Electrochemical Sensing in Elimination Voltammetry

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Abstract: Elimination voltammetry with linear scan (EVLS) represents the fast and simple improvement of linear-sweep or cyclic voltammetric results. Through the various elimination functions, EVLS offers a new tool not also for better understanding of complex electrochemical processes on the surface of working electrodes, but also for improving the sensitivity of electrochemical measurements. The paper is mainly focused on the elimination function 'E4', which eliminates the kinetic and charging current components and conserves the diffusion one from a total current measured. It was found that this function has the most sensitive and separation ability in the case of adsorptive electroactive species. The strength of combination of EVLS with an adsorptive stripping technique was demonstrated by the enhancement and resolution of overlapped reduction signals of adenine and cytosine residues in short synthetic oligonucleotides (ODN) and DNA at the hanging mercury drop electrode. Two EVLS modes — single and double — are presented and their detection capabilities in analytical systems discussed.

Keywords: Linear sweep voltammetry; Elimination voltammetry with linear scan (EVLS), Elimination function; Adsorptive stripping technique; Hanging mercury drop electrode; Oligonucleotides; DNA.

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Introduction

There is increasing evidence in number of papers using cyclic voltammetry (CV) due to the characterization of electrochemical systems by redox probe. In spite of the fact that CV is an extension of linear sweep voltammetry (LSV) in postponing of potential, LSV is used and therefore occurs less in papers compared to CV. According to Web of Science survey (1999-2009), the number of papers with “CV” or “LSV” in abstract, keywords and title is 10,907 and 697, respectively. The increased interest in voltammetry is well evident in Fig. 1.

![Figure 1](image)

**Figure 1.** The number of papers concerning the linear sweep voltammetry (LSV; black bars) or cyclic voltammetry (CV; gray bars) evaluated via the appearance in the abstracts, key-words, and titles of the respective papers being published within 1999-2009.

For the evaluation of voltammetric signals the theory and application of CV or LSV is well reviewed in several monographs [1-4]. Elimination voltammetry with linear scan (EVLS) represents the fast and simple improvement of linear sweep or cyclic voltammetric results. The EVLS can be helpful to evaluate voltammetric signals with higher sensitivity and selectivity. Its theoretical foundations were published in 1996 and from this time its verification [5,6] and applications have been published in many papers [7-38]. The EVLS were also reviewed in 2005 and 2007 [16,39]. To verify EVLS theory the first experiments were performed on a hanging mercury drop electrode [6,8,10,40].

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The following experiments showed that EVLS could be applied not only for electroanalytical
detection, but also for the study of electrode processes of inorganic and organic electroactive
substances at mercury \[6,10,12,13,18,26-28,33,39,40\] and graphite electrodes \[14,20,21,
24,25,32\].

Elimination Voltammetry with Linear Scan (EVLS) – Single Mode

The EVLS can be considered as a transformation of current-potential curves capable of
eliminating some selected current components, while conserving others by means of
elimination functions \[16,39,41\]. The EVLS procedure is simple and it is based:

(i) on the different dependence of current component on scan rate (e.g. the diffusion current
\(I_d\) depends on the square root of scan rate, the charging current \(I_c\) is directly proportional
to the scan rate and the kinetic current \(I_k\), which represents a chemical reaction in the
vicinity of electrode, is independent of the scan rate), and

(ii) on two necessary conditions:

- the total current is formed by the sum of the current components:
  \[I = \sum_{j=1}^{n} I_j = I_1 + I_2 + I_3 + \ldots + I_n = I_d + I_c + I_k + \ldots + I_n\]
  \((I_d - \text{diffusion}, I_c - \text{charging}, \text{and } I_k - \text{kinetic currents}),\)
- the current component, which is eliminated, has to be expressed as the product of
two independent functions, \(I_j = Y_j(E) \cdot W_j(\nu)\), where \(Y_j(E)\) is the electrode
potential function and \(W_j(\nu)\) is the scan rate function. The function \(W_j(\nu)\) has the
form of a certain power \(x\) of the scan rate and for each current component is valid
the equation: \(I_j = Y_j(E) \cdot \nu^x\). As already written in (i), the rate coefficient \(x = \frac{1}{2}\), \(x = 1\),
and \(x = 0\) for \(I_d\), \(I_c\), \(I_k\), respectively \[16,39,41\].

