Research Article

Integrated chip electrophoresis and magnetic particle isolation used for detection of hepatitis B virus oligonucleotides

Rapid and sensitive detection is a key step in the effective and early response to the global hazard of various viral diseases. In this study, an integrated isolation of hepatitis B virus (HBV)-specific DNA fragment by magnetic nanoparticles (MNPs) and its immediate analysis by microchip CE was performed. Microfluidic CE chip was used to accommodate the complete process of viral DNA isolation by MNPs including hybridization and thermal denaturation followed by CE separation. Beforehand, calibration curves of HBV fragments were constructed. For isolation by MNPs, specific streptavidin–biotin interaction was used to bind complementary HBV fragment to magnetic particles. After analysis of isolated HBV by regular MNPs method, innovative approach was performed. The commercial CE chip (Bio-rad) was successfully used to execute HBV fragment isolation. Detection using LIF with detection limit of 1 ng/mL was accomplished.

Keywords:
Automation / Electrochemical detection / Magnetic particle / Microfluidic Chip / Virus detection

 DOI 10.1002/elps.201200697

Additional supporting information may be found in the online version of this article at the publisher’s web-site

1 Introduction

Hepatitis A, B, C, D, and E viruses can cause the inflammatory illness of the liver and other health problems. Of the many viral diseases, few are of greater global importance than hepatitis B virus (HBV) [1–3]. According to World Health Organization, more than 2000 million people alive today have been infected with HBV. Of these, about 350 million remain infected chronically and become carriers of the virus [4]. Besides, every year there are over four million acute clinical cases of HBV, and one million people die from the severe pathological consequences of persistent HBV infections, which include the development of chronic hepatic insufficiency, cirrhosis, and hepatocellular carcinoma, per year. Rapid and sensitive detection is therefore a key step to effectively diagnose and stop the virus to break out in global scale. For this purpose, there is a need for a quick and cheap method to identify the disease [5–7].

Diagnosis of HBV can be divided according to level of HBV-DNA and/or HBV-RNA in serum, blood, or in liver [8, 9]. HBV-DNA levels in serum of hepatitis B surface antigen (HBeAg) positive cases are high enough to be detected even by less sensitive laboratory techniques [8]. HBeAg itself can be also used as a marker of HBV infection [10, 11]. By contrast, in most HBeAg negative patients, serum viral DNA can be only detected by PCR because HBV-DNA level is much lower compared to HBeAg positive cases [12]. HBV-DNA levels in serum and liver of patients with HBV infection without outer hallmarks are significantly lower to those found in serum and liver of HBeAg-negative patients and must be diagnosed using a highly sensitive techniques including quantitative real-time PCR [13, 14].

In spite of the great development of PCR on a chip [15–17], non-PCR “on a chip” techniques are also suggested for detection of HBV and its antigens. Oh et al. utilized carbon nanotube-based biosensor coupled with microfluidic apparatus for detection of HBeAg. This antigen can be also detected using biotin–avidin amplified magnetic immunoassay
with GoldMag nanoparticles [18]. Besides antigen, methods for detection of HBV-DNA mainly based on nanoparticles from various materials have been suggested [19, 20]. Generally, magnetic particles have proven to be an effective tool for a wide variety of analytes including small organic molecules [21,22], proteins [23–26], as well as nucleic acids [27–31]. Thus, the aim of this study was to utilize paramagnetic particles–based isolation step coupled with chip CGE for detection of HBV-DNA. The commercially available chip CE platform dedicated for analysis of DNA, RNA, and proteins was used to accommodate the isolation process carried by magnetic nanoparticles (MNPs).

2 Material and methods

2.1 Chemicals

To optimize and characterize the suggested procedure, a short genomic DNA sequence specific for HBV was chosen based on the published data [32]. HBV and biotinylated HBV oligonucleotides (ODN) were purchased from Generi Biotech (Czech Republic). The sequence of the reverse HBV fragments is following: \(5'\text{'GGCAAGGTTGAAAGTTGC-3'}\) and biotinylated sequence on 5’ tail: forward \(3'\text{CCGCTCCTACTTTTACAACG-5'}\). Original concentration of both single-strand ODN (ssODN) solutions used for dilution was 230 \(\mu\)g/mL. All DNA solutions needed for the experiment were prepared to ACS (American Chemical Society) purity. Other chemicals used in this study were purchased from Sigma-Aldrich (USA) in ACS purity unless stated otherwise.

