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Molar Mass Profiling of Synthetic Polymers by Free-Solution Capillary Electrophoresis of DNA−Polymer Conjugates

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The molar mass distribution of a polymer sample is a critical determinant of its material properties and is generally analyzed by gel permeation chromatography or more recently, by MALDI-TOF mass spectrometry. We describe here a novel method for the determination of the degree of polymerization of polydisperse, uncharged, water-soluble polymers (e.g., poly(ethylene glycol) (PEG)), based upon single-monomer resolution of DNA−polymer conjugates by free-solution capillary electrophoresis. This is accomplished by end-on covalent conjugation of a polydisperse, uncharged polymer sample (PEG) to a monodisperse, fluorescently labeled DNA oligomer, followed by electrophoretic analysis. The monodisperse, charged DNA "engine" confers to each conjugate an equal amount of electromotive force, while the varying contour lengths of the uncharged, polydisperse polymers engender different amounts of hydrodynamic drag. The balance of electromotive and hydrodynamic forces enables rapid, high-resolution separation of the DNA−polymer conjugates as a function of the size of the uncharged PEG tail. This provides a profile of the molar mass distribution of the original polymer sample that can be detected by laser-induced fluorescence through excitation of the dye-labeled DNA. We call this method *free solution conjugate electrophoresis* (FSCE). Theory-based analysis of the resulting electrophoresis data allows precise calculation of the degree of polymerization of the PEG portion of each conjugate molecule. Knowledge of the molecular mass of the uncharged polymer's repeat unit allows for direct calculation of the molar mass averages as well as sample polydispersity index. The results of these analyses are strikingly reminiscent of MALDI-TOF spectra taken of the same PEG samples. PEG samples of 3.4-, 5-, and 20-kDa nominal average molar mass were analyzed by FSCE and MALDI-TOF; the values of the molar mass averages, M_w and M_n, typically agree to within 5%. Measurements and molar mass calculations are performed without any internal standards or calibration. Moreover, when DNA−polymer conjugate analysis is performed in a chip-based electrophoresis system, separation is complete in less than 13 min. FSCE offers an alternative to MALDI-TOF for the characterization of uncharged, water-soluble polymers that can be uniquely conjugated to DNA.

The determination of polymer molar mass distribution is a critical step in evaluating the outcome of a polymerization reaction and in predicting the material properties of a given polymer sample. Common methods for profiling the mass distribution of synthetic polymers include gel permeation and ion-exchange chromatographies, as well as time-of-flight mass spectrometry. In all molar mass characterization techniques, a polydisperse polymer sample is first fractionated according to size, with varying degrees of resolution. The resulting fractions are then analyzed to determine molar mass as well as concentration or amount. By far, the most common technique used to characterize the molar mass distributions of synthetic polymers is gel permeation chromatography (GPC).[1] GPC involves fractionation of a polymer sample according to differences in coil radius, followed by comparison of component elution times to those of a well-characterized polymer standard. Then, using appropriate algorithms, the resultant chromatogram is deconvoluted and molar mass distributions estimated.

More recently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has emerged as a powerful tool to characterize mass distributions of synthetic[2] and natural biological polymers.[3] MALDI-TOF mass spectrometric techniques allow for direct measurement of molar mass for each species present in a given polydisperse polymer sample and, hence, for direct calculation of molar mass distribution. This technique offers the advantage that, although it requires calibration, it relies less heavily on comparison to polymer standards and can provide higher resolution separation than chromatographic techniques for many samples.

There are drawbacks to both chromatographic and mass spectrometric approaches to polymer characterization. GPC

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provides information on molar mass averages and polydispersity through comparison to standards. For rare or specialty polymers and copolymers, it can be difficult to locate the appropriate standards that are necessary to obtain the most accurate results with GPC methods. Additionally, GPC instruments usually employ relatively insensitive modes of detection (e.g., refractive index gradients or UV/visible absorbance) and hence require relatively large amounts of sample. A typical GPC analysis requires on the order of 1 h to perform. MALDI-TOF, in contrast, is exquisitely sensitive, requires only picomoles of sample, and can be performed in under 10 min. It provides extremely detailed information about a polymer sample, often giving single-monomer resolution of samples up to ~20 kDa in molar mass.[4] However, it suffers from the drawbacks that it requires a relatively pure sample, and the sample must be of a chemistry that will "fly" well in the TOF tube.[5] Additionally, the sample must be appropriately prepared with a given matrix and the characteristics that make a particular matrix optimal for a given sample remain poorly understood.[5] Finally, MALDI-TOF mass spectrometry currently does not produce accurate analysis of synthetic polymers of wide polydispersity (e.g., polydispersity of >1.2).[6]

Capillary electrophoresis (CE) is quickly becoming established as a powerful tool for the analysis of a variety of analytes, including chiral molecules,[7] proteins,[8] carbohydrates,[9] and DNA.[10−12] This technique, in its most basic terms, separates charged molecules on the basis of differences in the velocities at which they migrate in an electric field. Migration is quantitated in terms of electrophoretic mobility, μ, which is given by the ratio of electrophoretic velocity (cm/s) to electric field strength (V/cm). For a given molecule, one can also express the electrophoretic mobility as the ratio of the effective charge to the effective friction:

$$\mu = V/E = q/f \qquad (1)$$

where V is the steady-state velocity of the analyte, E is the electric field strength, q is the effective charge, and f is the effective friction coefficient. Thus, for separation to occur via electrophoresis, the charge-to-friction ratios or electrophoretic mobilities (μ) of the analytes must differ from one another.

For highly and uniformly charged polymers such as DNA or polystyrenesulfonates in most aqueous buffers, the charge-to-friction ratio of the polymer is essentially constant for any chain length. Thus, for these polymers, which are known as free-draining coils, high-resolution electrophoretic separation according to polymer chain length in free solution is not possible beyond a given, relatively short chain length.[12−14] In many applications, including polymer synthesis product profiling and DNA sequencing, it is critical to be able to separate molecules with high resolution based on polymer chain length. Size-dependent electrophoretic separation of free-draining coils may be achieved by performing electrophoresis in a polymeric sieving matrix or a cross-linked hydrogel.[12] The presence of a polymer network serves to increase the effective molecular friction coefficient of the analyte polymer and, more importantly, to change the dependence of electrophoretic mobility on chain length, allowing for separation.

