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Molecular weight analysis of polycations by capillary electrophoresis in a solution of neutral polymers

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Abstract

Under appropriate conditions, polyelectrolytes separate according to molecular weight during their electrophoretic migration through a dilute solution of inert neutral polymers. These separations facilitate a new capillary electrophoresis-based approach for high resolution and high throughput polyelectrolyte molecular weight analysis, one theoretically applicable to both polyanions and polycations. Although pioneered for DNA, a polyanion, the new method is discussed here in the context of synthetic polycations. Numerous experimental difficulties evolve from the introduction of positive solute charge, not the least of which is a strong tendency for solute adsorption on the negative capillary walls. The adsorption can be overcome by using a run buffer with a cationic surfactant that forms a dynamic yet stable positive wall coating. Feasible at nearly any pH, the surfactant approach enables robust and high-resolution polycation analysis. For illustration, we compare the separation at low pH of three protonated poly(2-vinylpyridine)s to the separation at neutral pH of the same polymers after quaternization. A good match is found. Further, electrophoretic analyses by the new method suggest how poly(2-vinylpyridine)s degrade/crosslink when exposed to quaternizing conditions for an excessive period.

Keywords: Capillary electrophoresis; Polyvinylpyridine; Polycations

1. Introduction

Synthetic polycations find extensive industrial application in technologies such as flocculation, coatings, and sludge-dewatering. Polycations with quaternized ammonium functionality also serve as model systems for probing polyelectrolyte phenomena in non-aqueous media and for following the impact of linear charge density on polyelectrolyte properties. Unfortunately, the strong electrostatic interaction of these positively charged polymers with most hydrophilic surfaces hinders their molecular weight analysis. Development of a molecular weight characterization method that overcomes this obstacle would not only benefit model studies but could also improve commercial processes that rely on these materials. Targeting polymer charge as a driving force for improved characterization methods, we previously explored the analysis of synthetic polyanions by various electrophoretic methods [1–3]. Here, we demonstrate a method to determine polycation molecular weight distributions via capillary electrophoresis. The electrophoretic measurements are then compared to size exclusion chromatography (SEC) measurements.

As nearly all surfaces in contact with water develop negative surface charge, to characterize polycations by conventional methods necessitates special efforts to control surface interactions. SEC packings for polycations, for example, are chemically modified to limit electrostatically driven solute adsorption. Even so, adsorption on the high surface area packings can still occur, making SEC tedious, expensive, and unreliable as compared to its application with polyanions or neutral polymers [4]. Another disadvantage of SEC is its limited molecular weight range; many polycations of commercial importance possess molecular weights above the SEC method's upper limit, roughly $2–5 \times 10^6$ g/mol. Standard electrophoretic methods can easily handle polymers of much larger size. Gel electrophoresis, typically applied in this capacity for anionic biopolymers such as DNA, remains unexplored for cationic polymers. However, both polyacrylamide and agarose gels, the traditional supports for gel electrophoresis, contain residual negative charges that irreversibly bind most polycations. Because of these sorts of experimental difficulties, molecular weight distributions for commercially produced synthetic polycations are generally unavailable.

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Beyond the oligomer size range, capillary electrophoresis in a free solution containing only electrolyte does not provide a size-based separation of uniformly charged polyelectrolytes [5–11]. Rather, polymer migration depends on each chain's linear charge density as well as the solution's ionic strength. Adding a low concentration of neutral polymer to the electrolyte alters this undesirable state in a fundamental and positive way. Now, efficient separations by molecular weight can be achieved for a variety of both synthetic and biological polyanions [3,12–17]. A match of polyelectrolyte and neutral polymer chain length seems to optimize polyelectrolyte molecular weight separation.

