Int. J. Electrochem. Sci., 8 **(2013)** 12628 - 12642

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Sequences of Pandemic-Causing Viruses Isolated and Detected by Paramagnetic Particles Coupled with Microfluidic System and Electrochemical Detector

Ondrej Zitka[1,2], *Sylvie Skalickova*[1], *Miguel Angel Merlos Rodrigo*[1,2], *Ludmila Krejcova*[1], *Pavel Kopel*[1,2], *Vojtech Adam*[1,2], *Rene Kizek*[1,2*]

[1] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union
[2] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union
[*]E-mail: kizek@sci.muni.cz

Received: 4 June 2013 / *Accepted:* 7 August 2013 / *Published:* 20 October 2013

Detection of the specific sequences of pandemic-causing viral nucleic acids is area attracting numerous scientists worldwide. In this study, we focused on the isolation and detection of oligonucleotide specific sequences of life-threatening viruses (HIV (GAGCAGTGGGAATA), Influenza (TAATAACCATTGGA) and Ebola (ACCTCACTAGAAAA)) by flow injection analysis with electrochemical detection coupled with paramagnetic particles based isolation. All of used viral genomic sequences contained A6 adenine repetition on the 5´end. This modification enables us to bind them on paramagnetic particle anchoring T25 (MPs-$(dT)_{25}$). The isolated sequences were subsequently hydrolysed by optimized method using microwaves to obtain single adenines. The obtained samples were detected by microfluidic tool based on stopped flow injection analysis with glassy carbon electrode in the presence of Cu(II)/or CuS nanoparticles to enhance sensitivity and to lower detection limit for adenine. Basic detection parameters as accumulation of CuS nanoparticles, flow influence and accumulation of the adenine deposition influencing the analytical response were also tested. We found that bases gave well developed signals with detection limit down to 30 ng per ml (50 pg per electrochemical cell) under the optimized conditions.

Keywords: Paramagnetic Particles Based Isolation; Microfluidics; Glassy Carbon Electrode; Copper Nanoparticles Modification; Viral Nucleic Sequences; Stopped Flow Injection Analysis; Microwave Hydrolysis

1. INTRODUCTION

Electrochemistry belongs to the most suitable methods for studying nucleic acid. The electrochemical methods are of great interest due to usability, easy sample manipulation,

miniaturization possibilities, high sensitivity and selectivity [1-7]. The solid electrodes as a glassy carbon (GCE) electrodes are widely used for construction of DNA biosensors [8] or for electrode surface modification, which can enhance the sensitivity of determination [9,10]. Nanoparticles belong to materials used for electrode surface modification [11]. Nanoscale materials modified electrodes were utilized for DNA bioassays [12-14]. In addition, multi-walled carbon nanotubes incorporated with poly(new fuchsin) composite film [15] and polythionine/NPAu/MWNTs modified electrode was utilized for adenine determination [16]. In addition, electrodeposited nano-copper oxides on glassy carbon electrode was successfully used for simultaneous detection of guanine and adenine [17]. It is well known that copper(I) and adenine forming the soluble complex, which is accumulated on the electrode surface, whereas Cu(I) ions are produced by electrochemical reduction of Cu(II). This process markedly increases the sensitivity of adenine determination [18]. However, such assays used for determination of nucleic acids must be coupled with suitable separation technology.

Microfluidics is emerging field in biomedicine and it have inspired the design of new microcarriers that efficiently encapsulate bioactive agents to enable efficient encapsulation and controlled release for biological applications [19-22]. Microfluidic lab-on-a-chip devices, which utilize paramagnetic particles (MPs), are promising tools for accurate and rapid cell sorting and counting. MPs surface modification and thereby an elimination of adsorption of undesirable biomolecules is the main advantage of the magnetic based separation [3,23-25].

Detection of DNA strands by probe-coated paramagnetic particles and electrochemical detection of hybridized strands using a hanging mercury drop electrode and combining this method with dynamic synthesis of probe can result in new approaches for detection of other infectious agents by lab-on- chip technology [26-32]. In addition, other method for detection of DNA virus influenza was optimized and based on cadmium sulphide quantum dots (CdS QDs)-protein complexes connected to MPs. The measurements were performed using differential pulse voltammetry (DPV) on the surface of a hanging mercury drop electrode (HMDE) and/or glassy carbon electrode (GCE) [33]. The objective of this study is detection of the viral nucleic acid fragment specific sequences of human immunodeficiency virus (HIV), influenza and ebola by flow injection analysis with electrochemical detection coupled with paramagnetic particles (MPs) based isolation of target sequences.

2. EXPERIMENTAL PART

2.1 Chemicals and pH measurement

Sequence of viral nucleic acid fragments containing of 20 nucleotides from the middle of the gene of target viruses as HIV 5´-AAAAAAGAGCAGTGGGAATA-3´ (Genbank number of HIV-1: JX848351.1), influenza 5´-AAAAAATAATAACCATTGGA-3´ (Genbank number of Influenza: CY125094.1) and ebola 5´-AAAAAAACCTCACTAGAAAA-3´ (Genbank number of Ebola: U23187.1) were synthetized by Sigma Aldrich (St. Louis, USA). NaH_2PO_4, Na_2HPO_4, KCl, NaOH, water and other chemicals were purchased from Sigma-Aldrich in ACS purity unless noted otherwise Distilled water was prepared in laboratory using AquaOsmotic 02 (AquaOsmotic, Tisnov, Czech

Republic) and subsequently rafined by Millipore RG (MilliporeCorp., USA, 18 MΩ) to deionised (MiliQ) water.

2.2 Preparation of nanoparticles

CuS nanoparticles were prepared according to the following procedure. Copper acetate monohydrate $Cu(OAc)_2 \cdot H_2O$ (0.02 g, 0.1 mM) was dissolved in ACS water (25 ml) and mercaptosuccinic acid (0.08 g, 0.53 mM) was mixed at room temperature and stirred for 30 min. During the time mercaptosuccinic acid dissolved. 0.5 ml of 1 M NH_4OH was added with stirring to yellow solution, followed by addition of sodium sulphide nonahydrate $Na_2S \cdot 9H_2O$ (0.012 g, 0.05 mM) in 24.5 ml of ACS water. Colour of solution turned to light brown. The mixture was stirred overnight at room temperature and finally it was stored in dark at 4 ºC.