However, previous reports have shown that through the attachment of a monodisperse, uncharged, frictional entity to one or both termini of DNA polymers, DNA *can* be separated by free-solution electrophoresis.[15−17] The functional premise of this method, which is known as end-labeled free-solution electrophoresis (ELFSE), is the alteration of the charge-to-friction ratio of the charged molecules through the conjugation of a monodisperse, uncharged "drag" macromolecule to charged DNA polymers of varying lengths. The uncharged "drag" macromolecule increases the overall friction coefficient of each conjugate polymer without increasing its electric charge. This addition of a fixed amount of drag to each conjugate renders the electrophoretic mobility of the composite polymer a function of the variable DNA chain length in free solution, allowing DNA size-based separation without the use of a polymeric sieving matrix. These studies have demonstrated that a "ladder" of DNA fragments differing in length by single bases can be separated with single-monomer resolution, up to a maximum chain length of ~120 bases.[17] These results indicate that ELFSE may be developed as a fast and effective method of DNA sequencing, which can be performed in microchannels in the absence of a polymeric sieving matrix.

Here, we present a method for the analysis of uncharged, polydisperse, water-soluble polymers that is similar in principle to ELFSE. We call this mode of separation *free-solution conjugate electrophoresis* (FSCE). Consider the following metaphor: Suppose that the charged polymer (e.g., DNA) is thought of as an "electrophoretic engine" and the uncharged drag polymer (e.g., poly(ethylene glycol), PEG) to which the charged polymer is conjugated as an "electrophoretic parachute". In FSCE, the DNA "engines" are monodisperse and hence of identical effective charge and, therefore, provide the same magnitude of electromotive force to each DNA−polymer conjugate during electrophoresis. However, each of the uncharged polymer "parachutes" is of variable contour length and thus exhibits a different amount of molecular friction or drag force. This creates a family of conjugates that each comprise the same electromotive force, yet have an amount of hydrodynamic drag force that is proportional to the contour length of the particular PEG parachute to which they are attached.[18] Thus, each conjugate exhibits a unique electrophoretic mobility during electrophoresis, enabling separation of the conjugates according to the size of the uncharged "parachute" polymer. ELFSE operates in the converse manner; identical parachutes are used to separate variably sized DNA "engines".

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Specifically, FSCE separations are accomplished by covalently attaching a monodisperse, fluorescently labeled DNA oligomer to one of the chain termini of each polymer in a polydisperse, uncharged polymer sample, such as PEG. While PEG in its underivatized form will not migrate by electrophoresis and can only be detected in aqueous solution by a sensitive refractometer (and then only at relatively high concentrations), conjugation to a fluorescently labeled DNA oligomer imparts PEG polymers with electrophoretic mobility as well as a label for sensitive detection via laser-induced fluorescence (LIF) at picomolar sample concentrations.

The separation of uncharged, polydisperse PEG samples by CE has been previously demonstrated by Bullock[19] through derivatization of the PEG sample with a UV-absorbing chromophore that additionally serves as a charged "engine" for free-solution separation. This technique was successfully applied for the separation of PEG oligomers with an average molar mass of up to ~3000 Da. However, in this study, a single, divalent, charged moiety was used to impart the uncharged PEGs with electric charge, an approach that limits the polymer molar mass range that can be analyzed because of the small overall charge of the engine. Here, we show that the use of a uniformly and highly charged oligomer, such as DNA, as the engine allows the FSCE method to be flexibly tuned to study polymers in various size ranges, although FSCE with any engine will have limits to the molar mass range that can be accurately analyzed.

In an approach similar to that of Bullock, Sudor and Novotny demonstrated free-solution CE separations of negatively charged oligosaccharides, derived from a partially hydrolyzed κ-carrageenan, through the covalent attachment of either a negatively or positively charged fluorescent label.[20] This conjugation simultaneously changed the charge-to-friction ratio of the oligosaccharide analytes and allowed for sensitive LIF detection.

In this report, we demonstrate use of the FSCE technique for high-resolution separation and effective mass spectrometry of PEG polymers of up to 20 kDa in molar mass. We show the effects of DNA engine chain length on the resolution and efficiency of the CE separation of PEGs of various average molar masses. Additionally, we present a mathematical model that enables simple and straightforward calculation of the degree of polymerization for the PEG component of each of the DNA−PEG conjugates. This allows for accurate and direct calculation of important polymer sample characteristics such as M_w, M_n, and polydispersity index (PDI), given the molar mass of the drag polymer's constituent monomer. The analysis of PEG with a nominal molar mass of 5 kDa can be performed in under 40 min in a capillary-based electrophoresis system and in under 13 min in a chip-based electrophoresis system. FSCE allows for complete polymer molar mass characterization with an extremely small amount of sample (<1 nmol) and without the need for external standards or calibration, as are required in both GPC and MALDI-TOF techniques. FSCE, here demonstrated for PEG samples, should also be applicable to a number of other important, water-soluble, uncharged polymers including oligosaccharides, carbohydrates, acrylamides, celluloses, and poly(vinyl alcohol)s, so long as appropriate conjugation chemistry between the uncharged drag polymers and the charged DNA engine can be devised. Additionally, it may be possible to separate polymers according to chain architecture or configuration as well as size using FSCE techniques. While GPC and MALDI-TOF will likely remain the most common methods of polymer molar mass characterization due to their applicability to a number of different polymer chemistries, we believe that FSCE can provide a method for high-resolution characterization of important specialty polymers where accurate molar mass characterization is crucial to the polymers' application.

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THEORY

The analytes in this study consist of fluorescently labeled single-stranded DNA (ssDNA) oligomers attached to polydisperse PEG polymers. We will refer to the ssDNA as the "engine" and to the PEG polymers as the "drag" entities. Long et al.[18] described a methodology for calculation of the electrophoretic mobility of a Gaussian polyampholyte chain of an unusual class, composed of different monomers having equal sizes but different electric charges and, hence, different electrophoretic mobilities. These polyampholytes could also be modeled as composite polymers with two different sections each with different electrophoretic mobilities. The electrophoretic mobility of the composite object is essentially an average of the mobility that each monomer would have in solution, provided that the *effective* monomer size is larger than the Debye length that characterizes the solution. Of course, we cannot assume that the engine and drag monomers have the same size here. To apply the equations of Long et al., it is thus necessary to define a unique effective hydrodynamic monomer, of size b_{eff}, that will apply to *both* polymers. We will call these effective monomers "blobs". There are many equivalent ways to construct these blobs. For example, a simple method is to use the hydrodynamic radius of the ssDNA oligomer as a measure of the size of the blobs. A detailed theoretical study of this problem will be published elsewhere. Here, we will simply define the blobs such that they contain α_{engine} ssDNA monomers and α_{drag} PEG monomers. Note that $\alpha_{engine} \neq \alpha_{drag}$ since the two polymers have different monomers and different degrees of polymer chain stiffness (i.e., different persistence lengths). The conjugate is thus made of $m_{drag} = M_{drag}/\alpha_{drag}$ drag blobs and $m_{engine} = M_{engine}/\alpha_{engine}$ engine blobs, where M_{drag} and M_{engine} are the number of monomers in the drag and engine sections of the polyampholyte, respectively. In other words, it is made of $m_{drag} + m_{engine}$ blobs of size b_{eff}.

