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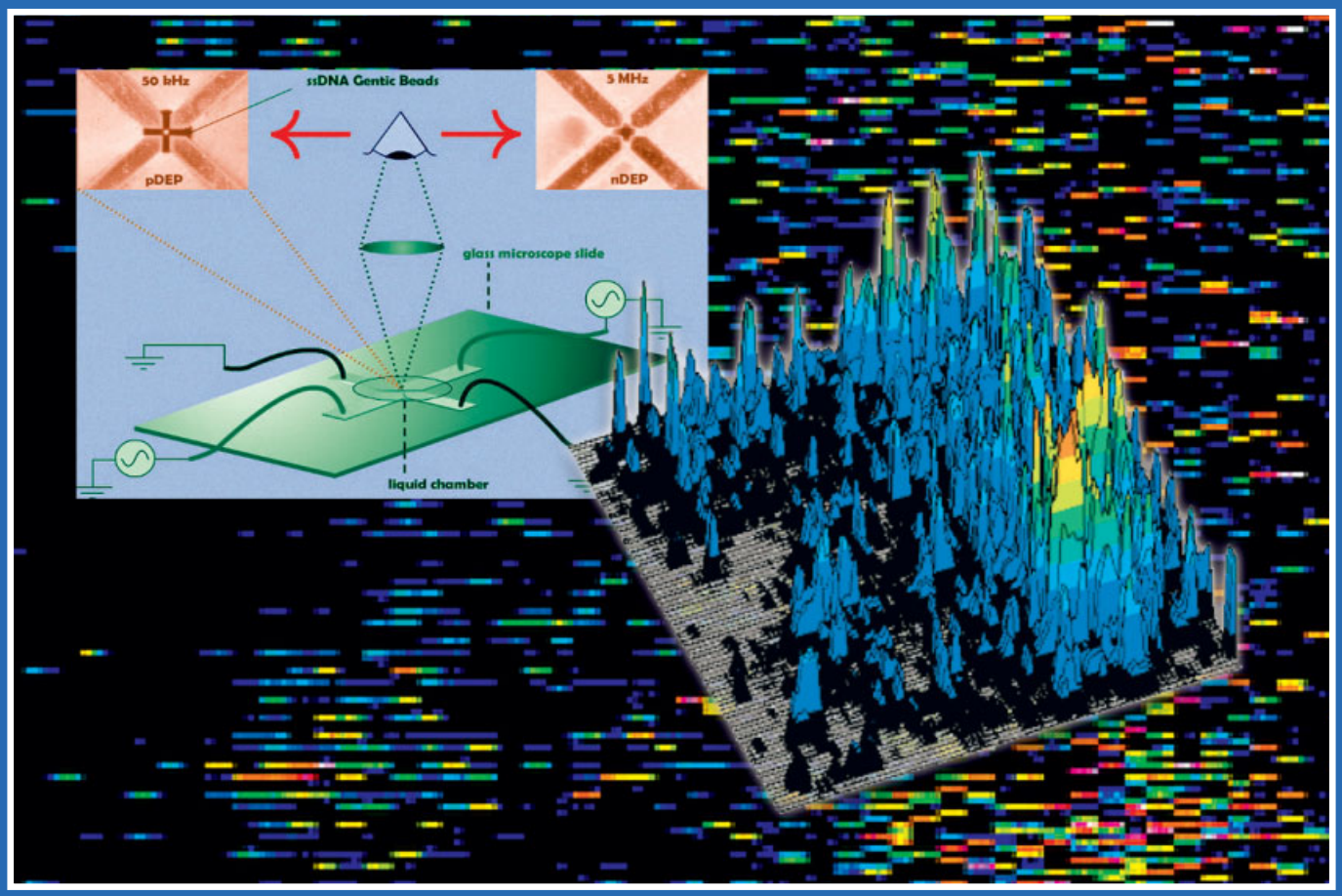

Miniaturization

Editors:
Frantisek Foret and Jörg Kutter

WILEY-BLACKWELL

ISSN 0173-0835 · ELCTDN 29 (24) 4807–5068 (2008) · Vol. 29 · No. 24 · December 2008

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Received July 10, 2008
Revised August 27, 2008
Accepted August 31, 2008

Research Article

Miniaturized electrochemical detector as a tool for detection of DNA amplified by PCR