2.3 MPs based viral nucleic acid fragment isolation by robotic pipetting station

Automatic pipetting station EP Motion 5075 (Eppendorf, Germany) with original devices (microplate holder, tips holder, tips (1000, 300, 50 µl), rack tubes, reservoir holder, container for used tips, thermo adapter, PCR plate 96, magnetic adapter) was used for the fully automated target nucleic acids isolation process according to procedure adopted from Huska et al. [34]. Volume of 10 µl of Dynabeads Oligo $(dT)_{25}$ (Invitrogen, Oslo) was dispensed in selected wells in the microplate (PCR 96, Eppendorf). Plate was subsequently transferred to the magnet and MPs storing solution was aspirated to waste. Subsequently, beads were further washed three times with 20 µl of phosphate buffer I (pH = 6.5, 0.1 M NaCl + 0.05 M Na_2HPO_4 + 0.05 M NaH_2PO_4). The hybridization was the next step. 10 µl of polyA-modified anti sense oligonucleotides and 10 µl of hybridization buffer (0.1 M phosphate buffer, 0.6 M guanidinium thiocyanate, 0.15 M Tris, pH = 7.5) were added in each well and then the plate was incubated (30 min, 25 °C, mixing). This procedure was followed by a washing (three times) with 20 µl of phosphate buffer I. Then 50 µl of elution solution (phosphate buffer II - 0.2 M NaCl + 0.1 M Na_2HPO_4 + 0.1 M NaH_2PO_4) was added into selected wells and plate was subsequently incubated (5 min, 85 °C, mixing). After elution step, the plate was transferred to the magnet. Supernatant was transferred to new wells after 120 s long incubation.

2.4 Chip electrophoresis

Analyses were carried out in DNA chip using automated microfluidic electrophoresis system Experion (Bio-Rad, USA) according to the manufacturer's instructions with supplied components: Experion priming station, Vortex station and Spin filters (Bio-Rad), and chemicals Experion DNA 1K reagents and supplied kit containing: DNA gel, DNA stain, DNA loading buffer and DNA ladder (Bio-Rad). Experion Software v. 3.2 (Bio-Rad) was used for processing of the data obtained. Briefly to the protocol, the priming was performed as follows: 9 µl of GS (gel mixed with fluorescent stain) was pipetted into the well labelled GS (gel priming well) and primed using the priming station. Loading of

the chip was done according following procedure. Firstly, 9 µl of GS was pipetted into the 3 other wells labelled GS. Then, 5 µl of loading buffer (10%) was placed into each sample well and the ladder well. Subsequently, 1 µl of DNA ladder was pipetted into the well labelled L and 1 µl of each sample was pipetted into sample wells. Before the CE analysis, the wells were carefully inspected to avoid air bubbles. The chip was vortexed for 60 seconds to ensure the mixing and immediately analysed on Experion electrophoresis station according to Vaculovicova et al. [35].

2.5 Viral nucleic acid fragment hydrolysis

Hydrolysis of samples was performed by adding 50 µl of 10 mM HCl to 50 µl of the sample contained adenine at concentration of 100 $\mu g.ml^{-1}$. The mixture was heated for 30 min at 800 W in microwave reaction system (Multiwave 3000, Anton Paar, Austria). Then, the sample was cooled to room temperature, and neutralized with sodium hydroxide. Samples were evaporated (Ultravap, Porvair, United Kingdom) to dry under N_2 and 60 °C on low DNA binding plate (Eppendorf). After that the samples were diluted in 0.05 M PBS buffer (pH 7) and aliquots were used for voltammetric measurements.

2.6 Stopped flow injection analysis coupled with differential pulse voltammetry

For electrochemical detection of adenine, miniaturized microfluidic system coupled with automated electrochemical detection was used [36]. The system is consisted of programmed syringe pump (Model eVol, SGE Analytical Science Pty Ltd, Australia), 3-way 2-position selector valve (made from 6-way valve) (Valco, Instruments Co. Inc., USA), dosing capillary, which is entered to the electrochemical flow cell (CH Instruments, Inc., USA) and a prototype of miniaturized micropotentiostat (910 PSTAT mini (Metrohm, Switzerland)). Programmed syringe pump enables precise sample injections (units of µl with error lower than 5 %). To prepare a fully automated system, switching valve enabling switching between the waste and sample flow was placed into the system. Flow cell in volume of 500 – 1000 nl with electrochemical detector (working electrode: glassy carbon electrode (GCE), auxiliary electrode: platinum, reference electrode: Ag/AgCl 3M KCl) was used for a measurement. The CuS nanoparticles film was firstly electrodeposited on GCE surface by maintaining potential of -0.4 V for 200 s in the presence of 0.1 M KCl solution containing 200 µM CuS nanoparticles. After that, the cyclic voltammetry was carried out the presence of 0.1 M NaOH solution repetitively for 20 cycles under potentials ranging from -0.5 to 0.3 V at 100 $mV.s^{-1}$.

The sample (10 µl) was injected by automated syringe (SGE Analytical Science, Australia) through flow cell in maximal applied speed of 1.66 µl/s. Supporting electrolyte for electrochemical detection of adenine was 0.05 M phosphate buffered saline (PBS, pH 7). Detection parameters of differential pulse voltammetry (DPV) were as it follows: initial potential 0 V, end potential 1.8 V, step potential 0.015 V, amplitude 0.1 V, scan rate 0.07 V, pulse period 200 s and equilibration time 20 s. The flow cell was cleaned by rinsing with 200 µl HNO_3 in water (10 % *v*/*v*), then with 200 µl of water.

Cleaning was applied after each measurement. The data obtained were processed by PSTAT software 1.0 (Metrohm, Switzerland). The experiments were carried out at 20 °C.

2.7 Stationary electrochemistry

The comparative electrochemical analysis using GC electrode was carried out with standard stationary electrochemical system consisted of electrochemical cell with three electrodes and the same potentiostat as used above (910 PSTATmini (Metrohm, Switzerland)). An Ag/AgCl/3M KCl electrode was used as the reference electrode and the glassy carbon electrode was used as auxiliary electrode. For data processing PSTAT software 1.0 (Metrohm, Switzerland) was employed. PBS buffer (0.5 M) was used as a supporting electrolyte. The supporting electrolyte was changed after each analysis. The parameters of the measurement were as it follows: Initial potential 0 V, end potential 1.8 V, step potential 0.015 V, amplitude 0.1 V, scan rate 0.07 V, pulse period 200 s and equilibration time 20 s.

The comparative analysis after nucleic acid hydrolysis by determination of adenine by differential pulse voltammetry was performed with 663 VA Stand (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing GPES 4.9 software was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%). Acetate buffer (0.2 M CH_3COONa + CH_3COOH, pH 5) as a supporting electrolyte was used. The supporting electrolyte was changed after each analysis. The parameters of the measurement were as it follows: purging time 120 s, deposition potential -0.9 V, accumulation time 240 s, equilibration time 5 s, modulation time 0.057 s, interval time 0.2 s, initial potential -0.9 V, end potential -0.3 V, step potential 0.00195 V, modulation amplitude 0.02505 V, volume of injected sample: 10 µl, volume of measurement cell 1 ml (10 µl of sample + 990 µl acetate buffer).

