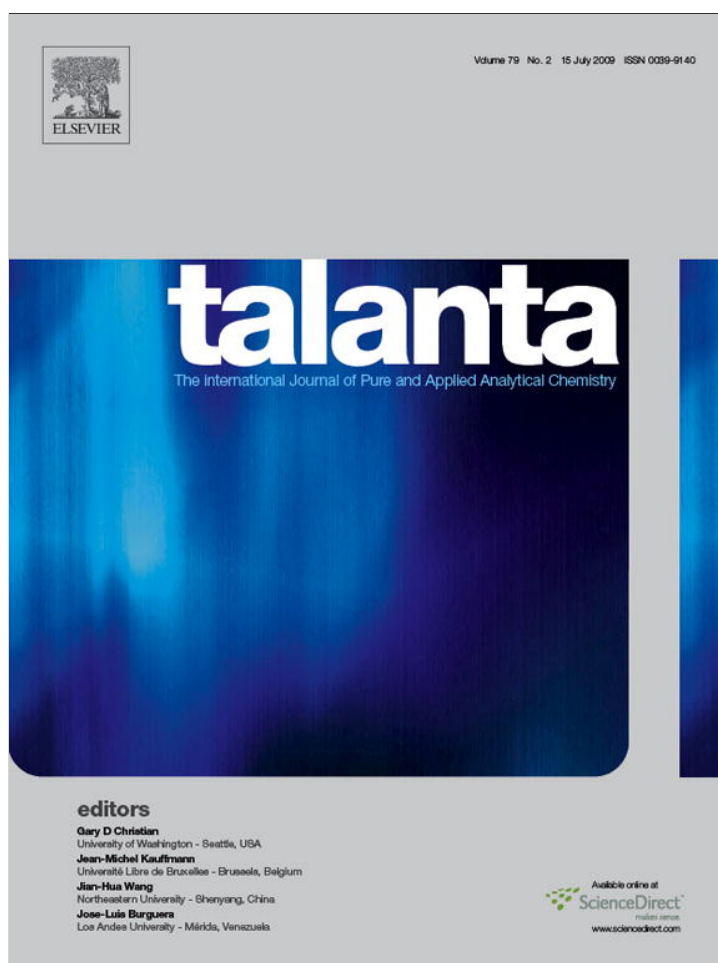


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Automated nucleic acids isolation using paramagnetic microparticles coupled with electrochemical detection

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ABSTRACT

Easy, efficient and low demanding separation of mRNA from biological material is needed to study gene expression and to use in chip technologies. It is common knowledge that each mRNA molecule contains sequence of 25 adenines. This feature can be used for binding mRNA on the surface of the particles coated by thymine chains. The present work reports on suggesting and optimizing of mRNA separation and detection from biological material via paramagnetic microparticles coupled with electrochemical detection. Primarily we optimized cyclic and square wave voltammetric conditions to detect poly(A), which was used as standard to mimic behaviour of mRNA. Under the optimized square wave voltammetric conditions (frequency 280 Hz, accumulation time 200 s, supporting electrolyte and its temperature: acetate buffer 4.6 and 35 °C) we estimated detection limit down to 1 ng of poly(A) per ml. To enhance effectiveness and repeatability of isolation of nucleic acid automated approach for rinsing and hybridizing was proposed. We optimized the whole procedure and experimental conditions. Using automated way of isolation and under optimized conditions the yield of poly(A) (isolated concentration of poly(A)/given concentration of poly(A)*100) was approximately 75%. The suggested and optimized method for poly(A) isolation and detection was utilized for the analysis of brain tissues of patients with traumatic brain injury. The total amount of isolated mRNA varied from 40 to 760 g of mRNA per g of brain tissue. The isolation of mRNA from six samples per run was not longer than 2.5 h. Moreover, we applied the optimized procedure on fully automated pipetting instrument to isolate mRNA. The instrument was successfully tested on the analysis of extracts from roots of maize plants treated with cadmium(II) ions.

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1. Introduction

Simple, rapid and reproducible isolation of nucleic acids with high purity is needed in a wide range of molecularly biological procedures. We are able to obtain nucleic acids with sufficient purity by the most commonly used method based on phenol–chloroform extraction; however, the whole procedure is laborious and time consuming. Due to this fact methods and approaches have been developing to shorten the time of isolation and to obtain nucleic

acids with sufficient purity to be analysed by polymerase chain reaction and other molecularly biological methods [1–4]. Nucleic acids isolation using paramagnetic or superparamagnetic particles (MPs) represents promising tool for this purpose [5–7]. MPs, whose size is ranging from nm to mm, respond to external magnetic field and facilitate bioactive molecules binding because of their affinity for the MPs modified surface made of biological components [8–11]. The paramagnetic properties of the particles enable us to use magnetic force for transferring of the beads or for rinsing of nonbinding, otherwise commonly interfering substances. Among other advantages of MPs easy-to-use, non-laborious relatively rapid sample preparation without centrifugation and dialysis compared to conventional purification techniques belong. The time needed to get target biomolecule is also reduced due to the fact that binding of the biomolecule by MPs can protect it against physical and biological damage, e.g. denaturation [12]. Physico-chemical prop-

Abbreviations: CV, cyclic voltammetry; SWV, square wave voltammetry; HMDE, hanging mercury drop electrode; MPs, magnetic particles; mRNA, messenger ribonucleic acid; NA, nucleic acid; poly(A), polyadenylic acid.

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erties of MPs (e.g. their size, surface topography) are important to evaluate their usage in biology [13–21]. The mostly used MPs in biosensors applications are superparamagnetic nanoparticles composed of ferrous oxide or ferric oxide [22]. Small particles with size from 1 to 10 nm are paramagnetic, but larger particles (mm) are ferromagnetic.

Nanoparticles have a lot of physico-chemical advantages [23,24]. Their size can be adapted to the extension and kind of a biological sample, which is a source of target biomolecules (e.g. proteins 5–50 nm, viruses 20–450 nm, cells 10–100 μm) [15,25,26]. Nanoparticles from ferric oxide also provide surface suitable for biomolecules binding. Methods of magnetic particles synthesis are largely discussed [9]. There are two ways to find how MPs can be modified. The first way is based on electric envelope layer for electrostatic adsorption of biomolecules. This method compared to conventional methods enables to avoid organic solvents. Matsunaga et al. modified the surface of MPs using electrically positive amino groups. Isolation was based on mutual electrostatic activities between negatively charged DNA and positively charged MPs [27]. The second way of paramagnetic particles surface modification is based on biomolecules anchored on particles. These biomolecules specifically bind target biomolecules [28–30]. On this method it is based also mRNA isolation. Oligo(deoxythymine)₂₅ anchored on the surface of MPs can be hybridized to mRNA molecule due to the presence of single chain sequence of tens adenines at the very end of all mRNA molecules [29].

MPs are commonly coupled with optical detection including fluorescent dyes. In addition, the isolated molecules can also be detected by electrochemical methods [28,31–35]. The main aim of this work was to automate isolation of mRNA using paramagnetic microparticles and detect the isolated nucleic acids by cyclic and square wave voltammetry both coupled with adsorptive transfer technique.