This paper reports on the analysis of specific sequence of Phage Lambda DNA amplified by PCR. Agarose gel electrophoresis, gel electrophoresis on chip and stationary electrochemical instrument were employed for detection of amplicons obtained after 2, 4, 6, 8, 10, 15, 20, 25, 30 and 35 cycles. In the case of agarose gel electrophoresis the lowest detectable amount of DNA was obtained after 15 PCR cycles. Gel electrophoresis on chip offers higher sensitivity because the lowest detectable amount of amplicons by this technique was obtained after eight PCR cycles. Further we employed square wave voltammetry and various working electrodes (hanging mercury drop electrode, screen-printed carbon electrode and carbon-nanotube-based screen-printed electrodes) to detect amplicons. Amplicons obtained even after two cycles were detectable at all electrodes. To improve the selectivity of electrochemical detection carbon nanoelectrodes were off-line coupled with gel electrophoresis. Into the agarose gel the electrodes were placed. Further the amplicons were loaded into agarose gel wells. DNA migrating to the detection place was electrochemically analyzed. Amplicons obtained after two cycles were detectable by this hyphenated technique.

Keywords:
Carbon nanotubes / DNA / Gel electrophoresis on chip / PCR / Voltammetry
DOI 10.1002/elps.200800445

1 Introduction

A technique called PCR, at which a DNA polymerase is used to amplify a piece of DNA by *in vitro* enzymatic replication [1], is widely used in many branches of science. Amplified DNA can be detected by using of various techniques. Gel electrophoresis is routinely used for the detection of DNA fragments but suffers from no automated analysis and low sensitivity. Advantages, disadvantages and pitfalls of this standard technique are reviewed by Muyzer and Smalla [2] and von Wintzingerode *et al.* [3]. For automated analysis of large sets of samples CE can be used [4]. CE can be coupled with various types of detectors such as UV—VIS, fluorescent, MS, electrochemical and others. CE and its application have been reviewed several times in the last 2 years [5–8]. CE coupled with PCR can be used in various fields such as forensic science [9], clinical diagnostics [10], DNA mismatch detection [11, 12], food control [13], *etc.* Moreover, there are other proposed methods and techniques for the analysis of target DNA sequences on chips [14].

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Abbreviations: AdTS, adsorptive transfer stripping**; CV,** cyclic voltammetry**; HMDE,** hanging mercury drop electrode**; SWV,** square wave voltammetry

This paper reports on the analysis of PCR-amplified DNA by using various techniques. We employ agarose gel electrophoresis, gel electrophoresis on chip (Experion System [15]) and stationary electrochemical instrument with various working electrodes (hanging mercury drop, carbon paste and carbon screen-printed electrodes) to detect DNA. Then we attempt to couple gel electrophoresis with carbon-nanotube-based screen-printed electrodes.

2 Materials and methods

2.1 Chemicals, material and pH measurements

All chemicals of ACS purity (chemicals meet the specifications of the American Chemical Society) were used and parafilm were purchased from Sigma Aldrich Chemical (Sigma-Aldrich, USA). Taq PCR Kit with controls was from New England Biolabs, USA. Deionized water underwent demineralization by reverse osmosis using the instrument Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified using Millipore RG (Millipore, USA, 18 MΩ) – MiliQ water. The pH value was measured using WTW inoLab (Weilheim, Germany).

2.2 Stationary electrochemical measurements

Electrochemical measurements were performed with AUTOLAB PGS30 Analyzer (EcoChemie, the 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 mm^2 or a carbon screen-printed electrode 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. Square wave voltammetric (SWV – square wave voltammetry) and/or cyclic voltammetric (CV – cyclic voltammetry) measurements were carried out in the presence of acetate buffer, pH 5.0. SWV parameters: potential step 5 mV; frequency 260 Hz (HMDE) or 50 Hz (carbon electrodes). CV parameters: scan rates 10, 20, 40 and 80 mV/s, potential step 5 mV and time of accumulation 120 s [16]. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 120 s. All experiments were carried out at room temperature.

2.3 Agarose gel electrophoresis

Agarose gel (1%) was prepared by boiling 1 × Tris-acetate EDTA buffer (40 mM Tris-acetate and 1 mM EDTA) for 15 min in a pressure cooker. Then the gel was cooled to 50°C and ethidium bromide was added (10 μL *per* 100 mL of the gel). Samples prepared with bromophenol blue were loaded onto a gel in 5 μL aliquots. The electrophoresis was run at 100 V for 60 min. The bands were visualized using gel projection system (Vilber-Lourmant, France).

2.4 Experion system

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad, USA) were carried out according to the manufacturer's instructions with supplied chemicals (Experion DNA 1 K analysis kit, Bio-Rad). After priming of the chip with gel and gel-staining solution in the diluted priming station sample, samples processed according to the manufacturer's instructions (9 μL) were loaded into sample wells. The chip with loaded samples was shaken for 60 s on Vortex-2 Genie (Scientific Industries, USA). The chip was placed into the electrophoretic instrument and measurement was carried out.