2.8 Spectrophotometric measurement

Spectra for determination of DNA concentration (260 nm) were recorded within the range from 200-500 nm on spectrophotometer SPECORD 210 (Analytik Jena, Jena, Germany) using quartz cuvettes (1 cm, Hellma, Essex, UK). Cuvette space was tempered by a thermostat Julabo (Labortechnik, Wasserburg, Germany) to temperature 20 °C.

2.9 Descriptive statistics and estimation of detection limit

Data were processed using MICROSOFT EXCEL® (USA). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (LOD, 3 signal/noise, S/N) were calculated according to Long and Winefordner [37], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

The viral genome contains long chains of DNA, where each bases are in different concentrations. In this study, we focused on adenine, which can be well detected by electrochemistry using solid electrodes in the presence of Cu(II) ions [38] as a hybridisation sensor on the principle of complementarity [39]. Carbon electrodes provides great potential for DNA virus biosensing, which is still emerging area from the end of the last century due to threat of virus pandemics [40]. Because of possibility of easy renewability of its surface as well as high sensitivity in the presence of Cu(II) ions it brings good tool for manufacturing low cost biosensors [41], which might be further useful for the diagnosis *in situ* [42]. Another advantage to sensitivity of adenine determination, we found that three of the most dangerous viruses contained different amount of adenine in its overall sequence (Fig. 1), which could be useful for their distinguishing.

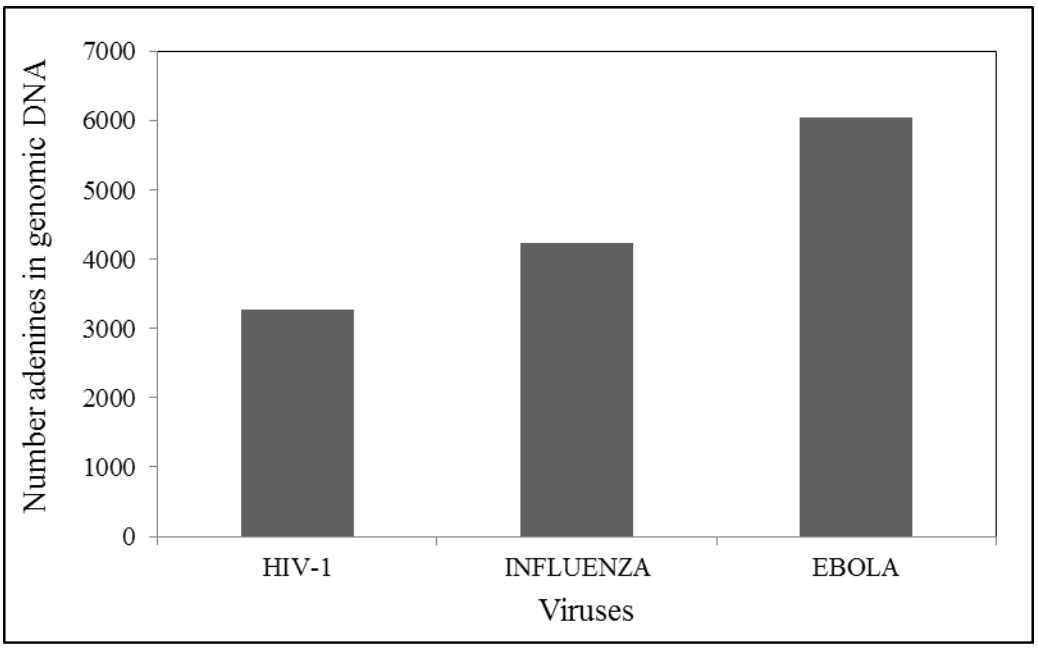

Figure 1. Number of adenines in genomic nucleic acid sequence of HIV-1, Influenza and Ebola viruses. Data was adopted from NCBI database.

3.1 GCE modification

Low cost and rapid detection of viral nucleic acid fragment could be live saving and beneficial in developing countries, where the incidence of these diseases belongs to the highest as well as there is critical insufficiency of well-equipped medical diagnostic tools. Considering low cost, robust and low demanding system for electrochemical detection, GC electrode represents useful for detection of all nucleotides [43] and is perspective for further modification to increase of selectivity of bases detection [44]. Numerous papers show that copper(II) ions can increase sensitivity of adenine detection, but almost all of them are based on the using of Cu(II) salts. Only two papers were based on the utilizing

of Cu nanoparticles for that purpose. The first work used oxide of copper created by electrodeposition on the surface of GC electrode for adenine detection with 6 nM detection limit [45] and the second one was based on nanostructured copper electrode (NSCuE) prepared by electroplating using scanning electron microscopy (SEM) [17]. In this study, we used CuS nanoparticles, which were prepared out of measuring device according to caption 2.2. The perspective of CuS modification of electrode is not only the enhancing the area of electrode, but the adenine creates the stable complex with Cu ions, which enable the sensitive detection [46]. The obtained liquid was injected to the electrochemical cell of the device suggested in our previously published paper [36] and the conditions of modification of the surface of the electrode was the same like as Zhang et al. found [17] with one difference that we used a solution of 200 µM CuS nanoparticles for nano copper oxide formation on electrode surface. It can be expected that during CV in the presence of NaOH solution CuS nanoparticles convert to CuO. At the potentials used for analyses, Cl^- anions present in analyte do not interfere as their oxidation occurs at much higher potential (1.36 V).