2. Materials and methods

All chemicals of ACS purity used and parafilm were purchased from Sigma–Aldrich Chemical Corp. (Sigma–Aldrich, USA) unless noted otherwise. Synthetic polyadenylic acid (poly(A)) was used as a standard. Stock standard solution of poly(A) (100 $\mu\text{g}/\text{ml}$) was prepared from lyophilized poly(A) (0.5 mg/ml, Mr = 400,000) with water of ACS purity (Sigma–Aldrich) and stored in dark at -20°C . The concentration of poly(A) was determined spectrophotometrically at 260 nm using spectrometer Spectronic Unicam (UK). The pH values were measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany). All the chemicals used for the preparation of plant cultivation media were purchased from Duchefa (Netherlands).

2.1. Instrumentation for poly(A) isolation

Isolation of poly(A) and mRNA was carried out using paramagnetic particles Dynabeads Oligo (dT)₂₅ (Invitrogen, USA) and magnetic stand Dynal Magnetic Particle Concentrator-S supplied by Dynal A.S (Norway). All experiments with paramagnetic particles were performed in RNA/DNA UV cleaner box UVT – S – AR (Biosan, Latvia). For centrifuging and vortexing of a sample multi-spin MSC-3000 centrifuge (Biosan, Latvia) placed in UV cleaner box was used. Denaturation was carried out at 85°C using the Thermomixer 5355 Comfort/Compact (Eppendorf, Germany).

The buffers used in our experiments were as follows: (a) phosphate buffer I: 0.1 M NaCl + 0.05 M Na₂HPO₄ + 0.05 M NaH₂PO₄; (b) phosphate buffer II: 0.2 M NaCl + 0.1 M Na₂HPO₄ + 0.1 M NaH₂PO₄; (c) acetate buffer: 0.2 M CH₃COOH + 0.2 M CH₃COONa. Hybridization solution: 100 mM Na₂HPO₄ + 100 mM NaH₂PO₄, 0.5 M NaCl,

0.6 M guanidinium thiocyanate, 0.15 M Trizma base adjusted by HCl on pH of 7.5.

2.2. Fully automated isolation of nucleic acids

Fully automated isolation was carried out on automated pipetting system epMotion 5075 (Eppendorf, Germany). The position of B4 is a magnetic separator (Promega). The positions of C1 and C4 can be thermostated (Eppendorf adapter PCR96). The pipetting provides a robotic arm with adapters (TS50, TS300 and TS1000) and Gripper (TG-T). The samples are placed in the position B3 in adapter Ep0.5/1.5/2 ml. Module Reservoir is located in the position B1, where washing solutions and waste are available. The device is controlled by the epMotion control panel. The tips are located in the A4 (eTips 50), A3 (eTips 300) and A2 (eTips 1000) positions. PCR 96 plates are used. The resulting volumes of collected samples ranged from 10 to 30 μl depending on the procedure.

2.3. Electrochemical analysis

Electrochemical measurements were performed with AUTOLAB PGS30 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/3 M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. Cyclic voltammetric or square wave voltammetric measurements were carried out in the presence of acetate buffer pH 4 or 4.6. CV parameters were as follows: potential step 5 mV, scan rates 10, 20, 40, 80, 160 and 320 mV/s. Square wave voltammetry parameters: potential step 5 mV and frequency 280 Hz. The samples measured by cyclic and square wave voltammetry were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s. The temperature of supporting electrolyte was maintained by the flow electrochemical cell coupled with thermostat JULABO F12/ED (Labortechnik GmbH, Germany).

2.4. In vitro plants cultivation

Maize (*Zea mays* L.) F1 hybrid Gila was used in our experiments. Maize kernels were sterilized with sodium hypochlorite (5%, *v/v*), washed with water and germinated on wet filter paper in special vessels at $23 \pm 2^\circ\text{C}$ in dark. After seven days, each maize seedling was placed into glassy test-tube containing Murashige Skoog medium with gerlit [36] and with addition of Cd(II)-ethylenediaminetetraacetic acid (Cd(II)-EDTA, 0, 5, 10, 25, 50 and 100 μM). Plants grown without Cd(II)-EDTA (0 μM) were used as a control. These seedlings were cultivated in Versatile Environmental Test Chamber (MLR-350 H, Sanyo, Japan) for five days with 14 h long daylight per day (maximal light intensity was 70 lx) at a temperature $23.5\text{--}25^\circ\text{C}$ and humidity 71–78%. Three plants each were harvested at certain time intervals (24, 48, 72, 96 and 120 h) during the experiment, and their roots were rinsed three times in distilled water and 0.5 M EDTA. In addition, each harvested plant was divided into leaves and roots. Fresh weight of the samples was measured immediately after the rinsing by using a Sartorius scale.

2.5. Preparation of plant tissues for mRNA isolation

Weighed plant tissues (approximately 0.2 g) were transferred to a test-tube, and liquid nitrogen was added. The samples were frozen to disrupt the cells. The frozen sample was transferred to mortar and grinding for 1 min. Then, 1000 μl of 0.2 M phosphate buffer (pH 7.2) was added to the mortar, and the sample was grinding

for 5 min. The homogenate was transferred to a new test-tube. The mixture was homogenised by shaking on a Vortex-2 Genie (Scientific Industries, New York, USA) at 4 °C for 30 min. The homogenate was centrifuged (14 000 × g) for 30 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Before the analysis the supernatant was filtered through a membrane filter (0.45 μm Nylon filter disk, Millipore, Billerica, Mass., USA).

2.6. Patients with brain disorders

We have investigated blood samples of patients with brain disorders. All patients ($n = 6$) received cranial computerized tomography (CT). Five patients were diagnosed as focal traumatic brain injury (TBI) according to the CT scan performed during admission, and one patient was diagnosed as cerebral stroke (sample 6CE). Four patients with TBI were submitted to neurosurgery (samples 1NM, 2SE, 3SEL, 4MA) due to expansive contusion with midline shift of more than 10 mm, and intra-cranial pressure elevation of more than 10 Torr, and one patient without expansive behaviour of contusion (sample 5HA) with suspicion on diffuse axonal injury. *Informed agreement:* The ethics committee at each institution approved this study. Written informed consent was obtained from all patients. The group II of controls ($n = 6$) included patients with non-traumatic diagnosis.

2.7. Preparation of brain tissues for mRNA isolation

To the sample of brain tissue (approximately 200 mg of tissue, obtained from patients with serious brain injury) guanidinium thiocyanate (4 M, pH 7.5) was added. pH of this solution was adjusted by 1 M Tris-HCl to 7.5. The cells were lysed by vortexing until the eye-observable precipitates disappeared. To the homogenate: (i) 50 μl 2 M of sodium acetate pH 4.1, (ii) 450 μl of phenol, (iii) 130 μl of chloroform:isoamyl alcohol (49:1, v/v) was added. This mixture was vortexed for 10–30 s until the emulsion was formed and then incubated for 15 min on ice. Then the sample was centrifuged for 15 min at 14,000 rpm, 4 °C. After the centrifugation the upper phase containing total RNA was collected. This phase was transferred to new test-tube prior to analysis by magnetic microparticles.