Consider first the general case where the drag polymer has an arbitrary charge. Since all the blobs have the same hydrodynamic radius b_{eff} (hence the same hydrodynamic friction coefficient), the equation for the mobility, μ, of the conjugate is given by the average value

$$\mu = \frac{m_{engine}\mu_{engine} + m_{drag}\mu_{drag}}{m_{engine} + m_{drag}} \qquad (2)$$

where μ_{drag} and μ_{engine} are the free-solution mobilities of the two types of polymers. We now go back to the real molecular parameters. Equation 2 can be rewritten as

$$\mu = \mu_{engine}\frac{M_{engine} + \beta M_{drag}\alpha}{M_{engine} + M_{drag}\alpha} \qquad (3)$$

Here, $\beta = \mu_{drag}/\mu_{engine}$ is the mobility ratio while $\alpha = \alpha_{engine}/\alpha_{drag}$ is a measure of the (hydrodynamic) size asymmetry between the two types of monomers. These two parameters have fixed values when working with two specific chemical components. This equation is general and can be used to understand the results obtained with different types of conjugates. In the case of an uncharged and flexible polymer such as PEG for which $\beta = 0$, it reduces to

$$\mu = \mu_{engine}\frac{M_{engine}}{M_{engine} + \alpha'} \quad (4)$$

where $\alpha' = M_{drag}\alpha$ is the effective friction added by the uncharged polymer of molecular weight M_{drag}. It is interesting to note that the friction generated by the neutral drag polymer is in fact proportional to its chain length ($\sim M_{drag}$) and not to its radius of gyration. The conjugation of "engine" and "drag" polymers no longer allows one to simply sum up the individual friction coefficients of both the "drag" and "engine" coefficients in a linear fashion; rather, the two entities form a single nonhomogeneous hydrodynamic unit that must be studied in terms of the hydrodynamic "blobs" that we have defined here. Therefore, we can interpret α as the effective friction coefficient per drag monomer.

Equation 4 can be used to analyze experimental data in order to obtain the degree of polymerization M_{drag} of the drag labels (we will see that eq 4 provides an excellent fit of the data). Since FSCE allows for single-monomer resolution of the DNA−polymer conjugates, a plot of $\alpha'(N) = M_{engine}[\mu_{engine}/\mu(N) - 1]$ vs peak number N should result in a straight line with a slope α; note that μ_{engine} is simply the mobility of the unlabeled DNA oligomer of size M_{engine} used in the experiment. Using the value of α, one can then calculate the degree of polymerization $M_{drag} = \alpha'/\alpha$ that corresponds to each peak. Alternatively, the α value, or the effective friction coefficient of one drag monomer, can be calculated as

$$\alpha = \frac{\alpha'_{N+j} - \alpha'_N}{j} \quad (5)$$

where α'_{N+j} and α'_N are the α' values of the $(N+j)$th and Nth peaks, respectively.

As each DNA−PEG conjugate has a single fluorophore bound to one terminus, the area of a given capillary electrophoresis peak can be used to determine the relative concentration of a particular DNA−polymer conjugate in the original sample. If pressure injection is employed, the peak area may be used directly to determine concentration. If, on the other hand, electrokinetic injections are used to introduce the sample into the capillary, the peak area must be corrected to account for injection-induced biases, which increase the concentration of analytes with higher mobilities introduced into the capillary.[21] With both molecular weight and relative concentration data for each species in the original mixture, the full molecular weight distributions, M_w, M_n, and the polydispersity index can be calculated easily and accurately.

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EXPERIMENTAL SECTION

Capillary Electrophoresis Analysis Conditions. All capillary electrophoretic separations were conducted in 25-μm-inner diameter fused-silica capillaries 100 cm in total length (95.6 cm effective length) (Polymicro Technologies, Phoenix AZ) filled with 1× TAPS (100 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) buffer, pH 8.5 (Amresco, Solon, OH). All buffers were prepared with 18 MΩ water (E-pure, Barnstead, Dubuque, IA). The internal surface of the capillary was dynamically coated with an adsorbed layer of a poly(dimethylacrylamide-*co*-allyl glycidyl ether) (epoxy-pDMA) to reduce electroosmotic flow mobility to negligible levels.[22] All analyses were performed with a BioFocus 3000 capillary electrophoresis system equipped with an LIF detector (BioRad, Hercules, CA). At the beginning of each day of analysis, the capillary was flushed for 15 min with 1 M NaOH, followed by a 5-min rinse with water, and then was flushed for 20 min with a 0.1% (w/v) solution of epoxy-pDMA in water. All rinses were done with a syringe pump, at a flow rate of 100 μL/h. Electroosmotic flow after capillary coating by this method was measured to be 3.0×10^{-6} cm^2/(V·s) by the method of Williams and Vigh.[23] Prior to each analysis, the capillary was flushed with several column volumes of the electrophoresis buffer. Immediately prior to sample injection, the injection end of the capillary was briefly dipped into pure water to remove any residual buffer salts on the outer surface of the capillary. The sample was introduced into the capillary by either an electrokinetic injection at 150 V/cm for 2 s or by pressure injection for 138 kPa·s. Electrophoresis was conducted at 300 V/cm until all peaks had eluted. Detection of the analytes was achieved with excitation of the 3′-fluorescein DNA label using the 488-nm line of an argon ion laser, and emission was detected at 521 nm.

