

Linear sweep polarographic determination of nucleic acids using acridine orange as a bioprobe

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Abstract: The interaction of acridine orange (AO) with double-stranded (ds) DNA in aqueous solution was investigated by linear sweep polarography (LSP) on a dropping mercury working electrode (DME). In pH 2.5 Britton–Robinson (B–R) buffer solution, AO had a sensitive linear sweep polarographic reductive peak at -0.89 V (vs. SCE), which could be greatly inhibited by the addition of dsDNA, with a positive shift of the peak potential. Based on the decrease of the reductive peak current, a new quantitative electrochemical determination method for dsDNA was developed with a linear range of 2.0 – 20.0 mg l⁻¹ and the linear regression equation: ΔI_p (nA) = 111.90 C (mg l⁻¹) + 125.32 ($n = 9$, $\gamma = 0.997$). The influences of commonly co-existing substances, such as metal ions, amino acid, etc., on the determination were also investigated. The method is sensitive, rapid and simple with good selectivity. The new proposed method was further applied to the detection of RNA and three synthetic samples containing dsDNA with satisfactory results. The binding number and the equilibrium constant between dsDNA and AO were calculated by an electrochemical method.

Keywords: acridine orange, nucleic acids, linear sweep polarography, electroanalysis.

INTRODUCTION

The electrochemistry of DNA was first discovered by Paleček *et al.* in the 1960s. DNA can produce redox signals at a mercury electrode and other solid electrodes, such as a metals, carbon and carbon nanotube modified electrodes.^{1–3} Some reviews have reported recent progress in the electrochemistry of DNA, electrochemical DNA biosensors, etc.^{4–7} The signals obtained from the working electrode were related to structural or conformational changes, hybridization and damage of DNA. The interaction of DNA with small molecules can result in the formation of DNA–molecule biocomplexes and have a large influence on the properties of DNA.⁸ Many analytical methods, such as spectrophotometry,⁹ fluorometry,¹⁰ light scattering,¹¹ etc., were also proposed for the detection of nucleic

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acids based on the interaction of DNA with organic dyes. Compared with these methods, electrochemical methods have some advantages, such as cheaper and smaller devices, a wider linear range and lower detection limit.¹² The explanation of the mechanism of the interaction of DNA with small molecules can further approach action mechanisms in cells or elucidate the binding process of drugs with DNA. Recently, the interaction of toxic chemicals and drugs with DNA was widely studied by an electrochemical method. Wang *et al.*¹³ established an electrochemical equation for examining the interaction of irreversible redox compounds with DNA. The electrochemical behavior of Hoechst 33258 and its interaction with DNA was investigated using cyclic voltammetry and other electrochemical techniques. The results showed that Hoechst 33258 binds tightly to the minor groove of DNA and covers four base pairs. Jiao *et al.* used some organic dyes, such as Toluidine Blue¹⁴ and Malachite Green¹⁵ for the detection of dsDNA based on the formation of electroinactive complexes after the mixture of dsDNA with dye in solution. Bard *et al.* reported the interaction of metal chelates of tris(1,10-phenanthroline)cobalt(III) with DNA in an aqueous medium by cyclic voltammetry and established a redox current equation for the intercalator–DNA complex. The result showed that after the addition of DNA, the change of the voltammetric responses of the reaction solution were caused by changes of the diffusion coefficient of the DNA complex.^{16–17} Paleček *et al.* studied the interaction between the anti-cancer drug mitomycin C and DNA by cyclic voltammetry on a hanging mercury drop electrode, which resulted in a decrease of the voltammetric current of the nucleic acid activated mitomycin C and a guanine oxidation signal.¹⁸

The interactions of the cationic dye acridine orange (AO, molecular structure shown in Fig. 1) with nucleic acids, such as dsDNA and RNA, have been carefully investigated by different methods.^{19–21} For example, AO has been used as a sensitive fluorescence probe for DNA. Bi *et al.*²² studied the interaction of flavonoids, such as quercetin, kaempferide and luteolin, with fish sperm dsDNA using AO as a fluorescence probe to distinguish the variation of spectroscopic characteristics. Cao *et al.*²³ applied the fluorescence energy transfer between AO and safranin T for the determination of DNA. Gherghi *et al.*²⁴ studied the interaction of AO and ethidium bromide (EB) with DNA in solution by alternating current voltammetry on a hanging mercury drop electrode based on the changes of the direct electrochemical responses of DNA at -1.20 V.

