INVESTIGATION OF CATECHIN AND ACRIDINE DERIVATIVES USING VOLTAMMETRIC AND FLUORIMETRIC DNA-BASED SENSORS

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Received November 5, 1999
Accepted April 25, 2000

Presented at International Conference on Modern Electroanalytical Methods, Seč, September 19–23, 1999.

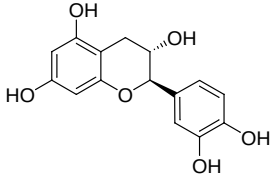
The preconcentration-differential pulse voltammetric detection scheme with a carbon paste electrode bulk phase modified with DNA was used for the investigation of behaviour and the determination of trace levels of catechin and acridine derivatives. The effect of electrochemical activation of the electrode and the DNA-type was examined. The results are compared with those obtained by fluorimetric measurement of the TO-PRO-3 dye : DNA complex in the presence of analytes. With voltammetric biosensors, the detection limits are in the nmol l^{-1} concentration region. Using competitive reagents, the intercalation of analytes to DNA is indicated.
Key words: DNA biosensors; DP voltammetry; Fluorescence DNA dye; Catechins; Acridines; Carbon paste electrodes; Electrochemistry.

Hybridization reaction, accumulation of specific guest molecules and sensitivity to cleaving agents rank among the main analytical features of DNA biosensors. DNA-modified mercury and carbon electrodes were introduced already in the 80's (refs[1–4]) and a graphite electrode supported DNA probe was named the DNA sensor[5]. Electrochemical methods are today established as important tools for the determination of nucleic acids and their interactions[6,7]. For the trace analysis of some important analytes such as drugs, pollutants and risk chemicals, a preconcentration ability of the double-stranded (ds) DNA attached to the electrochemical transducer is of interest[8–15]. Voltammetric signals of an electroactive analyte or special electrochemical marker as well as a constant-current chronopotentiometric stripping (CPSA) peak of the DNA guanine moiety were exploited in the

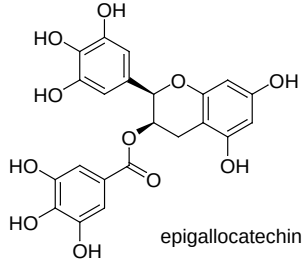
measurement. Simple preparation, high stability and large dynamic concentration range were shown as advantages of the dsDNA modified conventional as well as screen-printed electrodes[15,16].

Intercalative and non-intercalative (groove) binding of the analyte as the guest molecule to the dsDNA receptor are usually considered and multiple binding modes have been also found[15,17,18]. Recently, differences in DNA interactions in solution and at the electrode surface were reported. In the later case, a guest molecule is expected to bind only to the side of the DNA duplex oriented toward the solution phase while the opposite side of the DNA laying flat on the electrode is not accessible[13]. With respect to the surface morphology of the confined DNA-layer, the binding mode determined at other conditions (*e.g.* in solution or crystal structure) should be considered carefully. On the other hand, the DNA-derivatized electrodes provide a valuable methodology to examine DNA-bound redox reactions and DNA-mediated electron transfer[19].

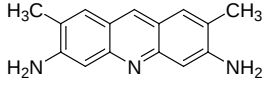
An optical biosensing strategy for detection of compounds with affinity for DNA was also introduced recently[20]. It is based on measuring changes in the fluorescence signal intensity of a monomeric derivative of Thiazole Orange, TO-PRO-3, complexed with DNA (ref.[17]). The optical sensor is capable of indirect detecting intercalating and groove binding compounds.