Regarding the sensitivity and instrumentation, it is convenient to use three voltammetric
currents measured at three scan rates with integer of 2, e.g., \(I_{1/2}\), \(I\), and \(I_2\) at \(\nu_{1/2}, \nu, \nu_2\).
The current \(I\) measured at \(\nu\) corresponds to the reference current \(I_{\text{ref}}\). For this integer
the six types of elimination functions were calculated. While the functions E1, E2, and E3, requiring
two voltammetric curves measured at two different scan rates \((\nu_{1/2}, \nu_{\text{ref}}\) or \(\nu_{\text{ref}}, \nu_2\)) eliminate
one current component and conserves the other (while the third component is distorted),
the functions E4, E5, and E6, needing three voltammetric currents measured at three different
scan rates, eliminate two current components, thus retaining one current component.
E1. Elimination of $I_k$ with $I_d$ conserved ($I_c$ distorted):

$$f(I) = -3.4142 I_{l/2} + 3.4142 I$$  \hspace{1cm} (1)

E2. Elimination of $I_c$ with $I_d$ conserved ($I_k$ distorted):

$$f(I) = 4.8284 I_{l/2} - 2.4142 I$$  \hspace{1cm} (2)

E3. Elimination of $I_d$ with $I_k$ conserved ($I_c$ distorted):

$$f(I) = 3.4142 I_{l/2} - 2.4142 I$$  \hspace{1cm} (3)

E4. Simultaneous elimination of $I_k$ and $I_c$ with $I_d$ conserved:

$$f(I) = -11.657 I_{l/2} + 17.485 I - 5.8284 I_d$$  \hspace{1cm} (4)

E5. Simultaneous elimination of $I_c$ and $I_d$ with $I_k$ conserved:

$$f(I) = 6.8284 I_{l/2} - 8.2426 I + 2.4142 I_d$$  \hspace{1cm} (5)

E6. Simultaneous elimination of $I_d$ and $I_k$ with $I_c$ conserved:

$$f(I) = 4.8284 I_{l/2} - 8.2426 I + 3.4142 I_d$$  \hspace{1cm} (6)

The best EVLS results are obtained using the function E4. For the irreversible current of a substance transported to an electrode surface by diffusion only, the EVLS function E4 causes a moderate improvement of reduction or oxidation signals, consisting in the peak height and in the resolution of overlapped peaks. For the irreversible current of a fully adsorbed substance, this function provides the characteristic signal of a peak-to-counter-peak form (confirmed by the theory) improving strongly the resolution and sensitivity \cite{10,16,23,26,28,33,35,39,40}. This signal, usually 10-20 times higher than corresponding voltammetric peak to be measured, is detectable without a base-line correction. It is successfully employed in the analysis of nucleic acids and short homo- or hetero-deoxyoligonucleotides (ODNs) containing adenine (A) and cytosine (C) \cite{10,13,17,18,26,28,39}. Moreover, the EVLS was found to increase the current sensitivity for A and C resolution and, thus, for the recognition of bases sequences in ODN chains.

