

CHROMSYMP. 699

THERMAL FIELD-FLOW FRACTIONATION OF WATER-SOLUBLE MACROMOLECULES

J. J. KIRKLAND* and W. W. YAU

E. I. du Pont de Nemours and Company, Central Research and Development Department, Experimental Station, Wilmington, DE 19898 (U.S.A.)

SUMMARY

While previous thermal field-flow fractionation (TFFF) separations have been carried out with mobile phases containing organic solvents, we have found that certain macromolecules can be fractionated in totally aqueous systems. A variety of macromolecules are separated with TFFF by applying a large thermal gradient across a single flowing mobile phase in a thin, highly polished channel, formed with parallel plates. Some non-ionic materials such as poly(ethylene oxides) and poly(ethylene glycols) are retained sufficiently in pure water to permit characterization. However, water-soluble ionic molecules such as sodium polystyrenesulfonates are normally poorly retained. We find that retention can be enhanced by adjusting the mobile-phase ionic strength to influence a change in molecular conformation from random-coil to rod-like structures.

Our studies suggest that the thermal diffusion coefficients for highly water-soluble macromolecules are often very small in pure water. Adjustment of the ionic strength of the mobile phase increases the thermal diffusion coefficients or decreases the normal diffusion coefficients of certain ionic macromolecules. This allows sufficient retention for practical characterization.

For useful separations in aqueous systems, the TFFF equipment should generate relatively large temperature differences between the channel faces. Fractionations in aqueous systems significantly expand TFFF utility.

INTRODUCTION

Previous publications have indicated that a wide range of synthetic polymers can be characterized by the high-resolution thermal field-flow fractionation (TFFF) method¹⁻⁵. Separations are performed with a single mobile phase in a thin, highly regular, open channel by applying a large thermal gradient across parallel plates⁶. Solute retention results from the redistribution of particles from fast- to slow-moving streams of the essentially laminar flow profile generated by the flowing liquid mobile phase between the parallel plates. Establishing a temperature differential between the two plates of the channel causes sample components to crowd against one wall. Larger sample components are pushed closer to the wall by this external force-field

and are carried downstream by slower-moving streams near the wall so that they lag behind and are eluted after smaller sample components.

We have determined that certain macromolecules can be retained and fractionated by TFFF in totally aqueous systems. Previously, TFFF separations have been carried out with organic mobile phases or mobile phases containing significant concentrations of organic solvents⁷; TFFF retention in purely aqueous systems has not been reported. Although significant retention of water-soluble macromolecules does not appear to be general, practical separations of a number of important water-soluble polymers have now been demonstrated. This report describes work carried out on such water-soluble macromolecular systems.

THEORY

The basis of TFFF has been detailed previously^{2,3,6-8}. Briefly, a temperature gradient is established between two parallel, closely spaced, highly polished, metal bars. Thermal diffusion forces a solute component in a liquid flowing stream towards one wall (presumably the cold wall) as a result of its interaction with the thermal gradient across the channel. Retention in TFFF can be expressed as⁹:

$$\lambda_T = \frac{D}{D_T W (dT/dx)} \quad (1)$$

where the dimensionless retention parameter $\lambda = V_o/6V_R$ for components retained at retention volumes V_R that are at least twice that of the channel dead-volume V_o ; D is the solute diffusion coefficient; D_T is the thermal diffusion coefficient; W is the channel width; and (dT/dx) is the temperature difference across the channel.

TFFF retention is complicated by the distortion of the normal laminar flow profile in FFF, resulting from the variable viscosity as specifically induced by the temperature gradient^{7,10}. Fortunately, however, distortion of the normal velocity profile in the channel, caused by the variable viscosity, does not have a significant effect on retention under usual experimental conditions. Therefore, eqn. 1 represents a useful retention relationship for many TFFF separations.

Eqn. 1 also assumes a linear temperature profile across the channel when, in fact, the thermal conductivity may vary slightly across the channel, causing additional distortions in the channel flow profile. However, the variable thermal conductivity across the channel appears to have relatively small effects, at least when organic mobile phases are used for the separations⁷. The effects of asymmetrical flow profiles and variable thermal conductivity effects in TFFF have not been studied with aqueous phases. Because the theory of TFFF for aqueous mobile phases is essentially unknown, we have chosen to carry out TFFF experiments largely on an empirical basis, with emphasis on developing practical approaches for characterizing water-soluble polymers.