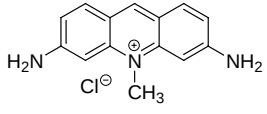
catechin



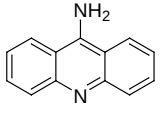
epigallocatechin



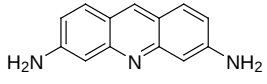
Acridine Yellow



acriflavine



9-aminoacridine



proflavine

We report here on the investigation of some catechin and acridine derivatives using the carbon paste electrode (CPE) bulk phase modified with DNA and the fluorimetric biosensor with the long-wavelength nucleic acid dye TO-PRO-3. The catechin derivatives were chosen as potential antimutagenic and anticancer agents present in tea. Preliminary optical experiments with tea extract indicate the presence of a compound strongly interacting with DNA (ref.[20]). Acridines are known to exhibit a broad spectrum of biological activities, including mutagenity (*e.g.* proflavine). Their derivatives are used as antibacterial and anticancer drugs[21]. Acridine dyes are considered as classic intercalators to DNA in solution. Numerous studies devoted to this topic were published and reviewed since 1960's (ref.[22]). By crystal structure analysis it was shown that Acridine Orange binds by intercalation in the cavity created by stretching the backbone of the two base pairs and the bond uses electrostatic and van der Waals interactions and minimization of exposed hydrophobic surface area[23].

The aim of this study was the investigation of voltammetric behaviour of catechin and acridine derivatives and the development of procedure for their determination using DNA-modified electrode. The results were validated by intermethodic comparison with fluorimetric assay. The binding mode of analytes was also considered.

EXPERIMENTAL

Apparatus and Reagents

A computerized voltammetric analyzer ECA pol, model 110 (Istran, Bratislava) with a modified CPE, a platinum auxiliary electrode and a silver/silver chloride (3 mol l^{-1} KCl) reference electrode were used for the measurement. A fiber fluorimeter (Royal Veterinary and Agricultural University, Copenhagen) with a 642 nm light-emitting laser diode was used and the signal output was read using a standard voltmeter.

The catechin and acridine derivatives were purchased from Sigma and used as received. Calf thymus DNA (high molecular, Catalog No. 124013) was obtained from Merck and used as received. The salmon sperm DNA (Catalog No. D 1626) was from Sigma and prepared as described previously[20]. The TO-PRO-3 dye was obtained from Molecular Probes (Eugene (OR), U.S.A.) and Doxorubicin from Sigma.

Stock solutions of $2.5 \cdot 10^{-3}$ mol l^{-1} catechin derivatives were prepared in 10% aqueous DMSO and $1 \cdot 10^{-2}$ mol l^{-1} solutions of acridine compounds were prepared in DMSO. Stock solutions of DNA (5 mg ml^{-1} calf thymus DNA or 250 μg ml^{-1} salmon sperm DNA) were prepared with a Tris-HCl–EDTA solution ($1 \cdot 10^{-2}$ mol l^{-1} Tris-HCl and $1 \cdot 10^{-3}$ mol l^{-1} EDTA, pH 8.0) and stored at –5 °C. The TO-PRO-3 stock solution was prepared as $1 \cdot 10^{-4}$ mol l^{-1} in 10% aqueous DMSO and stored at –20 °C. The stock solution of Doxorubicin was $7 \cdot 10^{-4}$ mol l^{-1} in distilled water. All other chemicals were of analytical reagent grade purity. Deionized, doubly distilled water was used throughout. The experiments were carried out at laboratory temperature (23 °C).

Preparation of the Modified Electrodes

For preparation of DNA modified CPEs, 0.500 g of graphite powder (Elektrokarbon, Topoľčany, Slovak Republic) was thoroughly mixed with 200 μl of the DNA stock solution and 1 ml of $5 \cdot 10^{-3}$ mol l^{-1} phosphate buffer (pH 7.0) and allowed to dry. The modified carbon powder was hand-mixed with 0.110 g of white pharmaceutical vaseline, a Teflon sleeve (2.3 mm i.d.) was filled with the paste and provided with a steel electric contact. The bare CPE was prepared by the same procedure omitting the treatment of the graphite powder with DNA.

Procedures

For a voltammetric measurement, a freshly polished DNA-modified CPE was activated electrochemically using a conventional anodic pretreatment of CPE by the constant-potential polarization at 1.7 V for 1 min in stirred phosphate buffer solution. For non-activated DNA/CPE, this pretreatment was omitted. The electrode was transferred into the test solution and the analyte was accumulated at a given potential (catechins at –0.350 V and acridines at –0.200 V) for a desired time (typically 60 or 90 s) under stirring. The DP voltammogram was recorded directly in the test solution using 100 mV pulse amplitude and 25 mV s^{-1} scan rate. After subtraction of the curve corresponding to the blank, the DP peak height was evaluated using standard software. The electrode surface was renewed mechanically after each measurement and the activation procedure was repeated.