Chip Electrophoresis Analysis Conditions. Chip-based separations were conducted using a proprietary breadboard electrophoresis system[24] (ACLARA BioSciences, Mountain View, CA). The channel was filled with 1× TTE buffer (100 mM Tris, 100 mM TAPS, 2 mM EDTA) with 0.3% (w/v) POP6 polymer (Applied Biosystems, Foster City, CA) as a dynamic channel coating to suppress electroosmotic flow. The sample was diluted to a concentration of 34 nM in electrophoresis buffer and added to the sample reservoir on the electrophoresis chip. The electrophoresis chips were manufactured of glass with a separation channel 18 cm in length. A confocal LIF detection system with a 488-nm excitation laser and a 520-nm emission detection system detected the analytes after they had migrated 18 cm through the electrophoresis channel. Samples were introduced into a 50-μm, offset "T" injection channel by applying a "pull-through" voltage of 125 V/cm for 1.5 min between the sample reservoir and the opposite buffer reservoir. Immediately following injection, the analytes were separated by applying a voltage of 240 V/cm across the 18-cm separation channel. Application of a "pull-back" voltage of 100 V/cm on the sample reservoir and its opposing buffer reservoir prevented the sample from migrating into the separation channel during electrophoretic separation. These separation voltages were selected because they were the maximum attainable with the power source available in the breadboard system. The separation voltage was applied until all peaks had eluted.

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MALDI-TOF Measurements. MALDI-TOF spectra were produced by the University of Illlinois at Urbana−Champaign mass spectrometry laboratory. The protocol was the same as described by other groups for the analysis of PEG polymers,[25] using α-cyanohydroxycinnamic acid as the matrix. Spectra were obtained on a PerSeptive Biosystems Voyager-DE STR instrument.

Sample Preparation. Bismaleimidopoly(ethylene glycol), M_w = 3400 and monomaleimidopoly(ethylene glycol), M_w = 5000 and 20 000 (Shearwater Polymers, Huntsville, AL), were each conjugated to poly(thymidine)deoxyribonucleic acid, 20 and 35 bases in length (Oligos, Etc., Wilsonville, OR). DNA samples were purified using gel electrophoresis by the vendor, to ensure that each DNA oligomer was absolutely monodisperse. Each DNA oligomer included a fluorescein dye on its 3′ terminus to allow for LIF detection and a thiol (SH) functionality on its 5′ terminus to allow for conjugation with the maleimide functionality of the PEG, forming a stable thioester linkage between the DNA and the PEG.

To ensure that the 5′-thiol of the DNA was optimally reactive with the maleimide terminus of the PEG, the DNA was first chemically reduced using the following protocol. A total of 12.8 nmol of the oligonucleotide was dissolved in 30 μL of 1× triethylammonium acetate (TEAA) buffer and 4.33 μL of 1 M $AgNO_3$ and incubated at 25 °C for 30 min. Next, 5.78 μL of 1 M dithiothreitol (DTT) was added and allowed to react with the DNA for 5 min. The sample was then centrifuged to remove the Ag−DTT precipitate; the liquid phase was aspirated and placed in a separate tube. The Ag−DTT precipitate was washed twice with 30 μL of TEAA buffer and centrifuged, and all three liquid phases were combined. The liquid containing the DNA was then gel-filtered on Centri-Sep columns (Princeton Separations, Princeton, NJ) according to the manufacturer's directions to remove any buffer salts and residual DTT. The eluents from these columns were combined, split into five equal aliquots, immediately frozen in liquid nitrogen, and lyophilized.

The DNA−PEG conjugation reaction was accomplished by dissolving a given PEG sample to a concentration of 12.8 mM in 0.1 M sodium phosphate buffer, 0.15 M NaCl, pH 7.2, and adding 10 μL of each of the PEG solutions to the lyophilized DNA powder from the above reduction protocol. This solution was incubated at room temperature for 24 h. Finally, the resulting mixture was diluted 1:300 in distilled, deionized H_2O immediately prior to injection. The reaction is outlined in Scheme 1.

Data Analysis. All peak parameters and migration times were determined with the PeakFit software package (SPPS, Inc., Chicago, IL).

RESULTS AND DISCUSSION

In this study, the FSCE separation mode afforded single-monomer resolution of DNA−PEG conjugates made with PEG samples of nominal average molar masses of 3.4, 5, and 20 kDa using electrophoretic DNA engines of 20 and 35 bases. In all cases, a Gaussian set of highly resolved peaks representing the distribution of PEG molar masses was produced by the electrophoretic analysis (e.g., Figure 1). Analysis of these electropherograms allows for precise and facile calculation of various properties of the size distribution (e.g., M_w and M_n) of the PEG species present in a given sample. Details follow.

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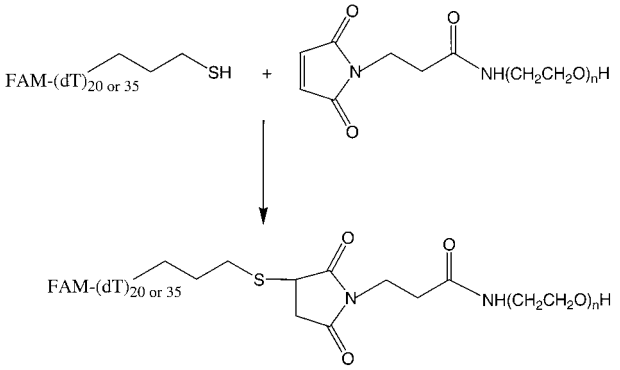
Scheme 1. Reaction of a Maleimide-Terminated PEG Polymer with a 5′-Thiol-Terminated ssDNA Oligomer



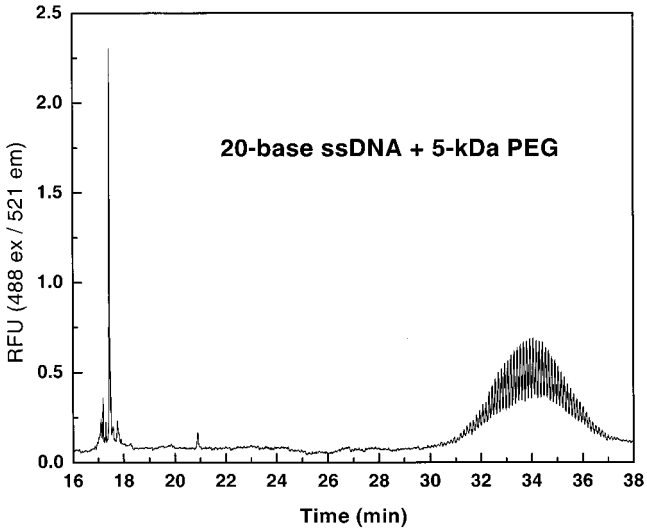


Figure 1. FSCE analysis of the 20-base DNA + 5-kDa PEG conjugate. Conditions: buffer, 1× TAPS; detection, LIF with 488-nm excitation, 521-nm emission; field strength, 300 V/cm; current, 5.6 μA; capillary, 25-μm i.d., 100-cm total length (95.6 cm to detector). Pressure injection for 138 kPa·s.