2.8. Statistical analyses

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences between mRNA content in roots of plants treated with cadmium(II) ions and control plants were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffeho test), which was applied for means comparison.

3. Results and discussion

More than 40 years ago Palecek discovered using oscillopolarography that nucleic acids gave two signals: (i) redox signal of adenine and cytosine, and (ii) oxidative signal of guanine [37–39]. Recently elimination voltammetry has been successfully utilized for resolution of reduction signal of adenine and cytosine [40–45]. Moreover it was published that cytosine, adenine, thymine and guanine gave signals at carbon electrodes [31,46,47]. Based on these promising milestones of electroanalysis of nucleic acids together with the fact that electrochemistry is still one of the most sensitive analytical technique, voltammetric methods can be

considered as a suitable tool for the detection of nucleic acids [48–50].

3.1. Electrochemical optimization detection of poly(A)

3.1.1. Electrochemical behaviour of poly(A) investigated by adsorptive transfer technique in connection with cyclic voltammetry

Nucleic acids can be accumulated onto surface of HMDE easily. Therefore, it is possible to use adsorptive transfer technique for their detection. The description of this technique follows. A working electrode with well-defined surface due to unique physico-chemical properties of mercury is renewed (Fig. 1A,a). An analysed sample in very low volume (5 μl) is introduced onto parafilm (Fig. 1A,b). The working electrode is immersed into the sample and nucleic acid is adsorbed for the desired time of accumulation (Fig. 1A,c). Then the working electrode is rinsed in water of ACS purity and supporting electrolyte (Fig. 1A,d). Finally the working electrode with adsorbed nucleic acid is transferred into electrochemical cell with supporting electrolyte and analysed (Fig. 1A,e). The principle of poly(A) isolation by using of paramagnetic particles is shown in Fig. 1B. Nucleic acids with homo-adenine sequence (poly(A)) are captured by the particles with chains of homo-thymidine anchored on their surface. Due to the principle of complementarity of nucleic acid bases hybridization of poly(A) with homo-thymidines takes place (Fig. 1B,a). Non-specifically linked molecules due to other types of attractive forces are washed due to rinsing step. Hybridized poly(A) is cleaved by heat treatment (Fig. 1B,b). Then, the particles are forced by magnetic field and only poly(A) containing solution is collected (Fig. 1B,c). The collected solution is analysed (Fig. 1B,d).

Cyclic voltammetry is a commonly used method for the analysis of DNA [29,30,43]. The ammonium formate is the most often used supporting electrolyte, but the mechanisms of electrode processes are still not clear. It is supposed that the supporting electrolyte provides proton, which is bound to DNA molecule and then the reduction of adenine and cytosine occurs. The dependence of the peak height of poly(A) (100 μg/ml) measured by cyclic voltammetry on scan rate was studied (Fig. 2A). The typical voltammograms of poly(A) measured at three scan rates (40, 80 and 160 mV/s) are shown in inset in Fig. 2A. The cyclic voltammetry gives well-developed symmetric reduction peaks within the potential range from –1.25 to –1.3 V. Peak height enhanced with increasing scan rate linearly according to the following equation $y = 74.943x + 6.0057$ and $R^2 = 0.9918$. Symmetry of the peak was poor at scan rate higher than 320 mV/s. Therefore we applied scan rate of 160 mV/s in the following experiments.

The time of nucleic acids accumulation onto the working electrode surface belongs to the one of the most important experimental conditions needs to be optimized. The sample (volume 5 μl) was accumulated onto the surface of HMDE for the 30, 60, 90, 120, 150, 180 and 210 s at open circuit. We found that the height of adenine peak enhanced with the time of accumulation up to 120 s, then gradually decreased (Fig. 2B). The observed decrease in adenine peak height at accumulation time higher than 120 s can be associated to length of poly(A) chains, which could form poly-layer structures on the surface of HMDE. The influence of the poly(A) concentration on the current response at the accumulation time of 120 s is shown in Fig. 2C. Redox signal of adenine was proportional to the concentration of poly(A) up to 35 μg/ml, then the signal enhanced slowly. The linear dependence was measured within the range from 0.4 to 25 μg/ml with equation $y = 1.204x + 888.5$, $R^2 = 0.992$ (Table 1). The obtained experimental data were repeatable very well with relative standard deviations 5.5% ($n = 5$).

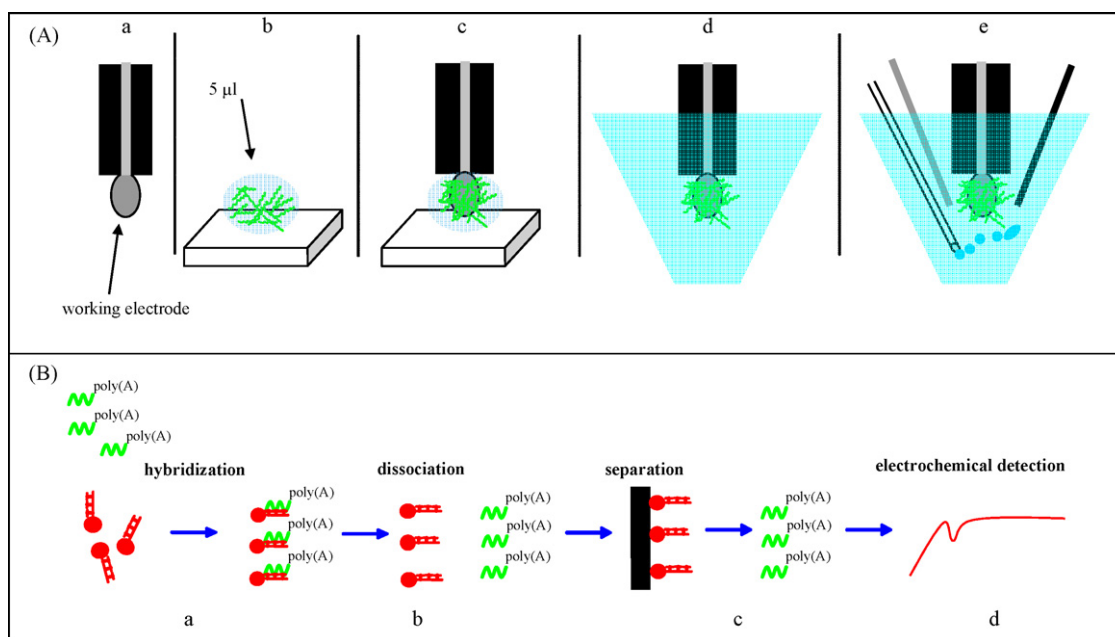


Fig. 1. (A) Electrochemical analysis of poly(A) and/or mRNA: (a) renewing of the hanging mercury drop electrode surface, (b) introducing of sample (5 µl) containing poly(A) and/or mRNA on parafilm, (c) adsorbing of poly(A) and/or mRNA in a drop onto the HMDE surface at open circuit, (d) rinsing electrode in water of ACS purity, (e) measuring in supporting electrolyte. (B) Scheme of poly(A) isolation by using of paramagnetic microparticles: (a) washing of the particles, adding of hybridization solution and a sample; (b) denaturation of hybridized nucleic acids chains at higher temperature; (c) forcing of the particles by magnetic field; (d) electrochemical detection an isolated nucleic acids.