Fluorimetric assay was performed as follows: to 1 248 μl of Millipore-purified water, 75 μl Tris-HCl (1.0 mol l^{-1}, pH 7.5), 3 μl EDTA (0.5 mol l^{-1}, pH 8) and 10 μl TO-PRO-3 ($1 \cdot 10^{-4}$ mol l^{-1}) were added, shortly vortexed, then 10 μl of salmon sperm DNA (250 μg ml^{-1} in Tris-HCl–EDTA buffer) was added, vortexed again and the mixture was allowed to equilibrate for 30 min in the dark. Then 54 μl of the equilibrated TO-PRO-3 mixture with DNA and 6 μl of analyte were pipetted into microfuge tubes and stored for 30 min in the dark. The samples were transferred to capillary tubes (40 μl) which were then slid onto the end of the optical fiber, the fiber was wiped clean with a tissue and the signal was read after 30 s. The measurements were performed in triplicate.

RESULTS AND DISCUSSION

Two DNA samples were tested: calf thymus (cth) DNA (the high molecular, for biochemistry) used as received and the salmon sperm (ssp) DNA which was purified as described previously[20]. The corresponding DNA-modified electrodes are denoted DNA_{cth}/CPE and DNA_{ssp}/CPE.

The effect of an electrochemical activation of the carbon surface at carbon paste[24] and carbon-based screen-printed[25] electrodes on the voltammetric response of analytes in solution is well known. At the activation the pasting binder is removed from the carbon particles and their surface becomes more hydrophilic. The constant-potential pretreatment of CPE at 1.7 V for 1 min is usually used prior to a surface-modification of CPE by the fixation of DNA from solution[8–10,13].

In this work, we investigate the bulk phase-DNA-modified CPE with DNA adsorbed at carbon particles prior to mixing with pasting binder. This approach allows to enhance the fixed amount of DNA and, consequently, the response when compared with the surface-modified CPE (ref.[14]). The effect of the electrochemical activation of the DNA/CPE was tested using the anodic pretreatment mentioned above. The sensor is denoted as activated electrode and it is compared with DNA/CPE without the anodic pretreatment (non-activated electrode). The role of activation may be in a better accessibility of the attached DNA to the analyte molecule.

Catechins

Figures 1 and 2 show DP voltammograms of catechin and epigallocatechin obtained with non-activated DNA_{cth}/CPE (curves *2*). For comparison, the voltammogram recorded with non-activated bare CPE is also presented (curves *1*). An enhancement of the signal using the DNA/CPE compared with bare CPE confirms an accumulation of the analytes in the DNA layer. The signal enhancement is higher at lower concentration of analytes. The voltammetric measurements were carried out immediately after the preconcentration step in analyte solution without transfer of the electrode into blank supporting electrolyte. The anodic DP signal of the DNA guanine moiety observed with DNA/CPE at 0.880 V does not change significantly in the presence of catechins.

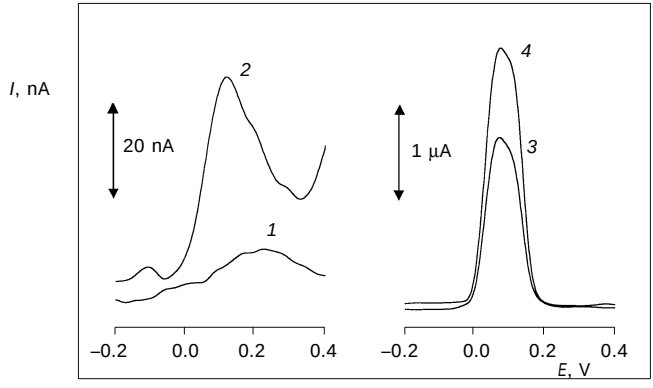


FIG. 1
DP voltammograms of catechin $5 \cdot 10^{-6}$ mol l^{-1} solution (corrected for blank) after 60 s accumulation at –0.350 V with non-activated CPE (*1*), non-activated DNA_{cth}/CPE (*2*), activated CPE (*3*) and activated DNA_{cth}/CPE (*4*) (0.05 mol l^{-1} phosphate buffer, pH 7.0, pulse amplitude 100 mV, scan rate 25 mV s^{-1})

The DP peaks of catechin derivatives obtained with the activated CPE and the activated DNA_{cth}/CPE are depicted in Figs 1 and 2, curves *3* and *4*. The peaks are of the same shape as with the non-activated electrode, however, the maximum peak currents are significantly higher. The determination of catechin was repeated twice a day within one week and the relative standard deviation (RSD) of 10% is comparable with that calculated for the signal of non-activated electrode. Hence, the activation procedure applied to DNA/CPE does not disturb the accumulation ability of DNA.

