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Electrochemical Characterization of PNA Oligonucleotide of Neuraminidase Gene

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This paper comes with the application of the Brdicka method for PNA testing and characterization. The classical approach based on the catalytic electroreduction of protons was used. In this study electrochemical determination of PNA in Brdicka solution was investigated by two techniques of differential pulse voltammetry (DPV) and DPV coupled with adsorptive stripping technique. The instrumentation of experiment was improved by manufacturing new holder for cooling electrochemical cell necessary for measurements in temperature sensitive electrolyte. This holder was made using 3D printing system from polylactide (PLA), biodegradable thermoplastic aliphatic polyester. AdT DPV produced four typical peaks (Co_1 peak at potential -1.00 ± 0.05 V, RS_2CO at potential -1.25 ± 0.05 V, Cat1 at potential -1.35 ± 0.05 V and Cat2 at potential -1.55 ± 0.05 V). Cat2 peak height was strongly depended on accumulation time, temperature of electrolyte, and concentration of PNA.

Keywords: PNA; Brdicka Electrolyte; 3D printing; Voltammetry; Adsorptive Transfer Technique

1. INTRODUCTION

Combining efforts of biochemist Peter Nielsen's and organic chemist Ole Buchardt's groups led to the investigation of peptide nucleic acid during the 1990s. It was suggested that α-helical poly-γ-benzyglutamate (PBG) with alternating nucleobases and acridine moieties could bind sequences selectively to duplex DNA by combining Hoogsteen base pair formation (the type of base pairing that occurs between adenine and thymine where adenine N7 acts as the hydrogen bonding acceptor rather than N1 of the Watson-Crick geometry) and intercalation with the helical backbone in the major groove because aromatic chromophores form stacked complexes with PBG. The proposed peptide

nucleobase compound was named peptide nucleic acid (PNA) [1,2]. Up to now, there have been published more than 130 000 articles about PNA. Application of PNA can be divided into four main categories, namely PNA as biosensor [3-5], PNA as a probe for diagnosis and detection [6-13], PNA as a tool for functional genomics and molecular biology [14-20], and PNA for antigene and antisense therapy [21-28].

PNA mimics DNA, in which the entire deoxyribose-phosphate backbone is replaced with a structurally homomorphous uncharged, achiral polyamide backbone composed of N-(2-aminoethyl)glycine units linked by peptide bonds (Fig. 1A). The different bases (purines and pyrimidines) are joined to the backbone by methylene carbonyl linkages [29], which is shown in Fig. 1A. Neutral property of peptide backbone eliminates electrostatic repulsion between hybridized strands. Some other properties of PNA are proper interbase distances, rigid amino bonds, and flexible aminoethyl linkers and finally intermolecular hydrogen bonding, which can increase affinity of binding between PNA and complementary DNA in comparison with binding of DNA with DNA [30].

Common methods for electrochemical detection of PNA are square wave voltammetry (SWV), cyclic voltammetry (CV) [31], and differential pulse voltammetry (DPV) in different buffer such as Tris-HCl containing NaCl [5,32] and/or phosphate buffer [33]. In this study, new method for electrochemical detection of PNA was investigated. DPV and DPV coupled with adsorptive transfer technique (AdT DPV) were employed for monitoring Brdicka reaction of PNA. The effect of accumulation time, temperature of Brdicka electrolyte, and concentration of PNA on electrochemical behaviour of PNA was described here.

2. EXPERIMENTAL PART

2.1. Chemicals

PNA oligonucleotide (ODN) was synthetized with cysteine groups (PNA sequence from N-terminus to C-terminus: cysteine-CCT CAA GGA G) by Biosynthesis (Biosynthesis, USA, Texas). Other chemicals were purchased from Sigma Aldrich (Sigma-Aldrich, USA) in ACS purity. Stock solutions (including PNA standard) were prepared with ACS water. Deionised water undergone demineralization by reverse osmosis using Aqua Osmotic 02 device (Aqua Osmotic, Tisnov, Czech Republic) and after that purified using Millipore RG (MiliQ water, 18 MΩ, Millipore Corp., USA). Deionised water was used for rinsing cell, washing and buffer preparation. pH value (Brdicka solution) and conductivity was measured using inoLab Level 3 (Wissenschaftlich-Technische Werkstatten; Weilheim, Germany).