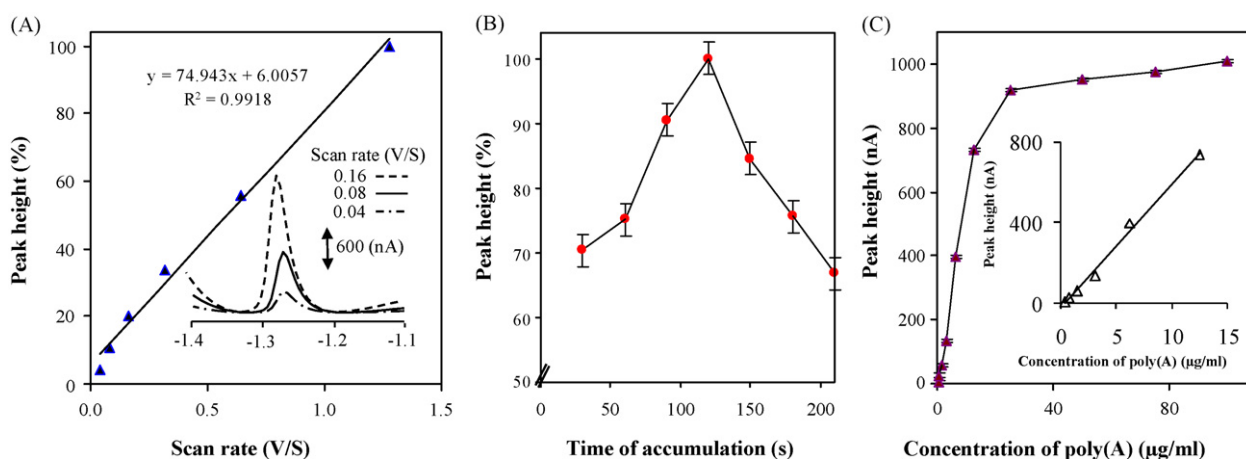


Fig. 2. Optimization of CV experimental parameters: the dependence of peak height on (A) scan rate, (B) time of accumulation and (C) concentration of poly(A). Concentration of poly(A): 100 µg/ml.

Table 1
Analytical parameters of poly(A) detection.

Data	Peak potential (V) ^a	Calibration curve (nA)	R ²	Linear dynamic range (µg/ml)	LOD (ng/ml) ^b	LOQ (ng/ml) ^c	R.S.D. (%) ^d
Poly(A) ^e	-1.4 ± 0.1	I = 1.204c + 888.5	0.9920	0.4–25	100	333	5.5
Poly(A) ^{120sf, g}	-1.4 ± 0.1	I = 56.91c + 7.14	0.9973	0.2–25	1	3	3.9
Poly(A) ^{60sf, g}	-1.4 ± 0.2	I = 36.89c + 4.52	0.9967	0.2–25	5	17	4.8
Poly(A) ^{30sf, g}	-1.5 ± 0.2	I = 19.86c + 6.67	0.9917	0.2–25	10	33	6.8

I: peak current (in calibration curve).

c Poly(A) concentration (in calibration curve).

^a Measured vs. Ag/AgCl/3 M KCl electrode.

^b Limit of detection estimated (3 signal/noise, S/N) was calculated according to Long and Winefordner [56], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

^c Limit of quantification estimated as (10 S/N) [56].

^d Relative standard deviation.

^e Poly(A) was measured by AdTS CV, time of accumulation 120 s.

^f Poly(A) was measured by AdTS SWV, time of accumulation as superscript.

^g Number of measurement (n = 5).

3.1.2. Electrochemical behaviour of poly(A) investigated by adsorptive transfer technique in connection with square wave voltammetry

Cyclic voltammetry is the most widely used technique for acquiring qualitative information about electrochemical reactions; however, it suffers from insufficient sensitivity and high detection limits. Therefore we selected much more sensitive method called square wave voltammetry (SWV) [51]. The comparison of poly(A) signals measured by CV and SWV is shown in Fig. 3A. The signal measured by CV was shifted for more than 50 mV to positive potentials. From the analytical point of view SWV signal was approximately two times higher compared to CV signal. Based on SW voltammetric analyses of DNA the highest current response was measured at 260 Hz [43,51]. We investigated the influence of frequency on poly(A) SWV signal (Fig. 3B). The highest signal was determined at a frequency of 280 Hz. Under higher frequencies the signal rapidly decreased. The difference between previously published value (260 Hz) and the optimized ones (280 Hz) can be probably associated with the influence of nucleic acids sequence. The dependence of peak height on accumulation time had similar trend compared to CV analyses with maximum measured at 200 s (Fig. 3C). Under higher accumulation times the peak height decreased. Further we focused our attention on studying the effect of temperature of supporting electrolyte on poly(A) signal. The highest peak was determined at 35 °C. Temperature higher than 40 °C resulted to the progressive destabilization of poly(A) molecule on the electrode surface. The increasing temperature enhances probability of irregular bases pairing (Fig. 3D). The significant role in nucleic acids detection also plays pH of supporting electrolyte. It clearly follows from the results obtained that the highest response

was measured in the presence of acetate buffer pH 4.6 (Fig. 3E). This phenomenon can be associated with the protonization of poly(A) molecule. Under the optimized conditions (frequency 280 Hz, accumulation time 200 s, supporting electrolyte and its temperature: acetate buffer 4.6 and 35 °C) we investigated the dependence of peak height on poly(A) concentration within the range from 0.02 to 12 µg/ml (Fig. 3F). The dependence was proportional to poly(A) concentration according to the equation $y = 56.91x + 7.14$, $R^2 = 0.9973$ with relative standard deviation 3.9% ($n = 5$). The detection limit of poly(A) was estimated as 1 ng/ml (2.5 pM). The other analytical parameters are shown in Table 1.

