Use of high-molecular-mass polyacrylamides as matrices for microchip electrophoresis of DNA fragments

DNA fragment analysis requires the use of polymer solutions as sieving matrices. Generally, such matrices are constituted of high-molar-weight polymers employed at a concentration higher than their entanglement threshold concentration. These polymer solutions are highly viscous and difficult to use in the narrow channels of a microchip. Ultralarge polyacrylamides synthesized via a nonconventional method, being the low-temperature plasma-induced polymerization (PIP), were used as DNA sieving matrices for microchip electrophoresis. The distinctive features of these polymers (ultralarge molecular mass and linearity) allow their use at a dilute concentration. Dilute PIP polyacrylamides revealed a constant value of resolution in a broad range of DNA fragment sizes (123 bp–1353 bp), thus proving to be effective in common genotyping applications. Moreover, the low viscosity of the dilute solutions enable it to be easier and faster in filling the channel between runs, thus enhancing the throughput of the microchip devices.

Keywords: DNA separation / Microchip electrophoresis / Miniaturization / Sieving matrices

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1 Introduction

Miniaturization is a trend in chemistry which has generated considerable interest over the last few years, and it has become a “hot topic” recently. There are many good reasons to prefer smaller-scale analysis, including high sample throughput, short analysis time, high separation efficiency, and low consumption of samples and reagents. Microfabricated devices for electrophoresis are in many cases the core of micrototal analysis systems (μ-TAS) in which different processes such as PCR reactions, immunoassays, protein digestion, and sample pretreatment are integrated in a single chip [1, 2]. Parallel analyses with multiple channels in one microchip are of great utility for increasing the analysis throughput. Microdevices are, more and more, routinely used in DNA analysis for the separation of restriction fragments and PCR products. The DNA fragments are separated by electrophoresis in a sieving medium which is able to induce a size-dependent separation of the different fragments which otherwise would have identical electrophoretic mobility. Although different sieving matrices have already been employed in the microchip format in research laboratories, the development of polymers suitable for large-scale application is still an active field of research. The rheological properties of most polymer solutions render them difficult to be used in the narrow channel of a microchip. High-pressure pumps and long flushing times are commonly required to properly fill microchannels with such viscous solutions. The analysis time in μ-TAS platforms is very short as compared to the time that is currently required for filling the channels with a viscous solution. For this reason it is important to reduce the time spent in prerun operations as much as possible. In addition, glass microchips with long separation channels are not disposable due to their cost, and the sieving matrix must be replaced before each run. This operation, in the case of a viscous solution, may cause a significant reduction of the chip’s lifetime.

Low-viscosity polymers fill microchannels more easily in a shorter time span without clogging them. Different strategies have been proposed to obtain high-performance DNA separations with a low-viscosity polymer. Thermoresponsive polymers, whose viscosity depends on temperature, have been proposed for microchannel DNA sequencing [3, 4]. Their use requires a spatial and temporal temperature dynamic control. In another interesting approach [5], a low-viscosity, dilute hydroxypropylmethylcellulose solution containing polyhydroxy additives has been used in microchip DNA separation. The additives do not influence the viscosity of the matrix, but they

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Abbreviations: EPDMA, epoxypolydimethylacrylamide; GPC, gel permeation chromatography; HEC, hydroxyethylcellulose; LPA, linear polyacrylamide; MALLS, multi-angle laser light scattering; PIP, plasma-induced polymerization

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do improve its sieving performance due to the formation of hydrogen bond interactions between polymer chains, additives, and DNA molecules. However, the efficiency that is obtained with these polymers is low as the DNA fragments undergo hydrogen bonding interaction with the matrix during electrophoresis.

It has been reported in several studies involving DNA and other polyelectrolytes that the high molecular weight of the matrix favors good separation [3]. Most studies on the influence of polymer $M_w$ on the network properties have converged in showing that a relatively low concentration of high-molecular-mass polymer chains is needed to separate large DNA fragments [6]. Constraint release and active network deformation provides a mechanism to explain the empirical observation that high-molecular-mass polymers perform better in CE of DNA than the same polymers of low molecular mass [7].