Electropherograms. Figure 1 presents a representative FSCE electropherogram of a DNA−PEG conjugate. The electrophoretic analysis of the 20-base DNA + 5-kDa PEG conjugates results in the expected, residual, unconjugated DNA peaks eluting first at 17.5 min, followed by the elution of a number of DNA−polymer conjugate peaks representing the oligomeric distribution of the PEG polymer sample between 30 and 37 min. Unconjugated DNA peaks were identified by spiking the sample with free DNA and observing which peaks displayed an increased signal in the resulting electropherogram. Each electropherogram shows strong DNA−polymer conjugate peaks representing the major species of PEG present in the original polymer synthesis mixture. These peaks elute with the expected Gaussian profile, representing the Gaussian distribution of the molar mass of the polydisperse PEG sample. The initial "free-DNA" peak in the electropherogram is used to calculate the free-solution mobility of unlabeled DNA, and hence is used as an "internal standard" in each electropherogram, allowing for the facile calculation of numerical molar mass distributions explained in the Theory section.

The yield of each conjugation reaction was determined by integrating the area under all the peaks in the electropherogram

Table 1. Yields of the DNA−PEG Conjugation Reaction[a] and Elution Time Reproducibility

conjugate	yield (%)	RSD of FSCE elution time (%)
20-base DNA + 3.4-kDa PEG	91	0.79
20-base DNA + 5-kDa PEG	78	0.45
20-base DNA + 20-kDa PEG	93	0.81
35-base DNA + 3.4-kDa PEG	92	0.60
35-base DNA + 5-kDa PEG	88	0.29
35-base DNA + 20-kDa PEG	78	0.36

[a] RSD refers to the relative standard deviation of the migration time for unconjugated DNA during three consecutive analyses.

and dividing the area of the peaks attributed to the DNA−PEG conjugates by the entire area of the electropherogram. The yields are shown in Table 1. Each of the conjugation reactions resulted in at least 78% of the DNA oligomer being conjugated with PEG polymers.

Electrophoretic analysis of each of the DNA−PEG conjugate samples was performed in triplicate. The relative standard deviation (RSD) in the electrophoretic mobility of the unconjugated DNA peak (residual free DNA without PEG attached) in each of the analyses is presented in Table 1. The initial DNA peak is observed in most electropherograms as a number of very closely spaced peaks. Of these multiple peaks, one peak, corresponding to a single ssDNA oligomer, is ∼10 times larger than the other three peaks. The multiplicity of free DNA species can be explained with two observations. First, the fluorescein dye that is covalently bound to the 3′ terminus of the DNA oligomers is present as two different isomers. Previous studies have shown that binding of small molecules to the termini of DNA can change the free-solution mobility of the DNA;[15] thus, two differing fluorescein isomers can lead to two, slightly offset peaks in free-solution electrophoresis. Second, the free-solution mobility of ssDNA fragments increases with increasing DNA lengths, up to ∼50 bases.[15] The DNA oligomers used in this study have thiolated termini that can oxidize to form disulfide dimers, creating two DNA species, one dimer and one monomer, each with two fluorescein isomers, thus accounting for at least four of the initial, unconjugated DNA peaks. Upon treatment of the sample with dithiothreitol, which is known to reduce disulfide bonds, the relative area of the peak corresponding to a disulfide-bridged DNA dimer to the monomer decreased (data not shown), in support of this hypothesis. In any case, the ssDNA monomer (the peak of interest) is by far the largest peak and is easily distinguished from minor species.

Molar Mass Calculations. To calculate the molar mass of the polymer using the theoretical arguments presented earlier in the paper, it is crucial that the friction coefficient, α', increases linearly with the number of monomers in the PEG portion of the conjugate. FSCE affords single-monomer resolution of the DNA−polymer conjugates, and thus, a plot of α' vs peak number, N, will yield a straight line with a slope of α. We point out that peak number, N, is merely a sequential integer counting of the peaks in their elution order and that the choice of which peak represents $N = 1$ is arbitrary and does not impact the calculation of the degree of polymerization of the drag polymer. A plot of α' vs N is presented in Figure 2 for the 20-base DNA + 5-kDa PEG conjugate and gives a straight-line fit with an $R^2 = 0.999$, a slope of 0.138, and an intercept of 14.69 (= 0.138 × 106.45). The intercept of

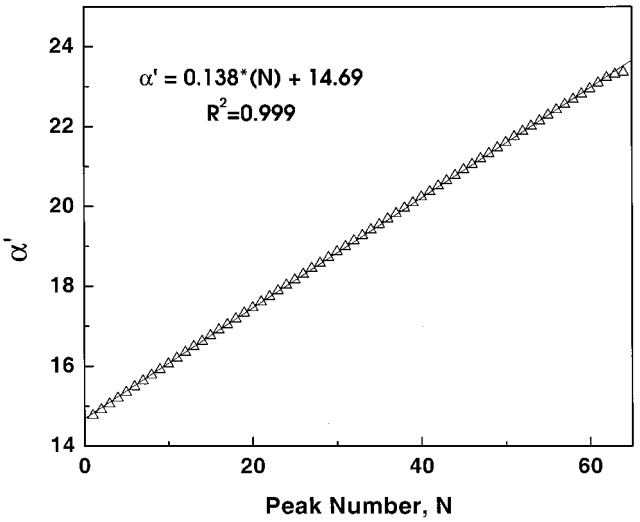


Figure 2. Plot of α' vs peak number for the FSCE analysis of a 20-base DNA + 5-kDa PEG conjugate, showing the linear scaling of α' with peak number.

0.138 × 106.45 shows that the peak we arbitrarily chose as $N = 1$ corresponds to a PEG drag polymer with 107 monomers. The high degree of linearity shows that α' does increase linearly with peak number, and because FSCE allows for single-monomer resolution, α' also scales linearly with number of monomers present in the PEG. This information, in addition to the theoretical discussion above, allows for calculation of the molar mass of a given peak in the 20-base DNA + 5-kDa PEG conjugate. An electropherogram for which the time axis has been transformed into a molar mass axis is presented in Figure 3a and is compared to the MALDI-TOF analysis of the same polymer sample in Figure 3b. As can be seen, FSCE results in a molar mass measurement that is in excellent agreement with the MALDI-TOF measurement. Many of the peaks in the MALDI-TOF spectrum appear as doublets that are separated by 23 atomic mass units, which are due to the presence of sodium adducts. The FSCE trace allows a much higher signal-to-noise ratio than is seen in a typical MALDI-TOF analysis. The M_w, M_n, and PDI of MALDI-TOF and FSCE analysis are in excellent agreement. Molar mass averages, as calculated for each of the conjugates used in this study, are presented in Table 2. Only FSCE peaks having areas greater than 2% of the largest peak in the analysis were included in molar mass calculations. We find that the molar mass calculation is insensitive to DNA oligomer length within the range used in this study and provides an accurate molar mass average calculation regardless of the size of the DNA oligomer used.