3.2. Semi-automated isolation of nucleic acids by using of paramagnetic particles

Analysis of nucleic acids represents still challenge for analytical chemistry and biochemistry especially in the field of RNA detection or determination of specific DNA sequences. From various types of RNAs mRNA is important due to the fact that relates with gene activity; however, their isolation is still laborious and time consuming. We utilized paramagnetic particles with homo-thymidine chain anchored on their surface for isolation of mRNA as it is shown in Fig. 1B. To enhance effectiveness and repeatability of isolation of nucleic acid automated approach for rinsing and hybridizing was proposed. The whole process is shown in Fig. 4. Paramagnetic particles (10 µl) are transferred with solution in which they are kept to a test-tube (A1). After this step the test-tube is placed to magnetic stand that separates paramagnetic particles from the solution of storage (A2). The solution is then pipetted away and 20 µl of washing solution (phosphate buffer I) is added (A3). Further the

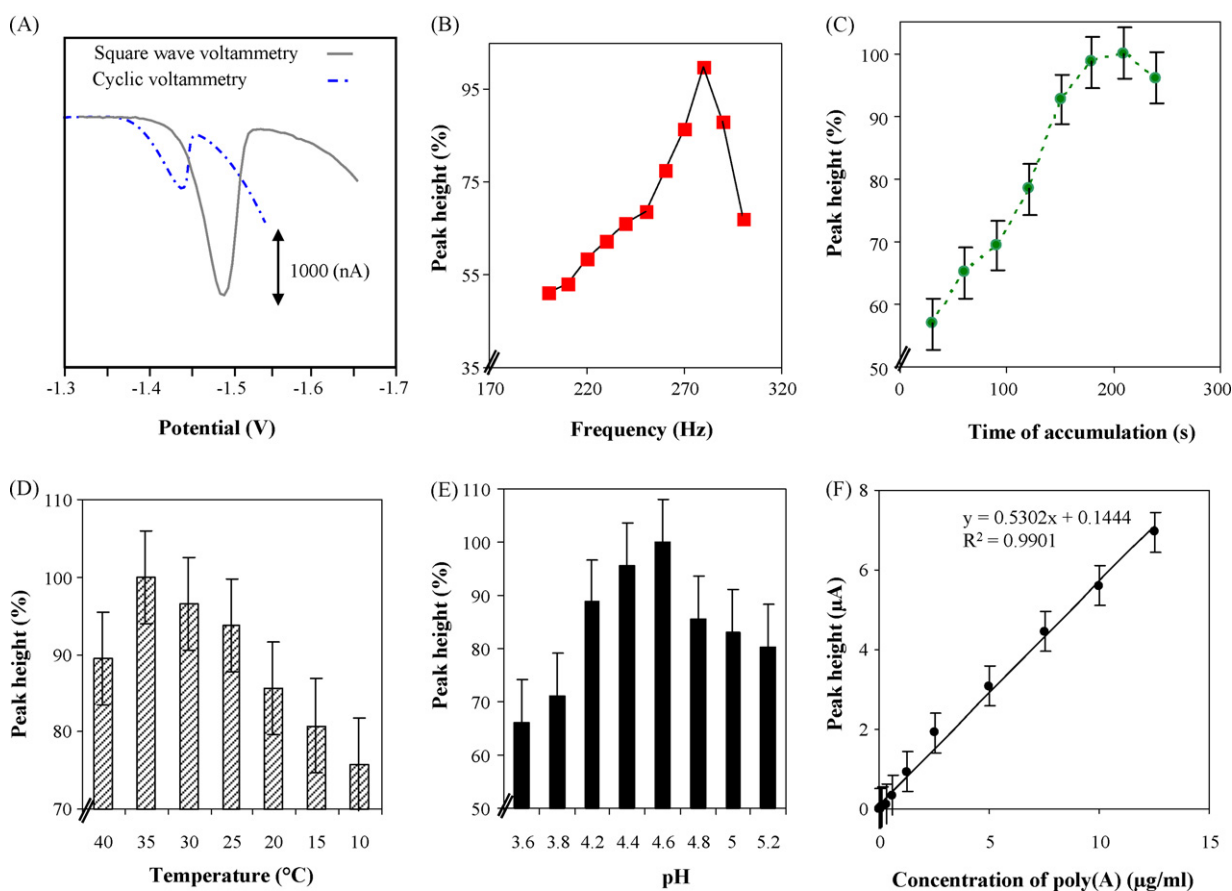


Fig. 3. (A) Cyclic and square wave voltammograms of poly(A). Optimization of SWV experimental parameters: the dependence of peak height on (B) frequency, (C) time of accumulation, (D) temperature of supporting electrolytes, (E) pH of acetate buffer and (F) concentration of poly(A). Concentration of poly(A) was 10 µg/ml except (F).

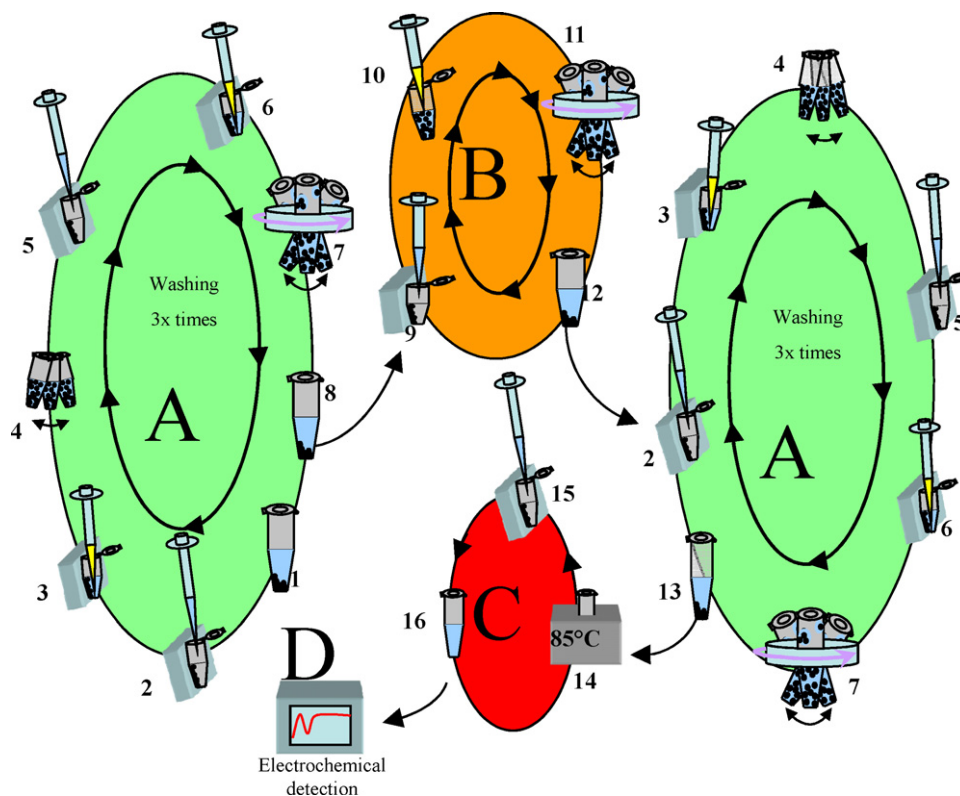


Fig. 4. Scheme of semi-automated isolation of poly(A): (A) rinsing before hybridization, (B) hybridization, (C) rinsing after hybridization, (D) denaturation and (E) detection.