In this work, the usefulness of high-molecular mass polyacrylamide in the separation of DNA fragments by microchip electrophoresis is demonstrated by comparing the sieving performance of polyacrylamides of various sizes which were obtained with different polymerization procedures. Among the different strategies to obtain polymers of high molecular weight, a nonconventional method, the low-temperature plasma-induced polymerization (PIP), provided polymers with the most suitable characteristics, both in terms of sieving capacity and rheological properties. The so-called “plasma-induced” polymerization process is essentially a nonconventional molecular polymerization in which the cold plasma is only used to produce the polymerization initiating species that generate, in the postpolymerization period, the “living macroradicals” (macroradicals with a very long life even at normal temperature) [8]. The polymer products are characterized by an “ordered” structure and by the following properties: ultrahigh molecular weights, very low polydispersity, and absence of branches and cross-links. Due to their unique features, these polymers provide an excellent separation of large-size DNA fragments when used at a very low concentration. The low viscosity of dilute solutions made filling the chip easier and faster, thus enhancing the throughput of the microchip device and avoiding the risk of separation failures.

2 Materials and methods

2.1 Chemicals and reagents

Tris(hydroxymethyl)aminomethane (Tris), N-tris(hydroxy-methyl)methyl-3-amino-propanesulfonic acid (TAPS), ammonium persulfate (APS), and the 123 bp DNA ladder were from Sigma (St. Louis, MO, USA). YO-PRO-1 was purchased from Molecular Probes (Eugene, OR, USA). Acrylamide and $N,N',N'-$tetramethylethylenediamine (TEMED) were from Bio-Rad Labs (Hercules, CA, USA). $N,N'$-Dimethylacrylamide was from Aldrich (Steinheim, Germany) and allyl glycidyl ether was from Fluka (Buchs, Switzerland).

2.2 Polymer synthesis

The experimental apparatus and general technique of PIP are described in [9–11]. Briefly, aqueous solutions of acrylamide of concentrations ranging from 8 to 20% w/v were introduced in a polymerization Pyrex ampoule, degassed by repeated freezing and thawing under vacuum, frozen in liquid nitrogen and finally sealed at different pressures (10$^{-3}$–10$^{-4}$ mbar). An electrical discharge was then operated between two external electrodes, situated on the ampoule and coupled to a high-frequency generator (13.5 MHz, 100 W). The discharge was operated during pre-established periods of time (60–120 s). After a postpolymerization period (generally 24 h), during which the ampoules were kept in the dark, their content was diluted with deionized water, extensively dialyzed, and lyophilized. Conventional polyacrylamide was synthesized in a two-necked round bottom flask which was equipped with a nitrogen inlet tube. After purging with $N_2$, 1 $\mu$L/mL of TEMED and 1 $\mu$L/mL of a 40% w/v APS water solution were added to a 6% w/v acrylamide solution. The polymerization was carried out overnight at room temperature. Epoxypolydimethylacrylamide (EPDMA) used as a dynamic wall coating was synthesized as previously described [12].

2.3 Polymer analysis

Intrinsic viscosity measurements were performed with a Cannon Fenske viscometer with a constant temperature bath maintained at 30°C. Flow times were manually measured by a 1/10 s mechanical stopwatch, from Hanhart (Franklin Lakes, NJ, USA). Polyacrylamide samples were dissolved in the solvent (degassed Milli-Q water) at room temperature by gently mixing. Solution concentration ranging from 2 to 1 g/L for linear polyacrylamide 1 (LPA1) and from 0.2 to 0.5 g/L for LPA2, LPA3, and LPA4, were prepared from independent weighing of polymer and solvent. For each polymer solution and solvent, average flow time values from six measurements were calculated. The solvent flow times varied from 88 to 90 s, while those of polymer solutions varied from 105 to 200 s. The dynamic viscosity of the different polymer solutions was determined with a P/ACE system MDQ capillary electrophoresis instrument from Beckman Coulter (Fullerton, CA, USA) equipped with a LIF detector system. A fused-
silica capillary (50 µm ID, 32 cm total length) was filled with 1% w/v LPA1, 0.2% w/v LPA2, LPA3, LPA4, and 1.5% w/v hydroxyethylcellulose (HEC) in 100 mM TTE. Pressure was applied to the inlet vial filled with the polymer solution added with a fluorescent marker. Pressure was applied until the column volume was replaced and the fluorescent front was detected at the detection window. The viscosity of polymer solutions were calculated from capillary dimensions, applied pressure, and replacement time with the help of the software CE Expert from Beckman Coulter (water κ= 1 cP). Polymer molecular weights were determined by gel permeation chromatography (GPC) equipped with multi-angle laser light scattering (MALLS) detection. Samples were dissolved in a buffer containing 0.1 mM NaCl, 50 mM NaH2PO4 and 200 ppm NaN3. All samples were prepared using a high-precision balance to weigh both solvent and polymer so that the final concentration could be estimated as accurately as possible. The polymer samples were fractionated by GPC prior to on-line MALLS detection using Shodex (New York, NY, USA) O'Hpack columns SB-806 HQ, SB-804 HQ and SB-802.5 HQ connected in series.

