′. The noncomplementary target (20 nc) is a 20 mer ODN with the following sequence: 5′ -biotin-GGGCAGGACCGGGCAGGACC-3′. All the electrochemical experiments were performed with a computer controlled PAR 273 EGG potentiostat, from Princeton Applied Research, in a deaerated one-compartment cell using a Pt working electrode (diameter 5 mm), a Pt wire as counter electrode and a KCl saturated Ag/AgCl reference electrode.

DNA adsorption onto polypyrrole modified Pt electrodes (obtained by cycling the potential between −0.35 and 0.7 V vs. Ag/AgCl three times in 20 mM pyrrole 0.1 M $LiClO_4$ aqueous solution at a scan rate of 20 mV s^{-1}) was proceeded in a PB 0.1 M pH 6 aqueous media containing 20 ppm of DNA by applying 480 s a potential of 0.5 V vs. Ag/AgCl.

Films of copolymers were deposited on Pt by scanning the electrode potential between −0.3 and +0.7 V at a scan rate of 20 mV s^{-1}. The electrolyte was a 0.1 M solution of lithium perchlorate containing 24 mM of pyrrole monomer and 5 μM of ODN functionalised pyrrole. Hybridisation was carried out by immersing the ODN modified electrodes in PBS (pH 7.4) containing 200 nM of ODN targets. The hybridisation lasted at room temperature for 20 min with shaking. After that, the electrode was washed three times with PBS to remove the unhybridised DNA. Denaturation was performed by washing the modified electrodes, respectively, in 0.1 M NaOH (for 30 s), distilled water and PB.

Intercalation was effected by immersion of the obtained modified electrode in stirred 0.1 M pH 7 PB solution containing 125 μM of **1** during 90 min. DPV were recorded by scanning the potential anodically from −0.45 to 0.2 V vs. Ag/AgCl after stabilization of the electrode at a potential of −0.45 V during 30 s. DPV parameters are 30 mV pulse amplitude and 50 ms pulse width. These experiments were effected in deaerated medium and by scanning the potential anodically to obtain well defined and stable signals However, one can note that the redox signal of the intercalator is not affected by the presence of oxygen that only decrease the signal to noise ratio without any influence on the intercalator specificity and electrochemical properties.

For binding studies, fluorescence data were recorded at room temperature with a LS50 Perkin-Elmer fluorometer. Excitation was set at 520 nm and fluorescence emission was monitored over the range 550–700 nm. Experiments were performed with a concentration of 12.2 μM base pairs for ds ADN, 5.94 μM for intercalator 1 and an ethidium concentration of 0.0–12.3 μM in a tris buffer pH 6.9.

Fluorescence microscopy images were recorded for 1 s with an epifluorescence microscope (BX 60, Olympus) equipped with a Peltier cooled CCD camera (Hamamatsu) and an image analysis software (Imagepro plus, Media Cybernetics) using streptavidin–phycoerythrin conjugate (Molecular Probe) as fluorescent probe.

UV–visible spectra were recorded with a UVIKON spectrometer in PB pH 7 0.01 M solutions.

3. Results and discussion

1 was characterized at a platinum electrode by cyclic voltammetry (CV) in deaerated phosphate buffer at different scan rates ranging from 50 to 200 mV s^{-1}(Fig. 1).

The CV of **1** exhibits one quasi-reversible redox wave at −0.24 V ($E_{1/2}$ vs. Ag/AgCl) at pH 7. The peak separation of about 60 mV indicates that one electron is involved in the reaction as confirmed by quantitative coulometry. The peak current varies linearly to the square root of the scan rate, indicating that the electrode reaction is due to the diffusion of **1** in the solution. The voltamperometric response of **1** is pH dependant and $E_{1/2}$ shifts to negative potentials with pH increasing following a slope of −60 mV per pH unit, thus