Chip-Based Separations. The 20-base DNA + 3.4-kDa PEG and 20-base DNA + 5-kDa PEG conjugates were also analyzed by chip electrophoresis. Chip-based electrophoretic separations offer the possibility of higher separation efficiencies and more rapid separations due to the narrower injection zones obtainable in these systems in comparison to capillary systems.[26] These narrow injection zones allow for the 3.4-kDa PEG to be separated with good resolution in a separation distance of only 18 cm, as seen in Figure 4. The decrease in distance required for separation allows the analysis to be complete in just over 8 min. Although the peaks are far from baseline-resolved, the use of a peak

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Table 2. Molecular Mass Estimates for the PEG Samples Studied in This Report, As Calculated According to the Equations Presented in the Theory Section[a]

	pressure injection				electrokinetic injection			
	M_w	M_n	PDI	% diff	M_w	M_n	PDI	% diff
3.4-kDa PEG analyzed with								
35-base DNA					4245	4159	1.02	9.3
20-base DNA	3970	3917	1.01	2.9	3860	3912	1.01	2.8
MALDI	3830	3805	1.01					
5-kDa PEG analyzed with								
35-base DNA	5839	5805	1.01	1.3				
20-base DNA	5919	5882	1.01	2.6	5775	5735	1.01	0.12
MALDI	5783	5728	1.01					
20-kDa PEG analyzed with								
20-base DNA	21212	21100	1.01	3.2	21400	21300	1.01	2.3
MALDI	21900	21800	1.01					

[a]% diff, the percentage difference for M_n between MALDI-TOF and FSCE methods. PDI, the polydispersity index. These estimates were obtained using pressure and electrokinetic injections.

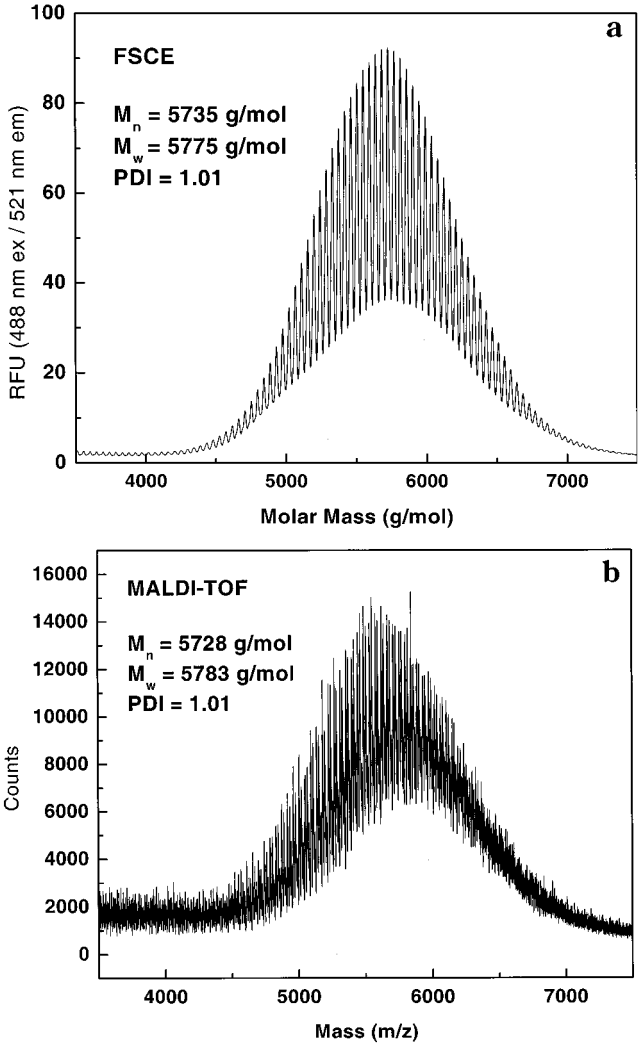

Figure 3. Comparison of FSCE (a) and MALDI-TOF (b) analyses of a 5-kDa PEG sample. The FSCE separation uses a 20-base ssDNA engine. FSCE analysis conditions are the same as in Figure 1.

extrapolation program, such as PeakFit, allows for deconvolution of the electropherogram into its constituent baseline-resolved peaks. After applying this deconvolution program, and using the

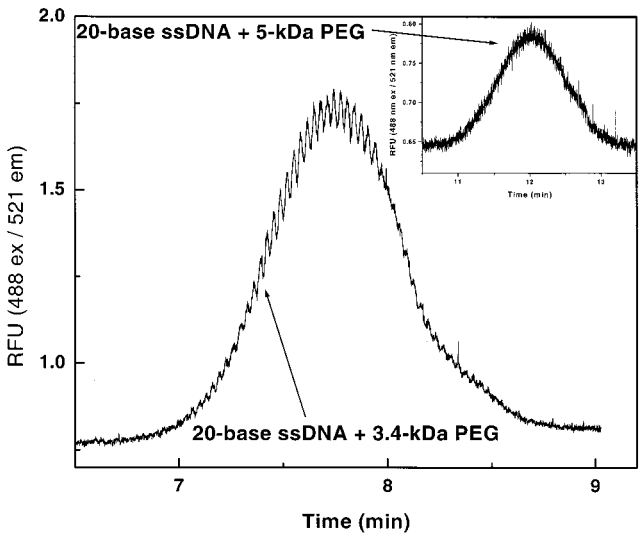

Figure 4. FSCE separation of 20-base DNA + 3.4-kDa PEG conjugate in chip electrophoresis device. Inset: 20-base DNA + 5-kDa PEG. Conditions: buffer, 1× TTE; detection, LIF with 488-nm excitation, 521-nm emission; field strength, 240 V/cm, current, 7 μA; channel, 18 cm total (18 cm to detector), trapezoidal cross section, 100 μm deep, 75 μm top width, and 50 μm bottom width.

relationships presented in the Theory section, the molar mass averages were calculated to be $M_w = 3976$ and $M_n = 3932$, giving a polydispersity index of 1.01. These measurements agree quite well with the MALDI-TOF measurements ($M_w = 3830$, $M_n = 3805$, and a polydispersity index of 1.01).