3.2 Analytical parameters of SFIA system

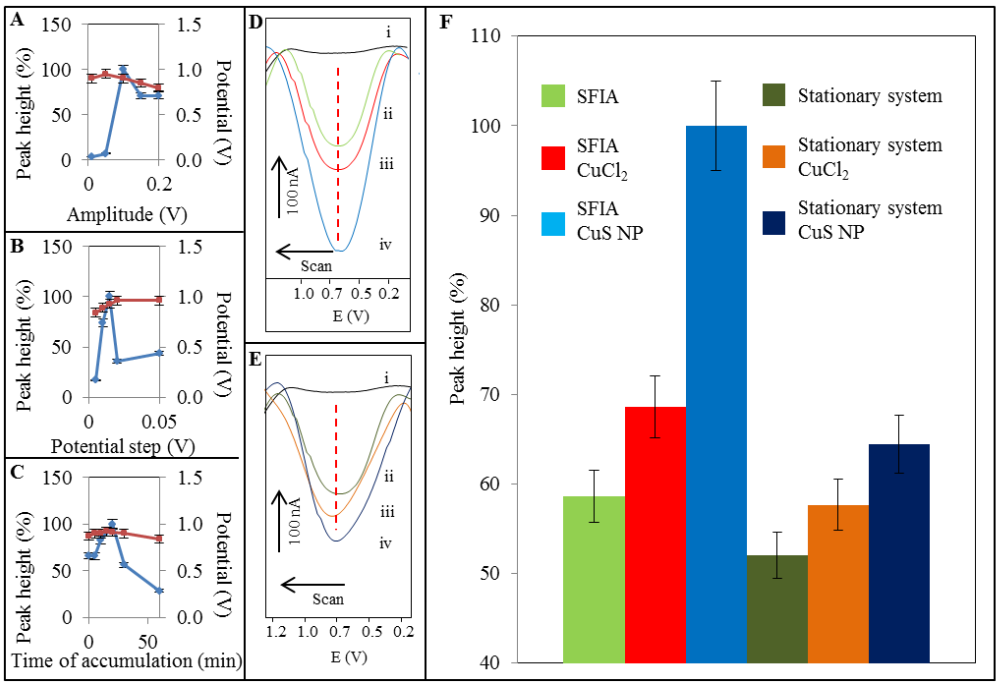

Figure 2. Optimization of electrochemical detection of adenine (100 ng per cell volume) using differential pulse voltammetry in the presence of 0.05 M PBS (pH 7), without electrode modification. Dependence of height of electrochemical signal (blue line) and potential (red line) on (**A**) applied amplitude (0-0.2V), (**B**) applied potential step (0-0.05) and (**C**) time of accumulation (0-50 min). Electrochemical signal showing responses of (i) blank and adenines measured at glassy carbon electrode (ii) without modification, (iii) with modification with 20 µM $CuCl_2$ and (iv) with modification with 200 µM CuS nanoparticles measured by (**D**) SFIA instrument and (**E**) standard stationary electrochemical instrument. (**F**) Comparison of adenine peak heights measured by SFIA and/or standard electrochemical instrument at modified or unmodified GCEs.

We attempted to develop a method based on CuS nanoparticles modified GC electrode to be able to distinguish various viral genes based on the overall concentration of adenine. Thus we had to optimise several parameters of differential pulse voltammetry (DPV). For obtaining the highest sensitivity of detection we observed the highest peak of adenine at amplitude as 0.05 V (Fig. 2A), potential step as 0.02 V (Fig. 2B) and time of accumulation as 20 s (Fig. 2C). After that we optimized the mentioned experimental conditions, we compared various ways of detection (i) without modification of electrode surface, (ii) with modification of electrode surface with $CuCl_2$ and (iii) with modification of electrode surface with CuS nanoparticles. We applied the same GC electrode in the SFIA system (Caption 2.7) (Fig. 2D) as well as in the standard three electrode system (Caption 2.8) (Fig. 2E) for better comparison of the results. The obtained electrochemical signals (nA) including RSD ± 5% (n = 5) are summarized in columns in Fig. 2F. We found that applied potential for accumulation of adenine on the surface of modified electrode and applied flow during modification, which is critical for preconcentration of analyte on the surface of the electrode in the microfluidic device, were the main factors influencing the height of adenine peak [17].

During the optimizing flow rate and accumulation potential, we found that the increasing flow rate enhanced the peak height under 0.4 and 0.5 V accumulation potentials. Application of 0.3 V and the highest flow rate caused decreasing of the peak (Fig. 3A). Based on these results, we used the best conditions including flow rate 1.66 µl per second during accumulation at 0.4 V. Under these conditions we obtained the calibration curve ranging from 0.37 to 11.72 ng per cell volume with linearity R^2= 0.9948 and LOD (3 S/N) as 50 pg per cell (Fig. 3B).

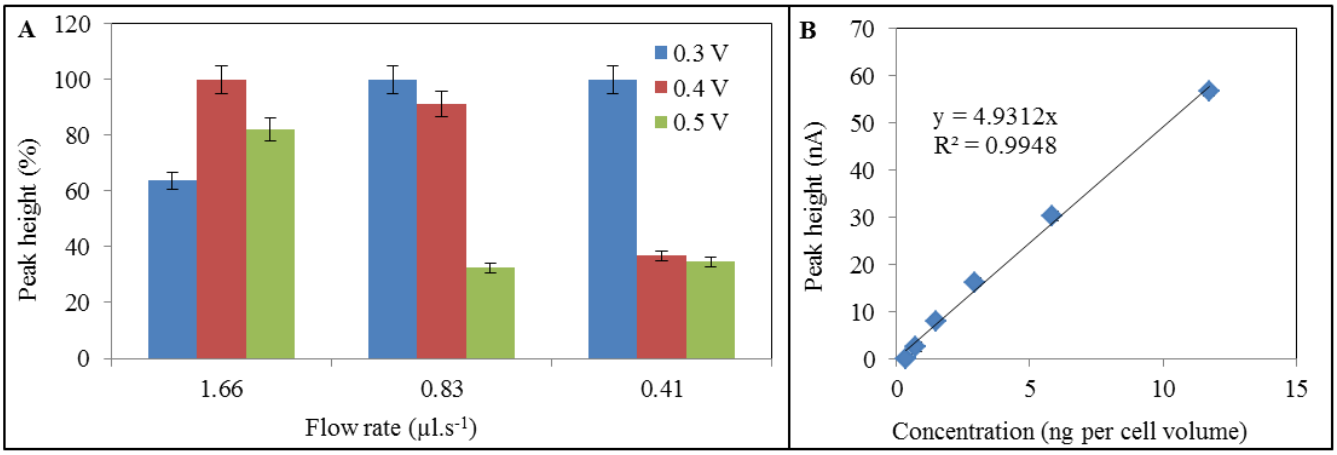

Figure 3. (**A**) Influence of flow rate (0.415, 0.83 and 1.66 µl.s^{-1}) and applied accumulation potential (0.3-0.5 V) on adenine (100 ng per cell volume) detection. (**B**) Calibration curve of adenine ranging from 0.37 to 11.72 ng per cell analysed by the optimized DPV. GC modified with 200 µM CuS particles was used as working electrode. Other experimental details are in Caption of Figure 2.

It clearly follows from the obtained data that modification of GC electrode by CuS nanoparticles can increase sensitivity of determination of adenine due to enhancing area of working electrode, which is caused by spherical shape of the nanoparticles. It also demonstrates that

microfluidic SFIA tool is suitable to be coupled with such modified GCE. However, there is necessity to isolate viral nucleic acid from the host. SFIA method needs at least 1.5 µl of the sample as a cell volume of the instrument for carrying out of the analysis, which is beneficial for using *in situ* [42].

3.3 Optimization of microwave assisted hydrolysis of viral nucleic acid fragment

We optimized the hydrolysis of the nucleic acid chain for detection of the adenine as marker of the various viral nucleic acid fragments of the three selected viruses. This might be done by different approaches as addition of $HClO_4$ and heating of the mixture with subsequent neutralization [38]. The hydrolysis of nucleic acids required a long time, which might be reduced using microwave assisted hydrolysis [47]. Based on the experimental conditions from Marrubini et al. [47], we selected microwave assisted hydrolysis, but we had to optimize the parameters of concentration of applied HCl, energy and also time of hydrolysis (Fig. 4).