We have found that broad molecular-weight (MW) distributions are best characterized by programming a temperature gradient (ΔT) decreasing during separation, so that increasing MWs are successively eluted in a reasonable analysis time¹¹. A convenient programming technique involves maintaining a constant ΔT for a time τ after sample injection, after which ΔT is exponentially decreased, also with a time

constant τ . As in non-aqueous systems¹¹, this approach also produces a linear plot of $\log MW$ vs. retention that provides accurate calibration measurements over a wide MW range for water-soluble macromolecules exhibiting TFFF retention. In this time-delay exponential force-field decay TFFF method (TDE-TFFF), a plot of $\log MW$ vs. retention exhibits a linear relationship¹¹:

$$\ln M = (1/\alpha) \ln \left(\frac{2.2\beta\tau F}{V_o(\Delta T)_o} \right) + t_R/\alpha\tau \quad (2)$$

where, M is the molecular weight, $\alpha = 0.5-0.6$ for random-coil polymer conformations, $\beta = b/D_T$ (b being a constant), F is the flow-rate (ml/min), $(\Delta T)_o$ is the initial temperature difference between the hot and cold blocks, and t_R is the retention time of the eluted component. The approach described by eqn. 2 provides an accurate calibration for molecular weight distribution (MWD) measurements over a wide MW range.

Giddings and co-workers^{8,9} have shown that the broadening of FFF bands may be described by the plate-height equation:

$$H = \sigma^2/L = \frac{2D}{R\bar{u}} + \chi \frac{W^2\bar{u}}{D} + \Sigma H_i \quad (3)$$

where H is the plate height (cm); σ is the standard deviation of the peak (cm); L is the length of the channel (cm); R is the retention ratio, V_o/V_R ; \bar{u} is the mean mobile-phase velocity; χ is a dimensionless parameter; D is the diffusion coefficient; and H_i is the plate height due to other band-broadening processes. The first term of eqn. 3 describes the effects of longitudinal diffusion, the second the mass transfer (non-equilibration) effect, and the third term is the sum of the other band-broadening processes due to incomplete particle relaxation, injection problems, sample polydispersity, etc.

EXPERIMENTAL

Equipment

TFFF experiments were performed with apparatus described previously¹¹. Briefly, the TFFF separating channel was formed between two copper alloy blocks with a total length of 55.5 cm and a width of 2.54 cm. The ends of this channel were narrowed at 45° angles to produce smooth inlet and outlet flow patterns. The channel itself was formed by a 0.025-cm thick spacer, cut from Mylar® polyester film (E. I. du Pont de Nemours and Company, Wilmington, DE, U.S.A.). The desired mobile-phase flow was generated by a syringe-type displacement metering pump, and a sample loop-type injection valve was utilized to inject the sample into the channel for analysis.

Temperatures of the hot and cold blocks were measured by thermocouples inserted into small wells, drilled to within 75 μm of the chromium plating on the surface of the copper blocks that formed the faces of the separating channel. Resistance heaters (5000 W) were used to power the hot block, and the cold block was cooled by a circulating 50% ethylene glycol-water mixture from a refrigerated bath.

TABLE I
 TFFF RETENTION OF MACROMOLECULES IN AQUEOUS MOBILE PHASE
 Conditions as in Fig. 1.

Macromolecule	MW or MW range	Mobile phase	Retention
Poly(ethylene oxide)	$2 \cdot 10^4$ – $1 \cdot 10^6$	Water	Strong
Poly(ethylene glycol)	$1.2 \cdot 10^4$	Water	Strong
Sodium polystyrenesulfonate	$1.8 \cdot 10^4$ – $1.2 \cdot 10^6$	0.001 – $0.1 M$ Tris– Na_2SO_4	Weak to moderate*
Polysaccharide	$3.8 \cdot 10^5$ – $2 \cdot 10^6$	Water; $0.01 M$ Tris– $0.1 M$ Na_2SO_4	Not observed
Polyacrylamide	$5.6 \cdot 10^6$	Water	Not observed
Poly(ethylene imine)	$5 \cdot 10^4$	Water	Very weak
Poly(vinyl pyrrolidone)	$3.6 \cdot 10^5$	Water	Strong
Gelatin	$> 10^6$	Water	Not observed
γ -Globulin	$1.6 \cdot 10^5$	$0.01 M$ Tris– $0.1 M$ Na_2SO_4	Not observed
Fibrinogen	$3.4 \cdot 10^5$	$0.005 M$ Tris– Na_2SO_4 ; water–DMSO** (1:1)	Not observed
MS 2 viral RNA	$1.2 \cdot 10^6$	Water; water–DMSO (1:1)	Not observed

* Depending on polymer conformation.