On the other hand, chip-based electrophoretic separation of the 20-base DNA + 5-kDa PEG conjugate resulted in one large Gaussian peak (inset Figure 4**)** that lacked the peak resolution seen for the 20-base DNA + 3.4-kDa PEG conjugate. Here we point out that this chip design is not optimized for FSCE; single-monomer resolution should be achievable on a chip with larger PEG polymer samples if chips are designed so that longer separation channels or narrower injection widths are employed. We are pursuing a detailed theoretical study of the importance of separation distance and injection width with respect to FSCE separation performance in microchannel electrophoresis.[29]

Effects of PEG Molar Mass. The analysis of higher molar mass polymer samples (>5 kDa in average molar mass) presents

challenges for both FSCE and MALDI-TOF. In Figure 5a, the electropherogram from the analysis of a 20-kDa nominal molar mass PEG pulled by a 20-base DNA engine is presented. While some degree of single-monomer resolution was achieved, the DNA−polymer conjugate peaks were very far from baseline-resolved. The 20-kDa PEG eluted as one large Gaussian peak with a sawtooth-like profile. However, the use of PeakFit allows one to deconvolute the electropherogram into its constituent peaks, the results of which are presented in Figure 5b. This deconvolution allows for relatively accurate estimation of the concentrations of each species in the electropherogram and, hence, direct calculation of molar mass averages. Alternatively, one can fit the entire Gaussian profile into one large single peak, without the need for peak deconvolution, and calculate the molar mass averages using mathematical techniques similar to those used in GPC analysis. Interestingly, the FSCE analysis of the 20-kDa PEG conjugate (Figure 5a) results in a significantly higher signal-to-noise ratio than the MALDI-TOF analysis of the 20-kDa PEG (Figure 5c). On the other hand, the molar masses of the FSCE analysis quantitatively agree with the MALDI-TOF analysis (Figure 5a and c).

Effects of DNA Chain Length. FSCE analysis was conducted with three different PEG samples that were each conjugated to 20- or 35-base DNA engines, depending upon the experiment. Compare the 20-base DNA + 5-kDa PEG analysis to the 35-base DNA + 5-kDa PEG analysis shown in panels a and b of Figure 6, respectively. The DNA−polymer conjugate peaks become more closely spaced and narrower as the DNA "engine" length is increased. As a result, the 20-base engine produces conjugate peaks that are more fully resolved, while the 35-base conjugate results in peaks that are more closely spaced and not as fully resolved. The average full width at half-maximum (fwhm) of the peaks in the analysis of the 20-base DNA + 5-kDa PEG is 5.0 s, while the 35-base DNA + 5-kDa PEG analysis produces peaks with a fwhm of 3.9 s. For a PEG sample of this size, the narrowing of peaks is not sufficient to offset the decrease in peak spacing. Hence, the analysis using the 35-base engine is complete in a shorter time period at the cost of reduced resolution. The 35-base conjugate still provides an equivalent amount of information about the polymer's molar mass distribution; however, the resolution is lessened and deconvolution of the peaks in the electropherogram is more challenging. This example illustrates the importance that the DNA oligomer engine be properly sized with respect to the PEG molar mass that is to be analyzed, if optimal resolution with FSCE is to be achieved. As the DNA oligomer length is increased for a given PEG size, there will be a point at which single-monomer resolution is completely lost, and the PEGs will elute as one, large, single, smooth Gaussian peak that will be difficult to accurately deconvolute. We believe that, for a given PEG sample, there will be an optimal DNA engine size for FSCE analysis. The determination of an optimal engine size, so that sufficient resolution can be achieved in an acceptable analysis time, is currently under investigation in our laboratories.[29]

Effects of Sample Injection Method. Table 3 presents the average plate efficiency in each FSCE analysis. The peaks in the electrokinetic (EK) injection analyses generally showed higher signal-to-noise ratio than those obtained by pressure injection. Previous reports have observed a phenomenon known as "sample stacking" with EK injections in CE.[27] This phenomenon occurs when samples enter into the capillary but do not migrate substantially into the lumen of the capillary due to differences in