2.5 PCR

The master mix was prepared for Taq PCR according to the kit with controls purchased from New England BioLabs (USA). Lambda DNA template – primer (forward): 5′-CCTGCTCTGCGCTTCACGC-3′; (backward): 5′-TCCGGATAAAAACGTCGATGACATTTTGC-3′ – was used, $MgCl_2$ (2 mM), dNTP (10 mM), primers (10 pM), and Taq polymerase was introduced in 0.2 μL aliquots. Eppendorf tubes were placed into the thermal cycler (Eppendorf Mastercycler Gradient, Germany). After the denaturization at 95°C for 2 min, 35 cycles were kept at 94°C for 15 s, at 55°C for 15 s and at 72°C for 45 s. Finally, it was kept at 72°C for 5 min and kept at +4°C. The amplicons were analyzed by agarose gel electrophoresis, Experion and electrochemistry.

2.6 Screen-printed carbon electrode preparation

2.6.1 Screen-printed carbon electrodes

The sensor was fabricated by using a standard thick-film technology process with screen-printing semi automat (UL 1505A, TESLA, CZ) and firing oven (TFF51, BTU International, USA). The thick-film technology materials used for reference (Ag/AgCl), and auxiliary electrode (platinum) were polymer pastes (DuPont, Czech Republic). Conductive layer, dielectric layer and auxiliary electrode were cermet pastes (ESL ElectroScience, UK). All pastes were fired according to the producer's recommendations described in datasheets. The reference electrode was calibrated against saturated Ag/AgCl electrode [17].

2.6.2 Carbon-nanotube-based screen-printed electrodes

The carbon multiwalled nanotubes (Sigma-Aldrich) was mixed with organic binder based on terpineol (Heraeus, Germany) and homogenized in mortar for 15 min. Printing was done using a semi-automated screenprinter and dried at 150°C as described in Section 2.6.1.

3 Results and discussion

In the last decade electrochemical instruments are used intensively for analysis of nucleic acids. The main trend in their usage is to miniaturize the detection system [18–28]. Coupling of separation and detection systems brings many advantages including much higher selectivity and sensitivity. Owing to very low amounts of biological samples, preconcentration or amplification techniques must precede own analysis. PCR belongs to the most commonly used method for amplification of DNA.

3.1 PCR

Few DNA molecules isolated from a sample of interest cannot be analyzed without PCR. Many authors attempt to miniaturize PCR instruments [29–32]. Here, the concentration of template DNA was selected at such a low level, which was under detection limits of all tested instruments. Therefore, it had to be amplified by PCR. To ensure repeatability of DNA amplification, a simple amplification program was proposed. The PCR cycle was run according to the scheme shown in Fig. 1. One cycle consisted of standard steps such as initialization, denaturation, annealing and

