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Plastic Microchip Electrophoresis for Analysis of PCR Products of Hepatitis C Virus

Yu-Hung Chen,[1] Wei-Chang Wang,[1] Kung-Chia Young,[2] Ting-Tsung Chang,[3] and Shu-Hui Chen[1*]

Background: **Electrophoresis on polymeric rather than glass microstructures is a promising separation method for analytical chemistry. Assays on such devices need to be explored to allow assessment of their utility for the clinical laboratory.**

Methods: **We compared capillary and plastic microchip electrophoresis for clinical post-PCR analysis of hepatitis C virus (HCV). For capillary electrophoresis (CE), we used a separation medium composed of 10 g/L hydroxypropyl methyl cellulose in Tris-borate-EDTA buffer and 10 μmol/L intercalating dye. For microchip electrophoresis, the HCV assay established on the fused silica tubing was transferred to the untreated polymethylmethacrylate microchip with minimum modifications.**

Results: **CE resolved the 145-bp amplicon of HCV in 15 min. The confidence interval of the migration time was <3.2%. The same HCV amplicon was resolved by microchip electrophoresis in <1.5 min with the confidence interval of the migration time <1.3%.**

Conclusion: **The polymer microchip, with advantages that include fast processing time, simple operation, and disposable use, holds great potential for clinical analysis.**

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Microchip electrophoresis is emerging as a highly promising method for rapid and sensitive analysis that is potentially well suited for clinical analysis. Whereas most of the reports on microfabricated electrophoretic devices have utilized glass or silica as substrates *(1–3)*, recent attention has been given to the use of polymeric microstructures. The polymer substrates are viewed as promising alternatives for the production of microfluidic systems *(4, 5)* because they are less expensive and easier to manipulate than silica-based substrates. Moreover, the inherent neutral hydrophilic nature of the polymer substrate allows direct use of the channel for clinical analysis of biomolecules without the need of surface modifications to reduce wall adsorption *(6)*. However, in the past 15–20 years, the use of glass tubing such as fused silica has become a well-established technique in capillary electrophoresis (CE).[4] The deprotonated silanol groups on the glass surface provide satisfactory electroosmotic flow for the fluid delivery and separation. Although polymer-based capillary tubing, such as polypropylene *(7–9)*, polytetrafluoroethylene *(10)*, and hydrophilic polymethylmethacrylate (PMMA) *(6)*, has been investigated for CE separations, experience with polymer-based microchip CE is limited. Analytical assays on these devices need to be explored before their usefulness can be further addressed.

One of the major clinical applications that uses electrophoresis is associated with PCR *(11)*. PCR enzymatically generates millions or billions of exact copies, thereby making genetic analysis of small samples a relatively simple process. It is widely used in molecular biology, with direct applications in the field of medical diagnosis. Conventionally, slab gel electrophoresis has been used for the analysis of PCR products for sizing, mutations, or polymorphisms, but the technique is time-consuming, labor-intensive, and nonquantitative. Because of the superior separation efficiency and speed in an automated format, CE is rapidly becoming an important tool for PCR analysis. However, as the electrophoresis technique advances to microchip devices, the use of microchip electrophoresis for DNA analysis is emerging as a promising method. The PCR-CE chip has also been microfabricated and investigated for some model reactions *(12–14)*.

[1] Department of Chemistry, National Cheng Kung University, Tainan 701, Taiwan.

Departments of [2] Medical Technology and [3] Internal Medicine, National Cheng Kung University Hospital, Tainan 701, Taiwan.

*Author for correspondence. Fax 886-6-2740552; e-mail shchen@mail.ncku.edu.tw.

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[4] Nonstandard abbreviations: CE, capillary electrophoresis; PMMA, polymethylmethacrylate; HCV, hepatitis C virus; HPMC, hydroxypropyl methyl cellulose; and EtBr, ethidium bromide.