When dilute, the neutral chains can be viewed as isolated molecular-sized obstacles that transiently entangle in pairwise fashion with the migrating polyelectrolytes; entangled pairs may then drift downfield some distance before the two disentangle. Polyelectrolyte molecular weight strongly affects the processes of entanglement and drift, explaining how separation by this parameter occurs [18,19]. When semidilute or concentrated, the neutral polymers may act more in the manner of a conventional gel support, and reptation-based migration models have been suggested [20]. The degree to which the neutral polymers modulate electrophoretic mobility depends not only on polyelectrolyte molecular weight but also on additional factors such as neutral polymer molecular weight and concentration [3,6,15,16,18,19]. With many of these factors poorly understood from a theoretical perspective, a molecular weight analysis by this approach necessarily relies on calibration by polyelectrolyte molecular weight standards, a significant drawback. Nevertheless, the neutral polymer approach is faster than gel electrophoresis, and unlike SEC, apparently does not encounter an upper molecular weight bound. For polycations, the tenuous nature of the neutral polymer separation media would suggest reduced aggregation and adsorption problems as compared to gel electrophoresis and SEC. The purpose of the current study is to evaluate this hypothesis.

With the neutral polymer solutions, polycation adsorption problems are certainly not eliminated, as negative siloxy charges populate the surface of the usual fused silica capillaries employed in capillary electrophoresis. However, various techniques for coating capillary surfaces have proven effective in preventing adsorption of ampholytic biopolymers (i.e. proteins) in free solution experiments [21–26]. These coatings render the capillary surface either positively charged or neutral, and several of the coatings can be cheaply and easily prepared. Coated capillaries can also be purchased from commercial vendors.

We have thus combined two advances in capillary electrophoresis, coated capillaries to prevent polycation adsorption and neutral polymer solutions to impart size-dependent mobilities, for the study of synthetic polycations. Preliminary publications by our group [27] and Clos and Engelhardt [28] reported the feasibility of this approach by demonstrating that separations of nearly monodisperse protonated poly(2-vinylpyridine)s [P2VPs] could be obtained in coated capillaries. The previous studies, however, were limited to low pH (in the range 2.5–3.0), a condition needed to achieve the protonation of an otherwise neutral polymer. Here, we extend the technique to include the examination of quaternized P2VPs at neutral pH, development of a more robust capillary coating procedure, and comparison of separations for protonated and quaternized polycations of the same chain length. Further, we illustrate the usefulness of the new method by examining broad molecular weight distribution samples produced by degradation/crosslinking of a narrow polydispersity starting polycation.

2. Experimental

2.1. Materials

Three nearly monodisperse P2VP samples (Scientific Polymer Products) and their quaternized counterparts are examined in this study. The nominal weight-average molecular weights of the P2VPs are 3.6×10^4, 4.0×10^5, and 1.2×10^6 g/mol, and each is characterized by $M_w/M_n \leq 1.1$. The neutral matrix polymer is pullulan (polymaltotriose) at a molecular weight of either 1.7×10^6 g/mol ($M_w/M_n = 1.14$) or 8.5×10^5 g/mol ($M_w/M_n = 1.14$) (Shodex standards available through Phenomenex). Buffer solutions are prepared from potassium hydrogen phthalate [KHP] (Fisher Scientific), hydrochloric acid [HCl] (Fisher Scientific), and tris(hydroxymethyl)aminomethane [Tris] (Sigma). For the non-quaternized P2VPs, water-solubility and a degree of protonation of $\cong 35\%$ [29,30] are ensured by use of KHP-HCl buffer at pH = 2.8 and $I = 0.01$ M. The quaternized P2VPs are examined at neutral pH, so a Tris–HCl buffer at pH = 7 and $I = 0.01$ M is selected.

