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Electroanalytical determination of d(GCGAAGC) hairpin

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Abstract

Hairpins (or hairpin-like structures) may play a major role in expansion events of triplet repeat expansion diseases (X syndrome, Huntington's disease, Friedreich's ataxia). The d(GCGAAGC) fragment has been found in the replication origins of phage ϕX 174 and herpes simplex virus, in a promoter region of an *Escherichia coli* heat-shock gene, and in rRNA genes. The paper deals with the application of electrochemical methods to the determination of the DNA heptamer—d(GCGAAGC) which forms very stable hairpin structure in aqueous solutions. On mercury electrodes, this hairpin provides voltammetric reduction signals of adenine and cytosine, and oxidation signals of guanine. Both signals have been studied by cyclic voltammetry (CV), linear sweep voltammetry (LSV), and elimination voltammetry with linear scan (EVLS) in dependence on pH, accumulation time, scan rate, and loop sequences. The EVLS in combination with the adsorptive stripping was employed to the determination of the detection limit (LD) of this mini-hairpin (2 nM). Multidimensional voltammetric data were worked up by Fourier Transform (FT) and for the first coefficient a confidence ellipse was calculated in order to drop out some outlier data. The same method was used also for detection limit determinations. The values of LD obtained by two approaches were compared.

Keywords: The DNA heptamer d(GCGAAGC); Mini-hairpins; Linear sweep voltammetry (LSV); Cyclic voltammetry (CV); Elimination voltammetry with linear scan (EVLS); Hanging mercury drop electrode (HMDE); Adsorptive stripping procedure

1. Introduction

Hairpins or hairpin-like structures consisting of single-stranded loop and base-paired stem regions occur naturally not only in single-stranded DNAs and RNAs but also in double-stranded DNAs [1,2]. They play a significant role in many biological processes. The shortest fragment d(GCGAAGC), containing tri-nucleotide (GAA) loop and two G–C pairs stem, participates on expansion events of triplet repeat expansion diseases (X syndrome, Huntington's disease, Friedreich's ataxia). The d(GCGAAGC) fragment has been found in the replication origins of phage ϕX 174 [3] and herpes simplex virus [4], in a promoter region of an *Escherichia coli* heat-shock gene [5], and in rRNA genes [6]. Recently, the structure of d(GCGAAGC) has determined by NMR spectroscopy [6] and refined using molecular dynamics [7].

It has been revealed that mini-hairpin molecules are stable and their thermodynamic stability depends not only on the sequences of the stem region but also on the sequences of the loop region [2,6]. The aim of this paper was not only the fast and economical determination of the hairpin in biological materials by means of electrochemistry with statistical approach, but also the possible help of voltammetric methods, including elimination voltammetry with linear scan (EVLS), to the study of the primary and secondary structure of mini-hairpins.

The EVLS [8] has been already applied to study adenine and cytosine reduction signals at the mercury electrode [9]. In comparison with the linear scan voltammetry providing only one unresolved peak, EVLS provides good resolution of individual peaks and significant increase of sensitivity. The best results are given by the function eliminating simultaneously the kinetic and charging currents (I_k and I_c) and conserving the diffusion current (I_d). The well-resolved peaks of adenine and cytosine in a wide concentration range were obtained, while the linear sweep voltammetry gave badly resolved peaks due to the hydrogen evolution. This function, eliminating kinetic and charging currents and conserving a diffusion current, was calculated for an adsorbed electroactive substance and experimentally verified for DNA and short synthetic oligonucleotides [10–14]. For the adsorbed electroactive substance, this elimination function provides the signal in a peak–counterpeak form. The-

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oretical and experimental transformation current–potential curve into this specific elimination signal is demonstrated in Fig. 1. In this figure, the curves were determined in the same way as in preceding papers [10,11,13], as well as the elimination voltammogram of oligodeoxynucleotide dA_9 in 0.2 M acetate buffer (pH 5.3). The ssDNA is strongly adsorbed on a mercury surface. Thanks to this adsorption the elimination signal corresponding to reduction of adenine and cytosine reduction peak keeps the peak–counterpeak [14]. The sensitivity of this elimination signal in the comparison with the measured voltammetric signal increased by 13 times. One reduction peak from adenine and cytosine residues and one oxidation peak of guanine residues were observed in the electroanalytical study of solutions of self-complementary decamer d(CCAGGCCTGG) [15].