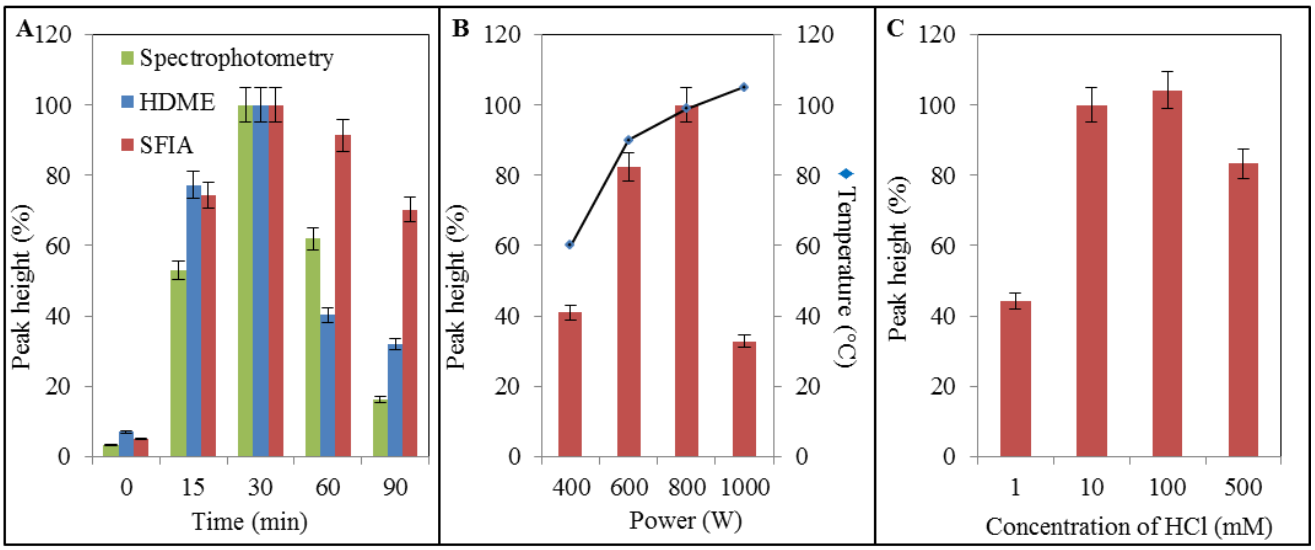

Figure 4. Optimization of hydrolysis of the mixture of HIV, Influenza and Ebola viral sequences (1:1:1, 100 µg.ml^{-1}). Influence of (A) HCl concentration the adenine peak was determined by SFIA (red column), electrochemical CSV using HMDE (blue column) and spectrophotometry (green column), and (B) power of microwave radiation (during experiment the temperature inside the vial did not exceed of 110 °C), and (C) time of hydrolysis on adenine peak height.

The first parameter, HCl concentration, was optimized and three methods, SFIA, spectrophotometry and standard electrochemical apparatus using HMDE, were used for determination of adenine concentration. The optimizing was carried out with the mixture of all three viral sequences in ratio 1:1:1 and applied concentration of each of the sequences as 100 µg.ml^{-1}. We found that the highest yield determined as the total concentration of adenine was found by SFIA method and two supported methods as 30 min (Fig. 4A). The increasing of the trend from 0 to 30 minute was caused by continuing of the cleaving of the bonds in the chain and after overcoming of the maximal yield the

decreasing of the signal may be as result of some damaging of free bases by microwaves in the acidic environment that leads to the digestion of the molecules. Nevertheless, the microwave irradiation power of 800 W was found as the optimal (Fig. 4B) and the optimal concentration of HCl was 10 mM (Fig. 4C), because we wanted to hold the concentration of HCl as low as possible. The using of small volume vials minimizing sample consumption was another advantage of our method [48,49].