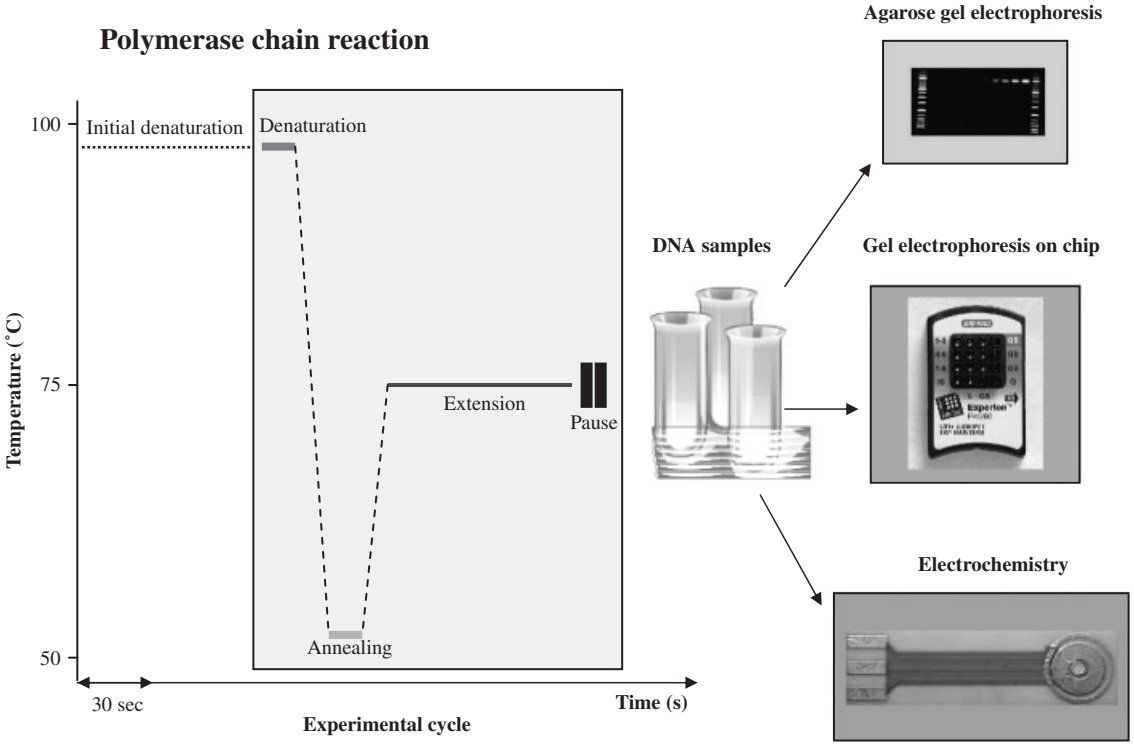

Figure 1. Scheme of PCR coupled with various types of detection systems (agarose gel electrophoresis, gel electrophoresis on chip, electrochemistry).

extension. At the end of 2nd, 4th, 6th, 8th, 10th, 15th, 20th, 25th, 30th and 35th cycles the PCR program was stopped to get a sample for analysis. The amplicons obtained were analyzed by (i) agarose gel electrophoresis, (ii) gel electrophoresis on chip and/or (iii) electrochemistry (Fig. 1).

3.2 Agarose gel electrophoresis

Primarily the amplicons were detected on agarose gel in the presence of intercalating substance ethidium bromide. Typical gel of amplified part of Phage Lambda DNA is shown in Fig. 2. The lowest detectable amount of DNA was obtained after 15 PCR cycles. It follows from the results that we had to do more than 20 cycles to detect a specific sequence, which could be laborious and time consuming. Moreover, processing of the data obtained and their quantification could be difficult. On the other hand, good repeatability and reproducibility belong to the main advantages of this technique.

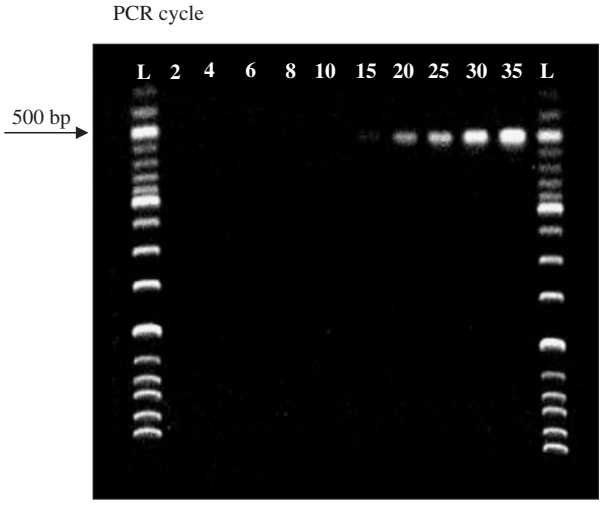

Figure 2. Agarose (1%, 1 × Tris-acetate EDTA buffer) gel of PCR products. DNA was stained by ethidium bromide (10 μL *per* 100 mL of the gel). Samples prepared with bromophenol blue were loaded onto a gel in 5 μL aliquots. Ladder (L) contains the following DNA fragments: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.5, 2, 3, 4, 5, 6, 8 and 10 kb. The experimental parameters were as follows: voltage 100 V, time 60 min.

3.3 Gel electrophoresis on chip

Recently, gel electrophoresis on chip instrument called Experion was suggested to analyze DNA. Chip technology is suitable for detection of smaller nucleic acid fragments as PCR products. Therefore, we used this technique to detect our amplicons. We expected enhancing of sensitivity of measurements. A typical record of analysis of a specific sequence of Phage Lambda DNA is shown in Fig. 3A.