Using our procedure, multidimensional data such as cyclic voltammetric curves are transformed into two-dimensional data by Fourier Transformation (FT) [16,17].

$$F_k = \sum_{j=0}^{m-1} A_j \cos\left(\frac{2\pi jk}{m}\right) + i\sum_{j=0}^{m-1} A_j \sin\left(\frac{2\pi jk}{m}\right)$$

For the first coefficient in FT ($k=1$), the transformed curve is represented by a single point with coordinates xf and yf, i.e. $F_1 = xf_1 + iyf_1$. For the calculation of the slope b_T, the orthogonal regression was used:

$$b_T = \frac{S_x - S_y}{S_{xy}} \pm \sqrt{1 + \left(\frac{S_x - S_y}{S_{xy}}\right)^2}$$

where:

$$S_x = \sum x_c^2 \qquad S_y = \sum y_c^2 \qquad S_{xy} = \sum x_c y_c$$
$$x_c = x - \bar{x} \qquad y_c = y - \bar{y}$$

The whole set of points is rotated to bring the regression line into the horizontal position, i.e. in the x-axis. New coordinates of points are now x_r and y_r.

$$[x_r y_r] = \begin{bmatrix} \cos(\varphi) & \sin(\varphi) \\ -\sin(\varphi) & \cos(\varphi) \end{bmatrix} \times [x_c y_c]$$

where: $\varphi = \tan(b_T)$. The points are surrounded by 95% (99%) confidence ellipses, it means that the half axes are twice (three) times the standard deviations Standard Error of Estimate Longitudinal (SEEL) and Standard Error of Estimate Transverse (SEET):

$$\mathrm{SEEL} = (P + M)/2$$

$$\mathrm{SEET} = (P - M)/2$$

$$P = \sqrt{\left(S_x + S_y + 2\sqrt{S_x \times S_y - S_{xy}^2}\right) \Big/ (n-1)}$$

$$M = \sqrt{\left(S_x + S_y - 2\sqrt{S_x \times S_y - S_{xy}^2}\right) \Big/ (n-1)}$$

The backward rotation can bring the points together with ellipses into the original position. The ellipse (95%) is characterized by its area of PL = 2 and the ratio of SEEL to SEET.

2. Experimental

2.1. Chemicals

Oligodeoxynucleotides (ODNs) were synthesized by VBC-Genomics (Wiena, Austria). Buffer components (CH_3COOH and CH_3COONa) were purchased from Sigma-Aldrich Chemical, USA (purity of ACS). All solutions

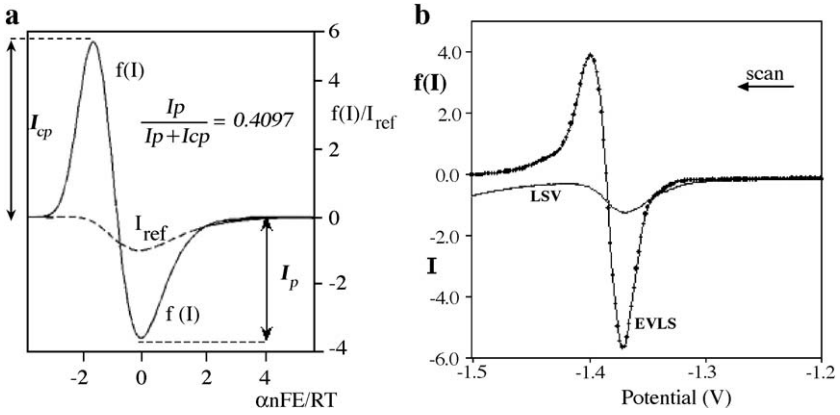

Fig. 1. Comparison of theoretical (a) and experimental (b) transformation of the irreversible current–potential curve. *f(I)*—elimination function for simultaneous elimination of kinetic and charging currents and conserving the diffusion current. (a) Dotted line—a reference voltammetric curve, full line—elimination curve. (b) Full line–reference voltammetric curve of oligodeoxynucleotide dA_9 (256 μg/ml in 0.2 M acetate buffer, pH 5.3) measured at reference scan rate ($v = 200$ mV/s), full lines with dotes—elimination curve.

were prepared using triply distilled water from quartz apparatus.