** Dimethyl sulfoxide

The temperature of both blocks was maintained by temperature controllers, the set-points of which were established remotely with a microcomputer, operated by software developed in our laboratory. Components eluted from the TFFF channel were monitored by means of a spectrophotometric detector or an interferometric refractive-index (RI) detector. Output from these detectors was handled by the microcomputer with software generated in our laboratory.

Chemicals

High-performance liquid chromatographic (HPLC)-grade solvents (Fisher Scientific, Pittsburgh, PA, U.S.A.) were used throughout the study. Distilled water was passed through a Milli-Q water system (Millipore, Bedford, MA, U.S.A.) for further purification. Polymer MW standards were obtained from Polymer Laboratories (Amherst, MA, U.S.A.). Other polymer samples were secured from Aldrich (Milwaukee, WI, U.S.A.).

RESULTS AND CONCLUSIONS

Although another team⁷ has noted that certain materials are not retained in TFFF when water was used as the mobile phase, we determined in preliminary studies that some water-soluble macromolecules are actually retained under certain operating conditions. Table I summarizes the qualitative aspects of TFFF retention for a variety of macromolecules in TFFF with aqueous mobile phases. Certain water-soluble synthetic polymers, such as poly(ethylene oxides) and poly(vinyl pyrrolidone), exhibit relatively strong retention, similar to that shown by synthetic polymers in organic mobile phases. On the other hand, other macromolecules in Table I show weak to moderate retention. Several compounds, largely of biological origin, are unretained under the experimental conditions used.

Since certain macromolecules exhibit retention in pure water, the obvious question arose as to whether such retention followed the same course as the retention of polymers in organic solvents. It was equally as important to find out whether this retention could be used for analytical purposes. Accordingly, we carried out studies to document the characteristics of compounds retained by TFFF in aqueous systems.

Non-ionic macromolecules

The exploratory studies summarized in Table I indicate that certain non-ionic polymers exhibit relatively strong TFFF retention in aqueous mobile phases; quantitative data can be obtained on such material. As predicted by eqn. 2, and in keeping with previous data obtained on polymers soluble in organic solvents¹¹, TDE-TFFF experiments with poly(ethylene oxide) standards produced data that show the expected straight-line relationship between \ln MW and retention time t_R (except, as anticipated, at very low retention near the V_0 value of the channel). For the plot in Fig. 1, the smallest component (MW = 12 600) was actually a poly(ethylene glycol). This material has the same molecular structure as a poly(ethylene oxide), but is a polymer formed from a different monomer. RI detection was quite straightforward in obtaining data for this plot; excellent sensitivity and a stable baseline were observed with pure water as the mobile phase.

