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Determination of the Compositional Distribution of Copolymers by Frontal Analysis Continuous Capillary Electrophoresis

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Frontal analysis continuous capillary electrophoresis (FACCE) was carried out for a series of random copolymers of an ionic monomer, sodium 2-(acrylamido)-2-methylpropanesulfonate (AMPS), and a nonionic monomer, acrylamide (AAM). The electropherograms appeared in order of anionic content and were generally sigmoidal, in contrast to that of hyaluronic acid (HA), which was abrupt and discontinuous. This difference could be related to the compositional heterogeneity of the copolymers, completely absent in the biopolymer. Through the range of copolymer composition (10−100% AMPS) the relationship between average mobility and nominal AMPS content could be described by a calibration curve, making it possible to deduce the compositional distribution of copolymer samples.

Copolymers that incorporate ionic residues are of great commercial importance, with large volumes produced for technologies such as water purification and sludge dewatering.[1] These copolymers combine either cationic or anionic monomers with less expensive comonomers, such as acrylamide; recently, synthetic methods have become more complex with the goal of incorporating hydrophobic substituents into some of the nonionc residues. Polymers with variable ionic content can also be prepared by reactions on nonionic starting materials, e.g. carboxymethylation of cellulose, sulfonation of polystyrene, or quaternization of polyamines, and these also are significant commercial materials.

Charge content is clearly a critical variable in such copolymers, and among the many techniques utilized to this end are elemental analysis and chemical titration methods. These, however, provide only the average composition with no information about compositional distribution. Of particular interest is the effect of this heterogeneity on differences in charge content and related properties among the distribution of polymer chains that make up the sample. This distribution is expected to arise as a result of the variation of composition with conversion that takes place during normal copolymerization of monomers with different reactivity ratios.[2] NMR studies can illuminate the monomer sequence distribution of copolymers, but the process of band assignments may be arduous. It is evident that a technique involving separations is required for the determination of charge heterogeneity.

Among separation methods, HPLC and SEC (size exclusion chromatography) comprise an array of versatile techniques ideal for the analysis and purification of soluble molecules ranging from biomacromolecules to synthetic polymers.[3] With on-line small-angle light scattering and dual concentration detection, SEC can allow determination of molar mass heterogeneity at each elution volume for some types of copolymers,[4] but such methods are not suitable for the separation of synthetic polymers with similar molecular weights but variable compositions. Cross-fractionation methods, such as coupled column chromatography using different stationary phases and mobile phases,[5] have been used to evaluate compositional heterogeneity, but these procedures are very time-consuming. Capillary electrophoresis (CE) provides a new approach to characterization of charged copolymers. CE exploits the tendency of different analytes to migrate at different velocities in an electric field and can provide high resolution with minimal diffusion. As such, it has considerable potential for characterization of ionic/nonionic copolymers.[6,7]

Recently, a new method, FACCE (frontal analysis continuous capillary electrophoresis), was developed in our laboratory for use

(1) See, for example: Farinato, R. S.; Huang, S.-Y.; Hawkins, P. Polyelectrolyte-Assisted Dewatering. In *Colloid-Polymer Interactions*; Farinato, R. S., Dubin, P. L., Eds.; Wiley-Interscience: New York, 1999; Chapteer 1.

(2) See, for example: Rosen, S. L. *Fundamental Principles of Polymeric Materials*; Wiley-Interscience: New York, 1993; p 203 ff.

(3) (a) Snyder, L. R.; Kirkland, J. J. *Introduction to Modern Liquid Chromatography*, 2nd ed.; John Wiley & Sons: New York, 1979. (b) Snyder, L. R. Gradient Elution Separation of Large Biomolecules. In *HPLC of Biological Macromolecules: Methods and Applications*; Gooding, K. M., Regnier, F. E., Eds.; Marcel Dekker: New York, 1990.

(4) (a) Dawkins, J. V. *Adv. Chem. Ser.* **1995**, *247*, 197. (b) Dumelow, T.; Holding, S. R.; Maisey, L. J.; Dawkins, J. V. *Polymer* **1986**, *27*, 1170. (c) Mrkvickova, L. *Macromolecules* **1997**, *30*, 5175.