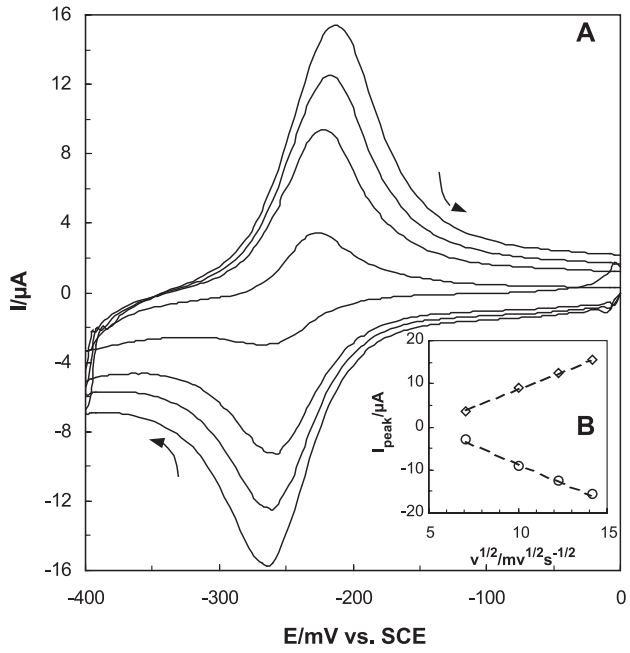


Fig. 1. CV of **1** (1 mM) in deaerated phosphate buffer aqueous solution (0.1 M pH 7) at Pt electrode for scan rates ranging from 25 to 200 mV s^{-1} (A) and dependency of I_{peak} with the square root of the scan rate (B).

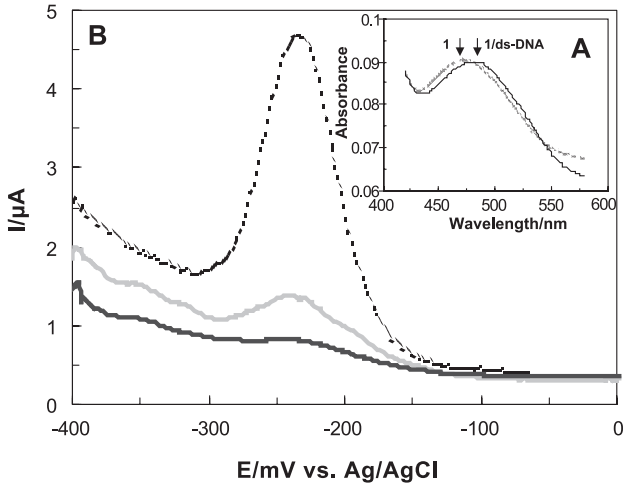


Fig. 2. (A) Absorbance spectra of **1** (50 µM) in phosphate buffer aqueous solution (0.1 M pH 7) before (dashed line) and after addition (dark line) of ds-DNA (0.25 mM base pairs). (B) DPV detection of **1** (125 µM) in deaerated phosphate buffer aqueous solution (0.1 M pH 7) in a DNA free PB solution (dashed line), after addition of ss-DNA (0.5 mg ml^{-1}, grey line) and ds-(0.5 mg ml^{-1}, dark line) DNA at a PPy modified GC electrode (3 mm diameter). DPV were recorded by scanning the potential anodically from −0.45 to 0.2 V vs. Ag/AgCl after stabilization of the electrode at a potential of −0.45 V during 30 s. DPV parameters are 30 mV pulse amplitude and 50 mV pulse width.

indicating the contribution of one H_3O^+ ion to the redox process (data not shown).

In order to visualise the effectiveness of the intercalation process within ds-DNA, we have recorded the UV−visible absorption spectra (Fig. 2A) of **1** (0.05 mM) in the absence (grey line) and presence (dark line) of salmon ds-DNA (250 µM base pairs) in PB media. **1** exhibits an absorption peak at λ_{max} = 470 nm (ε = 980 M^{-1} cm^{-1}). When ds-DNA was added into the solution, a red shift in the adsorption wavelength was observed (from 470 to 480 nm) thus highlighting that **1** interacts with ds-DNA [9]. Then, binding studies were carried out by a competitive displacement fluorometric assay with ethidium bromide. The binding constant of **1** was evaluated to $K_1 = 2.8 \times 10^5$ M^{-1}.

Otherwise, Fig. 2B displayed the electrochemical responses of **1** in a pH 7 PB solution prior (dashed line) and after addition of ss-DNA (grey line) or ds-DNA (dark line). In solution, the DPV response of **1** is centred at $E_{1/2}$ with an amplitude of 3.6 µA (Fig. 2B). After addition of ds-DNA, we have recorded the quasi-total disappearance of the DPV signal of **1**. This signal loss could be attributed to the decrease of **1** mobility after intercalation within ds-DNA. Otherwise, after addition of ss-DNA, the current amplitude was divided by a factor of 6 compared to **1** in DNA-free solution. This suggests nonspecific electrostatic interactions between **1** and ss-DNA, since the polyamine chain of **1** is protonated at pH 7, and/or π stacking with the unmatched guanine residues of the CT ss-DNA [10]. Nevertheless, since the interaction rate of **1** with ds-DNA, is higher than such recorded with ss-DNA, this new redox intercalator has been utilized for the detection of hybridised DNA adsorbed at electrode surfaces (Fig. 3A). For this purpose, a polypyrrole (PPy) thin film was electropolymerized onto the platinum electrode. Polyanionic ss-DNA or ds-DNA were then adsorbed onto the polycationic PPy film by oxidative adsorption at 0.5 V.