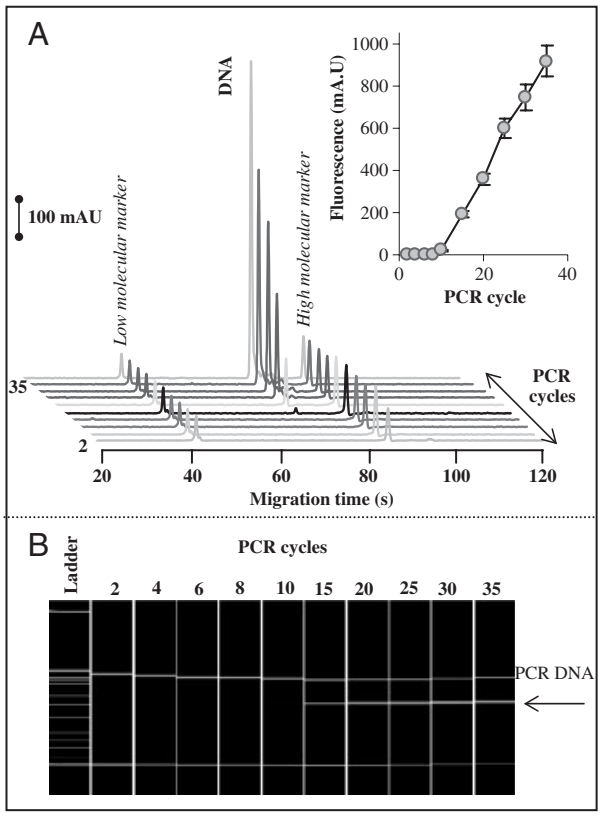

Figure 3. Gel electrophoresis on chip. (A) Electrophoreograms of DNA amplified by PCR measured by Experion system; inset: height of fluorescence signal of amplified DNA. (B) Virtual gel of PCR products obtained after different numbers of cycles.

We used two molecular mass markers for lighter (migration time 42 s) and heavier fragments (migration time 84 s). Amplicons were detected in 63 s. The signals corresponding to amplicons were well separated and developed with good symmetry. Measurements were well repeatable with relative standard deviation as 5.6% ($n = 30$). We found that the lowest detectable amount of amplicons was obtained after eight PCR cycles. The main increase in height of the signal corresponding to amplicons was determined from the 10th PCR cycle (inset in Fig. 3B). Because of Experion software we were able to make virtual gel (Fig. 3), where changes were also shown. The advantage of making a virtual gel is that we can compare more easily results obtained from standard gel electrophoresis and this instrument. Compared with standard agarose gel electrophoresis detection limit of gel electrophoresis on chip was almost two times lower. Therefore, this technique could be used in clinical diagnostics or forensic science.

3.4 Electrochemical detection of nucleic acids – mercury electrode

Several papers dealing with electrochemical detection of the amplified DNA considering their potential uses for real-time PCR were published recently [33, 34]. The electrochemical detection appears to have a potential to become a very sensitive analytical tool for nucleic acid analysis and also belongs to the most sensitive methods for detection of nucleic acids [35–37]. For this purpose different types of working electrodes (mercury or solid) can be used. Detection of nucleic acids at mercury electrodes was described by Professor Paleček more than 40 years ago [38]. Technical innovations, mathematical corrections and improvements of the detection technology allowed achieving a sensitive detection of nucleic acids using an HMDE. Moreover, to prevent interferences [39] and to enable analysis of very low volumes of a sample [40, 41], voltammetric methods can be coupled with adsorptive transfer stripping (AdTS) technique. The main improvement is based on electrode removal from a solution after accumulating of a target molecule on its surface, rinsing of the electrode and transferring to a pure supporting electrolyte, where no interferences are present (Fig. 4A). Typical AdTS cyclic voltammograms of amplified nucleic acids measured at various scan rates 10, 20, 40 and/or 80 mV/s are shown in Fig. 4B. The observed signals were well developed, symmetric and separated. The increasing of the sensitivity of the determination was reached by using SWV coupled with AdTS. The important parameter in adsorptive transfer technique is time of accumulation of the nucleic acid onto the electrode surface. To optimize this parameter we used amplicons obtained after 30 PCR cycles. The highest signal was measured after 60 s-long accumulation of the amplicons onto the surface of HMDE (Fig. 4C). The slight decrease in the analytical signal was observed with prolonged time of accumulation. This might be caused by the unorganized layer formation at the working electrode surface. We also tested accumulation dependences with lower concentration of amplicons. In that case enhancement of the signal with increasing time of accumulation was more distinct with maximum within the interval from 120 to 240 s (data not shown). This phenomenon relates with longer time needed to fully cover a surface of working electrode due to lower concentration of DNA in a solution.

The proposed detection of the amplified DNA can also be used for quantification of amplified DNA. The electrochemical signal of the amplified DNA increased linearly to the 10^{th} cycle (inset in Fig. 4D). From this point the observed signal increased exponentially. Moreover, the detection limit of the proposed procedure was lower compared with agarose gel or chip electrophoresis, because we were able to detect amplicons even from the second cycle (Fig. 4D). In spite of the fact that mercury electrode offers very high sensitivity to nucleic acids, mercury working electrode is not suitable for applications in automated detection systems because of the difficulty in handling the electrode due to their physico-chemical properties. Nevertheless, carbon electrodes have great potential to be miniaturized.