(5) (a) Gloeckner, G. *Trends Anal. Chem.* (Personal Ed.) **1988**, *7* (5), 169. (b) Gloeckner, G. *J. Appl. Polym. Sci.: Appl. Polym. Symp.* **1992**, *51*, 45. (c) Gloeckner, G. *J. Chromatogra.* **1991**, *550*, 629. (d) Dawkins, J. V.; Montenegro, A. M. C. *Br. Polym. J.* **1989**, *21*, 1. (e) Mori, S. *Adv. Chem. Ser.* **1995**, *247*, 211.

(6) Gao, J. Y.; Dubin, P. L.; Sato, T.; Morishima, Y. *J. Chromatogr., A* **1997**, *766*, 233.

(7) Hoagland, D. A.; Smisek, D. L.; Chen, D. Y. *Electrophoresis* **1996**, *17*, 1151.

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Table 1. Results of Elemental Analyses (S/C Ratios) and Copolymer Compositions for Part of the Copolymers Used

f(AMPS), mole fraction in feed	S/C ratio	*F*(AMPS), mole fraction in copolymer
0.1	0.085	0.11
0.3	0.19	0.30
0.5	0.26	0.49
0.7	0.31	0.65
0.9	0.36	0.88

in protein−polyelectrolyte binding studies.[8−10] In FACCE, sample introduction and separation are integrated into one process. The result is a reduction of diffusion-broadening effects and ease of quantitation of plateau heights for subspecies separated by electrophoresis. In this paper, we study the use of FACCE to determine the compositional heterogeneity of a series of random copolymers of acrylamide and a charged monomer, 2-(acrylamido)-2-methylpropanesulfonate (AMPS). We demonstrate the validity of this approach by comparison with a "copolymer" which is completely homogeneous with respect to composition, namely hyaluronic acid, and thereby also suggest the possibility of studying the compositional distribution of ionic polysaccharides of natural origin.

EXPERIMENTAL SECTION

Materials. Random copolymers of 2-(acrylamido)-2-methylpropanesulfonate (AMPS) and acrylamide (AAM) with AMPS mole fractions ranging from 10 to 100% were synthesized using the following procedures:[11] A predetermined amount of 2-(acrylamido)-2-methylpropanesulfonic acid was neutralized with Na_2CO_3 in DMF at 0 °C with stirring for 30 min. Predetermined amounts of acrylamide (AAm) and 2,2′-azobis(isobutyronitrile) (AIBN) (0.5 mol % based on the total monomers) were added to this solution, and the mixture was transferred into a glass ampule. The mixture was outgassed by five freeze−pump−thaw cycles on a high-vacuum line. Polymerization was carried out to high conversion at 60 °C for 12 h. The polymerization mixture was poured into an excess of ether to precipitate the resulting polymers. The polymers were purified three times by reprecipitation from an aqueous solution into excess acetone, allowed to air-dry, and dissolved in water. The aqueous solutions were dialyzed against pure water for several days and then lyophylized. Results of elemental analyses (S/C ratios) and copolymer compositions for some of the copolymers used are shown in Table 1. Table 1 shows that the average copolymer compositions are nearly equal to the monomer feed composition; therefore, these two variables were used interchangeably. SEC results for selected copolymers indicated molecular weights on the order of 200 000. Since the electrophoretic mobility of flexible linear polyelectrolytes is known to be essentially independent of molecular weight, more detailed molecular weight characterization was not performed.

Hyaluronic acid (HA) with a molecular weight of 1 200 000 was a gift from Shiseido Co. Analytical grade dibasic sodium phosphate was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ), and monobasic sodium phosphate was from Mallinckrodt Inc. (Paris, KY). Experiments were carried at pH 6.99 and ionic strength 0.05 M (sodium phosphate (NaH_2PO_4/Na_2HPO_4) buffer). At this pH, the carboxylic acid groups of HA are fully ionized. Milli-Q water was used throughout this work.