The DP peak potentials of 0.069 V with a shoulder at 0.110 V for catechin and –0.036 and 0.060 V for epigallocatechin were observed with activated DNA/CPE after 60 s accumulation at –0.350 V from $5 \cdot 10^{-6}$ mol l^{-1} solution, 100 mV pulse amplitude and 25 mV s^{-1} scan rate. The peak potential of catechin is shifted by +3 mV and that of epigallocatechin by –13 mV compared to bare CPE. The accumulation of catechins does not depend on the polarization potential in the range from –0.600 to 0 V. The DP peak height for $5 \cdot 10^{-7}$ mol l^{-1} catechin increases to 60 s accumulation time and then the dependence tends to level off.

About two-fold response values were obtained using the activated DNA_{ssp}/CPE when compared with DNA_{cth}/CPE. With respect to the twenty-fold lower DNA total amount in DNA_{ssp}/CPE than in DNA_{cth}/CPE, the preconcentration effect of purified salmon sperm DNA is much higher. This could be explained by a different purity degree of the DNA samples, partic-

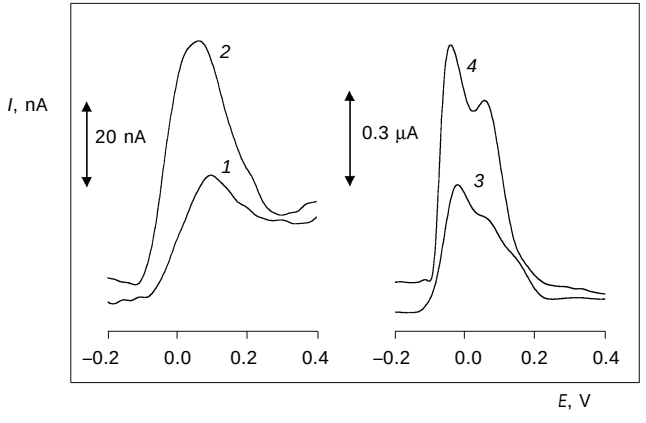


FIG. 2
DP voltammograms of epigallocatechin in $5 \cdot 10^{-6}$ mol l^{-1} solution (corrected for blank) after 60 s accumulation at –0.200 V with non-activated CPE (*1*), non-activated DNA_{cth}/CPE (*2*), activated CPE (*3*) and activated DNA_{cth}/CPE (*4*) (for other conditions see Fig. 1)

ularly regarding the content of proteins and single-stranded DNA. The purity of DNA samples was not characterized.

Linear calibration curves (correlation coefficient $R > 0.995$) were obtained for two concentration ranges of catechins ($2 \cdot 10^{-8}$ to $1 \cdot 10^{-6}$ and $4.5 \cdot 10^{-7}$ to $3.2 \cdot 10^{-6}$ mol l^{-1}). The detection limits (6σ) of $2.2 \cdot 10^{-9}$ and $8.9 \cdot 10^{-9}$ mol l^{-1} achieved after 60 s accumulation with activated DNA_{cth}/CPE were estimated from the signal-to-noise ratio for $1.3 \cdot 10^{-8}$ mol l^{-1} catechin and $4.8 \cdot 10^{-8}$ mol l^{-1} epigallocatechin, respectivelly. The repeatability of the measurement ($n = 7$) is given by the RSD of 8.4% for $8.4 \cdot 10^{-7}$ mol l^{-1} catechin and 10.5% for $9.0 \cdot 10^{-6}$ mol l^{-1} epigallocatechin at 60 s accumulation.