3.4 Liquid phase separation of viral nucleic acid fragment by oligo DBT$_{25}$

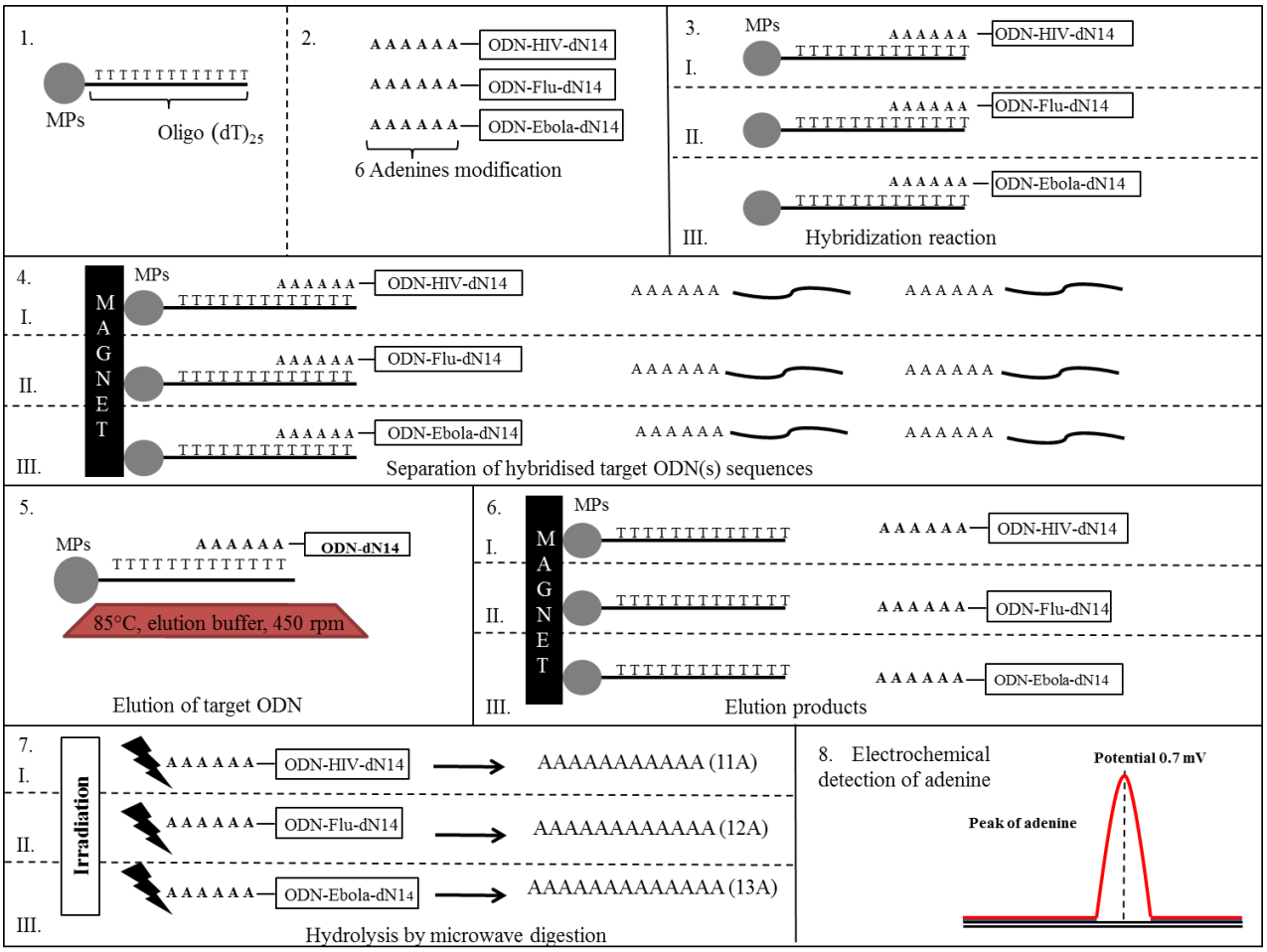

Figure 5. Scheme of MPs based isolation of different specific ODN sequences. (**1.**) MPs modified by Oligo(deoxythymine)$_{25}$. (**2.**) Three different specific ODN sequences derived from nucleic acid of viral disease: Ebola, influenza and HIV. (**3.**) Hybridization of MPs-(dT)$_{25}$ with (I) ODN-HIV-dN14, (II) ODN-Flu-dN14 and (III) ODN-Ebola-dN14. (**4.**) Forcing of MPs by magnet and washing the interferences. (**5.**) Elution by condition: 85 °C, elution buffer and 450 rpm. (**6.**) Products of elution after isolation (MPs and isolated specific sequences). (**7.**) Hydrolysis of viral ODN sequences by microwave digestion. (**8.**) Electrochemical detection was carried out with microfluidic tool based on stopped flow injection analysis using GC electrode.

We used the magnetic beads as so called stationary phase for isolation of target viral nucleic acid fragments from the sample. Our approach was adopted and modified from our previously

published papers [32,50]. All the steps of the isolation used in this study are shown in Fig. 5. Other authors also prefer for isolation and/or detection of the nucleic acid fragment of viruses different modification of MPs, gold NPs or they also uses aptamers based isolation. Aptamers (SELEX) can bind to their targets with high affinity and specificity due to their specific three-dimensional structures. Especially, RNA and ssDNA aptamers can differ from each other in sequence and folding pattern, although they bind to the same target [51,52]. Two materials of nanoparticles are commonly used as gold [53,54] and iron [55,56]. Using our iron oxide beads in combination with automatic pipetting facility, we were able to automatically isolate all viral fragments within ten minutes. The yield of the isolation was 68 % for HIV (n = 5), 74 % for influenza (n = 5) and 78 % for Ebola (n = 5).

3.5 Chip electrophoresis analysis

Gel capillary electrophoresis was used for analysis and verification of the isolated HIV, influenza and Ebola genomic nucleic acid fragments. The studied viral nucleic acid sequences had also a different number of the purine a pyrimidine bases next to the different number of adenines. As we observed from the different electrophoretic mobilities of all three isolated fragments, the ratio of pyrimidine and purine bases influenced the migration markedly (Figs. 6A, B and C). AG pairs were 17 in HIV sequence and 14 in Influenza and Ebola sequences. This explains the different charge of the fragments and the difference in the mobilities. HIV fragment had highest mobility (42.35 s, Fig. 6A) due to the highest amount of AGs and the Influenza and Ebola has 37.67 s and 37.58 s respectively due to lower and same amount of AGs (Fig. 6B and C, respectively).

3.6 Determination of the adenine content in hydrolysed viral genomic fragments

Twenty nucleotides long fragments of viral genomic sequences of HIV, Influenza and Ebola were firstly isolated using paramagnetic particles, analysed by the capillary chip gel electrophoresis (Figs. 6A-C) and hydrolysed under the optimized experimental conditions. After the evaporation of the hydrolysed sample at 60 °C under nitrogen we dissolved the solid sample in the analysing buffer and made the final analysis by the SFIA method using Cu nanoparticles. There were obtained the oxidation signals at potential 0.64±0.05 V (n = 5) in the anodic scan measured by DPV (Fig. 6D). The voltammograms were well developed. Analytical parameters of determination of viral nucleic acid genomic fragments are indicated in Tab. 1. Here reported values prove that major quality parameter as redox potential did not change. Concerning the detected concentration we observed different intensities of the signal for each separated sequence in the order HIV < Influenza < Ebola (Fig. 6E) with RSD not exceeding 10 %. These results are in good agreement with the content of adenines in the HIV, Influenza and Ebola viral sequences (Fig. 1).