In this study, the linear sweep polarographic method was employed to investigate the interaction of AO with dsDNA. It was found that the addition of dsDNA into AO solution greatly decreased the reductive peak current of AO with a positive shift of the peak potential, which are typical characteristic of the intercalation model of small molecules with DNA. By monitoring the electrochemical behavior of AO before and after the addition of dsDNA, the electrochemical parameters were calculated and compared. The decrease of reductive peak current

was further used to establish a sensitive detection method for microamounts of nucleic acids.

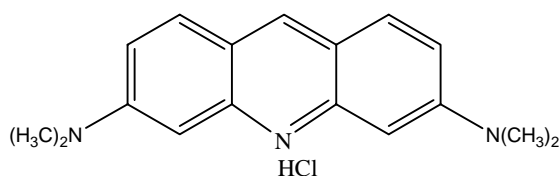


Fig.1. The molecular structure of acridine orange (AO).

EXPERIMENTAL

Instruments

A JP-303 polarographic analyzer (Chengdu Instrumental Factory, China) was used for polarographic detection with a drop mercury electrode (DME) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode. A DS model 2004 electrochemical analyzer (Shandong Dongsheng Electronic Instrument, China) was used for the cyclic voltammetric experiments with a DS-991 hanging mercury drop electrode (HMDE) as the working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode. All the measurements were carried out at a temperature of 25 ± 1 °C, except when otherwise stated. The UV-Vis absorption spectra were recorded on a Cary 50 probe spectrophotometer (Varian, Australia). A pHs-3C pH meter (Shanghai Leici Instrumental Factory, China) was used for pH measurements.

Chemicals

Double-stranded (ds) calf thymus DNA was obtained from Beijing Jinke Biochemical Company and used without further purification. A 1.0 g l^{-1} stock solution of dsDNA was prepared by dissolving the DNA in doubly distilled water and stored at 4 °C. The purity of the dsDNA was determined by the absorbance value of $A_{260}/A_{280} = 1.85$, which indicated that it was free of protein. The concentration of dsDNA was determined by measuring the absorbance at 260 nm. RNA was obtained from Sinopharm Chemical Reagent Co. Ltd, and the preparation procedure was the same as that of ds DNA. $1.0 \times 10^{-3} \text{ mol l}^{-1}$ acridine orange (AO, Shanghai Chemical Reagent Company) was prepared by directly dissolving 0.04380 g AO in water and diluting to 100 ml. A series of 0.2 mol l^{-1} Britton-Robinson (B-R) buffer solution was used to control the acidity of the interaction solution and also used as the supporting electrolyte for the electrochemical measurements. All the employed chemicals were of analytical reagents grade and doubly distilled water was used throughout the experiments.

Procedure

In a 10 ml flask, the interaction solutions were prepared with 2.0 ml pH 2.5 B-R buffer solution, 4.0 ml of $1.0 \times 10^{-4} \text{ mol l}^{-1}$ AO solution and different amounts of dsDNA solution. The mixture was shaken homogeneously, stood at 25 °C for 20 min and then subjected to the electrochemical studies. The potential range was selected in the range from -0.7 to -1.0 V and the peak current of AO at -0.89 V (vs. SCE) was recorded.

RESULTS AND DISCUSSION

UV-Vis absorption spectra

The UV-Vis absorption spectra of AO and its mixture with dsDNA are shown in Fig. 2, from which it can be seen that AO exhibits an absorption maximum at 495 nm with a shoulder at 488 nm (curve 1). When dsDNA was added into the

AO solution, the absorbance maximum decreased and was red-shifted by about 4 nm (curve 2 and 3). According to Barton,²⁵ the hypochromism and red shift of the wavelength are typical characteristics of intercalation of small molecules with dsDNA. Hence, the changes of the UV–Vis absorption spectrum of AO in the presence of dsDNA indicate that intercalation had occurred in the mixed solution.