The catechin derivatives displace the TO-PRO-3 dye from its complex with salmon sperm DNA. A calibration curve for fluorimetric measurement is shown in Fig. 3. The strong binding competition of catechins with the intercalator TO-PRO-3 can be taken as an indication of their intercalative binding. According to the structure of catechins this mode of interaction could be expected[26].

For charged guest ions such as metal complex compounds exhibiting a charge change accompanied with their electrochemical redox reaction, a shift (negative or positive) of the formal (half-wave) potential value reflects the ratio of association constants for the reduced and oxidized form of the metal complex and indicates a dominant mode of the interaction with DNA (electrostatic or intercalative)[18,27,28]. However, this is not the case with the oxidation of catechins and, therefore, no direct conclusions regarding the type of interaction can be done from voltammetric measurements.

Separate experiments were done investigating the effect of known intercalating drug Doxorubicin (ref.[29]) on the accumulation of catechin at the

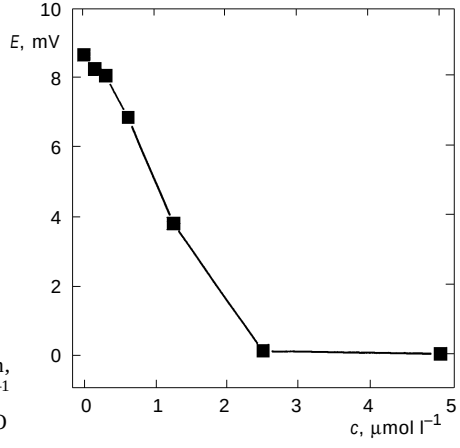


FIG. 3
Fluorimetric calibration plot for epigallocatechin, $6.7 \cdot 10^{-7}$ mol l^{-1} TO-PRO-3 dye, 0.050 mol l^{-1} Tris-HCl–0.001 mol l^{-1} EDTA, pH 7.5, 0.5% DMSO

activated DNA_{cth}/CPE. After the accumulation from an equimolar mixture of catechin with Doxorubicin and the transfer of the electrode to blank supporting electrolyte, the DP peak of catechin decreases to about 50% compared with the signal in the absence of Doxorubicin. This decrease is lower (to about 25%) that observed with bare CPE, where a strong adsorption of structurally similar drug Daunomycin was found[13,30]. It indicates a competitive preconcentration of catechin in DNA and may be considered as a confirmation of the intercalative DNA/catechin interaction.

Acridines

With respect to low solubility in aqueous solution, the acridine derivatives were investigated in 0.05 mol l^{-1} phosphate buffer solution (pH 7.0) with 0.5% DMSO. The compounds under study can be preconcentrated and oxidized with the DNA-modified CPE and they exhibit 50 to 100% enhancement of the anodic DP voltammetric peak with the non-activated DNA_{ssp}/CPE compared with the bare CPE. The signal increases with the electrochemically activated electrodes without significant change in the peak position and shape.

The DP voltammograms with activated DNA_{cth}/CPE (Fig. 4) consist of one peak at 0.842 V for 9-aminoacridine and two poorly separated peaks at about 0.6 and 0.7 V for all other derivatives (30 s accumulation at –0.200 V, 100 mV pulse amplitude and 25 mV s^{-1} scan rate). The peaks evidently correspond to oxidation of amino groups present in the molecule. The accu-