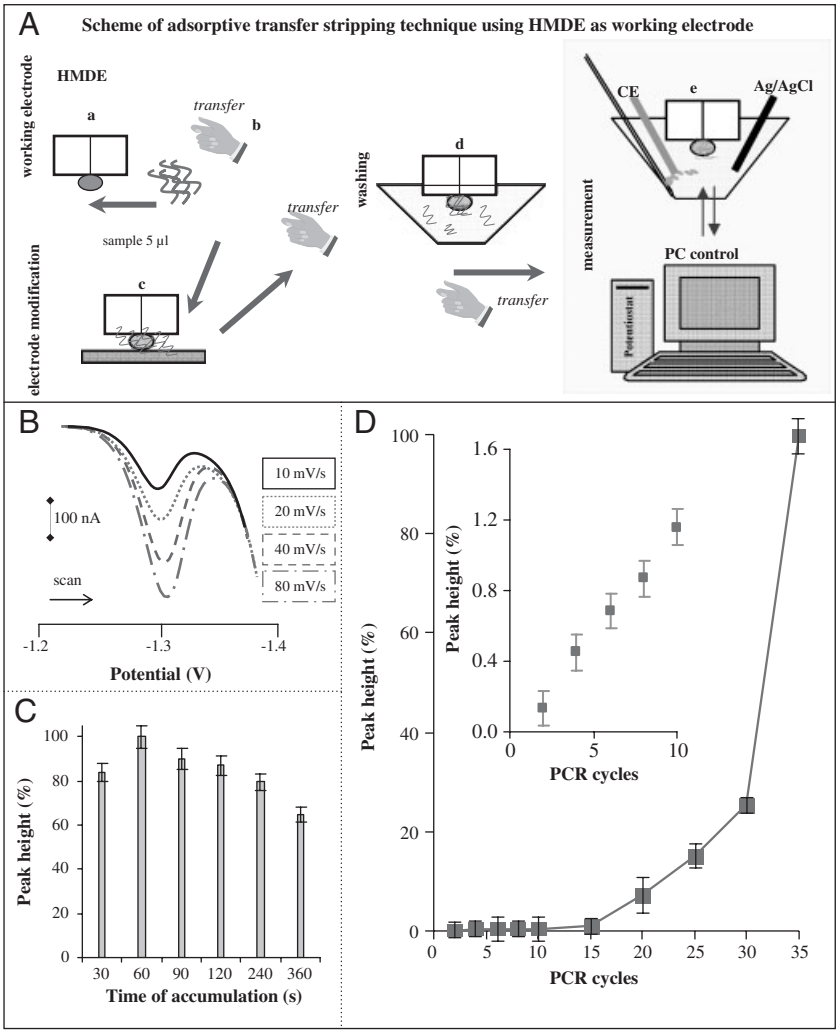

Figure 4. (A) The scheme of AdTS can be summarized in the following steps: (i) renewing of a surface of a working electrode; (ii) introducing of a sample on parafilm; (iii) adsorbing of a target molecule in a drop solution onto the surface at open circuit and/or superimposed potential; (iv) washing the working electrode in a solution; and (v) transferring of the washed electrode to a supporting electrolyte and measuring. (B) Typical cyclic voltammograms of PCR products. The following measurements were carried out by SWV. (C) Influence of time of accumulation of PCR products on the surface of HMDE on peak height. (D) Dependence of peak height on the number of PCR cycles (2–35 or 2–10 in the inset). Experimental parameters: working electrode HMDE. SWV parameters: potential step 5 mV, frequency 260 Hz. CV parameters: scan rates 10, 20, 40 and 80 mV/s, potential step 5 mV, time of accumulation 120 s. Peak height of 64 µA (C) and 232 µA ((D) and its inset) corresponds to 100%.

3.5 Electrochemical detection of nucleic acids – carbon electrode

We focused on solid electrodes in the following experiment. Electrodes prepared by screen-printing or photolithography are suitable for automated detection systems [42, 43]. In our initial experiments the amplified DNA was measured at screen-printed electrodes with working carbon electrode. Low sample volume (3 µL) was introduced onto the surface of the carbon electrode. Further, nucleic acids were accumulated for 120 s. The electrode was rinsed in distilled water and placed into the measuring cell. At the end of a measurement, the electrode was washed in distilled water, dried, shortly sonicated and used for another measurement. This recycling of electrodes was done five times without any detectable decrease in the current response. Dependence of the peak height on the number of PCR cycles is shown in Fig. 5A. The current responses are not as high as those measured at HMDE. This may be caused by lower sensitivity of carbon electrodes compared with HMDE [44]. The change in signal height of amplicons obtained after 30 cycles with increasing time of accumulation is shown in the inset of Fig. 5A. The peak height increased gradually up to an accumulation time of 240 s, and then a slight decrease was determined.