Fig. 2 shows the TDE-TFFF fractogram of a mixture of three poly(ethylene

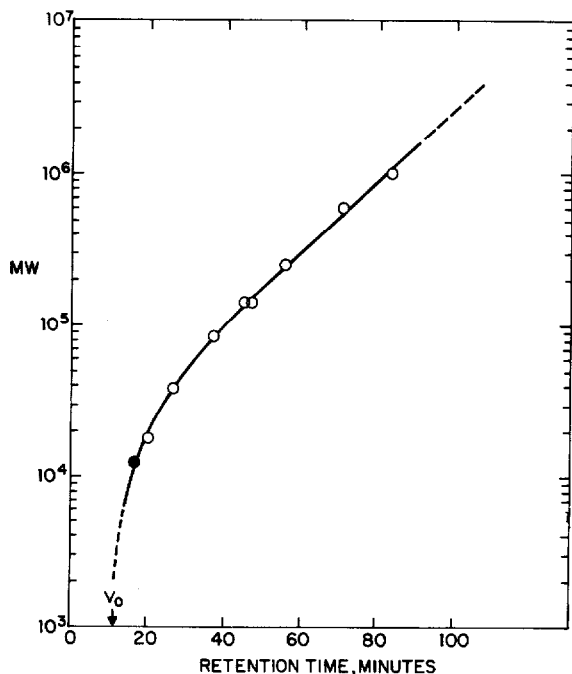


Fig. 1. Calibration plot for poly(ethylene oxide) standards by TDE-TFFF. Channel thickness, 254 μm ; mobile phase, water; flow-rate, 0.30 ml/min; initial hot block, 90°C; final hot block, 20°C; time delay constant, 20.0 min; time decay constant, 40.0 min; detector, RI; sample, 25 μl , 2 mg/ml each. (O) Poly(ethylene oxides); (●) poly(ethylene glycol).

oxide) standards in the MW range of $ca\ 4 \cdot 10^4$ to 10^6 . This separation was performed on a relatively thick channel (254 μm) for significant retention of lower-MW components. With this channel, an initial ΔT of 70°C was obtainable. Under the conditions shown in Fig. 2, the three poly(ethylene oxide) standards differing in average MW by a factor of $ca.$ 4–5, were separated essentially to baseline in a mobile phase of pure water.

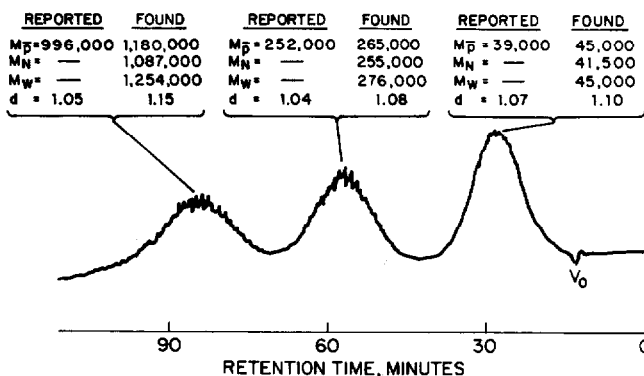


Fig. 2. TDE-TFFF fractogram of poly(ethylene oxide) standards. Conditions as for Fig. 1. $M_{\bar{p}}$ = peak molecular weight; M_N = number-average molecular weight; M_w = weight-average molecular weight; d = polydispersity, M_w/M_N .

Using the calibration of Fig. 1, we calculated the MWs for the individual poly(ethylene oxide) standards in the separation shown in Fig. 2. Values reported by the supplier of the standards agreed quite well with those calculated from the TDE-TFFF separation. We speculate that the manufacturer's values are probably slightly low, because the gel-permeation chromatography values are not corrected for instrumental band broadening. On the other hand, the MW values determined by TFFF show insignificant errors from band-broadening, because the resolution of this method is much higher.

To demonstrate the utility of the TDE-TFFF method for broad MWD water-soluble polymers, a sample of commercial poly(ethylene oxide) (Aldrich, Cat. No. 18199-4) with a reported MW of 200 000 was analyzed, as shown in Fig. 3. This sample exhibited an extremely wide MWD (polydispersity ≈ 6.3), with a calculated weight-average MW of 278 000. This value corresponds closely to the 200 000 \bar{M}_w value supplied by the manufacturer. The number-average \bar{M}_n value in Fig. 3 may be slightly in error because of a small overlap of the MWD with the V_0 peak. This error in \bar{M}_n could also affect the calculated polydispersity value. Nevertheless, it is apparent that the TDE-TFFF method is useful for characterizing water-soluble polymers of a broad MWD.