test-tube is moved from magnetic stand and shortly shook in the hands so that the paramagnetic particles are equally dispersed in washing solution (A4). After this step the washing process is repeated (A5–A6). The washing with apparatus multi-spin MSC-3000, in which the centrifugation and shaking can be changed, follows. Shaking was set to level hard and lasted for 20 s; centrifugation proceeded at centrifugal force 285 g for 1 s (A7). Steps nos. A2–A7 are three times repeated. After paramagnetic particles being washed they are ready for nucleic acid isolation (process of hybridization). When the washing solution is removed (A8–B9), the hybridization solution is added to the paramagnetic particles. Total volume of the hybridization solution with the sample was 30 μ l

(B10). The hybridization was proceeding on apparatus multi-spin MSC-3000 for the 40 min. The settings were as follows: 20 s of shaking (level soft) and centrifugation for 1 s at 285 g (B11). After the hybridization, the washing steps nos. A2–A7 were repeated three times. To the washed paramagnetic particles with bounded poly(A) phosphate buffer II (30 μ l) was added (A13). Then the test-tube was placed in the Thermomixer 5355. Denaturation was carried out at 85 °C for 5 min (C14). Under heat treatment poly(A) is released from the particles. After this step, paramagnetic particles were forced using magnetic stand and the solution containing only mRNA was transferred into new test-tube (C15–C16). The solution is subsequently analysed (D).

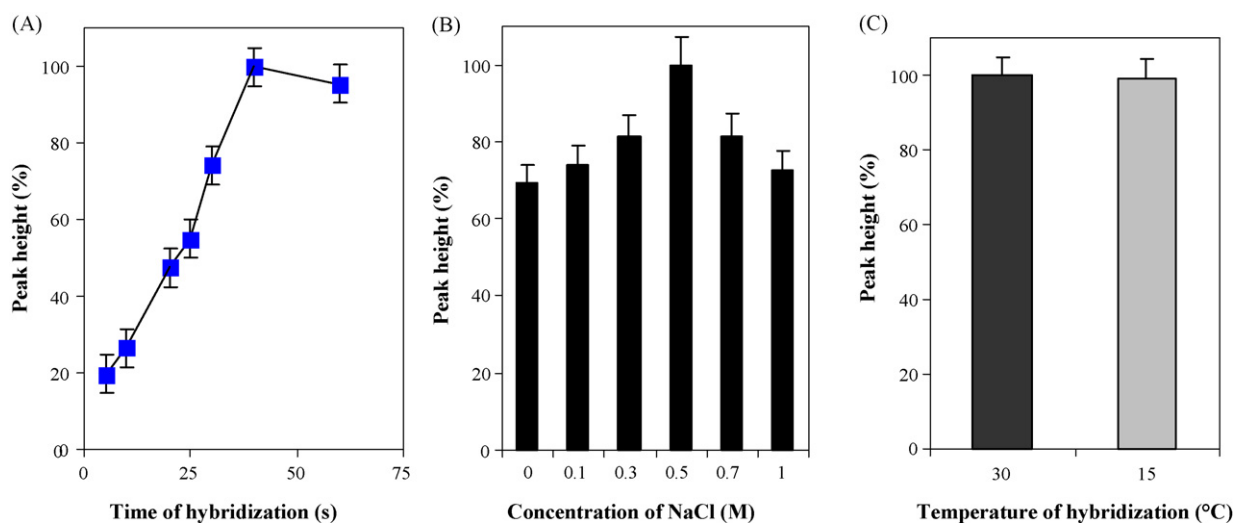


Fig. 5. Optimization of hybridization: (A) the dependence of hybridization time on poly(A) peak height, (B) the dependence of NaCl concentration on poly(A) peak height and (C) the dependence of temperature on poly(A) peak height.

3.2.1. Hybridization of poly(A) on paramagnetic particles

Hybridization of poly(A) to homo-thymine chains anchored on the surface of paramagnetic particles is the most important part of the whole isolation. Therefore we optimized experimental conditions to reach the highest yield of poly(A). There are many factors that affect the process of hybridization. The first parameter we investigated was length of hybridization. We found that the total amount of isolated poly(A) enhanced with increasing hybridization time. For the above-suggested semi-automated way of isolation (Fig. 4) the most suitable time for the most effective hybridization varied between 30 and 40 min (Fig. 5A). The yield of poly(A), which was defined as “isolated concentration of poly(A)”/“given concentration of poly(A)”, was approximately 50% ($n=15$). The ionic strength also markedly affects hybridization process. To mimic the influence of ionic strength on the hybridization we used NaCl. The highest peak was determined in the presence of 0.5 M NaCl (Fig. 5B). The differences in peaks measured in the presence of tested NaCl concentrations were not higher than 35%. Process of hybridization is also affected by temperature. In our experimental design it was possible to test two temperatures: 15 and 30 °C. Based on our results these two temperatures had no effect on the hybridization process (Fig. 5C).

Further we attempted to evaluate how the automation and optimization steps were effective (Fig. 6). The white columns show poly(A) (10, 50 and 90 µg/ml) detected without previous isolation. The development of peak heights corresponds to the typical concentration dependence shown in Fig. 3E. If we used the experimental procedure described by Palecek for poly(A) isolation [29], the yield was low (15%) with relative standard deviation 8%. Under the optimized conditions the yield of isolation enhanced up to 60% with relative standard deviation 10%. Using semi-automated way of isolation and under optimized conditions the yield was about 75% with the relative standard deviation below 6%. It clearly follows from the results obtained that automation step considerably reduced errors made by hand operating and thus enhanced yield of the isolation.

3.2.2. mRNA separation from samples of patients with traumatic brain injury

In many cases of traumatic injury there are no suitable markers available for the monitoring of brain damage and health state of

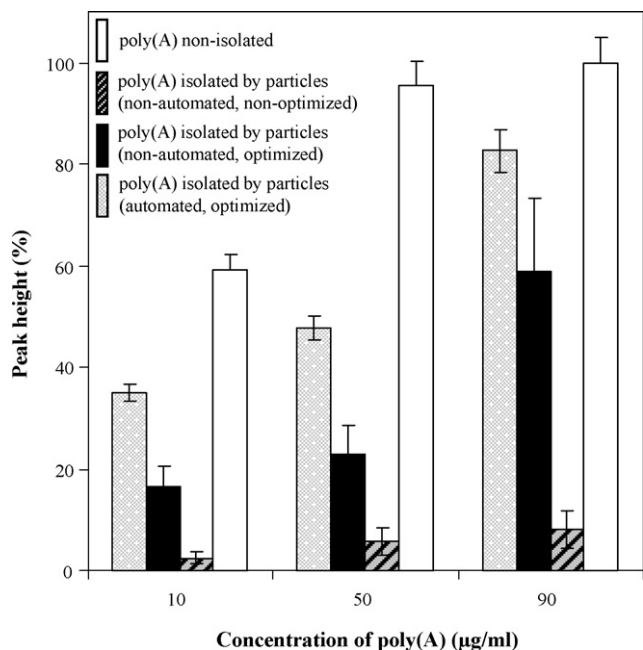


Fig. 6. The yield of isolation of various initial concentrations of poly(A) (10, 50 and 90 µg/ml).