The sensitivity of DNA determination can be improved by using nanomaterials for electrode fabrication. We used carbon nanotubes with size from 80 to 200 nm for fabrication of working electrode of screen-printed electrodes. Dependence of peak height measured at carbon-nanotube-based screen-printed electrodes on the concentration of amplicons is shown in Fig. 5B. It follows from the results obtained that the current response is higher compared with those measured at carbon screen-printed electrodes. Experimental data indicate that we are able to detect the signal of the amplified nucleic acid from the 2nd cycle of the PCR by using SWV and screen-printed electrodes with working electrode made from carbon nanotubes.

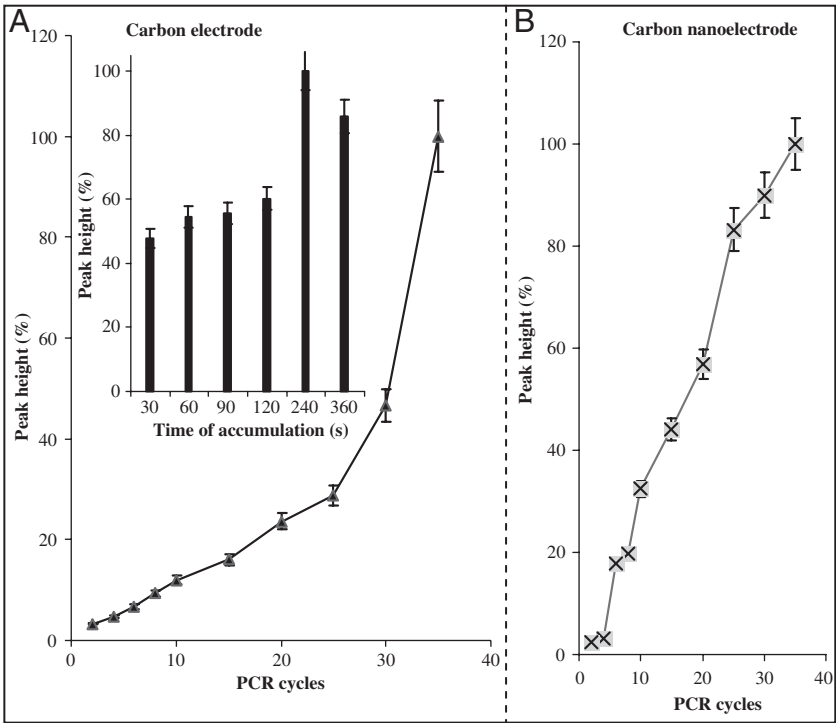

Figure 5. (A) Dependence of peak height on the number of PCR cycles (working electrode screen-printed carbon electrode); inset: influence of time of accumulation of PCR products onto the surface of screen-printed carbon electrode on peak height. (B) Dependence of peak height on the number of PCR cycles (working electrode carbon nanoelectrode). SWV parameters: potential step 5 mV, frequency 50 Hz. Peak height of 6.5 μA (inset in (A)), 15 μA (A) and 25 μA (B) corresponds to 100%.

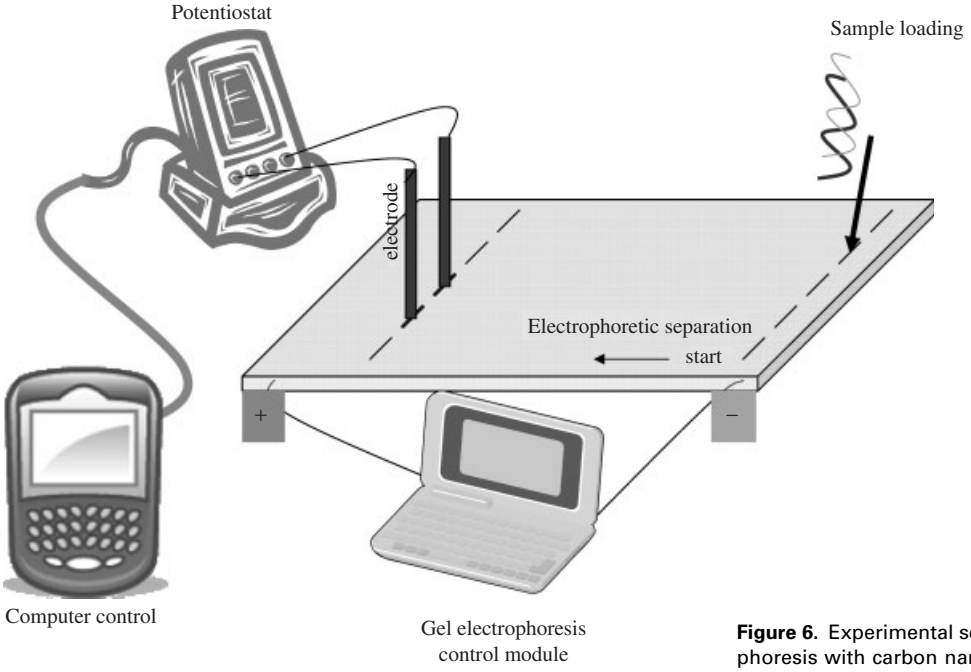

Figure 6. Experimental scheme of coupling of gel electrophoresis with carbon nanoelectrodes