2.4 Optical apparatus

The DNA separations were conducted in a home-made microchip electrophoresis apparatus. The optical setup (Fig. 1) is based on an ultrasensitive detector head equipped with a planar thin single photon avalanche diode (SPAD). A single-line argon laser with 15 mW power and 488 nm wavelength was used as an excitation source. The laser beam was filtered by way of an external interferential filter, centered on 488 nm; the resulting beam was then reduced in power by a variable dimmer and finally launched in a multimode fiber by means of a lens. At the other end of the fiber a lens collimator produced a 50 µm spot at a distance of 23 mm. The optical detection system employed a Zeiss Axiosvert S100 TV microscope. Light arising from the channel is collected by a 5 x objective and focused on the video output of the microscope, where the detector head is positioned. This head contains a notch filter centered on 488 nm and an interferential filter centered on 514 nm. The filtered light is then focused on the detector by a 40 x objective. The structure of the planar SPAD detector employed is described in [13]. The SPAD is operated with an over-voltage of 7 V above breakdown and is associated with a monolithic integrated active-quenching circuit (AQC) [13, 14].

2.5 Microchip electrophoresis

Glass microchips were purchased from Micralyne (Edmonton, Canada). The channels have a simple cross geometry; they have a diameter of 20 µm and lengths of 8.5 cm and 8 mm, respectively, and cross each other 5 mm away from the end of the longer one. The chip was glued on an aluminum support, to achieve an easier positioning on the optical plane. In order to provide external reservoirs and to easily fill the channels, a microchip holder was developed, starting from a 10 mm thick plexiglass plate. Electrosmotic flow in the separation channels was minimized using an EPDMA dynamic coating: microchip channels were flushed with 0.1 mM NaOH, treated for 10 min with a 0.1% w/v EPDMA solution and finally filled with the appropriate sieving matrix dissolved in the separation run buffer (100 mM, TAPS-Tris pH 8.5). All solutions were forced through the microchip channels by pressure with the aid of a motorized syringe connected with microchip reservoirs by the plexiglass chip-holder. For detection, the migrating 123 bp DNA ladder (0.1 µg/µL) was stained with the fluorescent intercalating dye YO-PRO-1 added to the sieving matrix at the final concentration of 1 nM. Electrical contacts were established on the chip by inserting Pt wires into the reservoirs. After loading the sample, 200 V (250 V/cm) were applied for 30 s between the reservoirs at the end of the short arm of the injection cross for injecting the samples into the separation channel. Separation was performed by applying 2000 V (235 V/cm) between the reservoirs placed at the end of the separation channel. The detection point was at 1 cm from the anode, so that the effective separation
length was 7 cm. The apparatus was under the full control of a PC equipped with an acquisition board and Lab View software (National Instruments, Austin, TX, USA). A controlled double 5 kV generator HV (Bertan) provided the electrophoretic voltages. A controlled relay board connected the chip electrodes to the proper power line or to ground, as required in the operation sequence.

