Electromigration behavior of polysaccharides in capillary electrophoresis under pulsed-field conditions

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ABSTRACT Various polysaccharides can successfully migrate through entangled polymer solutions during high-voltage capillary electrophoresis. For neutral polysaccharides, complexation with borate provides the electric charge needed for electromigration, while a fluorescent tag is needed to detect the solute bands with adequate sensitivity. At constant potentials between 50 and 300 V/cm, the charged polysaccharides undergo molecular stretching, resisting the desired separation according to their molecular mass. This problem can be overcome through the use of variable fields, pulsed along the separation capillary at a 180° angle. Variables of the pulsing experiment appear to have a profound influence on molecular shape rearrangements of polysaccharides with respect to the separation medium, as demonstrated here with highly efficient separations of polydextrans (8,000–2,000,000 Da).

The chemistry and biology of large glycoconjugate molecules are among the most important subjects of investigation in modern science. Besides the central roles that glycoconjugates play in biological recognition (1, 2) and pharmaceutical use (3) of the relevant biochemical processes, complex carbohydrates possess enormous potential as industrial polymers (4). Investigation of the properties of glycoconjugates and their correlation to chemical structure thus appears central to further advances in the field. Because of the enormous variation in glycoconjugate structures, such investigations place great emphasis on the development of powerful analytical methodologies (5, 6) featuring both a high component resolution and low detection limits.

High-performance capillary electrophoresis (HPCE) is a powerful analytical separation method with recent notable accomplishments, as evidenced particularly in the areas of nucleic acids (7–9), proteins (10–12), and chiral molecules (13–15). During the last 2–3 years, HPCE has also been applied to relatively small carbohydrate molecules, such as monosaccharides and oligosaccharides cleaved from model glycoproteins (16–18). To enhance the detection of carbohydrates, the compounds of interest were converted to their UV-absorbing aminopyriidylic (16) or 3-methyl-1-phenyl-2-pyrazolin-5-one (19) derivatives. Alternatively, fluorescent labeling with aminophthalenesulfonic acid reagents (20) and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) (17) can be used for measurements at greater sensitivity.

During HPCE of glycoconjugates, the medium inside the separation capillary is highly important. While the use of an open-tubular format appears suitable for the separation of relatively small oligosaccharides (16–19), gel-filled capillaries become useful in dealing with more complex oligomeric mixtures (21, 22). In most separations, borate complexation (16–18) is beneficial in imparting a convenient negative charge on the individual monomeric units of the oligosaccharide species. However, it also tends to cause an unfavorable charge-to-mass ratio, with the result that higher oligosaccharides may coelute in the free solution medium. In contrast, a dense gel network separates the oligosaccharides according to their molecular weight, and up to 70-meric mixtures have been adequately resolved (22) based on this mode of retention.

We have made attempts to increase the scope of HPCE applications to polysaccharides. Various fluorescently labeled (17) polysaccharides, such as chitosan, dextran, various water-soluble cellulose derivatives, etc., charged additionally through borate complexation, readily migrated in open tubular HPCE but showed little tendency to separate. In concentrated, immobilized polyacrylamide gels [which had been successfully applied (21, 22) to complex oligomeric mixtures], the gel network does not permit penetration of such large molecules. Thus, the polysaccharides and often even their enzymatic fragments merely concentrated at the column inlet, causing column clogging and decrease of current. As shown in this report, this basic problem can be overcome by causing the polysaccharides to migrate through solutions of entangled polymers (23, 24) that "yield" readily to such large molecules as they migrate toward the anode. However, the extent of separation according to mass seems to be complicated by molecular elongation, under conditions of high constant field, and the corresponding "reptation effect" (24, 25), in which the solute polymer undergoes a transition from its coiled form to an elongated molecule that snakes ahead through the network.