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In this study, DNA sizing of the hepatitis C virus (HCV) amplicon was attempted by CE with fused-silica tubing as well as by microchip electrophoresis on PMMA substrate. Initial results of the CE/laser-induced fluorescence method for the detection of HCV-specific reverse transcription followed by one-stage PCR amplification have been described briefly in the literature *(15)*. This method has also been validated and applied to diagnostic detection of 152 HCV amplicons *(16)*. Interinstrument and intercapillary reproducibility (CVs) of the migration time has ranged from 0.319% to 1.833%. Moreover, a complete agreement between the CE/laser-induced fluorescence and agarose gel electrophoresis/Southern blotting methods for product detection has been reported *(16)*. A two-stage PCR amplification that involves two pairs of primers deduced from the 5′ noncoding region of the HCV genome *(17)* was adopted in the present study. This two-stage amplification is reported to be 10-fold more sensitive than the one-stage assay *(17)* and has also been adopted for routine clinical diagnoses *(18)*.

Materials and Methods

CHEMICALS AND REAGENTS

Tris was purchased from Fluka, EDTA from Sigma, and boric acid from Janssen Chimica. Hydroxypropyl methyl cellulose (HPMC) with a viscosity range of 0.08-0.12 Pa · s (20 g/L in H_2O) was from Aldrich. DNA fragment calibrator ΦX-174-RF DNA digested by *Hae*III was from Amersham-Pharmacia Biotech. The intercalating dyes were ethidium bromide (EtBr) from Sigma and TOPRO-3 from Molecular Probes. All reagents were of the highest grade available. CE water was deionized distilled water filtered through a Barnstead E-pure system. The resistance of the water was >18.0 MΩ/cm^3.

PCR AMPLIFICATION

The serum of patients with HCV-related disease and hepatitis B surface antigen negative was collected from the hospital of National Cheng Kung University. The nucleic acid was then extracted from the serum, and cDNA was synthesized by reverse transcription. DNA amplification of HCV cDNA was performed by a two-stage PCR with two pairs of primers deduced from the 5′ noncoding region *(17, 18)*. The PCR products were analyzed by agarose gel electrophoresis as well as capillary and microchip electrophoresis. The protocols for the PCR and agarose gel electrophoresis methods have been described by Liou et al. *(18)*.

CE INSTRUMENTATION

The experiments were performed using Beckman P/ACE System 5500 equipped with a ultraviolet absorbance detector (Beckman Instruments). The inner walls of fused-silica capillaries (i.d., 50 μm; o.d., 375 μm; effective length, 40 cm; total length, 47 cm; Polymicro Technologies) were covalently bound with a non-cross-linked polyacrylamide according to procedures described elsewhere *(19)*. Unless specified, samples were injected by a stream of nitrogen gas at 3447.38 Pa (0.5 psi) for 20 and 10 s for the DNA marker and the HCV amplicon, respectively. Separations were carried out in the reversed polarity mode (−12 kV at the injector end; −255 V/cm). The detection wavelength was set at 254 nm throughout the experiment.

MICROCHIP SYSTEM

A schematic diagram of the device configuration is shown in Fig. 1. The channels were fabricated on PMMA plexiglass pieces ~2 cm in width, 10 cm in length, and 2.0 mm thick by a wire-imprinting method. The imprinting method was adopted from Martynova et al. *(20)* with some modifications *(21)*. The resulting channels had a rounded shape and were 75 μm in depth *(21)*. Two identical power suppliers (CZE 1000R; Spellman) were utilized to furnish the loading and separation voltages, respectively, and the power switching was controlled by a program written in LabView (National Instruments) running on a Pentium 75 MHz computer *(22)*. The sample loading was performed by applying −750 V (−150 V/cm) to the buffer channel (between reservoirs III and IV) for 0.15, with no voltage applied to the separation channel (between reservoirs I and II). The amplicons were diluted (threefold) with deionized water, and no desalting step was performed before the injection. [Note: the mismatch in ionic strengths between the sample and the buffer is