2.2. Quaternization of P2VP with dimethylsulfate

The quaternization procedure follows Noda et al. [31], using dimethylsulfate (Aldrich, 99 + %) as the quaternizing reagent and dimethylformamide (Aldrich, 99.8%) as the solvent. The reaction proceeds at room temperature in a 2.0 wt.% polymer solution prepared at a 10:1 molar ratio of dimethylsulfate to monomer repeat unit; all reactants and products are soluble in the homogeneous reaction medium. After a period of 1–24 h the quaternized polymer is precipitated into stirring acetone and dissolved in deionized water. The electrolyte level is raised to 1.0 M by addition of sodium chloride, and this salt solution of polymer is dialyzed extensively against 1.0 M sodium chloride to exchange the methylsulfate counterion with chloride. Further dialysis against deionized water provides a virtually salt-free poly(*N*-methyl-2-vinylpyridinium chloride) [PMVP-Cl] solution, as verified by conductivity measurements. The PMVP-Cl solution is then freeze-dried to produce a white, fluffy product. In the order of increasing molecular weight, the degrees of quaternization for the three

narrow polydispersity test polymers are 59, 54, and 50% (by elemental analysis); these values correspond to a quarternization reaction period of 1 h. In addition the 4.0×10^5 g/mol polymer is subject to quaternization periods of 4 and 24 h, producing degrees of quaternization of 60 and 64%, respectively.

2.3. Capillary electrophoresis

Capillary electrophoresis experiments are performed in a home-built apparatus [5] that encloses the capillary in a cooled environmental chamber. Two fans in combination with an air–water heat exchanger maintain the inner capillary temperature at 20 ± 0.5°C and eliminate undesirable Joule heating effects at electric field strengths less than 250 V/cm. P2VP and PMVP-Cl samples (0.2–1.0 mg/ml) are injected electrokinetically (1–5 s at 200 V/cm), and an ISCO CV^4 UV/Vis absorbance detector monitors sample elution at 265 nm. Acetone is co-injected as a neutral tracer to monitor the electroosmotic velocity. To eliminate anomalous signals from dust, all solutions are prefiltered through 0.22 or 0.45 μm Millex-GV or -HV syringe filters (Millipore).

The fused silica capillaries (50 μm internal diameter, Polymicro Technologies) must be modified to prevent polycation adsorption to the negatively charged inner silica surface. We employ for this task a cationic surfactant, cetyltrimethylammonium bromide [CTAB] (Aldrich), which forms a bilayer or hemimicellar coating on the capillary wall, reversing the wall's charge [23]. To form the CTAB layer, the capillary is first treated with 1.0 M potassium hydroxide (≥30 min), rinsed with deionized water (5 min), and then filled with a 0.5 mM CTAB/buffer solution (≥30 min). Finally, the coated capillary is equilibrated with this surfactant-containing buffer solution during application of an electric field, usually at 200 V/cm. The dynamic nature of the coating requires CTAB to be present at concentrations greater than its critical micelle concentration in the running buffer and sample solution vials. For the reported experiments, the CTAB concentration is 0.5 mM. The CTAB layer produces an electroosmotic velocity V_{osm} larger than the electrophoretic velocity V_{el} of the polycation but opposite in direction. To calculate V_{el}, the measured solute velocity is simply subtracted from V_{osm}. The electrophoretic mobility μ is then calculated according to its definition, $\mu = V_{el}/E$, where E is the applied electric field. Results are presented as properly normalized distributions of μ.

Fractions of the neutral and nearly monodisperse polysaccharide pullulan are prepared in the surfactant-containing buffer at pullulan concentrations below the critical overlap concentration c^*, a parameter defining the upper concentration limit of the dilute regime. Literature values [32] for both intrinsic viscosity $[\eta]$ and radius of gyration R_g are employed to calculate c^* ($c^* = [\eta]^{-1}$ or $c^* = 3M_w/4\pi R_g^3 N_A$); either approach yields $c^* \approx 5.6$ mg/ml for the 8.5×10^5 g/mol pullulan and $c^* \approx 3.5$ mg/ml for the 1.7×10^6 g/mol pullulan.