We wish to report here a novel and effective approach to overcoming the reptation behavior of polysaccharides. When a potential gradient along the separation capillary is periodically inverted at a 180° angle, the negatively charged polysaccharide molecules undergo shape transitions, which appear to favor separation according to molecular size. The general approach used here bears resemblance to a set of slab gel techniques pioneered by Schwartz and Cantor (26) and widely known under such names as "pulsed-field electrophoresis" or "field-inversion gel electrophoresis" (27). However, the results with polysaccharides we report here were obtained for a vastly different molecular-weight range, higher field strengths, and considerably shorter migration times than those typically observed with large DNA fragments.

MATERIALS AND METHODS

Apparatus. All measurements carried out in this work were obtained by using a home-built capillary electrophoresis/laser-induced fluorescence detection system described previously (28). The inner surface of the separation columns was modified by the attachment of linear polyacrylamide (29). Fused silica capillaries, 15–60 cm long (50-μm i.d.; 187-μm o.d.), were filled under pressure with appropriate polymer solutions, such as linear polyacrylamide, Instacryl (Internation...
nitional Biotechnologies), or Synergel (Diversified Biotech) of different concentrations. The separation column was enclosed in a Plexiglas box with an interlock safety system. The high-voltage dc power supply (Spellman High Voltage Electronics, Plainview, NY) used here was capable of delivering 0–30 kV. For the pulsed-field experiments, a 10-kV operational amplifier (model 10/10; Trek, Inc., Medina, NY) controlled with a function generator FG2 (Beckman Industrial, Emerson Electric, Brea, CA) was used. On-column fluorescence measurements were carried out with an argon-ion laser (model 543, Omnichrom, Chino, CA) used as a light source (5-mW power at 457 nm). An on-column optical cell was made by removing the polyimide coating from a short section of the fused silica capillary. The incident laser beam was aligned to its optimum position by adjusting the position of collecting optics between the flow cell and the detector. Fluorescence emission at 555 nm was collected through a 600-μm fiberoptic placed at a right angle to the incident laser beam. Signals isolated by a band-pass filter were monitored with a R928 photomultiplier tube and amplified with a model 128A lock-in amplifier (EG & G Princeton Applied Research, Princeton, NJ).

RC (resistance-capacitance) time constant of the system was approximately 3 orders of magnitude smaller than a duty cycle of pulses. The current was monitored at the ground end of the capillary with the oscilloscope.

Materials. Dextran standards (M, 8800; 39,100; 70,000; 503,000; and 2,000,000) were purchased from Sigma, and additional dextrans (M, 48,600; 273,000; and 667,800) were received from Fluka. A fluorogenic reagent, CBQCA, was synthesized in our laboratory (28). Acrylamide, ammonium persulfate, sodium borate, boric acid, sodium phosphate, ammonium chloride, and tris[(hydroxymethyl)aminomethane] (Trizma base), were analytical grade reagents also received from Sigma. Sodium cyanoborohydride was purchased from Aldrich. Potassium cyanide was purchased from Mallinckrodt. A carboxymethylcellulose sample was a gift from Astra/Hässle (Mölndal, Sweden). Cellulase (from Trichoderma viride; EC 3.2.1.4) and Mycodextranase (from Penicillium funiculosum; EC 3.2.1.61) were purchased from Sigma.

Cleavage of carboxymethylcellulose with the cellulase enzyme was performed in a 100 mM sodium phosphate buffer (pH 5.0) at a cellulase-to-substrate ratio of 1:100 and a temperature of 37°C. The enzymatic reaction was stopped after 1 hr by heating the mixture for 10 min at 100°C. The enzyme was then removed by passing the whole digest through a C18 cartridge (Millipore). The sample was reductively aminated as described (17, 21). Debranching of a derivatized polydextran (M, 48,600) with the mycodextranase enzyme was performed in a 100 mM sodium acetate buffer (pH 4.5) at the enzyme-to-substrate ratio of 1:1000 and a temperature of 37°C. The enzymatic reaction was stopped after 17 hr by heating the mixture for 10 min at 100°C. Derivatization by CBQCA has been described by Liu et al. (17, 21). Each sample was desalted by microdialysis on a Millipore MF membrane (0.025-μm pore size; Millipore) prior to sampling (30).