2.2 Streptavidin conjugated Fe\(_2\)O\(_3\) MNPs

For sequence-specific capturing of HBV fragments on magnetic particles, Dynabeads M-270 Streptavidin (Invitrogen, Norway) was purchased. Immobilization of biotinylated ssODN HBV was performed entirely according to following Dynabeads M-270 protocol (Invitrogen). Briefly, beads were resuspended in B&W Buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl) to a final concentration of 5 \(\mu\)g/\(\mu\)L (twice original volume). To immobilize, an equal volume of the biotinylated DNA was added in \(H_2O\) to dilute the NaCl concentration in the B&W Buffer from 2 M to 1 M for optimal binding. Incubation for 10 min at 20°C using gentle rotation ensured the quantitative immobilization. The biotinylated DNA-coated beads were separated with a magnet for 2–3 min, washed 2–3 times with B&W Buffer, and resuspended to the desired concentration. The whole procedure of MNPs preparation was performed inside DNA/RNA UV cleaner box (BioSan, Latvia).

2.3 MNPs isolation (classical procedure)

Isolation of DNA using MNPs was carried out according to method of Huska et al. [33]. The isolation took place inside DNA/RNA UV cleaner box (BioSan) with Magnetic stand for test tubes. Paramagnetic particles (10 \(\mu\)L) were transferred to a test tube and placed to the magnetic stand that separates paramagnetic particles from the storage solution. The solution was removed and 20 \(\mu\)L of washing solution (phosphate buffer I: 0.1 M NaCl + 0.05 M Na\(_2\)HPO\(_4\) + 0.05 M NaH\(_2\)PO\(_4\), pH 7.2) was added. Further, the test tube was moved from magnetic stand and shook that the paramagnetic particles were equally dispersed in washing solution. The washing process was repeated three times. The washing with apparatus multi-spin MSC-3000 (BioSan) followed enabling the centrifugation and shaking to be applied. Shaking was set to level hard and lasted for 20 s and centrifugation proceeded at centrifugal force 285 \(\times\) g for 1 s. When the washing solution was removed, the hybridization solution was added to the paramagnetic particles. The composition of hybridization solution was as follows: 100 mM Na\(_2\)HPO\(_4\) + 100 mM NaH\(_2\)PO\(_4\), 0.5 M NaCl, 0.6 M guanidinium thiocyanate, 0.15 M Trizma base adjusted by HCl on pH of 7.5. Total volume of the hybridization solution with the sample was 30 \(\mu\)L. The hybridization was carried out using apparatus multipin MSC-3000 (BioSan) for 40 min. The settings were as follows: 20 s of shaking (level soft) and centrifugation for 1 s at 285 \(\times\) g. After the hybridization, the washing steps with 20 \(\mu\)L of phosphate buffer I were repeated three times. To the washed paramagnetic particles with bounded HBV fragment, ACS water (30 \(\mu\)L) was added. Then, the test tubes were placed in the Thermomixer 5355 (Eppendorf, Germany). Denaturation was carried out at 85°C for 5 min. Paramagnetic beads were then separated from the denatured ssODN HBV using magnetic stand. The solution of ssODN HBV in ACS water (30 \(\mu\)L) was subsequently analyzed.

2.4 Stationary electrochemical measurements

Electrochemical measurements were performed with AUTO-LAB PGS30 Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode with a drop area of 0.4 mm\(^2\) as the working electrode was used. An Ag/AgCl/3M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. Square wave voltammetric measurements were carried out in the presence of acetate buffer pH 5.0 [33]. Square wave voltammetric parameters: potential step 5 mV, frequency 260 Hz, potential step 5 mV, time of accumulation 120 s. 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 20°C temperature. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed.

2.5 Experion system

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 labeled GS (gel priming well) and primed using the priming station. Loading of the chip was done according following procedure. First, 9 μL of GS was pipetted into the three other wells labeled GS. Then, 5 μL of loading buffer was placed into each sample well and the ladder well. Subsequently, 1 μL of DNA ladder was pipetted into the well labeled 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 s to ensure the mixing and immediately analyzed on Experion electrophoresis station.

3 Results and discussion

Miniaturization has become an important factor in all areas of modern society, reflected strongly in scientific research. Here, the idea of on-line coupling of isolation by MNPs with direct CE analysis was investigated. This approach has significant benefits for distinguishing between specifically MNPs-isolated DNA sequence and compounds isolated due to the nonspecific interactions. Moreover, CE analysis is providing additional information about the size and concentration of the isolated sequence, which can be used as a clear marker of the contamination.