2.4. Size exclusion chromatography

Following the procedures of Kato et al. [33], PMVP-Cl samples are examined by SEC with a single TSK gel GMPW column. To preclude undesired solution-packing interactions, these samples are eluted in an aqueous mobile phase containing 0.5 M acetic acid and 0.3 M sodium sulfate. Detection is by refractive index.

3. Results and discussion

Initial work [27] on protonated P2VPs was conducted in a commercial, poly(ethylene glycol)-grafted capillary (J & W Scientific, μSil-DB Wax) at low pH. Restricted in pH (2.5–5) and degraded with use, this capillary was of marginal utility for polycation studies. Searching for a more robust capillary, we followed several coatings procedures reported previously in the literature, including one based on chemical modification of siloxy groups [21] and one based on the physical adsorption of a cationic polymer [22]. Although the resulting coatings were perhaps suitable for analysis of small molecules or lightly charged proteins, neither proved reliable for the analysis of highly charged polycations. Searching further, the CTAB coating [23] was considered next. The dynamic and renewable nature of a surfactant coating could potentially ease capillary preparation and extend capillary lifetime. The pH stability of such a coating is another advantage, one allowing study, under other otherwise analogous conditions, of both protonated P2VPs at low pH and quaternized P2VPs at neutral pH.

3.1. Protonated P2VPs

Fig. 1 shows the μ distributions measured both in free solution and in a dilute pullulan solution for a mixture of the three protonated P2VPs. As expected, the samples elute simultaneously in free solution, reflecting the independence of μ on molecular weight in this environment. In a dilute ($c = c^*/2$) solution of high molecular weight (1.7×10^6 g/mol) pullulan, on the other hand, three distinct μ peaks are observed. The value of μ for each polycation decreases from its free solution value as a result of electric field-induced entanglements of the polycations with pullulan chains, a process affecting the migration of the highest molecular weight P2VP the most. Experiments performed on individual polycation solutions confirm the identification of peaks and that P2VP fractions migrate independently. Each peak is narrow, reflecting the low polydispersity of these polymers and the high resolution of the analysis. An analysis of parameters controlling peak width has not been attempted.

Clos and Engelhardt [28] also employed neutral polymer solutions to separate solutions of nearly monodisperse

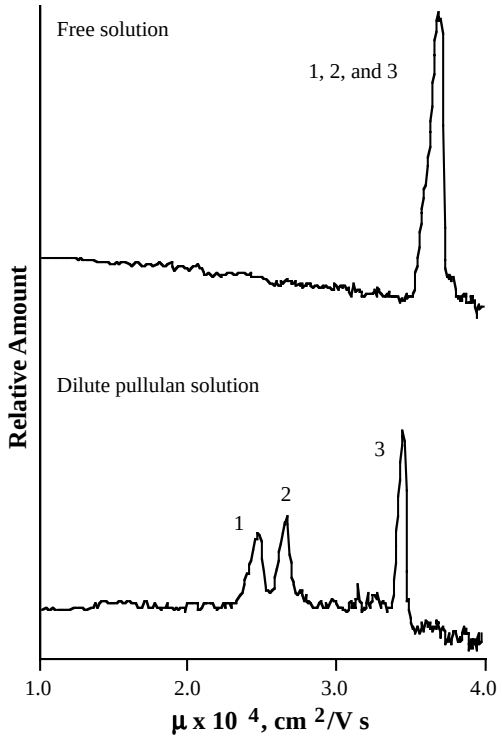

Fig. 1. Electrophoretic mobility μ for three protonated P2VPs, in free solution and in 1.7×10^6 g/mol $c^*/2$ pullulan solution: $1 = 1.2 \times 10^6$ g/mol; $2 = 4.0 \times 10^5$ g/mol; and $3 = 3.6 \times 10^4$ g/mol.