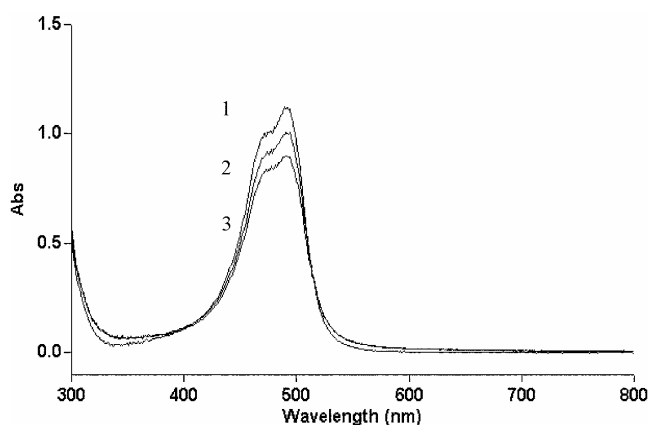


Fig. 2. The UV–Vis absorption spectra of AO and dsDNA in B–R buffer pH 2.5: 1) 4.0×10^{-5} mol l⁻¹ AO; 2) 4.0×10^{-5} mol l⁻¹ AO + 10.0 mg l⁻¹ dsDNA and 3) 4.0×10^{-5} mol l⁻¹ AO + 20.0 mg l⁻¹ dsDNA.

Cyclic voltammogram of AO and its mixture with dsDNA

The cyclic voltammograms of AO and its mixture with dsDNA are shown in Fig. 3. AO had a reduction peak at -0.89 V (vs. SCE) (curve 1) and did not have any oxidation peak in the potential range from -0.7 to -1.0 V. These results indicated that AO participated in an irreversible electrode process on the mercury electrode. After the addition of dsDNA into the AO solution, the peak current decreased and the peak potential moved positively (curve 2), which are typical characteristics of intercalation of small molecules with dsDNA.¹²

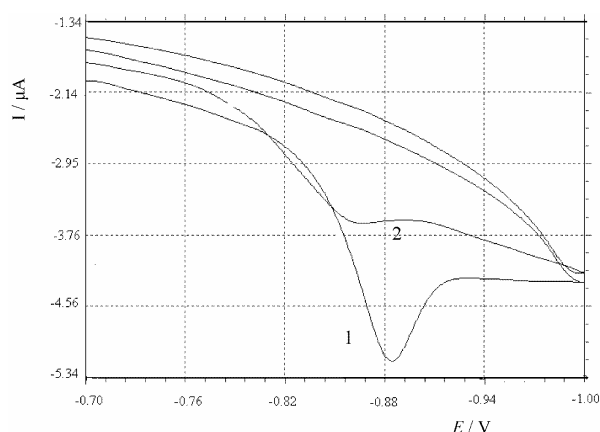


Fig. 3. Cyclic voltammogram of AO in B–R buffer pH 2.5: 1) 4.0×10^{-5} mol l⁻¹ AO and 2) 4.0×10^{-5} mol l⁻¹ AO + 10.0 mg l⁻¹ dsDNA.

The peak current increased with increasing scan rate and the relationship of peak current and scan rate is shown in Fig. 4. Irrespective of the presence of

DNA, a good linear relationship of the peak current vs. the square root of scan rate ($v^{1/2}$) was obtained, indicating that the electrode process was completely controlled by the diffusion of the electroactive substances to the surface of the mercury electrode.

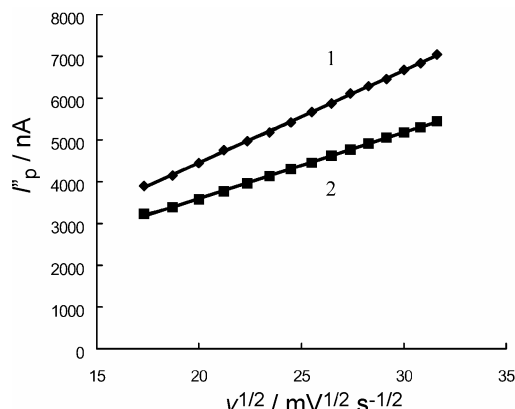


Fig. 4. The dependence of the peak current on the square root of the scan rate. Data taken from Fig. 3.

The electrochemical parameters of the reaction solution before and after the addition of dsDNA were calculated by the following equation for an irreversible electrode process:¹²

$$\ln I_p = -\frac{\alpha n F}{RT}(E_p - E_0) + \ln 0.227 n F A k_s c_0^*$$

where α is the electron transfer coefficient, k_s the standard rate constant of the surface reaction, E_0 the formal potential, n the electron transfer number, A the surface area of a mercury drop and c_0^* the concentration of AO.

According to the above equation, αn and k_s can be determined from the slope and the intercept of the linear equation of $\ln I_p$ against $(E_p - E_0)$. E_0 can be determined from the intercept of E_p vs. v plot by extrapolating the line to $v = 0$. By this method, the electrochemical parameters were calculated and the results are shown in Table I.