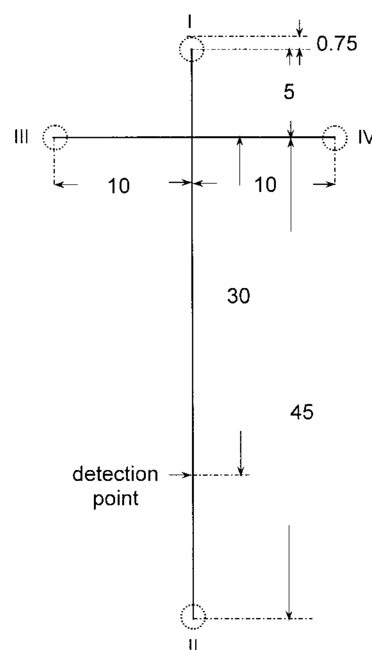

Fig. 1. Channel configuration of the microchip.

The buffer, analyte, and two waste reservoirs were indicated as *I*, *III*, *II*, and *IV*, respectively.

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likely to cause an inconsistency in the actual amount injected *(23)*]. Both the dilution and the desalting steps serve to reduce the ionic strength of the sample, and the desalting step may provide better sensitivity. For the separation, −1.0 kV (−200 V/cm) was applied to the separation channel with no voltage applied to the buffer channel. The fabricated device was used directly for chip electrophoresis without further modifications.

Signals were detected on the microchip via laser-induced fluorescence. The detection system was constructed through modifications of a commercial reflection microscope (Model BX40; Olympus). Briefly, a helium-neon laser with a wavelength of 632.8 nm (10 mW, model LHR-991; Melles Griot) was focused at a position 3 cm downstream from the cross-section within the channel by use of a ×50 (numerical aperture = 0.5) working distance objective. Fluorescence was collected by the objective and passed through a dichroic cube with a band-pass filter, followed by spatial filtering before photomultiplier detection operated at −725 V (model R928; Hamamatsu). Amplified photoelectron pulses were converted to an analog signal and acquired by a commercial interface (model 9524; SISC) running on the same computer as the voltage switching power supplies.

Results

The HCV fragment (145 bp) amplified by the two-stage method was shorter than that (308 bp) produced by the one-stage method *(15, 16)*. Therefore, the CE conditions published in the literature *(15, 16)* would not be directly transferable and would need to be modified to achieve an effective resolution. A polymer buffer solution is usually prepared to achieve an effective sizing, and its concentration can be varied to fit a particular separation. However, the solution viscosity increases as the polymer concentration increases. Ideally, one would like to choose a polymer solution with a low viscosity when going to a smaller mesh size. A few methylcellulose polymers, such as HPMC and hydroxyethyl cellulose, were investigated at several concentrations for the separation of the HCV amplicon. We found that HPMC at a concentration of 10 g/L provided the best resolution with an adequate viscosity. Moreover, intercalating dyes were added to the buffer solution in an attempt to increase the resolution *(15, 16)*. For the HCV amplicon, when EtBr was added, the peak height increased substantially and was accompanied by a slight increase in elution time. In addition, the separation was also attempted at 50 °C to reduce the elution time because the solute mobility would increase at a higher temperature. The elution time decreased with a concomitant increase of peak height. However, the resolution became poorer, and a longer equilibrium time before each electrophoretic run was required.

As seen in Fig. 2, the HCV amplicon was easily detected at a migration time of ~13 min when a physical gel solution composed of 10 g/L HPMC and 10 μmol/L EtBr in Tris-borate-EDTA buffer at 23 °C was used. There were no peaks during this time period in the negative control (Fig. 2). Identification of the amplified HCV fragment was further assessed by a DNA digest marker. Fig. 3 shows that the HCV amplicon 145 bp in size was eluted at a migration time between those of the 118- and 194-bp marker fragments. Moreover, analysis of >10 patient samples by both CE and agarose gel electrophoresis showed 100% correlation. The confidence interval of the migration time was 0.45% for within-day tests (n = 3) and 1.38% and 3.15% for five samples pooled from different days with the same and different columns, respectively.