protonated P2VPs. The main differences between their study and ours are the molecular weight and concentration range of the neutral polymer. For their neutral polymer, they used higher, non-dilute concentrations (5–9 wt.%) of a 7×10^4 g/mol dextran possessing a broad polydispersity. In a 5 wt.% solution of this material, they achieved good separation for P2VPs with molecular weights in the range $1–12 \times 10^4$ g/mol but poorer results at higher and lower molecular weight. Employing larger dextran concentrations, they increased resolution at lower molecular weights but never achieved good separation over the entire range studied ($0.15–173 \times 10^4$ g/mol). In contrast, we use a higher molecular weight and more dilute neutral polymer, thereby obtaining a better separation for the higher molecular weight P2VPs. These features are in accord with those described for polyanions in the literature [3,6,15,16,18,19] and recently highlighted in a model study with monodisperse polyelectrolytes and neutral polymers [3]. As the neutral polymer concentration approaches or exceeds c^*, lower molecular weight polyelectrolytes are hindered increasingly by entanglements with the neutral polymer, a factor that enhances polyelectrolyte separation. A comprehensive understanding of these molecular interactions is lacking.

3.2. Quaternized P2VPs

Fig. 2 shows that the three polymer samples subjected to quaternization for 1 h produce electrophoresis data similar to those for the same (protonated) polymers prior to quaternization. In free solution, all three PMVP-Cl samples elute as a single peak, whereas the dilute 1.7×10^6 g/mol pullulan solution causes a mixture to separate and yield three peaks. Except in buffer composition and polymer quaternization, these experiments are identical to the ones employed for the separations displayed in Fig. 1. Thus, the PMVP-Cl samples in free solution possess nearly the same values of μ as their protonated counterparts, a surprising finding given the differences in polymer charge density, either between protonated and quaternized polymer or between quaternized polymers of different molecular weight. This type of behavior has been noted before and rationalized through the concept of counterion condensation [34]. According to this concept, these highly charged polymers possess large enough charge densities to spur the condensation on the chain backbone of a sufficient number of small ions to yield an effective backbone charge density of one charge per Bjerrum length [34]. The counterion condensation concept does not predict explicitly the equivalence of protonated and quaternized polymers, but the structural differences between the two systems apparently are small enough to produce little discernable impact on μ. These trends reinforce the reliability of the new method for measuring polycation molecular weight in spite of minor variations in chain structure or charge density.

The three PMVP-Cl peaks produced by electrophoresis in the pullulan solution exhibit comparable widths and possess

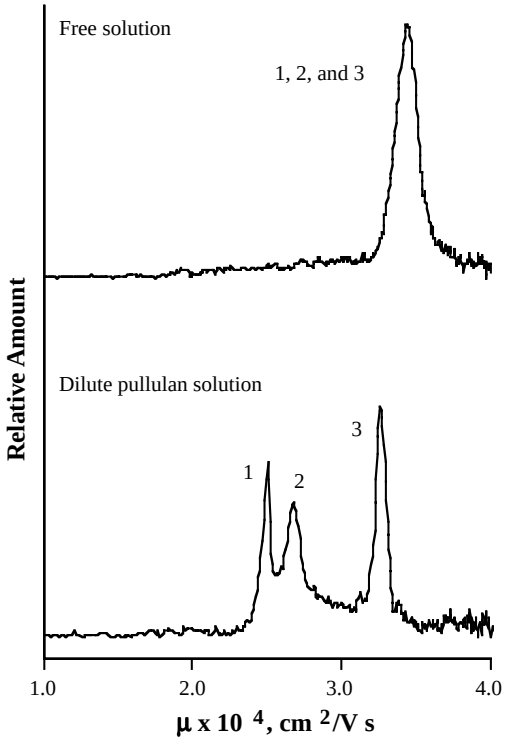

Fig. 2. Electrophoretic mobility μ for three PMVP-Cl samples, in free solution and in 1.7×10^6 g/mol $c^*/2$ pullulan solution; $1 = 1.2 \times 10^6$ g/mol; $2 = 4.0 \times 10^5$ g/mol; and $3 = 3.6 \times 10^4$ g/mol.