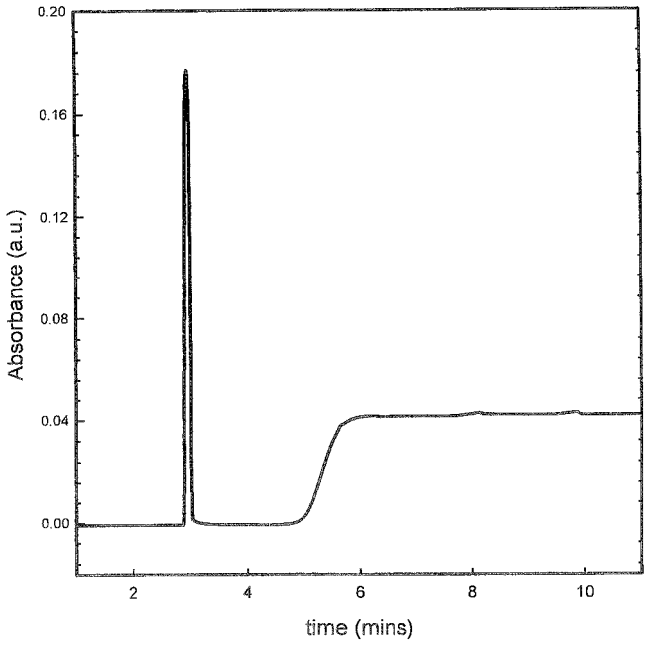

Figure 1. Typical FACCE electropherogram of poly(AMPS/AAM) copolymer with 20% AMPS. The sharp peak corresponds to the neutral marker and the plateau to the copolymer.

Capillary Electrophoresis. Capillary electrophoresis (CE) was carried out on a P/ACE 5500 instrument from Beckman (Fullerton, CA) using a 50 μm i.d. uncoated capillary, with a total length of 27 cm and an effective length (from the injection end to the detection window) of 19.25 cm. UV detection at 200 nm was employed. The voltage applied across the capillary was fixed at 6 kV, while the temperature was maintained at 25.0 ± 0.1 °C with Fluorocarbon coolant. For each CE experiment, a single 5 s injection of neutral marker (mesityl oxide) preceded the frontal analysis continuous capillary electrophoresis (FACCE). The detailed description of FACCE can be found elsewhere.[8,9] In FACCE, species separated by electrophoresis appear as discrete and progressive plateaus in the electropherograms. Shown in Figure 1 is a typical electropherogram in which the sharp peak corresponds to the neutral marker and the plateau to the copolymer.

The electrophoretic mobility, μ_E, was calculated by subtracting the electroosmotic flow (EOF) of the run buffer from the sample's apparent electrophoretic mobility as shown in eq 1,

$$\mu_E = \frac{v_0 - v_1}{E} = \frac{lL}{V}\left(\frac{1}{t_m} - \frac{1}{t_s}\right) \quad (1)$$

where v_0 and v_1 are the electroosmotic velocity and the solute velocity, respectively, E is the applied electric field strength, l is the effective length of the capillary and L is the total length of the capillary (both in cm), V is the applied voltage, and t_m and t_s are the retention times of the reference marker (mesityl oxide)

(8) Gao, J. Y.; Dubin, P. L.; Muhoberac, B. *Anal. Chem.* **1997**, *69*, 2945.
(9) Hallberg, R. K.; Dubin, P. L. *J. Phys. Chem.* **1998**, *102*, 8629.
(10) Gao, J. Y.; Dubin, P. L.; Muhoberac, B. *J. Phys. Chem. B* **1998**, *102*, 5529.
(11) Morishima, Y.; Ohgi, H.; Kamachi, M. *Macromolecules* **1993**, *26*, 4293.

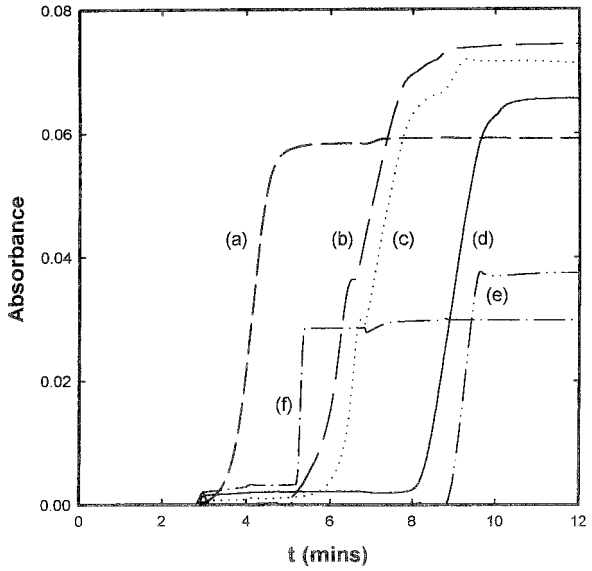

Figure 2. FACCE electropherograms for (AMPS/AAM)$_n$ copolymers containing 10 (a), 25 (b), 30 (c), and 50% (d) AMPS, along with the anionic homopolymer PAMPS (e) and hyaluronic acid (f).