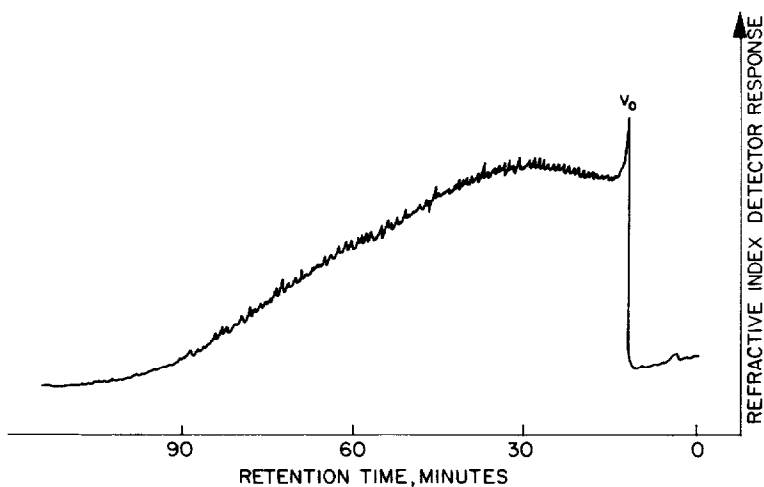


Fig. 3. Fractogram of commercial poly(ethylene oxide). Conditions as for Fig. 1, except: sample, 25 μ l, 8 mg/ml. Found: $\bar{M}_n = 43\,900$; $\bar{M}_w = 278\,000$; $\bar{M}_z = 53\,000$; $d = 6.3$.

As described by eqn. 3, the broadening of FFF bands, as described by plate height, is largely a function of mass-transfer effects. Band-broadening can be appreciable at higher mobile-phase velocities. However, the third term of eqn. 3 describes "extra-channel" band-broadening processes, including apparent broadening as a function of sample polydispersity. This term of the FFF plate-height equation can be significant in the case of a macromolecular sample of broad MWD.

To test the effect of mobile-phase velocity on apparent plate height, we performed a series of experiments using the 252 000-MW polyethylene oxide standard as a model for a narrowly dispersed sample in an aqueous mobile phase. Fig. 4 shows

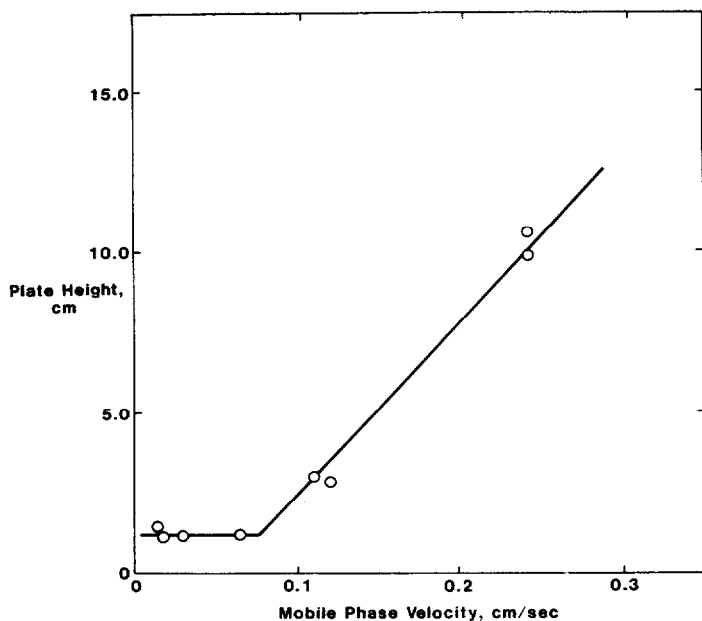


Fig. 4. Plot of plate height vs. mobile phase velocity for poly(ethylene oxide) standard. Conditions as for Fig. 1, except: mobile-phase velocity as shown; sample, 252 000-MW standard.

the plot of plate height vs. mobile-phase velocity, obtained for this sample with a constant ΔT of 60°C. As predicted by eqn. 3, the plate height for this polymer standard decreases with decreasing mobile-phase velocity, because of improved mass transfer. However, at velocities of *ca.* ≤ 0.07 cm/s, the apparent plate height for this polymer is relatively constant. This suggests that the contribution to apparent plate height at lower mobile-phase velocities is largely a function of sample polydispersity effects; band-broadening is dominated by partial fractionation of the individual components of this polymer standard, which shows a polydispersity of 1.08 by TFFF.

We conclude from this plate-height study that optimum resolution of components for this system will be obtained at mobile-phase velocities of *ca.* ≤ 0.07 cm/s. Under these conditions, corrections for instrumental band-broadening would be insignificant; best MW accuracy would be obtained.

In keeping with reports on polymers soluble in organic solvents⁸, retention ratio (*R*) values should remain constant for the water-soluble poly(ethylene oxide) when the velocity of the aqueous mobile phase is varied. Fig. 5 shows the effect of mobile-phase velocity on the *R* value of a 252 000-MW poly(ethylene oxide) standard. For this study, no relaxation or equilibrium time was used after injection of the sample into the channel.