3.6 Coupling of gel electrophoresis with carbon-nanotube-based screen-printed electrodes

Recently, use of carbon nanotubes as a material for fabrication of screen-printed electrodes was published by several authors [45–50]. In the following experiment carbon-nanotube-based screen-printed electrodes were off-line coupled with gel electrophoresis. Off-line coupling was performed as follows: Into the agarose gel the electrodes for monitoring of DNA presence were placed. Further, amplicons were loaded into agarose gel wells. DNA migrating to the detection place was electrochemically analyzed. Agarose

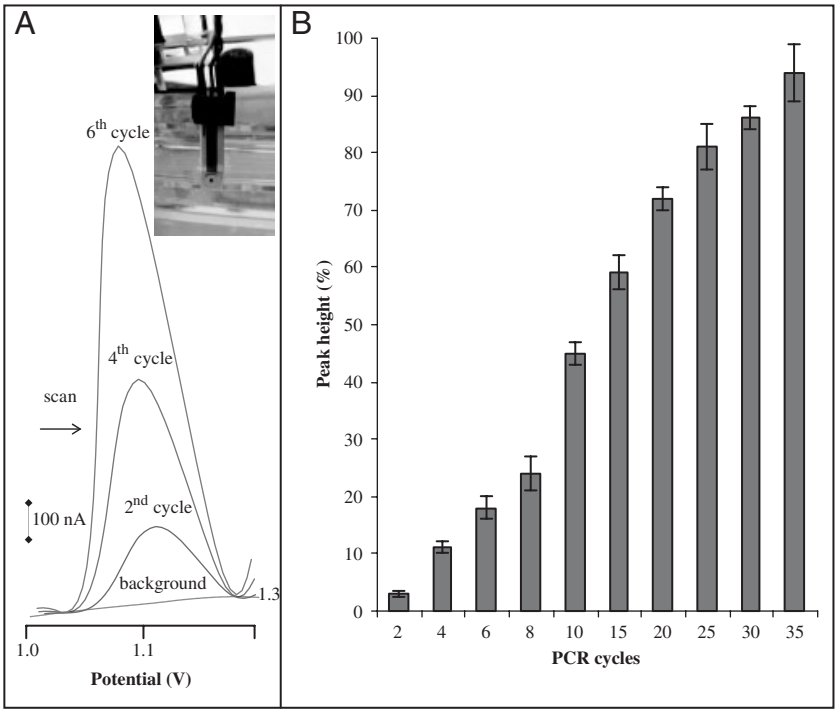

Figure 7. (A) Typical SW voltammograms of amplicons obtained after 2, 4 and/or 6 cycles, measured in agarose gel; inset: screen-printed electrode immersed in the gel. (B) Peak heights of PCR products separated by gel electrophoresis and measured by carbon nanoelectrode. Peak height of 20.5 μA corresponds to 100%.

gel electrophoresis was run for 5 min, and then stopped, and a measurement was carried out (Fig. 6). The electrochemical responses of PCR products obtained after 2, 4 and 6 PCR cycles are shown in Fig. 7A. The obtained signals were well developed and symmetric. When no DNA was present in the well on a gel, negligible signal of 1.1 V was detected. A photograph of the electrode placed into the agarose gel is shown in the inset of Fig. 7A. Dependence of the current response on the number of cycles is shown in Fig. 7B. When the PCR products were analyzed in the gel, it was possible to detect the signal of the amplified nucleic acid from the second cycle. The observed signal was proportional to the number of cycles. The separation on the agarose gel probably influences the amount of DNA migrating to the detection place, which could cause the observed differences between the results obtained by direct measurement in the solution and measurement in the agarose gel.

4 Concluding remarks

Coupling of separation and detection instruments can bring many advantages. Here, we show a possibility to off-line couple gel electrophoresis as a standard and reliable separation technique with carbon-nanotube-based screen-printed electrodes, which represents new trends in microanalysis. Owing to the selectivity of gel electrophoresis and sensitivity of the electrochemical detection we are able to detect low amounts of PCR products of Phage Lambda DNA. This coupling could be considered as another possibility to visualize gel.

The financial support from grant GA AV KAN208130801 is highly acknowledged.

The authors have declared no conflict of interest.

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