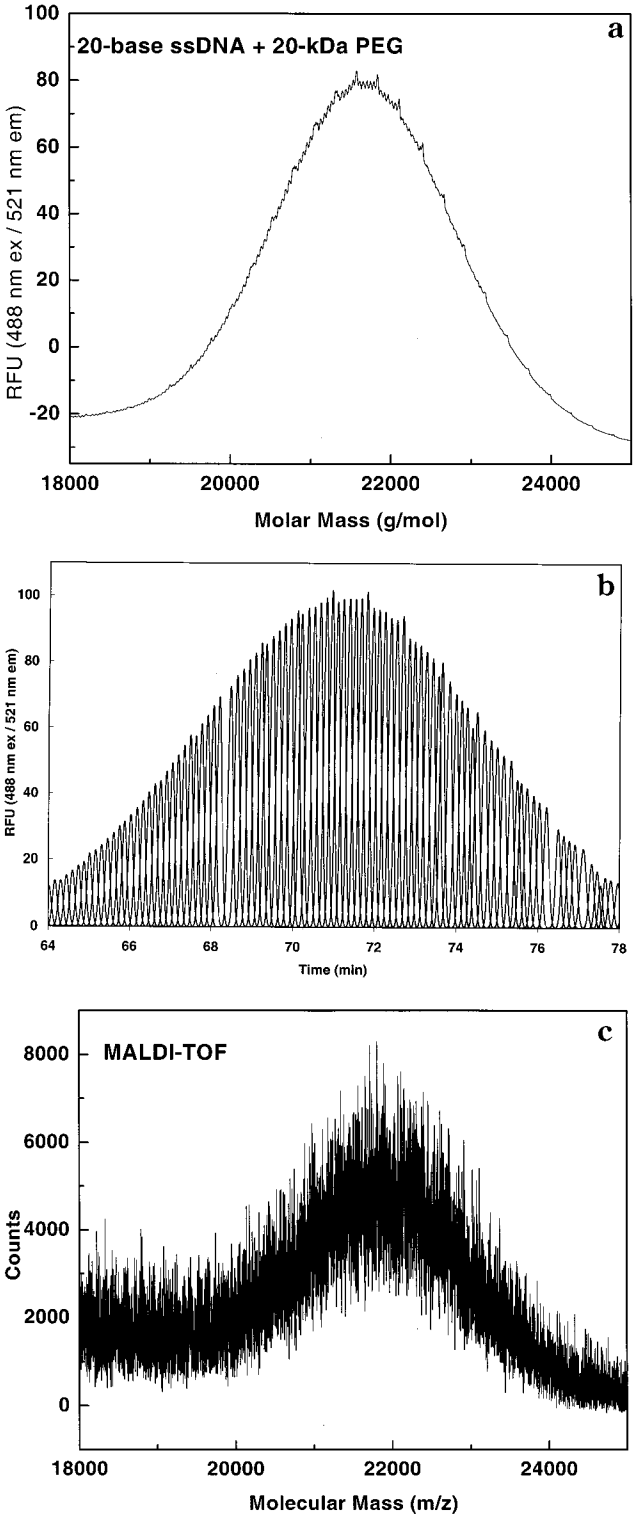

Figure 5. (a) FSCE analysis of 20-base DNA + 20-kDa PEG conjugates; conditions are the same as in Figure 1. (b) PeakFit analysis results of the 20-base DNA + 20-kDa PEG conjugate electropherogram. (c) MALDI-TOF analysis of the 20-kDa PEG sample.

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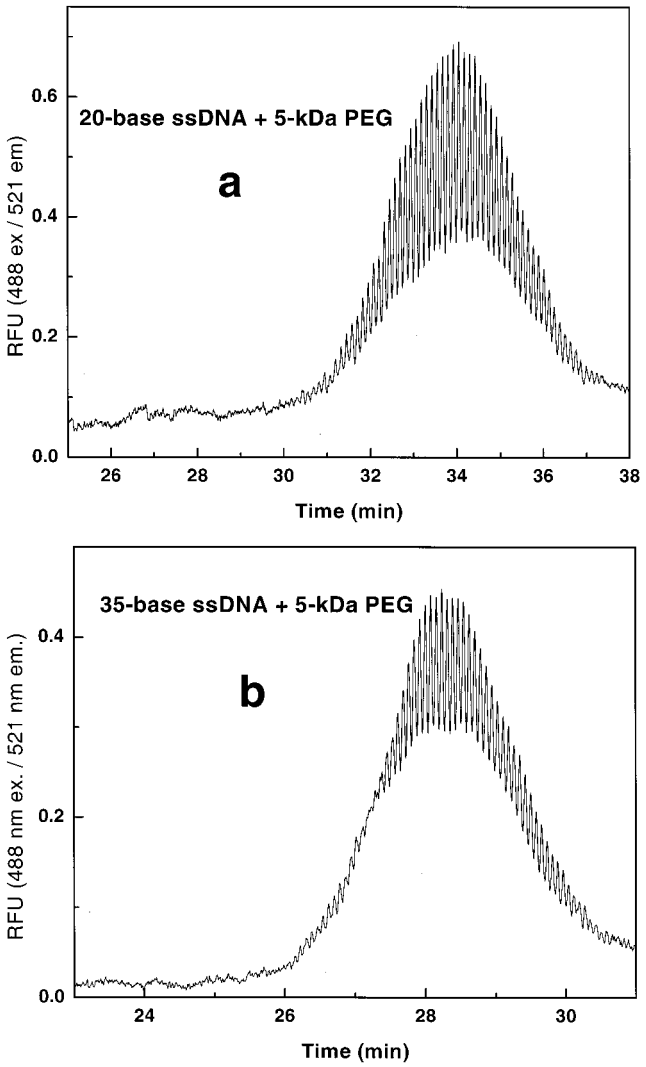

Figure 6. FSCE analysis of (a) 20-base DNA + 5-kDa PEG conjugates and of (b) 35-base DNA + 5-kDa PEG conjugates. Conditions are the same as in Figure 1.

Table 3. Average Plate Efficiencies of Conjugates Analyzed in This Study

conjugate	pressure injection	electrokinetic injection
35-base DNA + 5-kDa PEG	1 120 000	
20-base DNA + 5-kDa PEG	480 000	1 040 000
35-base DNA + 3.4-kDa PEG		664 000
20-base DNA + 3.4-kDa PEG	730 000	777 000

electrical conductivity between the sample solution and the buffer. Thus, sample stacking allows one to "concentrate" samples dissolved in low-conductivity buffers at the tip of the capillary. This allows for a greater amount of analyte to enter the capillary and results in a higher signal-to-noise ratio. This is not possible with pressure injections, where the analyte is introduced by simple hydrodynamic pumping of the analyte solution into the capillary.

While EK injections can provide higher signal-to-noise ratios, the mechanism of EK injections can also introduce a bias in the amount of each of the analytes introduced into the capillary.[28] This bias results in a disproportionately large amount of higher mobility analytes entering the capillary. Formally, EK injection-induced bias must be corrected to determine the relative concentration of a particular species in the original sample. However, this bias does not substantially alter the M_w, M_n, or PDI as determined by FSCE analysis of the narrow-polydispersity polymer samples in this study. Typically, the molar mass averages agree to within 2% between pressure and electrokinetic injection modes. For drag polymer samples with higher polydispersities, EK-induced biases may affect the accuracy of sample measurements to a greater extent.

CONCLUSIONS

We have presented a novel technique for the free-solution electrophoretic separation, sensitive detection, and mass determination of polydisperse, uncharged, water-soluble polymers. This mode of analysis results in single-monomer resolution of poly(ethylene glycol) polymers of up to 5 kDa in molar mass. Resolution with polymers up to 20 kDa in molar mass, although reduced relative to lower molar mass polymers, is still sufficient to allow for accurate molar mass characterization. FSCE plate efficiencies are typically in excess of 500 000 plates/m. Theoretical treatment of the data allows for easy and direct calculation of polymer molar mass distribution, M_w, M_n, and PDI. The FSCE technique gives results that are in excellent agreement with MALDI-TOF analysis for the polymers used in this study but yields a much higher signal-to-noise ratio and hence a clearer picture of the distribution of polymer molar mass. While GPC and MALDI-TOF will likely remain the most prevalent techniques of polymer molar mass characterization because of their wide applicability, we believe that FSCE can provide a method for high-resolution characterization of important specialty polymers, including PEGs which are used in pharmaceutical applications. Additionally, this technique does not require standards or calibration, as are needed in chromatographic and mass spectrometric methods, allowing for a completely self-consistent molar mass calculation. We believe that this analytical technique will be applicable for any uncharged, water-soluble polymer, so long as appropriate chemistry for the end-conjugation of the drag polymer to the DNA engine can be devised.

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