RESULTS AND DISCUSSION

The potential of entangled polymer matrices for biomacromolecular separations by HPCE has already been indicated in previous reports. Heiger et al. (31, 32) and Grossman and Soane (23) utilized such media to resolve DNA restriction fragments, while Ganzler et al. (12) reported effective separations of proteins more recently using a polysaccharide matrix. Besides the linear polyacrylamide, additional matrices, such as concentrated solutions of galactomannan (33), polyvinyl alcohol, polyethylene glycol, and so on, may become useful in HPCE. Additionally, the size of "dynamic pores" and the viscosity of the entangled polymer solutions at constant concentration can be varied by altering the length of the polymer chains. At first glance, our fluorescently labeled, negatively charged polysaccharides seemed to follow the example of well-behaved polynucleotides (7-9), as shown with the separation of cellulase-digested carboxymethylcellulose (Fig. 1). Additional polysaccharides (results not shown) yielded comparable results, indicating the potential utility of this approach for "finger-printing" various polysaccharides of pharmaceutical and industrial interest.

Fig. 1 shows at least a hint of sample polydispersity, but additional regularly spaced peaks appear as well. In further attempts at tentative assignment of molecular weight ranges for various hydrolyzed polysaccharides through a series of commercially available polydextran molecular weight standards, we failed to achieve the expected separation by size. In fact, in spite of their vast differences in size, each fluorescently labeled dextran migrated in two peaks. No separation due to molecular weight was achieved. This phenomenon can be attributed to a "biased reptation" behavior (25, 34, 35) on the part of the stretched-out macro-molecules.

Anomalous behavior of large DNA molecules, subjected to electric field inside a gel network, has been investigated for some time (36). Three different migration models have been developed for the behavior of large DNA in gel electrophoresis: the Ogston model; the reptation model; and the biased-reptation model. While in the Ogston model the polymer network is treated as a molecular sieve and a migrating solute is treated as a rigid spherical particle, in the reptation models the solute polymer undergoes a transition from its coiled form...
to an elongated (deformed) molecule that charges ahead through the network. As the electric field increases, the dependence of mobility on molecular weight becomes less pronounced. For the three polydextrans studied (molecular masses of 48,600; 667,800; and 2,000,000 Da), we plotted the electrophoretic mobilities of their first and second peaks as a function of field strength (Fig. 2). This figure seems to support the notion of molecular stretching under the condition of increasing potential (between 130 and 200 V/cm).

Here, the reptation behavior appears less evident for the set of slower zones than for the first peaks of the polydextran sample series. We offer the following tentative explanation: the first peaks represent linear dextran molecules that can exhibit classical biased reptation behavior, whereas the second peaks may be attributed to branched species of the same molecular weight, which are quite common with this polymer type (4, 37). When the branched molecules are subjected to an electric field, their hydrodynamic properties and frictional coefficients vis-à-vis the entangled polymer matrix are likely to result in slower migration. [Notably, the peak height ratio for the three dextrans was changed in favor of the first peak, assumed to be the linear molecule, when the debranching enzyme (mycodextranase) was used.]

When the same polydextrans were subjected to pulsed fields in the range of 1–10 Hz, their migration behavior was dramatically altered. A highly efficient separation according to increasing molecular weight is clearly seen (Fig. 3). Using the 3:1 forward/reverse regime of pulses, all polydextrans have sequentially reached the detector in 80 min. This is in contrast to the experience of DNA behavior in flat gels, where separations may take many hours or even days (38).