Fig. 3A gives the DPV responses obtained, respectively, for Pt/PPy/ss-DNA (grey line) and Pt/PPy/ds-DNA (dark

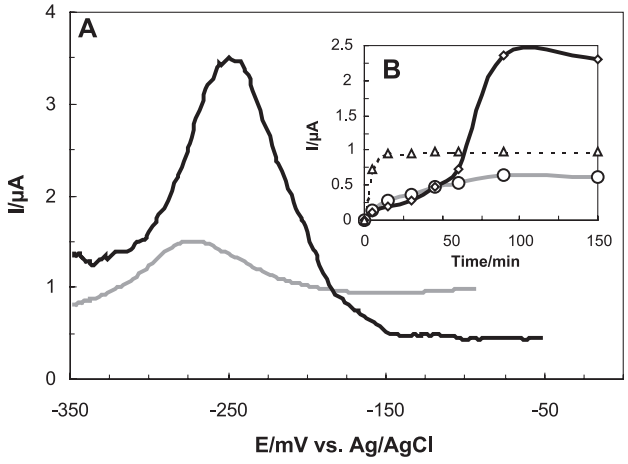


Fig. 3. (A) DPV detection of **1** in deaerated phosphate buffer aqueous solution (0.1 M pH7) after interaction with adsorbed ss-(grey line) and ds-(dark line) DNA at a PPy modified Pt electrode (5 mm diameter). Intercalation was effected by immersion of the obtained modified electrode in stirred 0.1 M pH 7 PB solution containing 125 µM of **1** during 90 min. Other experimental parameters as in (B). Evolution of the DPV amplitude with interaction duration for pure PPy film (dashed line), ss-(grey line) and ds-(dark line) DNA modified PPy.

line) after 90 min immersion in a 125 μM solution of **1**. Fig. 3 shows a slight shift (20 mV) of $E_{1/2}$ between the responses obtained for specific and nonspecific interactions. Such a behaviour could be related to the DNA microenvironment within the duplex [11] and, therefore, qualitatively highlights the effectiveness of **1** intercalation. In a thermodynamic point of view, the DPV signal allows us to determine the half wave potential of **1** after intercalation. Then, $E_{1/2}$, measured as the peak potential, takes a value of -250 mV similar to such obtained for the free molecule. This illustrates that there is no alteration of the electrochemical comportment, and especially reversibility and accessibility of **1** after intercalation. Moreover, we have verified by CV the linear variation of I_{peak} with the scan rate (not shown) indicating that **1** is effectively immobilized at the electrode surface and thus interacts with ds-DNA. As shown in Fig. 3A, the recorded amplitude for ds-DNA is about five times higher than such recorded for the single strand. This highlights the selectivity of the transduction process toward hybridised DNA and thus its reliability for the design of DNA sensors.

However, the intercalation process is rather slow since it takes nearly 90 min to reach completion while nonspecific interactions are completed within 50 min (Fig. 3B). Otherwise, the kinetic behaviour observed for ds-DNA shows two successive steps. The first step, from 0 to 50 min, presents the lower amplitude (e.g. 0.5 μA) and fits with the nonspecific interaction kinetic. Then, the second step, readily observable for durations longer than 50 min, may be associated to the intercalation process. It is also important to note that **1** can be adsorbed directly onto the pyrrole film (Fig. 3B dashed line). This adsorption is rapid and has reached a plateau after 15 min. In terms of DPV signal amplitude, we have recorded a peak current of about 1 μA which is sensitively higher (60 %) than such obtained for nonspecific interaction of **1** with ss-DNA. This indicates that **1** strongly adsorbed at the unmodified PPy surface, but this adsorption is significantly lowered by the presence of hydrophilic DNA molecules at the polymer surface. Otherwise, **1** may penetrate the polymer matrix despite the polycationic character of PPy which is maintained at its oxidised state during the intercalation process. Indeed these entrapped molecules may participate to charge transfer from the intercalated species in the ds-DNA to the platinum through the PPy film which is nonconductive at the detection potential.