2.2. Procedure

Voltammetric measurements—cyclic voltammetry (CV) and linear sweep voltammetry (LSV)—were carried out with the Autolab Electrochemical Analyzer (Ecochemie, Utrecht, the Netherlands) connected to the VA-Stand 663 (Metrohm, Zurich, Switzerland). The standard cell consisted of three electrodes including the working electrode (hanging mercury drop electrode—HMDE) with an area of 0.4 mm^2, the reference electrode (Ag/AgCl/3 M KCl), and the auxiliary electrode (platinum wire) was used. All solutions were deaerated with argon (99.99%) for at least 7 min prior measurement and blanketed with argon during measurements. Linear sweep or cyclic voltammetric curves were measured with the following parameters: initial potential − 0.1 V, end-point or switching potential − 1.75 V, equilibrium time 5 s and accumulation time 120 s (if it not presented otherwise). For elimination procedure, scan rates in two sequences with multiple integers of two, i.e. either 80, 160, 320, or 640 mV/s or 100, 200, 400 and 800 mV/s at constant potential step 2 mV were employed. Stock solutions of oligonucleotides were obtained by addition of triply distilled water to lyophilized samples of ODNs. Their concentrations were determined by absorption spectra in UV area. Concentrations of ODNs were calculated from the values of optical density and the average absorption molar coefficient (10 000 cm^2 $mmol^{-1}$) at 260 nm. Solutions of hairpins were prepared in water and for measurements small volumes were added to acetate buffer (0.1 M CH_3COOH + 0.1 M CH_3COONa). The pH of solutions was adjusted and checked by the IS-FET electrode (HOT-Line 3000-008, Roden, The Netherlands) connected with pH-meter TITAN 6000-062, Sentron (Roden). All experiments were carried out at room temperature.

The GPES 4.8 software (Ecochemie) for measurement and processing of recorded curves (smoothing using the Savitzky and Golay filter of level 4) [18] was employed. The resulting curves were exported into Microsoft Excel to calculate the elimination function.

2.3. Elimination procedure

Linear sweep voltammetric data obtained at scan rates $1/2v$, v, $2v$ were exported into Microsoft Excel (Microsoft, USA). The calculation of the chosen elimination function was made by means of Microsoft Visual Basic 6.0.

2.4. UV–Vis spectrophotometry

The UV–Vis spectra were measured using UV–Vis spectrophotometer (UNICAM 4UV Cambridge, UK) in the temperature-controlled quartz cuvette (0.1 cm) and were worked up using the software Visible Vision Unicam.

3. Results and discussion

The self-complementary heptamer d(GCGAAGC) measured on a hanging mercury drop electrode (HMDE) in acetate buffer produced cathodic and anodic voltammetric signals. The concentration of this heptamer forming the stable hairpin (HP 7) [1,2,6] was 100 nM. Two cathodic signals (~ − 1.35 V) correspond to the reduction of adenine (A) and cytosine (C) residues in the hairpin. Two anodic signals (~ − 0.2 V) arise through the oxidation process of a reduced guanine (G) intermediate. Reduction of G on a mercury electrode, at highly negative potentials close to background discharge, yields 7,8-dihydrogenguanine [19–21]. The oxidation of the G reduction product can be observed in cyclic or in anodic stripping modes when the electrode is shortly exposed to highly negative potentials prior to scanning to positive potentials [20,22]. It means that the reduction of guanine occurs prior to the oxidation step. The cyclic voltammograms (Fig. 2) were obtained by the adsorptive stripping technique with the accumulation time of 120 s. The adsorption performed at the initial potential of − 0.1 V (vs. Ag/AgCl/3 M KCl). After the adsorption of HP7, voltammetric curves were recorded at a scan rate ranging from 25 to 1000 mV per second in nonagitated solutions. A variation of the peak potential and the peak height of adenine (A), cytosine (C), and guanine (G) with an increasing scan rate is illustrated in two insets in Fig. 2. It can be seen that in the case of reduced species, A and C, the peak height increases and the peak potential shifts to the more negative values with increasing scan rate. On the contrary, the peak potential of oxidized G moved towards more positive values. This behavior is a concordance of irreversible electrode processes [23,24].

In order to select accumulation times (t_a) suitable for the detection of A,C, and G, the cyclic voltammetric measurement was carried out at the different t_a (not shown). The HP 7 (100 nM) was allowed to adsorb (from 10 to 400 s) on the electrode charged to the potential of − 0.1 V and then voltammetric curves were recorded at scan rate of 400 mV/s. The accumulation times of about 120 s were found advantageous for the measurement of both cathodic peaks and anodic peaks. Therefore, the t_a equal 120 s was used in all our experiments. The potentials and heights of the peaks varied not only with the scan rate and the accumulation time but also with the pH. Cyclic voltammograms of HP 7 were recorded at four different values of the pH (from 5.2 to 5.9); it was found that the height of oxidation signals was affected by pH more significantly than one of the reduction peaks.