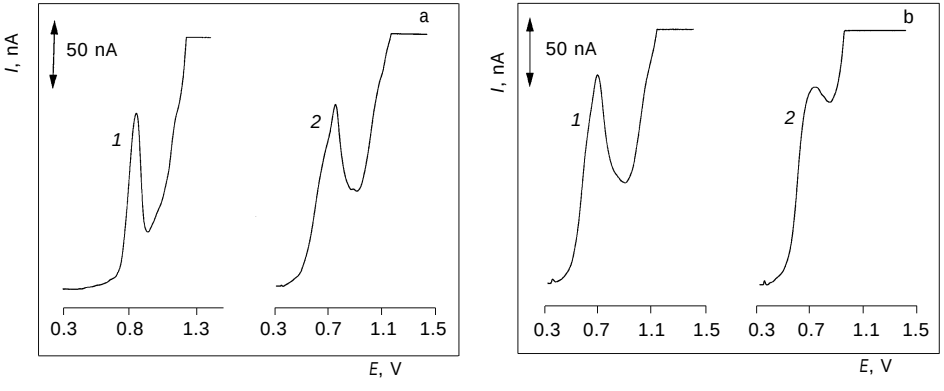


FIG. 4

DP voltammograms of acridines in $8 \cdot 10^{-7}$ mol l^{-1} solution after 30 s accumulation at –0.200 V with activated DNA_{cth}/CPE: a 9-aminoacridine (*1*), proflavine (*2*); b Acridine Yellow (*1*), acriflavine (*2*) (0.05 mol l^{-1} phosphate buffer, pH 7.0, 0.5% DMSO, for other conditions see Fig. 1)

mulation of acridines is not significantly influenced by the electrode potential in the range from –0.400 to +0.300 V. The DP peak height *vs* accumulation time dependences are linear to 90 s (the accumulation potential of –0.200 V) and then they exhibit a saturation. Considering similar signals with DNA_{cth}/CPE and DNA_{ssp}/CPE at lower DNA content in the later sensor, the high efficiency of the salmon sperm DNA sample was found again.

The calibration curves obtained with the activated DNA_{cth}/CPE and non-activated DNA_{ssp}/CPE are shown in Fig. 5. The differences are due to the electrode activation and not due to the use of two DNA/CPEs with different DNA samples. The activated electrodes are more sensitive to low concentrations of acridines, however, the response *vs* concentration dependences are much narrower than with the non-activated electrodes. Linear calibration curves (R = 0.992 to 0.999) were obtained with non-activated sensors for the concentration range from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-7}$ mol l^{-1} and 90 s accumulation.

The detection limits (6σ) of $2.6 \cdot 10^{-9}$, $8.3 \cdot 10^{-9}$, $8.9 \cdot 10^{-9}$ and $1.2 \cdot 10^{-8}$ mol l^{-1} were estimated from the signal-to-noise ratio for $1 \cdot 10^{-8}$ mol l^{-1} proflavine, Acridine Yellow, 9-aminoacridine and acriflavine, respectively, and 90 s accumulation with the activated DNA_{cth}/CPE. The repeatability of the voltammetric measurements (n = 9) is characterized by the RSD values of 7.7–13.7% for $8 \cdot 10^{-7}$ mol l^{-1} acridines (30 s accumulation) and 18.1–19.9% for $8 \cdot 10^{-8}$ mol l^{-1} acridines (90 s accumulation). The signal

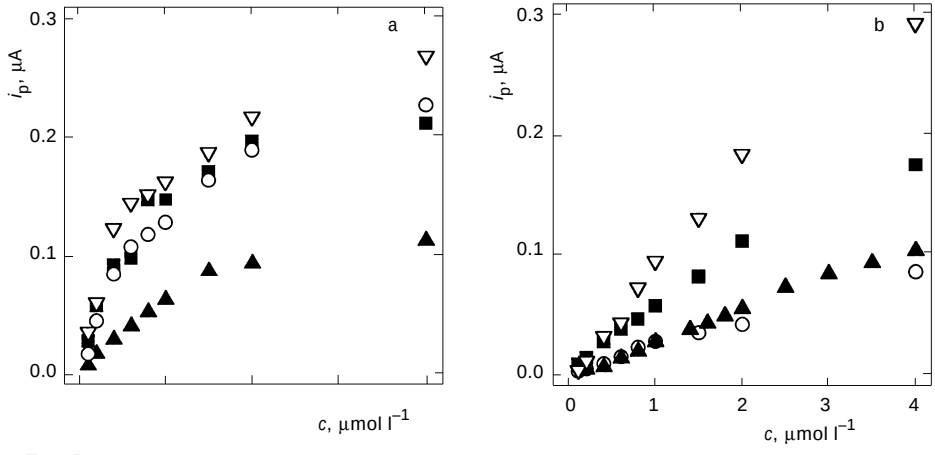


FIG. 5

Calibration data for acridines with activated DNA_{cth}/CPE (a) and non-activated DNA_{ssp}/CPE (b): 9-aminoacridine (○), proflavine (■), Acridine Yellow (▽), acriflavine (▲) (for other conditions see Fig. 4)

was stable to 95% after storage of the biosensor at 4 °C for 3 months using a usual mechanical regeneration of the electrode surface.