the patients. S100B protein might be one of such markers [52–54]. Moreover, changes in gene expression profile a set of genes transcripts for cell cycle control, inflammation, cell proliferation and oxygen free radical scavenger proteins, growth inhibitory factor; metallothionein-III, p53 antigen, p53-induced protein, interleukins, TNF receptor associated death domain, and FAS soluble protein was shown in patients with traumatic brain injury [54]. To follow these changes sample of pure mRNA is needed. Not only the level of mRNA can be considered as one of the markers of traumatic brain injury, but also the isolated mRNA can be used for more specialized processing to detect specific sequence of a gene associated with traumatic brain injury. Moreover, the enhancing of mRNA level in few hours after traumatic brain injury was discussed [55]. The suggested and optimized methods for poly(A) isolation and detection was utilized for the analysis of brain tissues of six patients with traumatic brain injury. Traumatic damaging of brain tissue measured by nuclear magnetic resonance (abnormal signals are white-highlighted) is shown in inset in Fig. 7. mRNA was successfully isolated from all analysed samples by the semi-automated above-described method. Their quantity was determined by SWV. Well-developed peaks about -1.6 V were observed in the SW voltammograms shown in Fig. 7A. The total amount of the isolated mRNA varied from 40 to 760 µg of mRNA per g of brain tissue (Fig. 7B). Based on the spikes of poly(A) to real samples we investigated the influence of matrix on the electrochemical response of isolated mRNA. The recovery was within the interval from 94 to 115% (Table 2).

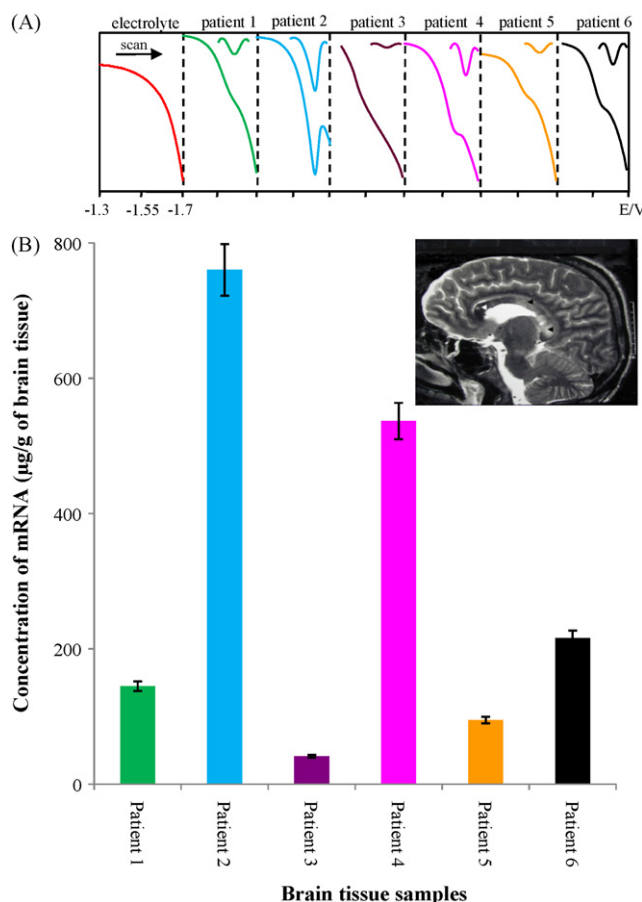


Fig. 7. mRNA separation from patients with traumatic brain injury. (A) Typical SW voltammograms of eluates (20 µl) obtained from the brain tissues. (B) The content of the isolated mRNA in brain tissues obtained from patients; inset: traumatic damaging of brain tissue measured by nuclear magnetic resonance.

Table 2
Detect mRNA vs. brain tissues.

Sample ^a	Peak current (nA) ^b	Peak potential (mV) ^c	Recovery (%) ^d
Patient 1	280 ± 20	-1.56 ± 0.02	105.5
Patient 2	205 ± 50	-1.59 ± 0.04	98.7
Patient 3	100 ± 5	-1.58 ± 0.03	94.6
Patient 4	1060 ± 20	-1.58 ± 0.02	110.8
Patient 5	220 ± 10	-1.56 ± 0.03	115.0
Patient 6	500 ± 20	-1.55 ± 0.02	108.8

^a Each sample was measured in triplicates.

^b The height of SWV signal after baseline correction (GPES 4.9, EcoChemie, Netherlands).

^c Measured vs. Ag/AgCl/3 M KCl electrode.

^d Addition of poly(A) (10 µg/ml) to a sample after isolation.

3.3. Fully automated separation of nucleic acids by using of paramagnetic particles

In the following experiments we have been carried out with fully automated isolation of poly(A) by using a robotic device epMotion (Fig. 8). Pipetting of liquids and moving of PCR plates provide *x*, *y*, *z* moving arm controlled by a microprocessor (Fig. 8A). The work sequence is shown in Fig. 8B. The eluate obtained is analysed electrochemically. For our purposes the following sequence was proposed: (i) paramagnetic microparticles (10 µl) are pipetted into the PCR plate; (ii) a washing cycle is three times repeated (50 µl); (iii) the sample (poly(A), 10 µl) and hybridization solution (30 µl) are pipetted into PCR plates with paramagnetic microparticles; (iv) 15 min long hybridisation is carried out at 25 °C; (v) a washing cycle is three times repeated (50 µl). The PCR plate is

later on moved on thermostated position, where the denaturation of bound nucleic acid chain takes place. The yield of poly(A) separation (25 µg/ml) was 40% with R.S.D. 6.5% (*n* = 10). Fully automatic process leads to timesavings and, in particular, reduces experimental errors. Among the technical problems, which are still associated with the use of this procedure, the evaporation of the sample analysed belongs. We have been investigated the isolation of poly(A) on DBT and/or ROCHE magnetic microparticles by using of the fully automated technique. The yield of poly(A) isolated by DBT microparticles was approximately 40%. Using these microparticles the dependence of peak height on concentration of isolated poly(A) (0–100 µg/ml) was investigated and is shown in Fig. 9A. In inset of this figure, strictly linear part of this dependence is shown. The yield of poly(A) isolated by ROCHE microparticles was approximately 25%. Moreover the isolated amount of