One of many advantages of elimination voltammetry (EVLS) is the ability to increase sensitivity of a voltammetric signal. Therefore, the EVLS was employed for the determination of detection limit of HP 7. The function $f(I)$, which eliminates simultaneously the charging and kinetic currents (I_c, I_k) and conserves the diffusion current (I_d), was formed by the following linear combination: $f(I) = -11.657I_{1/2} +$

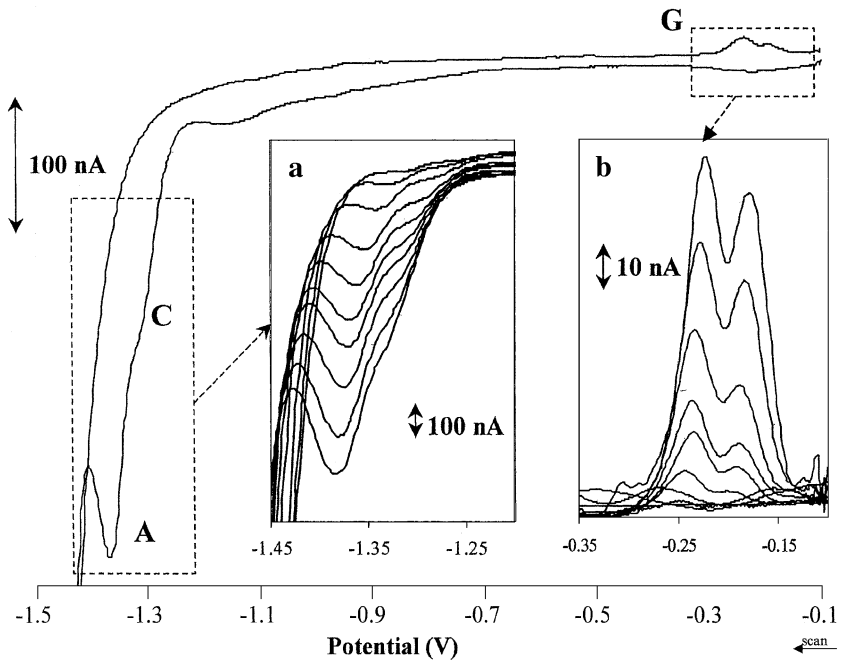

Fig. 2. Cyclic voltammogram of HP 7 (100 nM) obtained by adsorptive stripping technique (AdS) in 0.2 M acetate buffer, pH 5.32. Scan rate 400 mV/s, initial potential $E_i = -0.1$ V, switching potential $E_s = -1.75$ V, accumulation time $t_a = 120$ s (with stirring), time of equilibration 2 s (at E_i without stirring). Inset (a): cathodic peaks of adenine (A) and cytosine (C), inset (b): anodic peaks of guanine (G) recorded at different scan rates (from 25 to 1000 mV/s).

$17.485I - 5.8284I_2$, where I is reference current measured at the reference scan rate, $I_{1/2}$ and I_2 are the total currents measured at half and double reference scan rate. This elimination function giving rice to the characteristic signal (peak–counterpeak) for totally adsorbed electroactive substance [10–12] is very important from an analytical point of view. It significantly increases the sensitivity of the voltammetric measurement and enables the resolution of overlapped signals. The resulting elimination peak, which resembles a derivation signal, has been shown to be useful