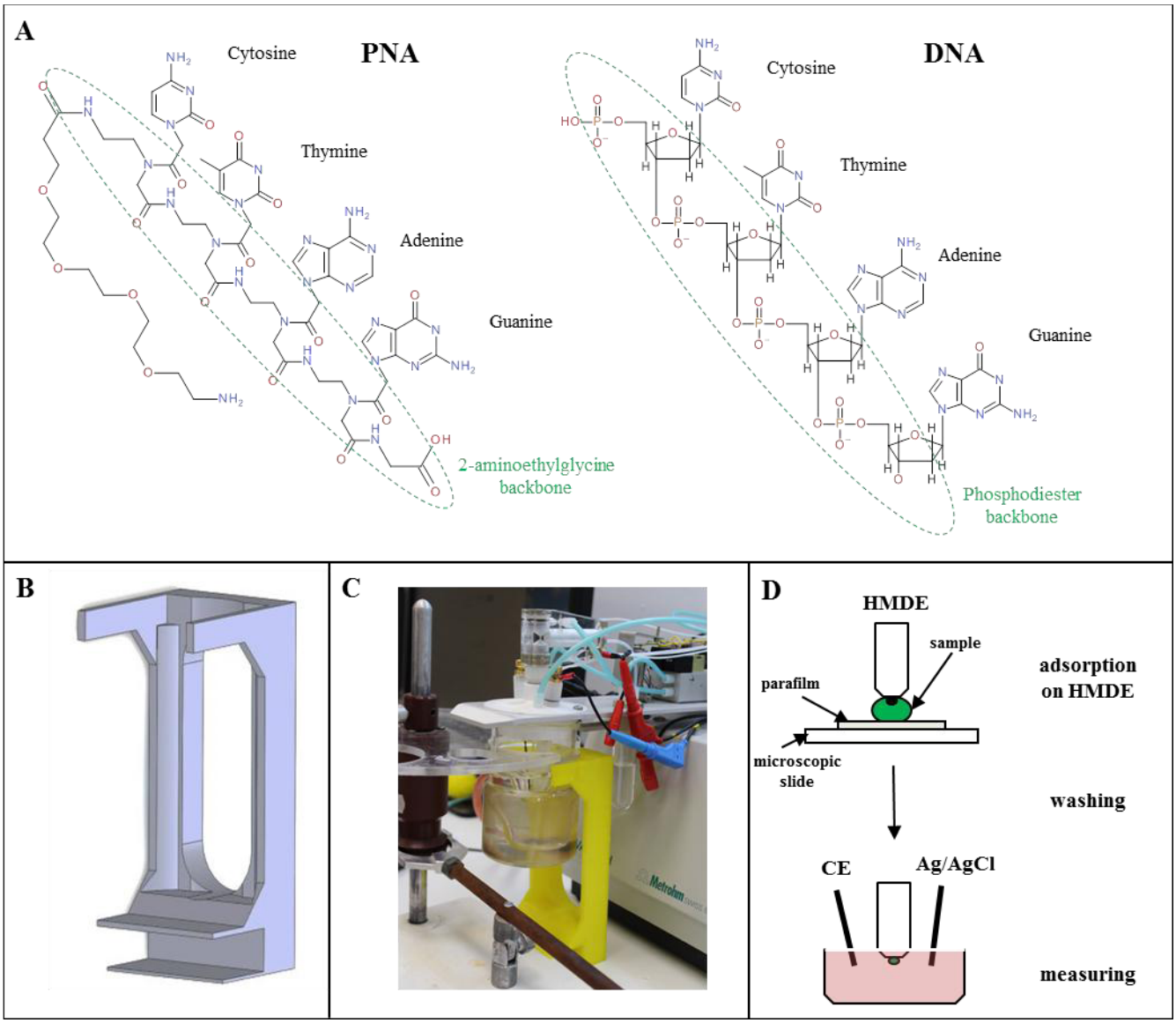

Figure 1. (**A**) Structure of PNA and DNA. (**B**) Scheme of 3D printed holder. (**C**) Real picture of holder application at electrochemical measurement by adsorptive transfer technique (AdT). (**D**) Scheme of AdT DPV (5 µl of PNA is placed on the parafilm and working electrode is immersed in the sample drop containing PNA, the PNA-modified HMDE is rinsed in miliQ water and the absorbed complex is analysed in the presence of Brdicka electrolyte).