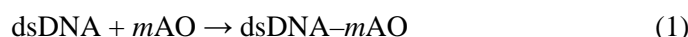
TABLE I. The electrochemical parameters of AO in the absence and in the presence of dsDNA

Parameter	AO	AO-dsDNA
E_0 / V	-0.975	-0.955
αn	0.504	0.253
k_s / s^{-1}	0.357	0.220

Comparing the two groups of the parameters, the values of αn and k_s in the absence and presence of dsDNA show distinct differences; hence the formed AO-dsDNA supramolecular complex was electroactive. In the mixed reaction solution, the diffusion coefficient of AO-dsDNA complex was decreased, which resulted in the decrease of the reduction peak currents.

Measurement of stoichiometry of dsDNA–mAO supramolecular complex

After the method proposed by Li,²⁶ it was assumed that AO and dsDNA produced a single complex of dsDNA–mAO. The binding number (m) and the equilibrium constant (β_s) between dsDNA and AO could be determined by the following method:



The equilibrium constant of the reaction, β_s is:

$$\beta_s = \frac{[\text{dsDNA–}m\text{AO}]}{[\text{dsDNA}][\text{AO}]^m} \quad (2)$$

The following equations can be deduced:

$$\Delta I_{\max} = kc_{\text{dsDNA}} \quad (3)$$

$$\Delta I = k[\text{dsDNA–}m\text{AO}] \quad (4)$$

$$[\text{dsDNA}] + [\text{dsDNA–}m\text{AO}] = c_{\text{dsDNA}} \quad (5)$$

Therefore:

$$\Delta I_{\max} - \Delta I = k(c_{\text{dsDNA}} - [\text{dsDNA–}m\text{AO}]) = k[\text{dsDNA}] \quad (6)$$

Introducing Eqs. (1), (3) and (5) gives:

$$\log [\Delta I / (\Delta I_{\max} - \Delta I)] = \log \beta_s + m \log [\text{AO}] \quad (7)$$

where ΔI is the difference between the peak current of the sample and blank, ΔI_{\max} corresponds to the maximum value of the difference of the peak currents, c_{dsDNA} , $[\text{dsDNA–}m\text{AO}]$, $[\text{dsDNA}]$ corresponds to the total, bound and free concentration of dsDNA in the solution, respectively.

From Eq. (7), the relation of $\log [\Delta I / (\Delta I_{\max} - \Delta I)]$ with $\log [\text{AO}]$ was calculated and plotted with the linear regression equation as $\log [\Delta I / (\Delta I_{\max} - \Delta I)] = 1.113 \log [\text{AO}] + 4.963$ ($n = 7$, $\gamma = 0.995$). From the intercept and the slope, a value of $m = 1$ and $\beta_s = 9.183 \times 10^4$ were deduced, which indicates that a stable 1:1 complex of dsDNA–AO was formed under the selected conditions.

Second-order derivative linear sweep voltammogram

In order to improve the detection sensitivity, second-order derivative linear sweep polarography was used for nucleic acids and their determination. A typical linear sweep voltammogram of the AO–dsDNA reaction system is shown in Fig. 5. Curve 1 is the voltammogram of B–R buffer, for which no redox response appeared. Curve 2 is the voltammogram of AO in B–R buffer solution and a sensitive linear sweep reduction peak at -0.89 V (*vs.* SCE) appeared, which was due to the electrode reduction of the pyridine group. After the addition of dsDNA into the AO solution, the peak current decreased greatly with a positive shift of the peak potential (curve 3 and 4), which indicates that dsDNA intercalation with AO could lead to decrease in the electrochemical response. With increasing amount

of added dsDNA, the greater was the decrease in the peak current, a fact which could be employed for the determination of dsDNA.

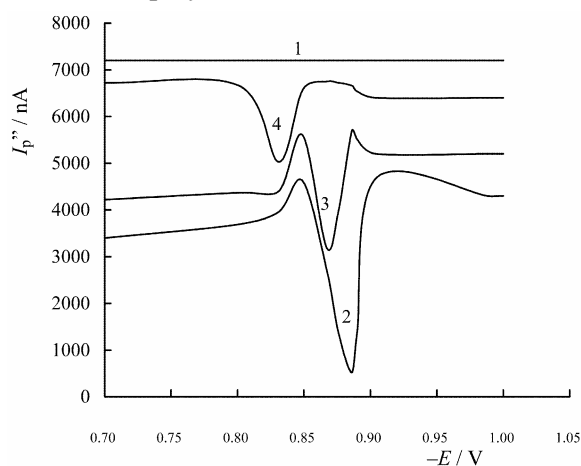


Fig.5. Second order derivative linear sweep voltammogram of 1) B-R buffer pH 2.5; 2) B-R buffer pH 2.5 + 4.0×10^{-5} mol l^{-1} AO; 3) B-R buffer pH 2.5 + 4.0×10^{-5} mol l^{-1} AO + 10.0 mg l^{-1} dsDNA and 4) B-R buffer pH 2.5 + 4.0×10^{-5} mol l^{-1} AO + 15.0 mg l^{-1} dsDNA.