For 9-aminoacridine a competition with the equimolar concentration of Doxorubicin was observed with DNA_{cth}/CPE leading to about 50% decrease in the acridine signal. No change of the acridine signal was found with bare CPE. From this experiment it can be deduced that both substances interact with the receptor by intercalation.

Calibration curves for the fluorimetric measurement are shown in Fig. 6. From these curves, the relative binding affinities of the acridine derivatives to salmon sperm DNA can be estimated as follows: 9-aminoacridine < proflavine < Acridine Yellow < acriflavine. The affinities are closely related to the presence of primary and tertiary amino groups in the acridine derivatives. Based on the TO-PRO-3 displacement by compounds under study and taking into account the similar behaviour of Acridine Orange (ref.[31]), which is known to intercalate to DNA (ref.[23]), the intercalation of acridines to DNA in solution seems to be confirmed. The analyte binding to DNA is evidently supported by charged amino groups. These results correspond with those obtained voltammetrically with the DNA_{ssp}/CPE. The lowest slope of the calibration curve for 9-aminoacridine can be explained by the poorest binding of this compound as well as the presence of only one electroactive primary amino group in its molecule.

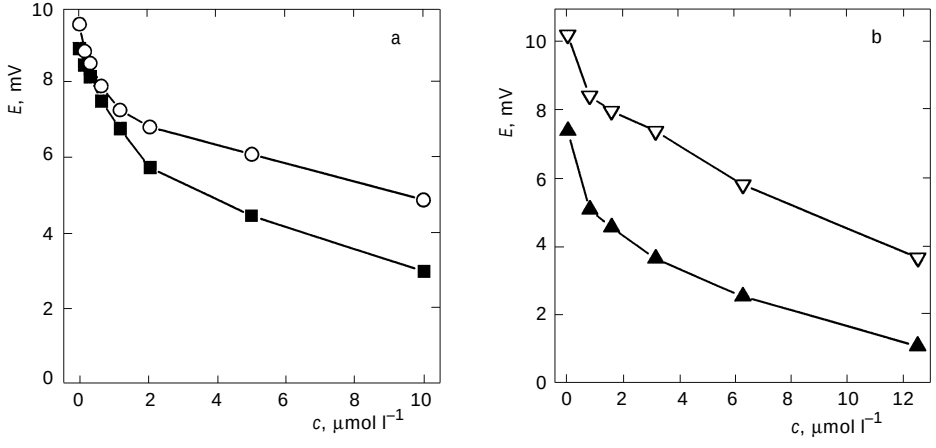


FIG. 6

Fluorimetric calibration curves for acridines: a 9-aminoacridine (○), proflavine (■); b Acridine Yellow (▽), acriflavine (▲) ($6.7 \cdot 10^{-7}$ mol l^{-1} TO-PRO-3 dye, 0.05 mol l^{-1} Tris-HCl–0.001 mol l^{-1} EDTA, pH 7.5, 0.5% DMSO)

CONCLUSION

Trace levels of electroactive catechin and acridine type compounds can be determined using DP voltammetric and fluorimetric procedures with DNA biosensors. Conventional anodic activation of the surface of bulk phase modified carbon paste electrode improves the contact of analyte with DNA-receptor attached to carbon particles prior to the paste preparation and enhances the current response. The repeated activation does not change the accumulation ability of DNA. Salmon sperm DNA was much more effective for the accumulation of analytes than calf thymus DNA. This could be explained by different purity of the DNA samples. The results obtained with DNA/CPE can be used for development of disposable DNA-modified screen-printed electrode.

Interaction of analytes with DNA in solution as well as at the electrode surface was also investigated. The intercalative binding of the compounds under study to DNA helix is indicated indirectly by the competitive binding with the fluorimetric DNA dye TO-PRO-3 and with the DNA intercalator Doxorubicin. For interpretation of results, morphology of the surface-confined DNA has to be taken into account.

This work was supported by the EU project INCO-COPERNICUS No. ERBIC15CT960804 and the Grant Agency of the Slovak Republic. The authors also wish to thank Ms M. Orlická for her assistance.

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