2.2. Electrochemical detection of PNA

PNA was analysed by two techniques of differential pulse voltammetry and differential pulse voltammetry coupled with adsorptive transfer technique. All measurements were performed with an AUTOLAB Analyzer (EcoChemie, Netherlands) connected to a 663 VA Stand instrument (Metrohm, Herisau, Switzerland). It was equipped with a standard cell consisting of three electrodes, measurement cell and cooled sample holder set at 4 °C (Julabo F25, JulaboDE, Seelbach, Germany). The three-electrode system consisted of a hanging mercury drop electrode (HMDE) with a drop area of

0.4 mm^2 as the working electrode, an Ag/AgCl/3M KCl reference electrode and a platinum electrode acting as the auxiliary. Software GPES 4.9 was used for data analysis. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 %) saturated with water for 60 s. The Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq)$ + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of -0.7 V; end potential of -1.8 V; modulation time 0.057 s; time interval 0.2 s; step potential 0.005 V; modulation amplitude 0.025V. Time of accumulation and temperature of the supporting electrolyte were optimized.

2.3. 3D printing technique for the second holder manufacturing

The second holder was designed with the purpose of filling the distance between cooled electrolyte solution vessel and surface of the table. The first step of fabrication of the second holder was its 3D processing in the modelling program Solidworks. The product was exported in STL format and further edited in netFabbprogramme (Parsberg, Germany), which allowed elimination of cranny or wrongly oriented triangles. The corrected model (in STL format) was opened in the program G3DMAKER (DO-IT, Czech Republic) and 3D printing was controlled by EASY3D MAKER (DO-IT). After the above-mentioned corrections, the model was ready for printing. Holder of the size [x, y, z] 80/100/186.75 mm was printed with an accuracy of [x, y, z] 0.1/0.1/0.25 mm for 263 minutes. As material polylactide (PLA) from DO-IT, which was applied by extrusion (melting head) at temperature 212 °C on a heated surface (55 ° C), was used. 3D structure of the second holder was shown in Fig. 1B.

3. RESULTS AND DISCUSSION

The Brdicka reaction is frequently employed electrochemical method for determination of paptieds including glutathione [34-36], and/or proteins [37] including metallothionein [38]. Such determination give four various signals called Co_1, RS_2CO, Cat1, and Cat2 [39-41]. Signals of Cat1 and Cat2 correspond to the reduction of hydrogen at the mercury electrode. Another signal, which is appeared at the potential about -1.0 V, relates with the reduction of the RS_2CO complex. In addition, Co_1 signal could result from reduction of $[Co(H_2O)_6]^{2+}$ [42]. Reactions behind these peaks are due to interaction of $[Co(NH_3)_6]Cl_3$ with thiol groups of protein. Because of the fact that PNA contains thiol group it was assumed that PNA could also give similar signals. Nevertheless, Brdicka reaction is still hard to use for automatic and in situ analysis in spite of the advantages of this method, and therefore, some technical solutions how to suggest some instruments to enable us to use Brdicka reaction in the mentioned application are searched for. 3D printing is a rapid prototyping method that makes 3D objects from computer models built by subsequent overprinting. 3D printing technology is commonly used in industry, medical, and transport sectors in scientific applications [43-45]. 3D printing has also been explored for 3D printed integrated reaction ware for chemical synthesis and analysis [46].

In this study we suggested and developed a new holder for attachment of electrochemical cell cooling another electrochemical cell containing electrolyte and optionally a sample. The function of a holder (Figs. 1B and 1C) was tested by electrochemical detection of PNA in temperature sensitive Brdicka electrolyte [42]. For electrochemical detection, two common methods were applied, DPV and AdT DPV [47]. The scheme of both methods is shown in Fig. 1D.

3.1. Brdicka reaction of PNA

The difference between DPV and AdT DPV analysis was in the accumulation step and was described in previously published papers [42,48-50]. Using DPV method, the sample was accumulated in the electrochemical cell and then measured, whereas using AdT technique the sample was placed on the parafilm, and accumulated (Fig. 1D). After accumulation step, a hanging mercury drop electrode (HMDE) with accumulated sample was rinsed in water and then immersed in electrolyte and measured (Fig. 1D).