Primarily, the detailed characterization of HBV-specific ODN was performed and due to its size (20 bp) the comigration with CE lower marker had to be addressed. Both, the lower marker and upper marker are present in the loading buffer supplied by the chip CE manufacturer and required for the normalization of the migration times of DNA analytes. DNA loading buffer is used for accurate alignment of electropherograms and for calculation base pair length of the target molecule. The lengths of markers in loading buffer are 15 bp and 850 bp. Nevertheless, due to the size of lower marker (15 bp) and HBV ODN (20 bp), the peaks of these two analytes comigrated (Fig. 1A). It was found out that dilution of the loading buffer to 5% of its original concentration does not have any effect on the electrophoretic properties (sensitivity or detection limit); however the resolution of the lower marker and the HBV ODN peak increased significantly (Fig. 1B). Original loading buffer concentration for ladder chip well was not changed.

The calibration curves of HBV ODNs, biotinylated HBV ODNs, and double-stranded ODN (dsODN) were measured (Fig. 2A–C). Linear responses of the chip-based CE to the concentration of ssODN HBV, biotinylated ssODN HBV, and dsODN HBV fragments were determined with coefficients of determination $R^2 = 0.9918, 0.9903$, and 0.9949, respectively (Fig. 2D). LODs of 5, 25, and 1 ng/mL were calculated for ssODN HBV, biotinylated ssODN HBV, and dsODN HBV, respectively. Remarkably higher LOD determined for biotinylated ssODN HBV leads to the assumption that biotin present in the structure of ODN influences the fluorescent labeling during the chip-CE analysis. The RSD of migration time and peak height were determined as 4.0 and 12.9%, respectively.

To verify the performance of the isolation by MNPs, the procedures were performed in the test tubes according to the protocol described by Huska et al. [33]. Briefly, surface of MNPs (10 μL) was modified by streptavidin and streptavidin–biotin interaction the biotinylated ODN (10 μg/mL) complementary to HBV-specific ODN (1, 5, 10 μg/mL) was immobilized on the surface (30 min, 40°C). After addition of the HBV-specific ODN, hybridization took place and using magnetic field remaining solution was removed (washing step). Subsequently, the elution (water) from the MNPs surface was carried out by the increasing temperature to 85°C (5 min). Finally the isolated HBV ODN was removed to clean test tube (10 μL). Isolated HBV fragments were loaded on chip and successfully analyzed using standard protocol for Experion Chip electrophoresis station. Lowered concentration of loading buffer (5%) was used in all chip wells except ladder well.

The isolation was first carried out in the test tubes and the isolated ODN were analyzed by chip-CE to verify the procedure. The brief summary of the procedure is shown in Fig. 3 and results obtained by this protocol are represented by the calibration curve shown in Fig. 3B. As expected, the increasing original concentration of HBV ODN (the amount of ODN entering the isolation procedure) resulted in increased fluorescence signal of the ODN isolated and analyzed by CE.
The coefficient of determination $R^2 = 0.9861$ showed good linearity of the response. The comparison of the original HBV ODN concentration and isolated concentration is shown in Fig. 3C. Also the isolation efficiency is added showing the fact that for lower concentrations of HBV ODN the isolation procedure is more effective than for higher concentrations. This is probably due to the saturation of the particle surface. When 7300 ng/mL was entering the isolation procedure the efficiency of only 22% was reached, however for 910 ng/mL of ODN entering the protocol, the efficiency of 78% was obtained. This fact may be explained by the saturation of the MNP's surface. To confirm the results, an independent method, square wave voltammetry, was employed. The correlation of both methods is shown in Fig. 3D. The slope of the curve is 1.1603 and the coefficient of determination $R^2 = 0.8967$. 

Figure 2. (A) Chip-CE analysis of ssODN HBV ($c = 0.05, 0.1, 0.2, 0.4, 0.9, 1.8, 3.5, 7 \mu g/mL$). (B) Chip-CE analysis of ssODN HBV + biotin ($c = 0.3, 0.6, 1.3, 2.5, 5 \mu g/mL$). (C) Chip-CE analysis of dsODN HBV + biotin ($c = 0.15, 0.3, 0.6, 1.3, 2.5, 5 \mu g/mL$). (D) Fluorescent calibration curves of analyzed oligonucleotides. For conditions see Section 2.