It has been shown that the EVLS in combination with an adsorptive stripping (AdS) procedure is a promising tool for achieving a very good resolution for the electrode processes, for qualitative and quantitative analysis of ODNs or their components, as well as for identification of the ODN structures.
Elimination Voltammetry with Linear Scan (EVLS) – Double Mode

In spite of the great advantages of single mode, EVLS can be applied in double mode. The peak-to-counter-peak signal has a much higher current sensitivity in the case of double EVLS [27]. The single mode of EVLS function eliminating two currents requires three voltammetric curves measured at three different scan rates. When this elimination procedure is repeated three times using LSV curves measured at five different scan rates \(v_{1/4}, v_{1/2}, v, v_2, v_d\), e.g. 10, 20, 40, 80, 160 or 50, 100, 200, 400, 800 mV/s, the double EVLS function E4 is obtained, where where \(I_d \neq 0\); \(I_k = 0\); \(I_c = 0\) [27]. It was found that amplification of double EVLS/single EVLS signals by a factor of 4 to 20 can be expected. The elimination function E4 in double mode is expressed by:

\[
double f(I)_{E4} = 135.9 I_{1/4} - 407.7 I_{1/2} + 441.6 I - 203.8 I_2 + 33.97 I_4
\]  

(7)

In contrast to the single EVLS, its double mode provides both higher sensitivity and better resolution of the overlapped voltammetric signals. This is illustrated in Fig. 2, showing the reduction of A and C residues in the hetero-ODN chain. The reduction involves the adsorbed state, when single- and double-EVLS signals have the shape of the peak-to-counter peaks.

Figure 2.
Linear sweep and elimination curves of hetero-oligonucleotide 5’- CCC AAA CCC-3’ on HMDE in phosphate buffer (pH 6.0) for single (●) and double (◇) EVLS modes. Reference LSV curve (200mV/s) — ; time of accumulation 90 s at accumulation potential –0.1V vs. Ag/AgCl/3M KCl, scan rates: 50, 100, 200, 400, and 800 mV/s.
Our results showed that EVLS in connection with the adsorption procedure (adsorptive stripping or adsorptive transfer stripping-AdS or AdTS) is a useful tool for qualitative and quantitative studies of short ODN with adenine, cytosine and guanine [13]. According to recently published results the EVLS has been successful in the separation of reduction signals of nonamers with three A’s and six C’s. The best results have been obtained for nonamer, where three A’s have central position between the three C (Fig. 2) [26]. The aim of the contribution is the application of EVLS:

(i) for the analysis of ODN containing only one A and eight C’s (ODN 5’- CCC ACC CCC -3’) as a verification of single and double mode of EVLS with the substantial increase of current sensitivity as a answer to the question if EVLS is able to separate the A signal under selected experimental conditions, and

(ii) for the analysis of DNA isolated from Neuroblastoma cells in both adsorptive and non-adsorptive mode as a natural sample.

Experimental

Chemicals and Reagents

Chemicals used were purchased from Sigma Aldrich Chemical Corp. USA (purity: ACS). Phosphate buffer, of 0.1 M NaH₂PO₄ + 0.1 M Na₂HPO₄, was used. All solutions were prepared using deionized ACS water (Sigma Aldrich). The pH of the phosphate buffer was determined by pH-meter Präcitronic (type MV870, Germany) using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3M KCl) was regularly calibrated by a set of WTW buffers (Weilheim, Germany). Synthetic hetero-oligodeoxynucleotide (nonamer, 5’- CCC ACC CCC -3’) was purchased from Thermo Electron, Ulm, Germany. The stock solutions of the ODN was prepared with ACS water (Sigma-Aldrich) and stored in the dark at -20 °C. Working solutions were prepared daily by dilution of the stock solutions.

Neuroblastoma Cell Line

UKF-NB-3 Neuroblastoma cell line, established from bone marrow metastases of high risk neuroblastoma, were a gift of J. Cinatl (J. W. Goethe University, Frankfurt, Germany). Cells were cultivated in IMDM cultivation medium (KlinLab Ltd, Prague, Czech Republic)
supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/ml of penicillin and 100 µg/ml streptomycin (PAA Laboratories, Pasching, Austria) and subcultivated in four-day intervals. The proper cultivation was performed in atmosphere-controlled incubator (at 37°C and with 5% CO₂).