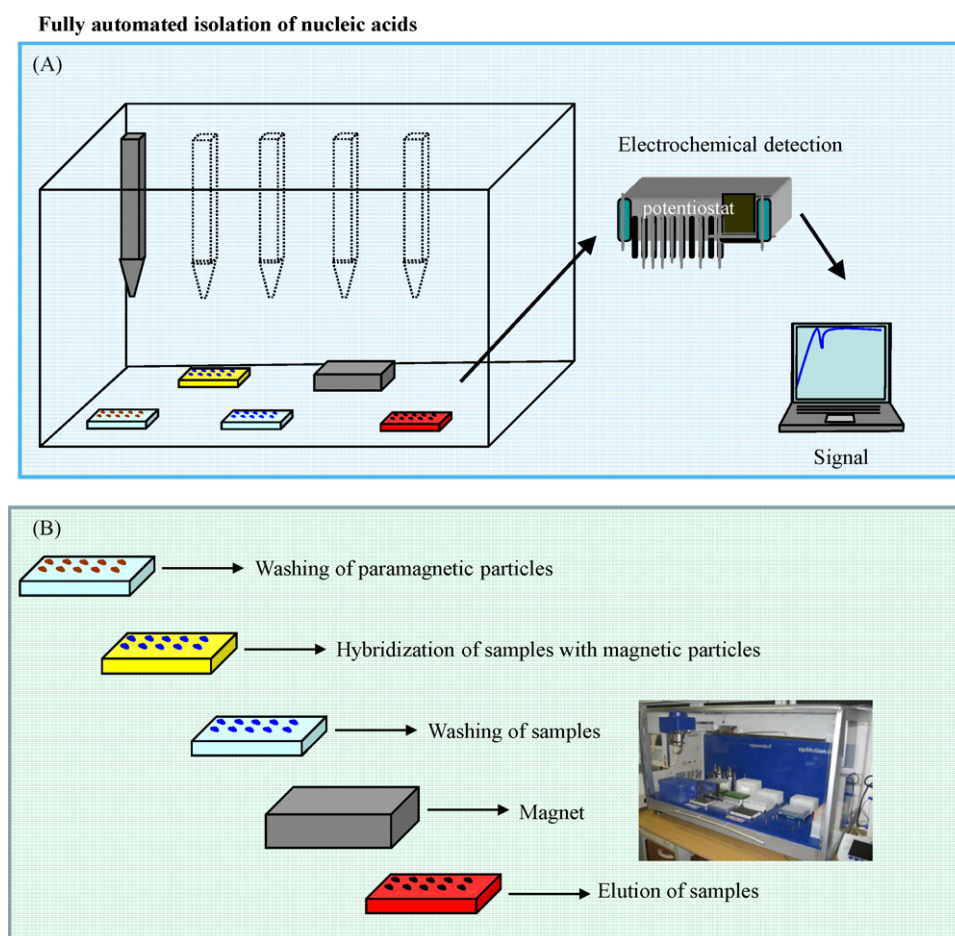


Fig. 8. Scheme of fully automated instrument for isolation of mRNA. (A) The arrangement of working tools. Electrochemical analyser is off-line connected. (B) Sequence of steps for the analysis of real samples.

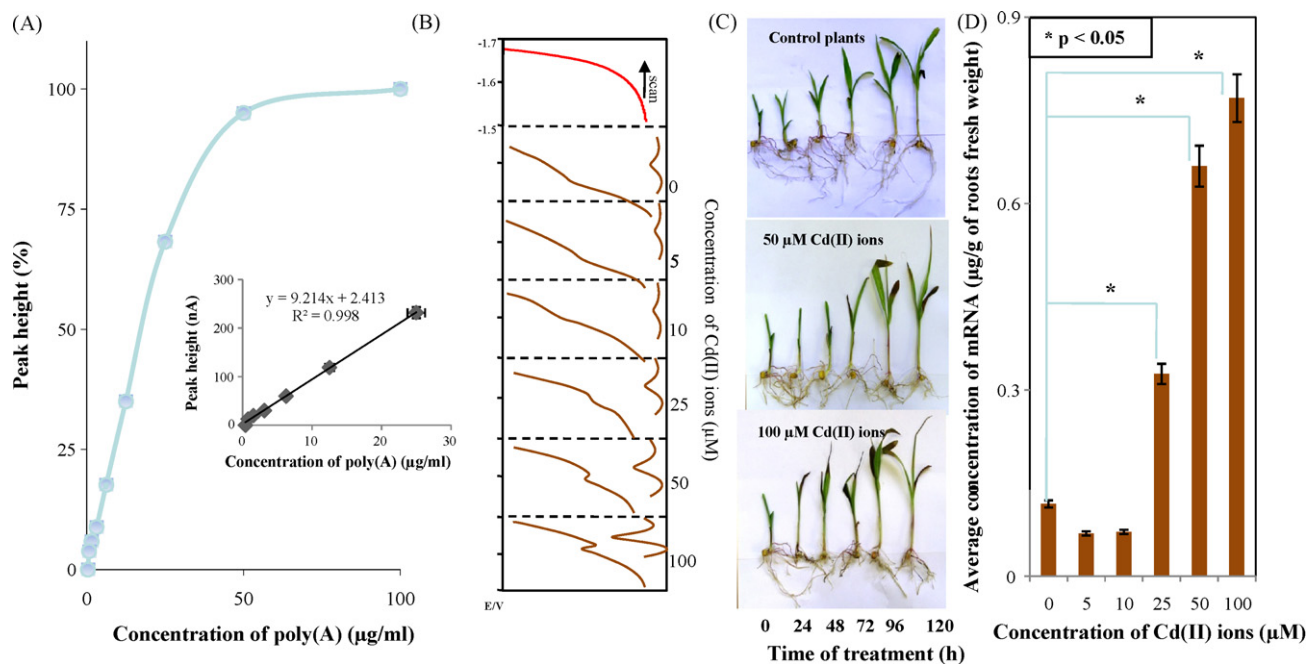


Fig. 9. (A) The dependence of height of poly(A) after its isolation by using fully automated instrument; inset: the linear part of this dependence. (B) Typical SW voltammograms of extracts from tissues of plants treated with cadmium(II) ions. (C) Photographs of maize plants treated with cadmium(II) ions (0, 50 and 100 μM). (D) The content of the isolated mRNA from roots extracts. *Differences with $p < 0.05$ were considered significant.

poly(A) was not proportional to the initial concentration of these molecules.

The method of fully automated isolation of mRNA was utilized for the analysis of tissues of maize plants exposed to cadmium(II) ions. Roots of the plants treated with particular concentration of cadmium(II) ions were sampling for five days. mRNA was isolated from the extract prepared from all roots belonging to one particular concentration of heavy metal. Typical voltammograms of the isolated mRNA are shown in Fig. 9B. In all samples it was possible to detect mRNA. Photographs of maize plants treated with cadmium(II) ions are shown in Fig. 9C. It follows from the photographs that plants are losing weight in roots, leaf surface is reduced and content of chlorophyll is decreased with the increasing concentration of cadmium(II) ions and time of the exposition. All of these negative processes lead to the gradual exhaustion of a plant. We found that the average level of mRNA in samples decreased slightly in the lower concentrations applied. Since the applied concentration of 25 μM, the significant increase of mRNA levels was observed (Fig. 9D). The dramatic increase in mRNA levels can be associated with the increased metabolic activity in plants of maize to overcome the adverse effects of heavy metal ions.

4. Conclusion

We succeeded in suggestion, optimization and automation of the method for mRNA isolation using paramagnetic microparticles. The main advantage of our method is its rapidity. The isolation of mRNA from six samples per run is not longer than 2.5 h. The proposed method was tested on brain tissues and maize roots.

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