3.2. Electrochemical detection of PNA by DPV in the electrochemical cell

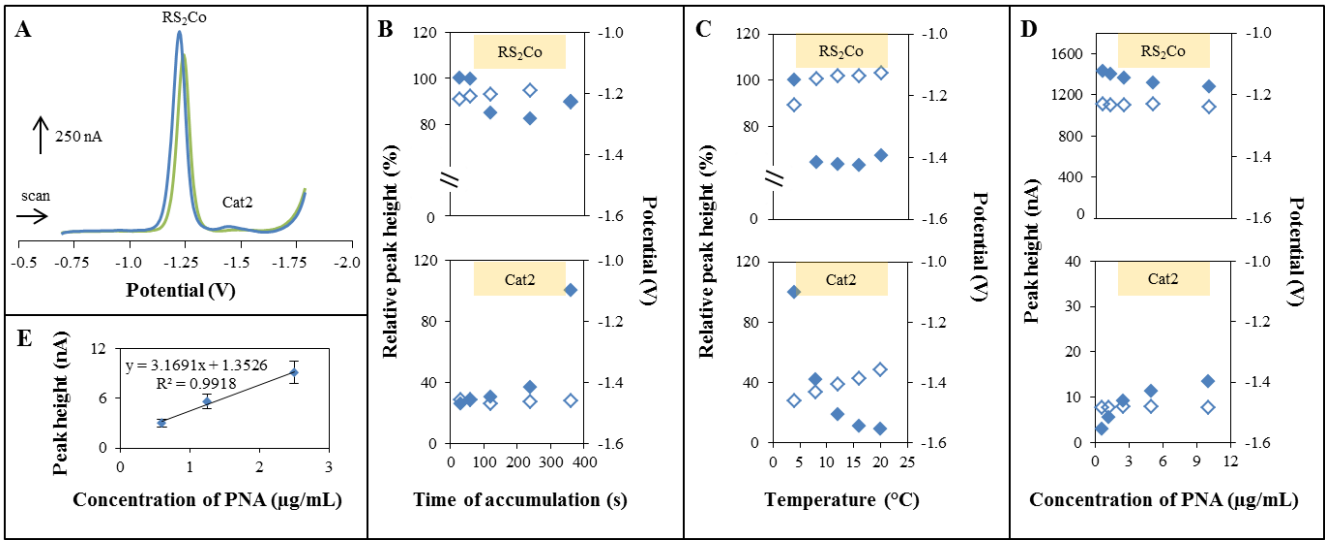

Figure 2. Electrochemical characterization of PNA by DPV method (filled dot: relative peak height or peak height, non-filled dot: potential). (**A**) Typical DP voltammogram of PNA (blue line) and Brdicka electrolyte (green line). (**B**) Dependence of relative heights of peaks RS2Co and Cat2 (related to the maximal values) and their potentials on time of accumulation; concentration of PNA 10 µg/ml. (**C**) Dependence of relative heights of peaks RS2Co and Cat2 (related to the maximal values) and their potentials on temperature of Brdicka electrolyte; accumulation time 360 s, concentration of PNA 10 µg/ml. (**D**) Dependence of heights of peaks RS2Co and Cat2 and theirs potentials on concentration of PNA (µg/ml); accumulation time 360 s, temperature of electrolyte 4 °C. Parameters of DPV were as follows: purge time 60 s, modulation time 0.057 s, interval time 0.2 s, initial potential -0.7 V, end potential -1.8 V, step potential 0.005 V, modulation amplitude 0.025 V, n = 3.

DPV is the most general electrochemical method for detection various compounds (e.g. drugs [51], oligonucleotides [52], metal ions [53]). Typical DP voltammogram of PNA measured in Brdicka electrolyte is shown in Fig. 2A. There are detected two peaks (RS_2CO at potential -1.25 ± 0.05 V and Cat2 at potential -1.50 ± 0.05 V). Co_1 and Cat1 peaks did not appear due to low concentration of thiols. The effect of three different parameters (accumulation time, temperature of electrolyte, and concentration of PNA) on Brdicka reaction of PNA was tested.

Fig. 2B showed dependence of relative height and potential of RS_2CO and Cat2 peak on the accumulation time of PNA onto the surface of HMDE. Concentration of PNA was 10 μg/ml and accumulation time of 30, 60, 120, 240, 360 s was tested. Relative height of Cat2 peak increased according to the increase of accumulation time because concentration of PNA corresponds to the height of Cat2 peak [39-41]. Cat2 peak gave the highest value at accumulation time of 360 s. Relative height of Cat2 peak increased about 63% from 240 s to 360 s of accumulation time of PNA. Therefore, 360 s of accumulation time was chosen for measurement of the influence of temperature of electrolyte on Cat2 peak height. Dependence of relative height and potential of RS_2CO and Cat2 peak on temperature of electrolyte is shown in Fig. 2C. Five different temperatures (4, 8, 12, 16 and 20 °C) were tested. Concentration of PNA was 10 μg/ml. Height of both peaks decreased with the increasing electrolyte temperature. The highest value of Cat2 peak was thus detected at $4^{\circ}C$. Finally, the influence of the concentration of PNA on peaks height was monitored. Dependence of relative height and potential of RS_2CO and Cat2 peak on concentration of PNA is shown in Fig. 2D. Accumulation time of 360 s and concentrations of PNA 0.6, 1.25, 2.5, 5, and 10 μg/ml were used for the measuring of the dependence. Relative Cat2 peak height increased according to the increased concentration of PNA. Linear dependence (Fig. 1E) was observed below the concentration of PNA of 2.5 μg/ml where dependence was as follows: $y = 3.17x + 1.35$, $R^2 = 0.992$, $n = 3$. RS_2CO decreased with the increasing concentration of PNA because RS_2CO peak corresponds to reaction between $[Co(NH_3)_6]^{2+}$ complex and thiols group of PNA. Change of potential of RS_2CO and Cat2 peak with the changing of three tested parameters was negligible. It means that accumulation time, temperature of electrolyte, and concentration of PNA parameter doesn't affect position of two observed peaks.