μ values similar to those of their protonated counterparts; therefore, we conclude that the quaternization reaction did not significantly alter the average molecular weight or broaden the polydispersities of these samples. However, the situation changes when quaternization reaction times are larger. To investigate the ability of our fractionation method to distinguish between polycations possessing broad molecular weight distributions, three samples of the 4.0×10^5 g/mol P2VP quaternized for reaction times of 1, 4, and 24 h are compared. Degrees of quaternization are similar despite the differences in reaction time. In free solution experiments all three samples provide narrow peaks of similar μ, as shown in Fig. 3. However, in dilute ($c = c^*/2$) solutions of 8.5×10^5 g/mol pullulan, the detrimental effect of longer quaternization reaction time is plainly evident. Fig. 4 shows that at a reaction period of 1 h the μ peak remains nearly as narrow as in Fig. 1. However, as reaction time increases to 4 and then 24 h, the peak broadens and μ shifts to higher values.

The broadened μ peak and the higher μ values indicate that the PMVP-Cl samples are significantly altered as reaction time increases. Additional insight into the alteration is offered by SEC. Fig. 5 displays chromatograms for the same polymer samples and surprisingly reveals that more lengthy quaternization times can produce chains with apparent sizes both larger and smaller than the original chains. The most plausible explanation for this broadening is the presence of a crosslinking side reaction, one that acts intramolecularly to reduce chain size and intermolecularly to increase chain

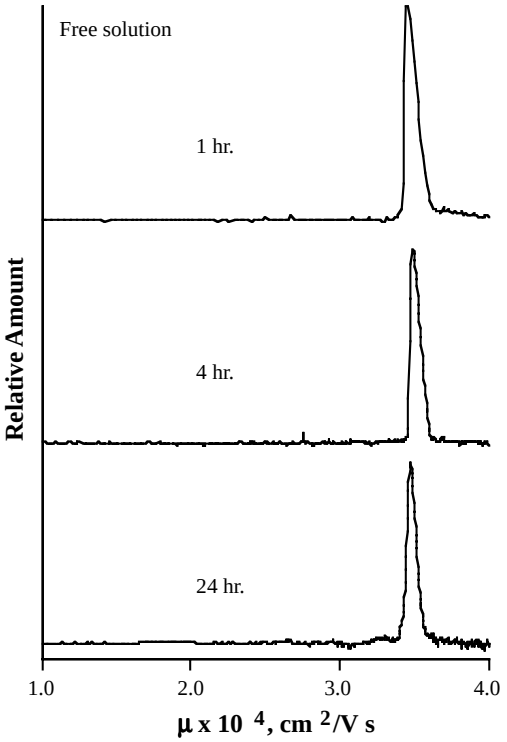

Fig. 3. Electrophoretic mobility μ for three 4.0×10^5 g/mol PMVP-Cls in free solution.

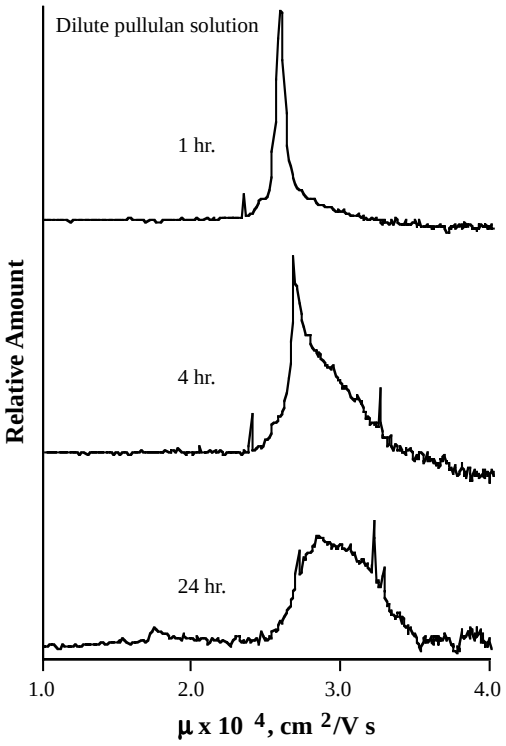

Fig. 4. Electrophoretic mobility μ for three 4.0×10^5 g/mol PMVP-Cls in 8.5×10^5 g/mol $c^*/2$ pullulan solution.