Optimal of reaction conditions

The influence of pH on the binding reaction was investigated and the results are shown in Fig. 6. The highest value of the difference of peak current was observed at pH 2.5, hence this pH was chosen for the assay. The amount of 0.2 mol l^{-1} B-R was varied and in the volume range 1.0 to 3.0 ml, the peak current remained constant, hence 2.0 ml of B-R buffer solution was selected.

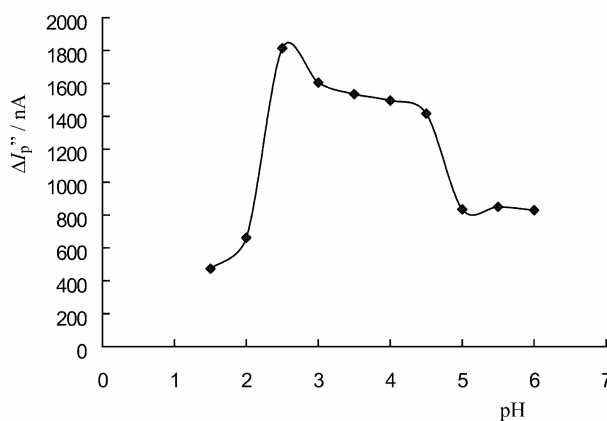


Fig.6. Influence of the pH of the B-R buffer solution on the binding reaction. The solution contained 3.0×10^{-5} mol l^{-1} AO + 10.0 mg l^{-1} dsDNA.

The concentration of AO on the binding reaction was examined by fixing the dsDNA concentration at 10.0 mg l^{-1} . As shown in Fig. 7, the peak current increased with increasing AO concentration and reached maximum at 4.0×10^{-5} mol l^{-1} AO. Hence, an AO concentration of 4.0×10^{-5} mol l^{-1} was used in this experiment.

The binding reaction occurred rapidly at 25 °C and reached equilibrium in about 20 min. The peak current remained constant for about 2 h and showed good stability.

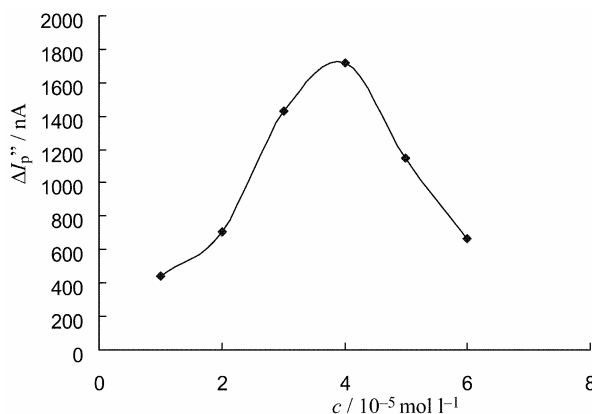


Fig.7. The influence of AO concentration on the current peak in B-R buffer pH 2.5 containing 10.0 mg l^{-1} dsDNA and different concentration of AO.

Optimal of detection conditions

The experimental conditions, such as dropping mercury standing time and the scan rate, on the peak currents were varied. With increasing the mercury drop standing time and the scan rate, the peak current increased correspondingly. The results were recorded at a standing time of 10 s and at a scan rate of 700 mV s^{-1} .

Calibration curves

Under the selected condition, the proposed electrochemical method was applied to the determination of dsDNA and RNA. The decrease of the peak current was proportional to the concentration of the nucleic acids and the analytical results are shown in Table II. It can be seen that this method had good sensitivity and a wide linear range.