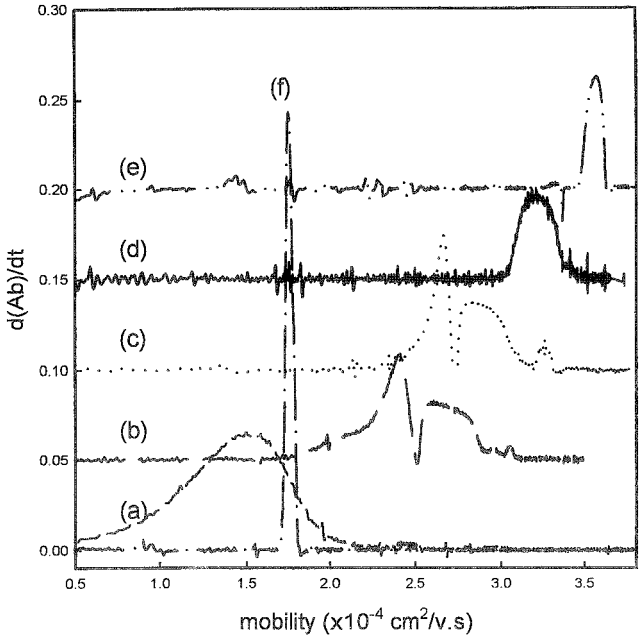

Figure 3. Mobility distribution obtained by differentiation of the electropherograms of Figure 2 via eq 1: (a) 10% AMPS; (b) 25% AMPS; (c) 30% AMPS; (d) 50% AMPS; (e) pure PAMPS; (f) hyaluronic acid.

and the sample, respectively. Multiple runs showed reproducibility of μ_E of ±2%. For copolymers that exhibited multinodal distribution, we used the retention time at half-height to calculate the average mobility.

RESULTS AND DISCUSSION

Figure 2 shows the FACCE electropherograms for AMPS/acrylamide copolymers containing 10, 25, 30, and 50% anionic residues, along with the anionic homopolymer (PAMPS). In striking contrast to the copolymer results, which appear as sigmoidal or more complex traces, the electropherogram for hyaluronic acid (HA) is essentially discontinuous and abrupt, with the solute eluting as a single front. This result can be understood as a consequence of the compositional homogeneity of HA, which contains precisely one ionophore per disaccharide unit, i.e., ca. one charge per 12 Å contour length at pH >6. The corollary conclusion is that the gradual FACCE patterns for the copolymers are reflections of compositional heterogeneity, which must arise from changing conditions during the progress of the polymerization reaction.

By means of eq 1, retention times can be converted to mobilities, larger retention times corresponding to larger mobilities because electrophoretic migration in this case is in the opposite direction to the EOF. Since absorbance is proportional to solute mass concentration, each FACCE electropherogram represents a cumulative distribution of the components of varying mobilities in each sample. These components arise from the usual compositional polydispersity of random copolymers and the "composition drift" during the course of polymerization.[2] However, in some cases the polymerization process is sufficiently nonideal as to produce nodes in the cumulative distribution, most obvious in the case of 30% AMPS (see Figure 2c). Differentiation of the electropherograms leads to the more familiar differential distributions shown in Figure 3. Again we note the strikingly narrow distribution for HA; its breadth probably results from instrumental limitations. In principle, similar curves could be obtained by single-