3.3. Electrochemical determination of PNA by AdT DPV

AdT coupled with Brdicka reaction seems to be very promising tool for detection of thiol rich compounds [35]. AdT method was used for measurement of dependence of accumulation time, temperature of electrolyte, and concentration of PNA on Brdicka reaction of PNA. Fig. 3A showed an obtained DP voltammogram of PNA (red line). There are four detected peaks (Co_1 peak at potential -1.00 ± 0.05 V, RS_2CO at potential -1.25 ± 0.05 V, Cat1 at potential -1.35 ± 0.05 V and Cat2 at potential -1.55 ± 0.05 V). Dependence of relative height and potential of CO_1, RS_2CO, Cat1, Cat2 peak on time of accumulation is shown in Fig. 3B. Concentration of PNA was 1 μg/ml and accumulation time of 30, 60, 120, 240, and 360 was tested. Like DPV method, relative Cat2 peak height increased according to the increase of accumulation time. Accumulation time of 360 s was optimized and used for next experiments because Cat2 peak was the highest at accumulation time of 360 s. Next

experiment tested the influence of electrolyte temperature on sensitivity of the measurement. The increase of temperature of the electrolyte led to the decrease of Cat1 and Cat2 peak height. Dependence of relative height and potential of CO_1, RS_2CO, Cat1, Cat2 peak on temperature of electrolyte is shown in Fig. 3C. Relative Cat1 and Cat2 peak height both decreased for more than 80 % when temperature of electrolyte increased from 4 to 8 °C. Last experiment of this study was measuring the influence of concentration of PNA on the heights of the mentioned peaks. Dependence of height and potential of CO_1, RS_2CO, Cat1, Cat2 peak on PNA concentration is shown in Fig. 3D. The increasing signal of Cat2 peak was observed when concentration of PNA increased.