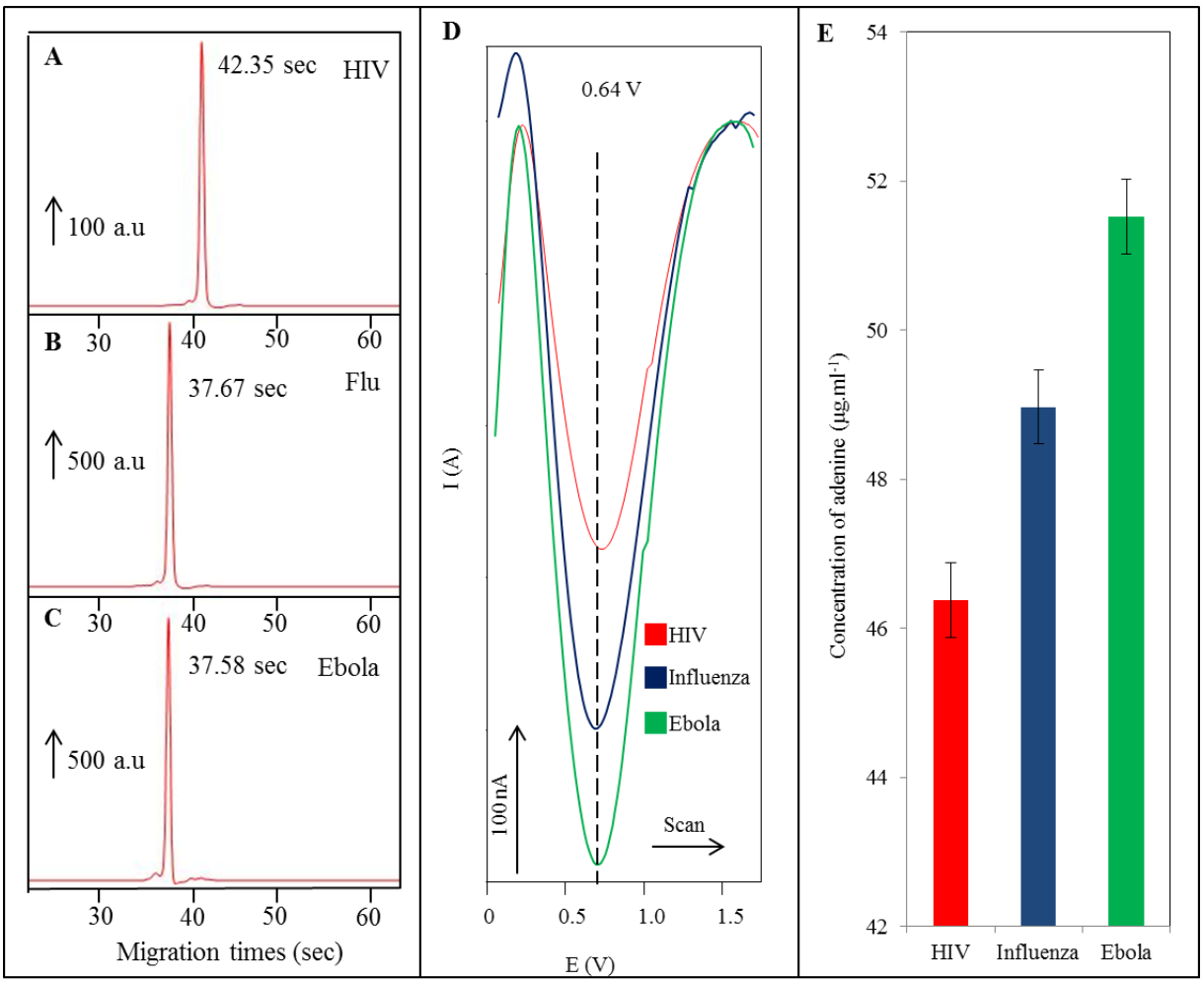

Figure 6. Chip-CE electropherograms of (**A**) ODN from HIV-1 virus, (**B**) ODN from Influenza virus and (**C**) ODN from Ebola virus after the hybridization. (**D**) Concentration of adenine after isolation of HIV, Influenza and Ebola viral sequences by paramagnetic particles followed by hydrolysis and analysed by the optimized SFIA with GC electrode modified with Cu nanoparticles. (**E**) Concentration of adenine determined in ODNs for HIV-1, Influenza and Ebola viruses. The data was obtained using SFIA system with GC electrode modified with CuS nanoparticles.

Table 1. Analytical parameters for adenine determination by SFIA.

Viruses	Width	Charge	Potential	Height	Concentration	RSD
	µC	µC	V	nA	µg.ml^{-1}	%
HIV	0.70	0.74	0.65	69.08	46.4	6.5
Influenza	0.73	0.70	0.64	72.03	49.0	5.8
Ebola	0.70	0.67	0.64	72.61	51.6	6.4

4. CONCLUSIONS

We show new approach for the identification of three very dangerous viruses based on the identification of adenine content using advantages of electrochemistry with advanced materials

isolation approach. Combining advanced biological electrochemistry detection methods with MPs separation techniques has tremendous potential for realization of an integrated system for pathogen detection [57]. Further, we show that the suggested electrochemical method is able to distinguish between various numbers of adenines in three different viral nucleic acid fragments. It is shown that sensitivity of CuS nanoparticles modified GC electrode is enough for such kind of task and further with subsequent methodologically improvements it might be considered for identification of all virus sequence based on the difference of adenine concentration.

ACKNOWLEDGEMENTS
Financial support from CEITEC CZ.1.05/1.1.00/02.0068, NanoBioTECell GA CR P102/11/1068 and IGA IP16/2013 is highly acknowledged.

References

1. T. Yumak, F. Kuralay, M. Muti, A. Sinag, A. Erdem and S. Abaci, *Colloid Surf. B-Biointerfaces*, 86 (2011) 397.
2. S. W. Dutse and N. A. Yusof, *Sensors*, 11 (2011) 5754.
3. K. F. Lei, *Recent Pat. Nanotechnology*, 7 (2013) 81.
4. H. Craighead, *Nature*, 442 (2006) 387.
5. J. M. Rothberg, W. Hinz, T. M. Rearick, J. Schultz, W. Mileski, M. Davey, J. H. Leamon, K. Johnson, M. J. Milgrew, M. Edwards, J. Hoon, J. F. Simons, D. Marran, J. W. Myers, J. F. Davidson, A. Branting, J. R. Nobile, B. P. Puc, D. Light, T. A. Clark, M. Huber, J. T. Branciforte, I. B. Stoner, S. E. Cawley, M. Lyons, Y. T. Fu, N. Homer, M. Sedova, X. Miao, B. Reed, J. Sabina, E. Feierstein, M. Schorn, M. Alanjary, E. Dimalanta, D. Dressman, R. Kasinskas, T. Sokolsky, J. A. Fidanza, E. Namsaraev, K. J. McKernan, A. Williams, G. T. Roth and J. Bustillo, *Nature*, 475 (2011) 348.
6. S. Brunklaus, T. E. Hansen-Hagge, J. Erwes, J. Hoth, M. Jung, D. Latta, X. Strobach, C. Winkler, M. Ritzi-Lehnert and K. S. Drese, *Electrophoresis*, 33 (2012) 3222.
7. F. J. Shang, E. Guihen and J. D. Glennon, *Electrophoresis*, 33 (2012) 105.
8. J. Wang, S. P. Li and Y. Z. Zhang, *Electrochim. Acta*, 55 (2010) 4436.
9. J. Hubalek, J. Prasek, D. Huska, M. Adamek, O. Jasek, V. Adam, L. Trnkova, A. Horna and R. Kizek, in J. Brugger, D. Briand (Editors), Proceedings of the Eurosensors XXIII Conference, Elsevier Science Bv, Amsterdam, 2009, p. 1011.
10. H. S. Wang, H. X. Ju and H. Y. Chen, *Anal. Chim. Acta*, 461 (2002) 243.
11. M. Pumera, A. Merkoci and S. Alegret, *Electrophoresis*, 28 (2007) 1274.
12. M. Pumera, S. Sanchez, I. Ichinose and J. Tang, *Sens. Actuator B-Chem.*, 123 (2007) 1195.
13. Y. P. Song, M. Feng and H. B. Zhan, *Prog. Chem.*, 24 (2012) 1665.
14. I. Palchetti and M. Mascini, *Anal. Bioanal. Chem.*, 402 (2012) 3103.
15. C. Tang, U. Yogeswaran and S. M. Chen, *Anal. Chim. Acta*, 636 (2009) 19.
16. H. Y. Liu, G. F. Wang, D. L. Chen, W. Zhang, I. Li and B. Fang, *Sens. Actuator B-Chem.*, 128 (2008) 414.
17. X. Y. Zhang, X. Liang, M. Xu, X. Bao, F. W. Wang and Z. S. Yang, *J. Appl. Electrochem.*, 42 (2012) 375.
18. L. Fojt and S. Hason, *J. Electroanal. Chem.*, 586 (2006) 136.
19. K. K. Liu, R. G. Wu, Y. J. Chuang, H. S. Khoo, S. H. Huang and F. G. Tseng, *Sensors*, 10 (2010) 6623.
20. T. S. Shim, S. H. Kim and S. M. Yang, *Part. Part. Syst. Charact.*, 30 (2013) 9.