3 Results and discussion

The aim of this work was to assess the performance of low-concentrated polymer solutions in the separation of double-stranded DNA fragments by microchip electrophoresis. The separation of a 123 bp ladder was carried out in a microchip, using a set of polyacrylamides obtained by different synthetic approaches whose characteristics are reported in Table 1. Suppression of EOF and analyte wall interaction was obtained by means of a dynamic coating made by a copolymer of dimethylacrylamide and allyl glycidyl ether (EPDMA), recently introduced for capillary channels. Polymers LPA1 and LPA2 were synthesized via a conventional polymerization procedure, whereas the polymers LPA3 and LPA4 were obtained by PIP, a method introduced by Osada and co-workers [15]. The so-called “plasma-induced” polymerization process is, essentially, a nonconventional molecular polymerization in which an ampoule, connected to a vacuum line, is placed between external electrodes. Plasma is generated by operating an electrical discharge for 30 s up to several minutes after evacuation of the reactor. The active polymerizing species generated in the plasma come into contact with the surface of the monomer phase, diffuses into it and initiates polymerization. Only the initiation reaction takes place in the gas-phase, whereas growth and termination reactions take place in the monomer solution. The polymer produced by PIP are characterized by an “ordered” structure and by the following properties: ultrahigh molecular weights, very low polydispersity, and absence of branches and cross-links. Recently, we have reported on the advantages of these polymers as DNA sieving matrices in capillary electrophoresis [16]. The $M_w$ of LPA3 and LPA4 could not be determined by GPC-MALLS being out of the range of applicability of this technique. The only information provided by GPC-MALLS is that the $M_w$ is higher than 10 MDa. In GPC, the polymer sample flows under pressure through a column filled with a porous gel packing. However, polymers with a gyration radius larger than the pore radius are completely excluded from the pores of the packing and not fractionated. As a consequence, polymers that are excessively large in coil radius, to be properly fractionated by GPC columns, are not accurately analyzed by tandem GPC-MALLS [17]. Therefore, these polymers were characterized by intrinsic viscosity. This constant is related to the molecular weight of the polymers through the Mark-Houwink-Sakurada equation:

$$[\eta] = KM_w^a$$

where $[\eta]$ is the intrinsic viscosity, $K$ and $a$ are empirical constants that are specific for a given polymer, solvent and temperature, and $M_w$ is the viscosity average molar mass. The Mark-Houwink-Sakurada equation is a widely used method to estimate the average molar mass of polymer samples, but is restricted to the analysis of polymer samples for which Mark-Houwink-Sakurada constants are tabulated or have been measured in the same solvent [8]; unfortunately, for ultrahigh-molecular-weight polyacrylamides $K$ and $a$ are unknown [18]. Although intrinsic viscosity shows that the PIP polymer has a higher $M_w$ than compared to the polymers obtained by conventional polymerization protocols, it was not possible to calculate their $M_w$ from the Mark-Houwink-Sakurada equation.

The different polymers under investigation were also characterized by measuring their dynamic viscosity in a capillary. The values of dynamic viscosity of each polyacrylamide solution are reported in Fig. 2. The dynamic viscosity of a HEC solution is also reported as a reference. The values were measured by replacing the solution in the capillary with a fluorescent solution of the same polymer contained in an external vial to which pressure is applied. Using a Beckman capillary unit and software developed by Beckman for that purpose, it was possible to compare on a relative scale the dynamic viscosity of the polymer solutions used as sieving matrices in a microchip. The experiment provides information on the time required by the different solutions to fill the microchannel under identical operative conditions. It is well known that a solution of polyacrylamide behaves as a non-Newtonian (shear-thinning) fluid [19]: its viscosity decreases as the applied shear stress increases. We found that the dynamic viscosity of solutions of high $M_w$ polyacrylamides (LPA2, LPA3, LPA4) at 0.2% w/v concentration is lower than that of 1% w/v LPA1 and 1.5% w/v HEC. When the injection pressure was increased, the viscosity of the high-molecular-mass polymers decreased more steeply than that of shorter chain polymers. This shear-thinning behavior is a

<table>
<thead>
<tr>
<th>Polymerization process</th>
<th>Intrinsic viscosity (dL/g) at 30°C</th>
<th>$M_w$ (MDa)</th>
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<tbody>
<tr>
<td>LPA 1 Solution</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td>LPA 2 Solution</td>
<td>10.5</td>
<td>7.0</td>
</tr>
<tr>
<td>LPA 3 Plasma</td>
<td>15.5</td>
<td>$&gt;10$</td>
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<tr>
<td>LPA 4 Plasma</td>
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suitable characteristic of a polymer for microchip electrophoresis. Filling the microchannel with diluted, high-molecular mass polyacrylamides requires a significantly shorter time than filling with more concentrated polymers.