Electrophoresis in pulsed fields is known to be affected by a number of variables: pulse frequency and amplitude, angle of pulsing, sinusoidal bias, and pulsing (forward/reverse) regime, among others (38). For example, when we changed the time ratio for the forward/reverse pulses from 3:1 (Fig. 3) to 2:1, the separation became different (Fig. 4). Slower migration occurred in spite of a change in polyacrylamide concentration from 5% to 4%. The extremely narrow peaks observed in the last two figures were anticipated, as diffusion

**Fig. 2.** Dependence of the electrophoretic mobilities of three polydextrans on a constant electric field strength; mobility is shown in $[\text{cm}^2/\text{V}\cdot\text{s}] \times 10^4$ units. Electrophoretic conditions were as follows: capillary, 50-µm i.d., 70-cm total length (55-cm effective length), coated with linear polyacrylamide, and filled with linear polyacrylamide (4% T; 0% C); buffer, 50 mM sodium borate/50 mM boric acid/100 mM Tris, pH 8.8; electromigration injection, 5 sec (1 kV); applied voltages, 85 V/cm, 130 V/cm, 170 V/cm, 210 V/cm, and 255 V/cm; dextrans, $M_r$ 48,600, $M_r$ 667,800, and $M_r$ 2,000,000; and sample concentration, 0.5 µM.

**Fig. 3.** Separation of the polydextran standards by pulsed-field capillary electrophoresis. Peak assignments are 1, 39,100 Da; 2, 70,000 Da; 3, 503,000 Da; and 4, 2,000,000 Da. Electrophoretic conditions were as follows: capillary, 50-µm i.d., 29-cm total length (20-cm effective length), coated with linear polyacrylamide, and filled with linear polyacrylamide (5% T; 0% C); ratio of forward-to-reverse times, 3:1; applied voltage, ±10 kV; and frequency, 3 Hz. Other conditions are the same as in Fig. 2.

of the large polydextran molecules is slow and the viscosity of our separation medium is relatively high. Frequency of pulsing voltage was briefly optimized, yielding the best separations at 3 Hz. The amplitude of pulsing voltage was
kept constant for both the forward and reverse modes. When the forward-biased mode was replaced with a zero-integrated field mode (36), only the first two polydextrans of the series (M, 8800 and 39,000) reached the detector in 120 min. It is known from the slab gel electrophoresis of DNA samples (36) that the zero-integrated-field mode provides better resolution for smaller polymers, while the migration rates of larger molecules decrease dramatically. When the same experiment is performed in an uncoated capillary (migration driven primarily through the electroosmotic flow), both peaks for each dextran can then be seen (Fig. 5). Under some experimental conditions, sample polydispersity may be observed, as demonstrated with a relatively small (39,100 Da) dextran (Fig. 6).

Slab gel electrophoresis using pulsed fields has now been widely documented in the separation of large DNA molecules (27), and, more recently, proteins in the presence of sodium dodecyl sulfate (SDS) (39). The utility of pulsed HPCE has been suggested for DNA restriction fragment analysis (31, 32, 40) but with no convincing results demonstrated. In illustrating the value of this technique for the separation of polysaccharides, we report here a successful, albeit preliminary, application of pulsed fields to HPCE.

The biased-reptation effect that we seem to have experienced with the borate-complexed model polysaccharides in this work appears to parallel a behavior of DNA at high field strengths (25). However, the major, somewhat unexpected, result of our experiments is that the reptation behavior and its manipulation by the pulsed field occurs at vastly different molecular weight ranges. [The effects on DNA are typically seen in the kilobase-to-megabase region (36).] We attribute this tentatively to a substantial difference in molecular flexibility between nucleoideps and polysaccharides. Polysaccharides are generally viewed as very rigid macromolecules (4), and it is quite possible that the internal repulsion caused by the borate negative charges on the adjacent residues may further modify the polymer backbone. Further experimentation in this area is clearly suggested, as it may become possible to use the complexation effect in concert with the pulsed-field operation to fine tune separations of hard-to-resolve molecular species.

While the experiments reported here were limited to polysaccharides, they provide hope for future studies on the pulsed-field HPCE of other large biopolymers and molecular aggregates. However, advances in mass spectrometry may also be necessary if the molecular weights of sample components are to be accurately assessed.

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