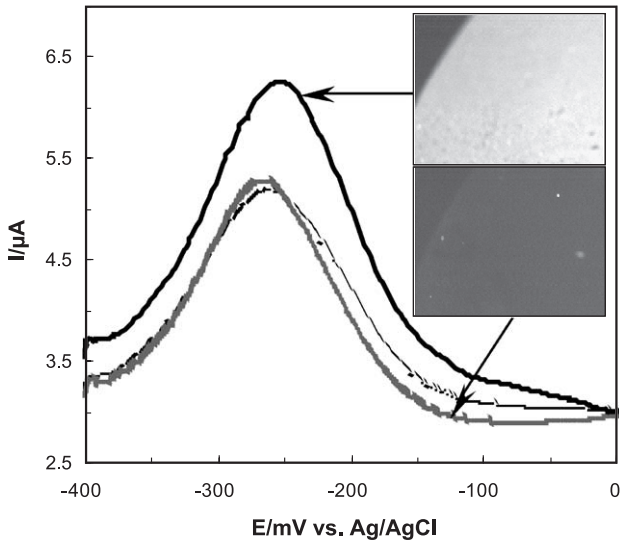


Fig. 4. DPV electrochemical detection of immobilized ODN probe hybridisation using **1** as electroactive label for the complementary 40 mer ODN (dark bold line), a noncomplementary 20 mer ODN (grey bold line) and pure PPy-ODN film (dark fine line). Hybridisation, intercalation and detection conditions as in Fig. 3. Fluorescence detection of DNA hybridisation using streptavidin–phycoerythrin conjugate. The fluorescent probe is coupled to the target ODN after hybridisation through the biotin–avidin bound.

Since **1** present a good selectivity toward ds-DNA, we have envisaged to design DNA sensors based on polypyrrole technology. For this purpose, adsorbed DNA was replaced by a synthetic ODN probe of medical interest covalently linked to the polymer matrix. Such a chemistry has been developed by Livache et al. [8] and, briefly, consists of the electrocopolymerization at electrode surfaces of an ODN functionalised by a pyrrole moieties through the phosphoramidite chemistry with unsubstituted pyrrole. This methodology allows to address complex surfaces and microelectrodes without loss of the ODN biological activity toward its complementary strand. We have synthesized here PPy-ODN films by electrocopolymerization of a solution containing a ratio of 1 ODN for 4500 pyrrole monomers. Such a ratio allows to obtain optimised fluorescence responses but it could be enhanced to a ratio of 1/700 without degradation of the ODN probe recognition capabilities toward its complementary target [12].

Fig. 4 displays the DPV responses of **1** after specific interactions with hybridised ODN probes (dark line), in the presence of noncomplementary 20 mer ODN (grey line) and finally after adsorption on the PPy-ODN matrix (dashed dark line).

Nonspecific responses due to **1** adsorption onto the PPy-ODN film in the presence and in the absence of noncomplementary strand present the same amplitudes and peak potential. This indicates that there is no induced variation of the nonspecific signal due to noncomplementary probes adsorption. Then, for DNA sensing applications, following base line correction, the subtraction of the response of **1** adsorption to the DPV obtained for hybridisation may be sufficient to obtain a quantitative response.

The DPV response obtained after ODN probes hybridisation with their complementary 40 mer ODN is more intense than the nonspecific ones and present a positive shift of potential already observed in Fig. 3. This highlights the effectiveness of the intercalation process. However, the selectivity of the electrochemical response toward ODN

hybridisation is relatively limited as the ratio of specific vs. nonspecific responses takes a value of 1.3. Thereby, the hybridisation process on ODN films was imaged by fluorescence microscopy. As displayed in Fig. 4 fluorescence intensity is higher for the hybridised ODN. Such a fluorescence response proofs the reliability of the electrochemical response for the detection of DNA hybridisation.

4. Conclusion

We have designed a new DNA intercalator which present suitable redox properties for the electrochemical detection of DNA hybridisation. This intercalator has been characterized in terms of signal selectivity in presence of ds-DNA or ss-DNA. Finally, this redox intercalator has been successfully used as electrochemical label in DNA sensor. However, the observed signal selectivity is rather low and further investigations are currently undergoing to enhance the sensor response, for example by studying the influence of the ODN probe density or by modifying **1** to enhance the DNA binding constant.

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