Concerning the electrophoretic performance of the polymers under investigation, our experiments confirm the general knowledge that low-concentrated solutions of long-chain polymers provide good resolution of large DNA fragments. Figure 3 shows the separation of the 123 bp ladder obtained with (a) LPA2, (b) LPA3, and (c) LPA4 used at 0.2% w/v concentration. Seven fragments are baseline-resolved using LPA2, whereas LPA3 and LPA4 provide a baseline resolution of 8 fragments. In all three cases, the run time is less than 80 s. These three polymers possess a high $M_w$ and their sieving performance is strictly correlated to intrinsic viscosity ($\eta$).

Constraint release and active network deformations occur in polymer solutions and the intensity of these phenomena depends on the stability of the polymer network which is directly related to the length of the polymer chains. A decrease in network stability, which is generally observed with low-molecular-mass polymers, leads to a poor sieving performance and to low separation efficiency as a result of a larger effective pore size [7]. If the size of the polymer is not appropriate, the increase of polymer concentration is not a good strategy for separating large DNA fragments as demonstrated by the separations of a 123 bp ladder in (a) 1% w/v and in (b) 0.2% w/v LPA1, reported in Fig. 4. When the polymer is used at a concentration of 1% w/v, a baseline separation of seven fragments (123–861 bp) of the ladder is obtained in 120 s. By reducing the concentration of this polymer to 0.2% w/v, only three fragments (123–369 bp) were separated at the baseline. A complete loss of resolution was observed for the largest fragments.

A plot of resolution per base is reported in Fig. 5 which summarizes the sieving performances obtained with the four polymer samples. "Resolution per base pair", $R_{bp}$, was measured according to the following equation [20]:

$$R_{bp} = \frac{\Delta X}{\Delta N W_W}$$

where $W_W$ is the peak width in mm at the maximum half height, $\Delta X$ or “peak spacing” is the distance in mm between two contiguous peaks, and $\Delta N$ is the molecular size increment of the two corresponding fragments. LPA1 shows a superior resolution from 123 bp to 615 bp fragments when used at 1% w/v, but no resolution is observed for DNA fragments larger than 984 bp. Only the three high-$M_w$ polymers (LPA2, LPA3, LPA4) are able to resolve fragments larger than 984 bp with only the PIP polymers LPA3 and LPA4 being able to separate fragments up to 1353 bp. Increasing LPA1 concentration
from 0.2% to 1% w/v did not prove to be an effective strategy to increase the separation of large DNA fragments that could only be resolved by polymers of higher $M_w$.

In order to assess how selectivity and efficiency influence the resolution provided by the various polymers, peak spacing and width were separately investigated. Figure 6 reports peak spacing versus DNA size obtained with the different polymers. The higher-molecular-mass polymers provided a higher selectivity in the electrophoresis of large fragments due to the rigidity of the network formed by their chains.

The peak width obtained in the separation of the 123 bp ladder using the four polymers tested at a concentration of 0.2% w/v is reported in Fig. 7. LPA1, the polymer with the lowest molecular mass, provides an efficient separation of short DNA fragments, whereas the efficiency decreases for large fragments. LPA2 provides a constant efficiency for the whole range of sizes, whereas LPA3 and LPA4, the two PIP polymers, provided the best efficiency for the largest DNA fragments. Dilute, high and ultrahigh $M_w$ matrices are useful for separating both, small and large DNA fragments, as they provide a moderate, yet constant resolution in a wide range of fragment sizes. This feature is useful in restriction fragment mapping and DNA genotyping. The possibility of using dilute polymer solutions is of great interest in microchip electrophoresis, where the viscosity of the polymer solution complicates the analytical procedure. This approach represents a valid isocratic alternative to the gradient of linear polymer matrices which was recently proposed by Zhang et al. [21] and overcome the band compression phenomenon observed in the separation of large DNA fragments, which is obtained with the Agilent 2100 Bioanalyzer kit by Nachamkin et al. [22].

4 Concluding remarks

In this work, dilute solutions of high-molecular mass polyacrylamides have been investigated for their use in the separation of double-stranded DNA by microchip electrophoresis. They revealed a moderate, yet constant value of resolution in a broad interval of DNA fragment sizes (123–1353 bp). This work has proven the usefulness of these polymers in the common genotyping applications demonstrating that microchip electrophoresis could potentially replace conventional gel electrophoresis DNA fingerprinting. The low concentration at which these polymers are used facilitates flushing and filling the channel with the polymer matrix, thus enhancing the general throughput of microdevices.

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5 References