21. P. S. Doyle, J. Bibette, A. Bancaud and J. L. Viovy, *Science*, 295 (2002) 2237.
22. S. M. Desmarais, H. P. Haagsman and A. E. Barron, *Electrophoresis*, 33 (2012) 2639.
23. C. P. Gooneratne, I. Giouroudi and J. Kosel, *Sens. Lett.*, 10 (2012) 770.
24. A. H. C. Ng, K. Choi, R. P. Luoma, J. M. Robinson and A. R. Wheeler, *Anal. Chem.*, 84 (2012) 8805.
25. V. Adam, D. Huska, J. Hubalek and R. Kizek, *Microfluid. Nanofluid.*, 8 (2010) 329.
26. K. Fatemi, H. Ghourchian, A. A. Ziaee, S. Samiei and H. Hanaee, *Biotechnol. Appl. Biochem.*, 52 (2009) 221.
27. D. Huska, V. Adam, J. Hubalek, L. Trnkova, T. Eckschlager, M. Stiborova, I. Provaznik and R. Kizek, *Chim. Oggi-Chem. Today*, 28 (2010) 18.
28. D. Huska, V. Adam, S. Krizkova, J. Hrabeta, T. Eckschlager, M. Stiborova and R. Kizek, *Chim. Oggi-Chem. Today*, 28 (2010) 15.
29. J. Prasek, D. Huska, O. Jasek, L. Zajickova, L. Trnkova, V. Adam, R. Kizek and J. Hubalek, *Nanoscale Res. Lett.*, 6 (2011) 1.
30. J. Chomoucka, J. Drbohlavova, M. Masarik, M. Ryvolova, D. Huska, J. Prasek, A. Horna, L. Trnkova, I. Provaznik, V. Adam, J. Hubalek and R. Kizek, *Int. J. Nanotechnol.*, 9 (2012) 746.
31. D. Huska, O. Zitka, O. Krystofova, V. Adam, P. Babula, J. Zehnalek, K. Bartusek, M. Beklova, L. Havel and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 1535.
32. L. Krejcova, D. Huska, D. Hynek, P. Kopel, V. Adam, J. Hubalek, L. Trnkova and R. Kizek, *Int. J. Electrochem. Sci.*, in press (2013).
33. L. Krejcova, D. Dospivova, M. Ryvolova, P. Kopel, D. Hynek, S. Krizkova, J. Hubalek, V. Adam and R. Kizek, *Electrophoresis*, 33 (2012) 3195.
34. D. Huska, J. Hubalek, V. Adam, D. Vajtr, A. Horna, L. Trnkova, L. Havel and R. Kizek, *Talanta*, 79 (2009) 402.
35. M. Vaculovicova, K. Smerkova, J. Sedlacek, J. Vyslouzil, J. Hubalek, R. Kizek and V. Adam, *Electrophoresis*, in press (2013).
36. O. Zitka, S. Krizkova, L. Krejcova, D. Hynek, J. Gumulec, M. Masarik, J. Sochor, V. Adam, J. Hubalek, L. Trnkova and R. Kizek, *Electrophoresis*, 32 (2011) 3207.
37. G. L. Long and J. D. Winefordner, *Anal. Chem.*, 55 (1983) A712.
38. F. Jelen, B. Yosypchuk, A. Kourilova, L. Novotny and E. Palecek, *Anal. Chem.*, 74 (2002) 4788.
39. J. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1989.
40. J. Wang, X. H. Cai, G. Rivas, H. Shiraishi, P. A. M. Farias and N. Dontha, *Anal. Chem.*, 68 (1996) 2629.
41. N. Aladag, L. Trnkova, A. Kourilova, M. Ozsoz and F. Jelen, *Electroanalysis*, 22 (2010) 1675.
42. W. G. Lee, Y. G. Kim, B. G. Chung, U. Demirci and A. Khademhosseini, *Adv. Drug Deliv. Rev.*, 62 (2010) 449.
43. A. M. Oliveira-Brett, J. A. P. Piedade, L. A. Silva and V. C. Diculescu, *Anal. Biochem.*, 332 (2004) 321.
44. S. Shahrokhian, S. Rastgar, M. K. Amini and M. Adeli, *Bioelectrochemistry*, 86 (2012) 78.
45. K. Klosova, N. Serrano, O. Salyk and L. Trnkova, *Curr. Nanosci.*, 7 (2011) 984.
46. H. Shiraishi and R. Takahashi, *Bioelectrochem. Bioenerg.*, 31 (1993) 203.
47. G. Marrubini, P. Fattorini, C. Previdere, S. Goi, S. S. Cigliero, P. Grignani, M. Serra, R. Biesuz and G. Massolini, *J. Chromatogr. A*, 1249 (2012) 8.
48. P. Majzlik, A. Strasky, V. Adam, M. Nemec, L. Trnkova, J. Zehnalek, J. Hubalek, I. Provaznik and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 2171.
49. D. Hynek, L. Krejcova, S. Krizkova, B. Ruttkay-Nedecky, J. Pikula, V. Adam, P. Hajkova, L. Trnkova, J. Sochor, M. Pohanka, J. Hubalek, M. Beklova, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 943.

50. L. Krejcova, D. Hynek, P. Kopel, V. Adam, J. Hubalek, L. Trnkova and R. Kizek, *Chromatographia*, in press (2013).
51. J. Liu, M. X. You, Y. Pu, H. X. Liu, M. Ye and W. H. Tan, *Curr. Med. Chem.*, 18 (2011) 4117.
52. K. M. Song, S. Lee and C. Ban, *Sensors*, 12 (2012) 612.
53. Z. Z. Wang, K. Shang, J. Dong, Z. Q. Cheng and S. Y. Ai, *Microchim. Acta*, 179 (2012) 227.
54. E. O. Ganbold, T. Kang, K. Lee, S. Y. Lee and S. W. Joo, *Colloid Surf. B-Biointerfaces*, 93 (2012) 148.
55. R. Taube, Q. Zhu, C. Xu, F. Diaz-Griffero, J. Sui, E. Kamau, M. Dwyer, D. Aird and W. A. Marasco, *PLoS One*, 3 (2008) 1.
56. A. Sakudo, Y. Tanaka and K. Ikuta, *Neurosci. Lett.*, 494 (2011) 237.
57. Q. Ramadan and M. A. M. Gijs, *Microfluid. Nanofluid.*, 13 (2012) 529.