Analysis of the same HCV amplicon was further attempted using microchip electrophoresis fabricated on PMMA substrate. As shown in Fig. 4, using the same separation medium except that 10 μmol/L EtBr was replaced by 1 μmol/L TOPRO-3 for fluorescence detection, 10 of 11 DNA fragments could be resolved in <2.5 min on the untreated PMMA chip. The electropherogram was much simplified because of the lack of fluorescence

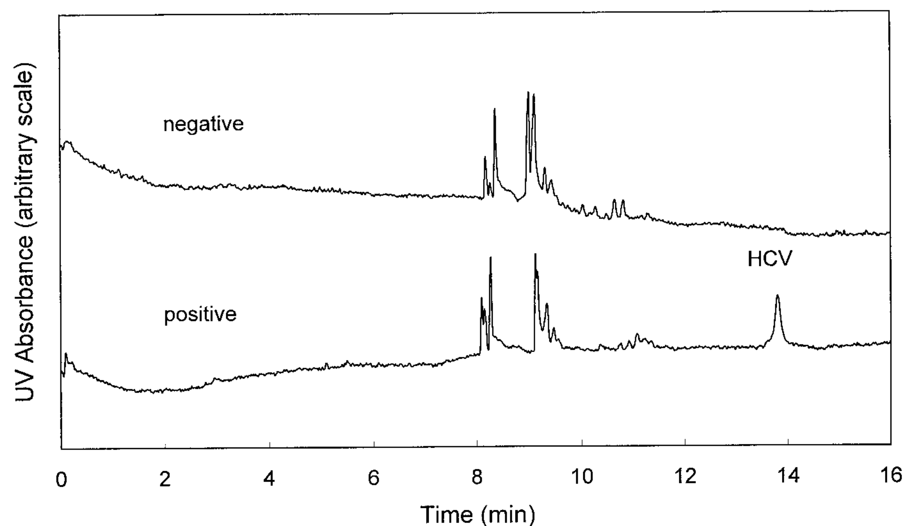

Fig. 2. Capillary electropherograms of the amplified HCV products from serum samples with negative (*top electropherogram*) and positive (*bottom electropherogram*) response.

The separation buffer was composed of 10 g/L HPMC and 10 μmol/L EtBr in Tris-borate-EDTA buffer (100 mmol/L Tris-borate, 5 mmol/L EDTA, pH 8.2). *UV*, ultraviolet.

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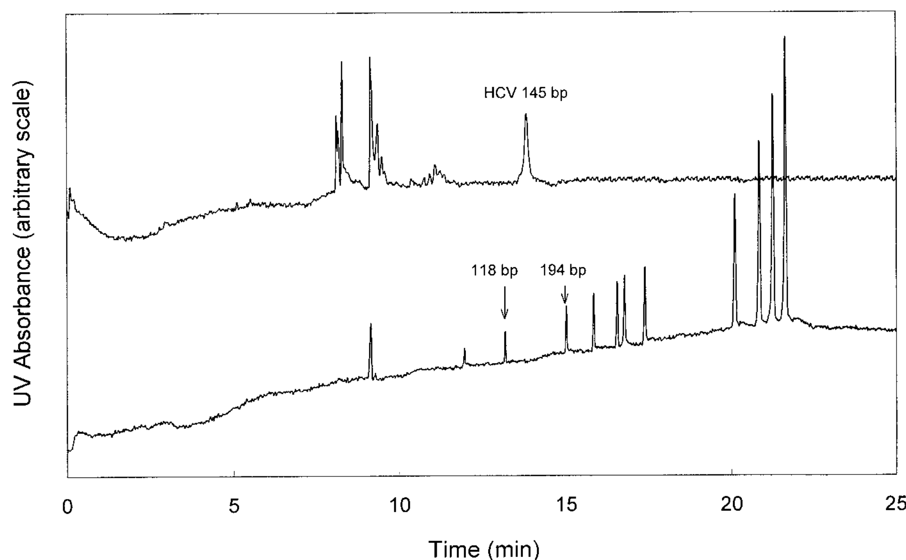

Fig. 3. Size confirmation of the amplified HCV products (145 bp) using CE.