**DNA Isolation**

DNA isolation was done using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to manufacturer’s instructions. The cells were trypsinized, harvested and centrifuged. Nuclei Lysis Solution (600 µl) was added and pipetted to lyse the cells until no visible cell clumps remained. RNase Solution (3 µl) was added to the nuclear lysate and sample was further incubated for 15 minutes at 37°C. Then the sample was cooling to room temperature. Protein Precipitation Solution (200 µl) was added and vortexed. In the following step sample was chilled on ice for 5 minutes and centrifuged. Supernatant containing DNA was removed and transferred into a microcentrifuge tube containing 600 µl of isopropanol. Solution was gently mixed until the white thread-like strands of DNA form a visible mass. Centrifugation followed and obtained supernatant was carefully decanted. 600 µl of 70% (v/v) ethanol were added and centrifugation again proceeded. Subsequently ethanol was aspirated and the pellet was air-dried. Polymerase chain reaction (PCR) quality water (100 µl) was added and DNA was incubated at 65°C for 1 hour. Concentration and quality of DNA were measured by means of spectrometry.

**Electrochemical Measurements**

Linear sweep voltammetry (LSV) measurements were performed with AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm² was employed as the working electrode. An Ag/AgCl/3M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing the software GPES 4.9 supplied by EcoChemie was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 90 s. All experiments were carried out at room temperature. LSV experimental parameters: start potential 0V, vertex potential –1.8 V, step potential 2 mV, scan rates 50, 100, 200, 400, 800 mV/s in the case of ODN and 40, 80, 160, 320 mV/s in the case of DNA. If the adsorptive stripping was applied, then the accumulation time is 90s.
The time of equilibrium was 5 s. All experiments were carried out at room temperature in phosphate buffer with 0.3 M ammonium formate (pH 6.3) for ODN and 0.2 M acetate buffer (pH 5.0) for DNA. The raw data were treated using the Savitzky-and-Golay filter (level 4) integrated to the GPES software (EcoChemie). The data processing and statistic analysis were performed by Excel (Microsoft, USA).

Results and Discussion

The detection of hetero-ODNs and DNA at a mercury electrode surface consists in the reduction responses of A and C at negative potentials (about –1.3 V vs. Ag/AgCl/3M KCl) represented usually by one overlapped voltammetric peak. The reduction of A and C in this common peak cannot be distinguished by conventional electrochemical methods, such as linear sweep, square wave, and differential pulse voltammetry. The LS and EVLS voltammograms of the ODN (5´- CCC ACC CCC -3´) in the presence of 0.3 M ammonium formate and 0.05 M phosphate buffer (pH 6.5) are shown in Fig. 3.

![Figure 3. Linear sweep and elimination curves of hetero-oligonucleotide 5´- CCC ACC CCC-3’ on HMDE in phosphate buffer (pH 6.5) for single ◆ and double ◊ EVLS modes. Reference LSV curve (200 mV/s) — — ; time of accumulation 90 s at accumulation potential –0.1 V vs. Ag/AgCl/3MKCl, scan rates: 50, 100, 200, 400, 800 mV/s.](image-url)
The curves were measured after adsorption of ODN at -0.1 V for 90 s. Figure 3A shows the single mode (Eq. 4) and Figure 3B shows the double mode of the elimination function E4 (Eq. 7), which eliminates the charging and kinetic currents and conserves the diffusion current.

Compared to ODN 5´-CCC AAA CCC-3´ (see Fig. 2), the EVLS is not able to separate reduction peaks A and C in either modes. According to the latest results of our electrochemical hetero-ODN research the separation of reduction peaks of A and C is also influenced by the position of base in the ODN chain (middle and end position) and by ODN secondary structure, which is determined by pH and solution components (not shown).