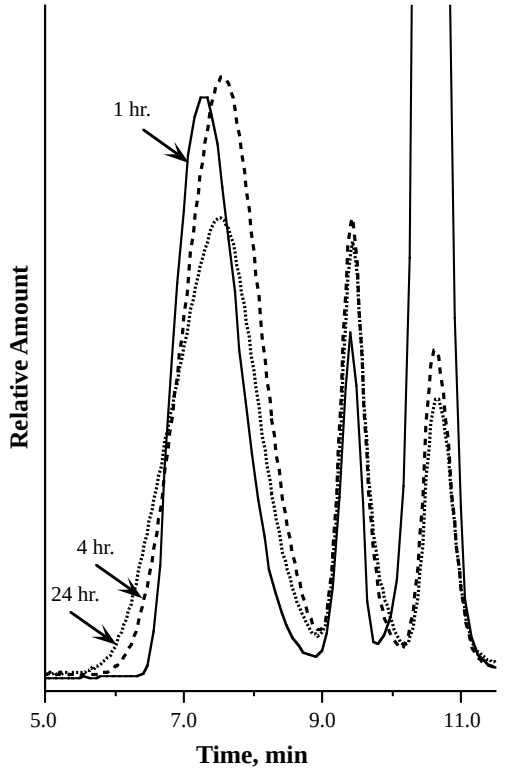

Fig. 5. SEC chromatograms for the three PMVP-Cls of Fig. 4. The peaks associated with polymer appear in the range of 6–9 min; extraneous peaks at times beyond 9 min are artifacts characteristic of the mobile phase.

size. A possible mechanism for crosslinking is the nucleophilic attack by an unsubstituted pyridine on a pyridine previously activated by quaternization [35]. Other quaternization reactions cause similar degradation/crosslinking of P2VP, although the mechanisms for the effect have not been specified [31]. Investigators relying on PMVP-Cl as a model polyelectrolyte rarely have checked for the presence of degradation/crosslinking. The comparison of electrophoretic and chromatographic data indicates that the two methods manifest degradation/crosslinking differently. An insensitivity of the electrophoretic approach to the larger size of crosslinked aggregates can be understood in terms of the proposed entanglement model for the separation mechanism [19].

4. Summary

Two recent advances in capillary electrophoresis techniques enable us to separate water-soluble polycations according to molecular weight. Using cationic surfactant coatings that block adsorption of the polycations to the negatively charged capillary surface, reproducible and high-resolution experiments can be performed at low and intermediate pH. We believe these developments make possible the routine molecular weight characterization of highly charged polycations. The biggest remaining challenge lies in detection, facilitated here by the strong intrinsic UV absorptions of P2VP and PMVP-Cl.

To produce molecular weight dependent values of μ, we perform electrophoresis in dilute solutions of a nearly monodisperse neutral polymer. At fixed neutral polymer molecular weight and concentration, quaternized P2VPs possess the same electrophoretic mobilities as their protonated counterparts. Counterion condensation or some similar effect apparently suppresses μ shifts due to small changes of structure or charge density. Different choices of neutral polymer and cationic surfactant are obviously possible, perhaps allowing for greater optimization than achieved here. Analysis of the degraded/crosslinked PMVP-Cl samples illustrates the type of application that might be expected. Subtle changes in the molecular weight distribution resulting from different reaction conditions are clearly discerned with these materials.

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