Figure 3. (A) Scheme of the procedure used for ODN isolation in tubes, according to Huska et al. [33], chip analysis according to manufacturer instructions. (B) Calibration curve of the isolated ssODN HBV by MNPs. (C) Efficiency of the isolation (original/isolated). (D) Correlation of the chip-CE analysis and electrochemical analysis.
3.1 Suggestion of in-chip DNA isolation assay

The optimized isolation procedure was implemented into the in-chip DNA isolation process. The MNP-based isolation process was carried out in side of the microwells of the CE chip. The magnetic beads placed in the microwell of the chip can be easily manipulated by magnetic field by placing a strong magnet under the chip. It was proved that the MNPs do not interfere with the CE because the MNPs with immobilized DNA probe do not migrate through the microfluidic channel during electrophoretic analysis. Therefore, there is no need of removing them from the sample well when targeted sequence is released by thermal denaturation and CE separation can be performed immediately. CE separation of the MNP-immobilized probe without an addition of the complementary sequence provides only baseline noise without significant DNA signal.

To perform the in-chip isolation, the heating/cooling/magnetic system was constructed. The scheme of the device is shown in Supporting Information Fig. 1A. This device contains a metallic plate with Peltier Thermo-Element underneath, ensuring elution (denaturation) of ODN HBV. The temperature is precisely controlled with the deviation of 0.3% by the digital thermometer to ensure the optimal temperature for DNA elution from the magnetic particles. This part is exchangeable by the strong square-shaped magnet to manipulate the particles. The top part of the device (Supporting Information Fig. 1A-1)—the plastic chip holder is shown in Supporting Information Fig. 1B in detail. Also the electronic diagram is included (Supporting Information Fig. 1C).

3.2 Application of in-chip DNA isolation assay

Subsequently, complete isolation on MNPs in the chip wells, denaturation, and analysis on chip CE was carried out [34]. It was found that streptavidin MNPs do not migrate through the channels of the chip when filled with supplied gel and therefore when the isolation is carried out in the chip well there is no need to remove the particles prior to the CE separation. Based on this knowledge, we carried out the isolation procedure by MNPs coated with streptavidin inside chip wells. The whole experiment is schematically described in Fig. 4. Briefly, the chip was primed on priming station. The solution of 5 μL of nanoparticles already modified with biotinylated DNA probe, complementary to test DNA sequence were placed into the microwell of the chip. The solution of MNPs was washed two times with 10 μL of 0.05 mM phosphate buffer (pH 7.2). For this purpose, square-shaped magnet was placed under the glass chip to immobilize the MNPs and solution was removed. After washing step, 5 μL of HBV ODN, 1.25 μL of sodium chloride (0.5 M), and 3.75 μL of hybridization solution were added and hybridization took place at 20°C for 40 min. Solution was carefully mixed with pipette tip every 5 min. Then, after hybridization, the particles were immobilized by the magnet and the unhybridized solution was removed. And the MNPs in the wells were washed again two times with 10 μL of 0.05 M phosphate buffer (pH 7.2) using magnet. Nonbounded ssODN were removed from chip. Finally, 10 μL of ACS water was added to chip wells containing MNPs. Denaturation was carried out on heating/cooling device at 85°C for 5 min. To prevent the evaporation during the heating, the application of the mineral oil was considered;
however, no difference between results with and without the oil was observed. For the analysis, only 1 μL of the sample was kept in the chip well to keep the consistent volume inside the chip well. Other chemicals from Experion DNA 1K analysis kit were added (gel stain, 5% loading buffer, DNA 1K ladder). Chip was vortexed and analyzed using CE instrument. Acquired peaks in electropherograms show successful isolation and detection of desirable ODNs (Fig. 5).

4 Concluding remarks
Integration of the sample preparation procedure with the analytical process increases the analytical throughput and therefore decreases the analysis time as well as its costs. In line with the general trend in miniaturization utilizing microfluidic chips has been gaining popularity especially due to their advantages, compared with common methods. These are extremely low consumption of samples as well as other chemical compounds, fast separation time, and in many cases cost efficiency of disposable chips when mass fabricated [35]. In this study, CE chip was used to accommodate the complete process of DNA isolation by MNPs including hybridization and thermal denaturation followed by CE separation and demonstrates the benefits of such on-line coupling. This study is aiming to exhibit the possibility of use of a commercial chip CE system, primarily developed for routine RNA, DNA, and protein separation, as an integrated platform for sample pretreatment namely MNPs-based DNA isolation followed by immediate CE analysis. This method is simple, rapid, and effective and its applicability to the analysis of virus-specific sequences is apparent. The advantage of this approach can be seen in easy automation where switching of the magnetic field as well as solution manipulation can be computer controlled.

The financial support from NanoBioTECell GA CR P102/11/1068 and CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. The author M.R. wishes to express her thanks to project CZ.1.07/2.3.00/30.039 for financial support.

The authors have declared no conflict of interest.

5 References