Table I. Comparison of LSV and EVLS reduction signals of A and C in nonamer (5´- CCC ACC CCC -3´)

<table>
<thead>
<tr>
<th>(v_{\text{ref}}) [mV/s]</th>
<th>(I_{\text{LSV}}) [(\mu A)]</th>
<th>single (I_{\text{EVLS}}) [p-cp]*</th>
<th>double (I_{\text{EVLS}}) [p-cp]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.105</td>
<td>0.822</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0.172</td>
<td>1.679</td>
<td>29.43</td>
</tr>
<tr>
<td>400</td>
<td>0.585</td>
<td>3.580</td>
<td></td>
</tr>
</tbody>
</table>

* peak-to-counter-peak distance

Fig. 3 also illustrates the substantial increase of sensitivity of the common signal A and C for both elimination modes. Table I quantifies this fact for three reference scan rates. It can be argued that the multiplier of peak height corresponds to the value about seven. If we compare the reference value (c-cp) for single (1.679) and c-cp value of double mode (29.43), we obtain the multiple 17.53.

The application of EVLS for the analysis of DNA isolated from Neuroblastoma cells in non-adsorptive mode is shown in Fig. 4; namely, with scan rate of a) 40, 80, and 160 mV/s or b) 80, 160, and 320 mV/s. Each EVLS function increases the original reduction signal (ref.) but E1, E2, E3 eliminating one current component in lesser extent than E4, E5, E6 eliminating two current components. The highest sensitivity provides the function E4 and this is especially true in the case of pre-adsorbed DNA on mercury electrode surface.
Figure 4. Linear sweep and elimination curves of DNA on HMDE in acetate buffer (pH 5) for all elimination functions (Eqs. 1-6), time of accumulation 0 s, scan rates: (a) 40, 80, 160 mV/s and (b) 80, 160, 320 mV/s.

Figure 5. Elimination curves of DNA on HMDE in acetate buffer (pH 5) for all EVLS functions (Eq. 1-6). Reference LSV curve (160 mV/s); time of accumulation 90 s at accumulation potential –0.1V vs. Ag/AgCl/3MKCl, scan rates: 80, 160, 320 mV/s.
The elimination transformation corresponds to the signal in the form of peak-to-counter peak. From an analytical point of view, this EVLS E4 transformation gives rise to well-shaped and easy-to-evaluate voltammetric signals. The differences between non-adsorptive and adsorptive mode are well evident (compare Figs. 4a and 4b with Fig. 5).

The course of E5 and E6 in the opposite direction than the E4 function supports the theory and their peak-to-counter peaks are about ten times higher than the original LSV peak. The EVLS E4 is again recorded with the highest sensitivity and its peak-to-counter peak is twenty two times higher than the original LSV peak. The behaviour of all six elimination functions can indicate the changes in the electrode mechanism due to the altered experimental conditions, where one current component becomes dominant and other recessive.

Conclusions

In this contribution, the applicability of modern voltammetric procedures has been verified based on elimination voltammetry with linear scan (EVLS) for one synthetic short oligonucleotide (5’- CCC ACC CCC -3’) and natural sample (DNA of Neuroblastoma cells). In the ODN experiment, both single and double modes of EVLS were used; both EVLS and AdS EVLS (without and with accumulation) being applied in the DNA experiment. It has been shown that, in electrochemical analysis, the EVLS takes the main position.

Also, it has been demonstrated that the EVLS may positively affect the detection capabilities of the analytical system itself via improvement of the separation of the overlapped signals, depending upon the experimental conditions chosen. According to such differences in the resultant separation, one could evaluate changes in the secondary structure of ODNs.

The elimination procedure proposed herein is of continuing interest and it can be anticipated that new results will contribute to the overall enhancement of the sensitivity of some voltammetric sensors.

Acknowledgements

The authors would like to express their gratitude to the Ministry of Education, Youth and Sports of the Czech Republic and to the Grant Agency of the Czech Republic for the financial support (projects №: BIO-ANAL-MED LC06035, INCHEMBIOL MSM0021622412, MUNI/A/0992/2009, and GA ČR 102/08/1546).
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