Bottom electropherogram, ΦX-174-RF DNA (total concentration, 5×10^4 μg/L) digested with *Hae*III; *top electropherogram*, HCV amplified products from serum samples. The CE separation buffer was the same as in Fig. 2. *UV*, ultraviolet.

signal of some unwanted species. The only unresolved fragments (271 and 281 bp) could be resolved by increasing the HPMC concentration at the expense of analysis time. Despite that, the 145-bp HCV amplicon could be easily identified in <1.5 min under the separation conditions described for the DNA marker (Fig. 4). It was also noticed that the sensitivity of the described method using microchip electrophoresis (Fig. 4, HCV amplicon diluted threefold before injection) was increased compared with that of CE (Figs. 2 and 3). Tentative comparisons showed that the detection limit of the microchip method was approximately two orders of magnitude lower than that of CE method. Fig. 5 shows that consecutive injections and separations were completed by voltage switching alone, with a relative SD <1.3% for the migration time. The chip-to-chip variations were also small: One PMMA chip could perform >100 analyses of the HCV amplicon without significant changes of the obtained electropherograms.

Discussion

The improved sensitivity with the use of microchip electrophoresis compared with CE could be partly attributed to the use of fluorescence detection and the much shorter separation time. The use of near-infrared dye (TOPRO-3) as the intercalator coupled with a He-Ne laser provided several advantages compared with other visible intercalators coupled with an argon ion laser: (*a*) the near-infrared region is inherently low in biological interferences and thus allows reduced background noise and a lower detection limit compared with those using visible dyes (*21*, *24*); and (*b*) the near-infrared-emitting semiconductor laser diodes, which are inexpensive, small, reliable and compact in size, may be used for the miniaturization of chip-based devices.

As demonstrated above, the use of the PMMA chip increased the separation speed by one order of magnitude. No column coating is needed, which would otherwise require tedious derivatization procedures or the

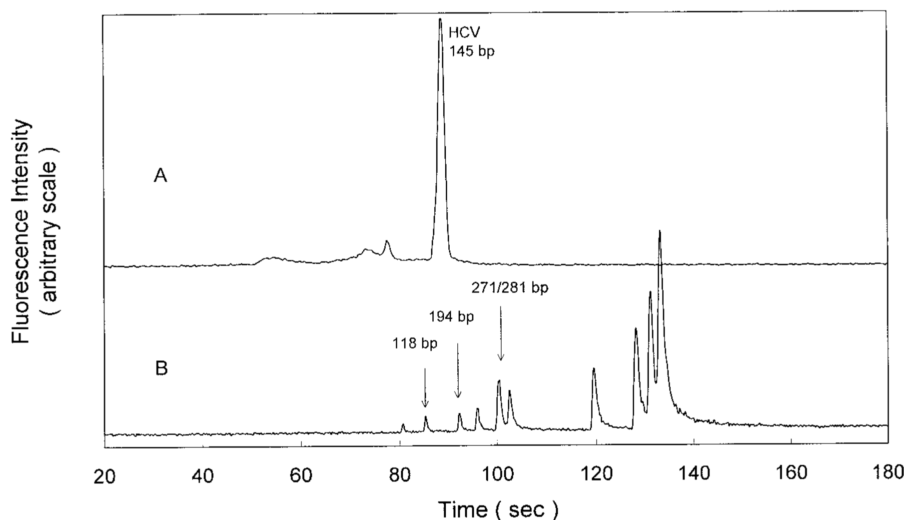

Fig. 4. Microchip electropherograms of the amplified HCV products (145 bp, diluted threefold; *top electropherograms*) together with ΦX-174-RF DNA (total concentration, 5×10^4 μg/L; *bottom electropherogram*) digested with *Hae*III.