TABLE II. Analytical parameters for different nucleic acids

Nucleic acid	Standard regression equation	Linear range mg l^{-1}	Detection limits mg l^{-1}	Regression coefficient (γ)
DNA	$\Delta I_p'' (\text{nA}) = 111.90c (\text{mg l}^{-1}) + 125.32$	2.0–20.0	0.051	0.997
RNA	$\Delta I_p'' (\text{nA}) = 131.84c (\text{mg l}^{-1}) + 88.46$	1.0–20.0	0.020	0.998

Influence of interferences

The influence of metal ions, amino acids, detergents, *etc.*, on the determination of 10.0 mg l^{-1} dsDNA was investigated and the results are shown in Table III. It can be seen that some metal ions and amino acids interfered with this assay. However, the detergents had a great influence on the determination, which was due to the adsorption of the detergent on the surface of the mercury electrode.

Sample determinations

Three synthetic samples containing dsDNA and different coexisting substances were determined by the proposed method and the results are shown in Table IV. It can be seen that the results are satisfactory with the recovery in the range of 101.5–105.6 %.

TABLE III. Effect of co-existing substances on the determination of 10.0 mg l⁻¹ DNA

Co-existing substance	Concentration mg l ⁻¹	Relative error %	Co-existing substances	Concentration mol μl ⁻¹	Relative error %
L-Arginine	2.0	-1.51	Mg ²⁺	2.0	3.43
L-Leucine	2.0	1.87	Ni ²⁺	2.0	0.72
L-Glutamine	2.0	-4.15	Pb ²⁺	2.0	-0.53
L-Cysteine	2.0	0.74	Cu ²⁺	2.0	1.81
L-Glycine	2.0	-1.87	CATB ^a	2.0	20.23
L-Threonine	2.0	3.00	SDS ^b	2.0	19.26
Citric acid	2.0	-1.70	β-CD ^c	2.0	11.11

^aCetyltrimethylammonium bromide; ^bsodiumdodecylsulfate; ^cβ-cyclodextrin.

TABLE IV. Analytical results of dsDNA in synthetic samples (*n* = 5)

Sample	Foreign co-existing substances	Added mg l ⁻¹	Found mg l ⁻¹	Recovery %	RSD %
1.	L-Arginine, L-Tyrosine, Ni ²⁺ , Zn ²⁺	10.0	10.15	101.5	5.46
2.	L-Arginine, L-Tyrosine, Fe ³⁺ , Zn ²⁺	10.0	10.56	105.6	5.02
3.	L-Arginine, Urea, Ni ²⁺ , Zn ²⁺	10.0	10.52	105.2	5.11

CONCLUSIONS

In this work the interaction of AO with dsDNA was studied by linear sweep polarography and the electrochemical parameters were calculated and compared. The results indicate that an electroactive complex was formed, which resulted in a decrease of the diffusion coefficient of the complex and a decrease of the reduction peak current. The positive movement of the reduction peak potential of the reaction system indicated that AO can intercalate with dsDNA. Based on the decrease of the peak current, a sensitive electrochemical method was developed for the determination of nucleic acids, such as dsDNA and RNA. The proposed method is sensitive, practicable, reproducible and applicable to the determination of synthetic samples.

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ИЗВОД

ОДРЕЂИВАЊЕ НУКЛЕИНСКИХ КИСЕЛИНА ПОМОЋУ АКРИДИН–ОРАНЖ БИО-ПРОБЕ ПОЛАРОГРАФИЈОМ СА ЛИНЕАРНО ПРОМЕНЉИВИМ ПОТЕНЦИЈАЛОМ

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Испитивана је интеракција акридин–оранжа (АО) и дуплекс–ДНК у воденом раствору, коришћењем поларографије са линеарно променљивим потенцијалом и капљуће живине електроде. У Britton–Robinson пуферском раствору, рН 2,5, АО показује редукциони струјни

врх на $-0,89$ V (према ЗКЕ) који се додатком дуплекс-ДНК значајно инхибира и помера ка позитивнијим потенцијалима. На основу смањења редукционог струјног врха развијена је нова квантитативна електрохемијска метода одређивања дуплекс-ДНК са опсегом линеарности $2,0-20,0$ mg l^{-1} и једначином линеарне регресије: ΔI_p (nA) = $111,90C$ (mg l^{-1})+ $125,32$ ($n = 9$, $\gamma = 0,997$). Такође је испитиван утицај супстанци које су уобичајени пратиоци (јони метала, аминокиселине) на тачност методе. Метода је осетљива, брза, једноставна и одликује се добром селективношћу. Такође је примењена на детекцију РНК и на три синтетичка узорка који су садржали дуплекс-ДНК и у свим случајевима су резултати били задовољавајући. Електрохемијском методом је израчунат и број молекула АО везаних за дуплекс-ДНК као и одговарајућа константа равнотеже.

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