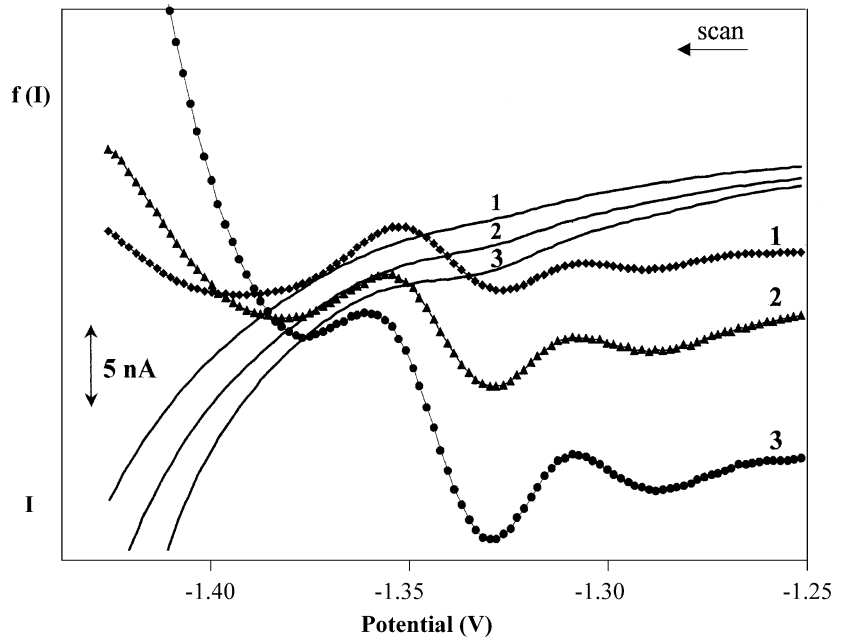

Fig. 3. The determination of detection limit of HP 7 in 0.2 M acetate buffer (pH 5.32) using EVLS. Linear sweep voltammograms (full lines) are reference curves (200 mV/s) measured for three concentration additions of HP 7 (2, 4, and 8 nM). EVLS curves (full lines with dots) are calculated by means of the function eliminating simultaneously kinetic and charging currents and conserving the diffusion current. For EVLS calculation, three LSV curves at scan rates 100, 200, and 400 mV/s were employed. Other experimental conditions were same as in Fig. 2.

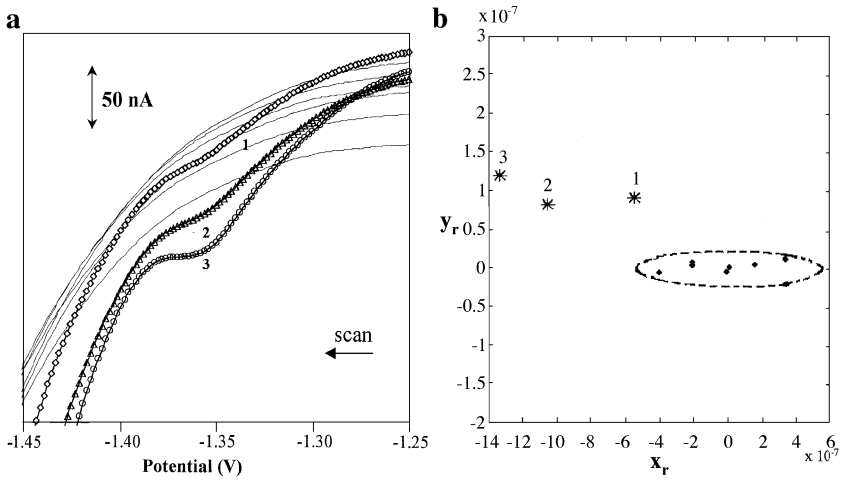

Fig. 4. Visualization of detection limit using a 95% confidence ellipse (CE). For CE, eight voltammetric curves of 0.2 M acetate buffer (pH 5.32) as a supporting electrolyte were used; three additives of HP 7 are shown by points 1, 2 and 3 with concentrations 2, 4, and 8 nM, respectively. Scan rate was 400 mV/s. For other experimental conditions, see Fig. 2.

in the analysis of DNA [10]. In addition, it is advantageous in the resolution of overlapped reduction signals of adenine and cytosine in synthetic short oligodeoxynucleotides [14]. The EVLS was applied in the resolution of cathodic signals of purine and pyrimidine bases which are detected at very negative potentials near to the supporting electrolyte discharge [9]. Fig. 3 shows the cathodic part of cyclic voltammograms measured for different low concentrations of HP 7 in buffer solution. Unlike the less-sensitive adsorptive stripping linear sweep voltammetry (AdS LSV), the adsorptive stripping elimination voltammetry with linear scan (AdS EVLS) enabled the determination of HP 7 in nanomolar concentrations, the detection being 2 nM. The ratio between the height of peak and the height of peak–counterpeak is characteristic parameter of elimination signal which can more specify electrode process. It means that the equation for this ratio for adsorbed substance undergoing to irreversible electrode process can be expressed as $I_p/(I_p+I_{cp})$, where I_p and I_{cp} are the heights of peak and counterpeak, respectively (Fig. 1a). The mean value determined for adenine residues was 0.406 which is in very good agreement with the theoretical value (0.4097) [14].

The detection limit of HP 7 was confirmed by using confidence ellipse. Fig. 4a shows eight linear sweep voltammetric curves of the acetate buffer (0.2 M; pH 5.32) as a supporting electrolyte. The first Fourier coefficients [17] after the transformation of the curves are visualized by the points in Fig. 4b surrounded by confidence ellipse. The ratio of the shorter to larger axis is about 0.04 indicating some deterministic effect in the repeated measurements of the supported electrolyte. In spite of this fact, the addition of the hairpin can be easily distinguished—in Fig. 4a by curves marked by different characters and in Fig. 4b by points 1, 2, and 3 for the concentrations 2, 4, and 8 nM, respectively, of the hairpin added. It follows that the limit of the detection is below the lowest concentration used.

Our results showed that EVLS in connection with the adsorption procedure is a useful tool for qualitative and quantitative studies of short oligodeoxynucleotides like hairpin d(GCGAAGC) DNA heptamer.

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