The separation buffer was composed of 10 g/L HPMC and 1 μmol/L TOPRO-3 in Tris-borate-EDTA buffer (100 mmol/L Tris-borate, 5 mmol/L EDTA, pH 8.2).

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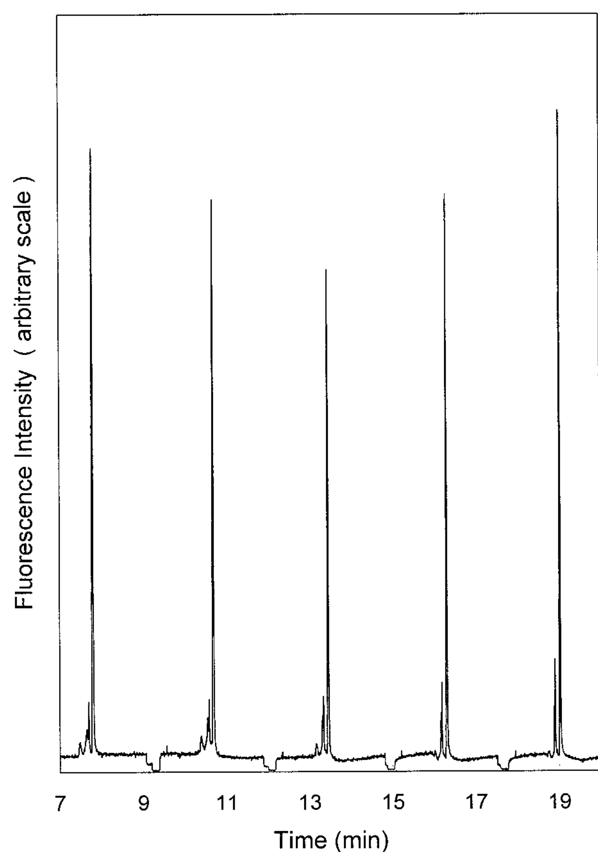

Fig. 5. Consecutive injections and separations of the HCV amplicon (diluted threefold) by voltage switching.

The separation buffer was the same as in the legend for Fig. 4.

purchase of a coated fused-silica column. The major concern in using the wall-derivatized column is that the silyl-oxygen bond is likely to be washed off because of the reversible hydrolysis, which leads to a limited column lifetime, especially at high pH values. For the PMMA microchip, >100 repetitive injections appear to be acceptable. In addition, mass production of disposable devices should also be possible because of the low cost of PMMA material. These features are well suited for clinical analysis, which requires the handling of a large amount of biological fluids. However, the disadvantages of the use of PMMA material include low resistance to organic solvents and high temperatures (>90 °C). Furthermore, the optical transmittance of PMMA at wavelengths below 250 nm is low. These disadvantages might hinder the fabrication of a monolithic PCR-CE device on PMMA substrate because of the requirement for PCR thermal cycling up to 90 °C. In addition, certain applications, such as the use of low wavelength optical measurements and nonaqueous buffer systems, for the separation may also be prohibited. However, creative fabrication methods or the use of other polymer materials may overcome these problems. Moreover, many electrophoresis methods have been developed that incorporate aqueous phases at room temperature together with optical measurements within an acceptable wavelength range of PMMA. This study did demonstrate that clinical assays, such as the sizing and detection of the HCV amplicon, established on silica tubing using CE could be transferred directly to PMMA chips with tremendous gains in speed of processing, ease of operation, and use of disposables.

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