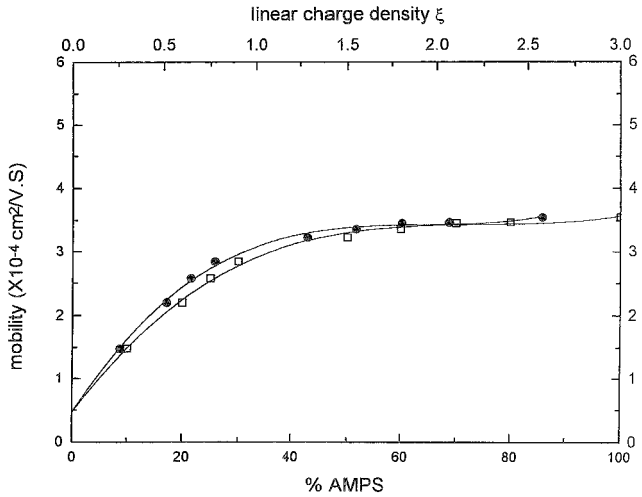

Figure 4. Dependence of mobility on percent AMPS (empty squares) and linear charge density ξ (solid circles).

injection CE. We find however that diffusion-broadening effects are reduced with FACCE and that the heights of the plateaus for subspecies separated by electrophoresis are more easily obtained than the peak areas.

The relationship between mobility and copolymer composition is obtained by calibration with copolymers, as shown in Figure 4, which plots the mean mobility (from the half-height position of the electropherogram) as a function of percent AMPS. Both the mean mobility and percent AMPS are average values, so that the calibration curve is only an approximation of the ideal one, which would be obtained using compositionally monodisperse copolymers. Nevertheless, the rather good fit to a smooth function suggests that this may be a reasonable approximation of the "true" calibration.

The decrease in slope at ca. 50% AMPS is a consequence of counterion condensation. After this point, the mobility only slightly

increases with AMPS composition. Thus, the utility of this approach is limited to the characterization of copolymers with AMPS composition less than 50% or with linear charge density less than 1.5. The concept of counterion condensation has been the subject of intensive theoretical studies[12−14] and has been proved to lead to a good picture of the electrostatic interactions in real polyelectrolytes.[15,16] As stated by Manning,[17] when the dimensionless linear charge density ξ of the polyion exceeds the reciprocal of the valence of the counterions Z, counterions will condense along the polyion contour length and so effectively reduce ξ to $|Z|^{-1}$ (i.e., unity in this case). ξ is defined in SI units as

$$\xi = \frac{e^2}{4\pi\epsilon_r\epsilon_0 k_B T b} \tag{2}$$

where e is the elementary charge of a proton (C), ϵ_r is the dimensionless dielectric constant of the solvent, ϵ_0 is the permittivity of vacuum ($CV^{-1}\ m^{-1}$), k_B is the Boltzmann constant (J/K), T is the temperature (K), and b is the average charge spacing along the polyion chain (m). The linear charge density of vinyl polymers with a charge spacing of 2.55 Å is $\xi = 2.8$, so values of ξ for copolymers were obtained by multiplying 2.8 by the mole fraction of AMPS. The results shown in Figure 4 can be used to portray a discontinuity near $\xi = 1$, as predicted by the Manning theory. However, as observed previously by us[6] and others,[7] the mobility displays a modest increase with polymer charge content even beyond $\xi = 1$. Thus, compositional polydispersity can still be observed in, e.g, the 50:50 copolymer. It may be noted that apparent extrapolation to nonzero mobility has also been previously observed, but no explanation is currently available.

Conversion of the differential curves of Figure 4 into compositional distributions could obviously be accomplished via the calibration curve. However, as noted, the compositional breadths of the copolymers themselves introduce some uncertainty into the calibration curve, so that more detailed analysis awaits the preparation of low-dispersity copolymers via low-conversion polymerization.

The procedure described is readily applicable to a number of commercial synthetic materials arising from copolymerization of ionic and nonionic monomers. Also of particular interest is the possibility of novel information about the composition of ionic polysaccharides of biological origin.

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(12) Fixman, M. *J. Chem. Phys.* **1969**, *51*, 924.
(13) Klein, B. K.; Anderson, C. F.; Record, M. T. *Biopolymers* **1981**, *20*, 2263.
(14) Ramanathan, G. V. *J. Chem. Phys.* **1983**, *78*, 3223.
(15) Manning, G. S. *Annu. Rev. Phys. Chem.* **1972**, *23*, 117.
(16) Ise, N.; Okubo, T. *Macromolecules* **1978**, *11*, 439.
(17) Manning, G. S. *J. Chem Phys.* **1969**, *51*, 924.