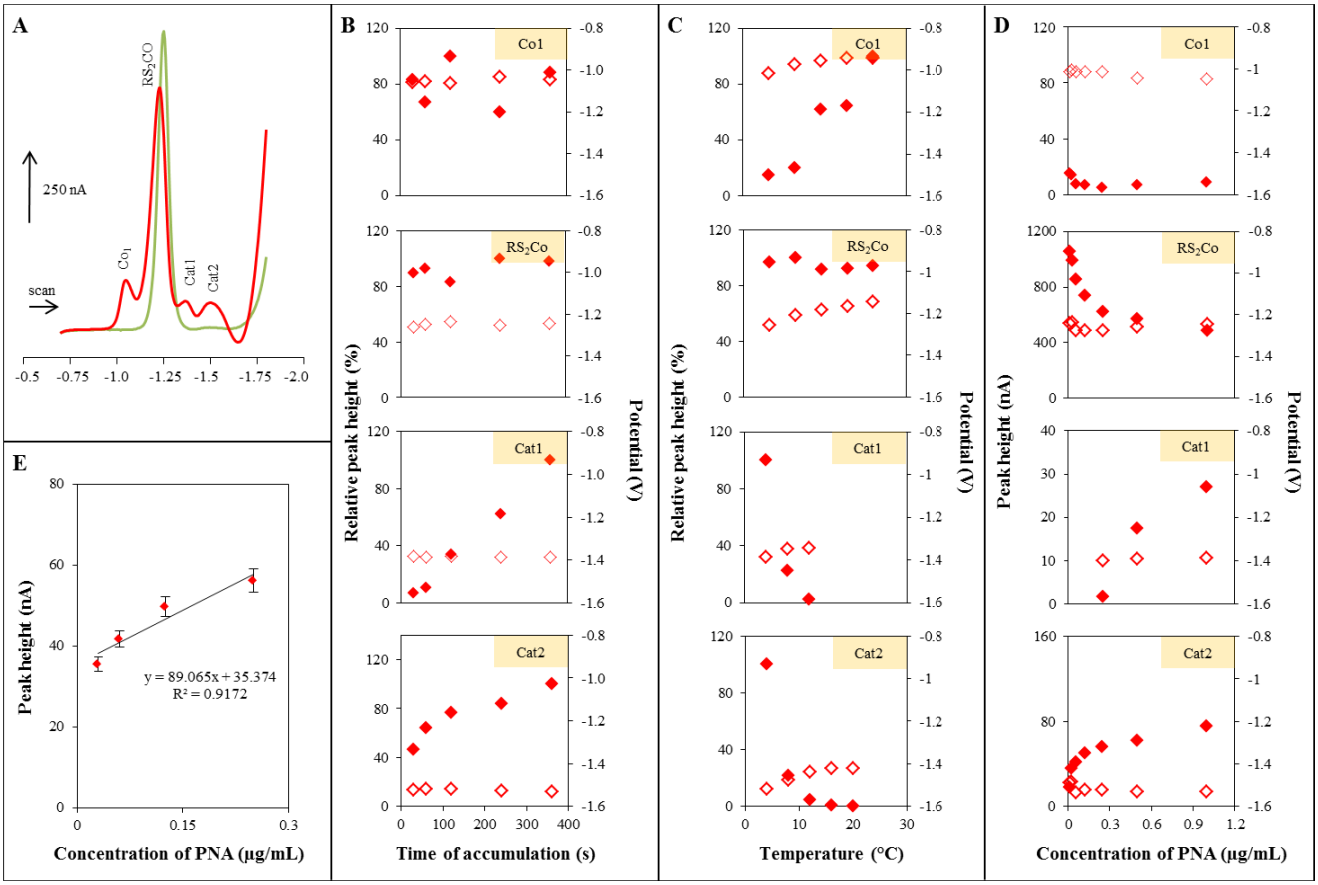

Figure 3. Electrochemical characterization of PNA by AdT DPV method (filled dot: relative peak height or peak height, non-filled dot: potential). (**A**) Typical DP voltammogram of PNA (red line) and Brdicka electrolyte itself (green line). (**B**) Dependence of relative heights of peaks Co1, RS2Co, Cat1 and Cat2 (related to the maximal values) and their potentials on time of accumulation (concentration of PNA 1 µg/ml). (**C**) Dependence of relative heights of peaks Co1, RS2Co, Cat1 and Cat2 (related to the maximal values) and their potentials on temperature of Brdicka electrolyte (concentration of PNA 1 µg/ml , accumulation time 360 s). (**D**) Dependence of concentration of PNA 1 µg/ml on concentration of PNA; accumulation time 360 s, temperature of electrolyte 4 °C. Parameters of AdT DPV were as follows: purge time 60 s, modulation time 0.057 s, interval time 0.2 s, initial potential -0.7 V, end potential -1.8 V, step potential 0.005 V, modulation amplitude 0.025 V, n = 3.

Linear dependence (Fig. 3E) was observed below the concentration of PNA of 0.25 µg/ml where dependence was as follows: y = 89.07x + 35.37, R^2 = 0.917, n = 3. Like DPV method, RS_2CO

peak height decreased according to the increase of concentration of PNA. However, decreasing speed of RS_2CO peak measured by AdT DPV method is faster compared to DPV method. Furthermore, accumulation time, temperature of electrolyte, and concentration of PNA parameter also doesn't affect position of four peaks, which are monitored by AdT DPV analysis.

4. CONCLUSIONS

Brdicka reaction of PNA was investigated in this study. It was tested coupling of adsorptive transfer technique with DPV to enhance the sensitivity. AdT DPV produced four typical peaks (Co_1 peak at potential -1.00 ± 0.05 V, RS_2CO at potential -1.25 ± 0.05 V, Cat1 at potential -1.35 ± 0.05 V and Cat2 at potential -1.55 ± 0.05 V). Cat2 peak height was strongly depended on accumulation time, temperature of electrolyte, and concentration of PNA. Furthermore, new holder prepared by three dimensional printing technology was designed and manufactured for facilitation of experiments and better adjustment of experimental conditions.

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CONFLICT OF INTEREST:
The authors have declared no conflict of interest.

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