These studies suggest that compounds retained by TFFF in aqueous mobile phases show the same retention characteristics as for separations in organic mobile phases.

As indicated in Table I, other non-ionic water-soluble polymers also exhibit significant retention in pure water. For example, Fig. 6 shows the TDE-TFFF sep-

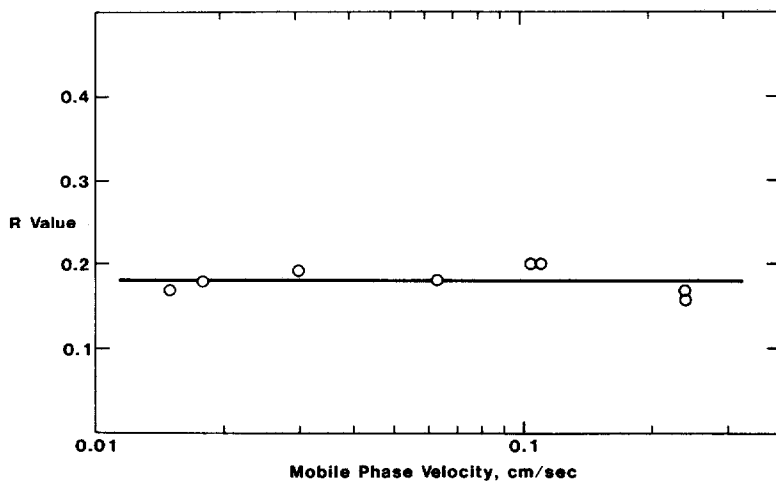


Fig. 5. Effect of mobile-phase velocity on retention ratio R . Conditions as for Fig. 4.

aration of a commercial poly(vinyl pyrrolidone). Quantitative characterization of this polymer was not attempted, since appropriate narrow MWD standards were not available. Nevertheless, it is apparent that such materials could be readily characterized by TFFF, with water as the mobile phase, by using the same techniques as those described earlier for poly(ethylene oxides).

Ionic macromolecules

Preliminary studies (Table I) showed that sodium polystyrenesulfonate (SPS) standards were retained in aqueous media; however, retention at high ionic strength was slight. In such media, SPS is known to exist in a random-coil state¹². In this condition, the polymer diffusion coefficient is sufficient to resist effectively the thermal diffusion force-field, so that retention is poor.

On the other hand, we believed that by operating at lower ionic strengths, this ionic polymer could be made to "unfold" to a more rod-like conformation. In this state, increased retention should be obtained, since the diffusion coefficients of the

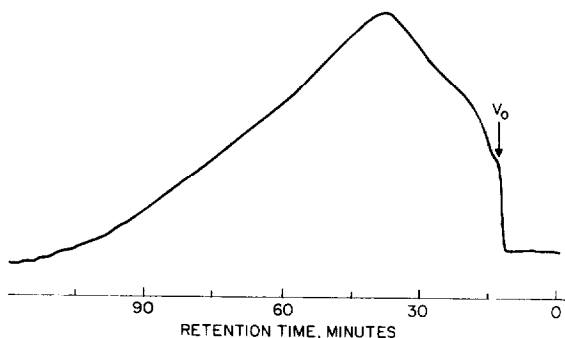


Fig. 6. Fractogram of commercial poly(vinyl pyrrolidone). Conditions as for Fig. 1, except: sample, 25 μ l, 5 mg/ml.

unfolded structure should be smaller relative to that of the random-coil. At comparable TFFF force-fields, this poorly diffusing molecule then would be less able to resist the force-field across the channel; it would be forced closer to the channel wall, remain in slower flow streams, and be eluted later.

Although, as anticipated, the retention of SPS standards can be significantly increased by carrying out TFFF with mobile phases of very low ionic strength, unfolding of SPS standards in such media appears to be variable. Reproducibility of retention data is also relatively poor, as indicated in Fig. 7. Large variability in the retention of higher-MW SPS standards was observed. This suggests that the folding/unfolding process is either very slow or irreproducible at very low buffer concentrations (e.g. 2 mM of both Tris and sodium sulfate). Alternatively, it is also possible that electrostatic repulsions of the ionic polymer at very low buffer concentrations strongly affect the osmotic pressure and inhibit the establishment of a reproducible concentration within the channel¹³. Data for lower-MW SPS standards are more reproducible, indicating that the unfolding process is less inhibited.

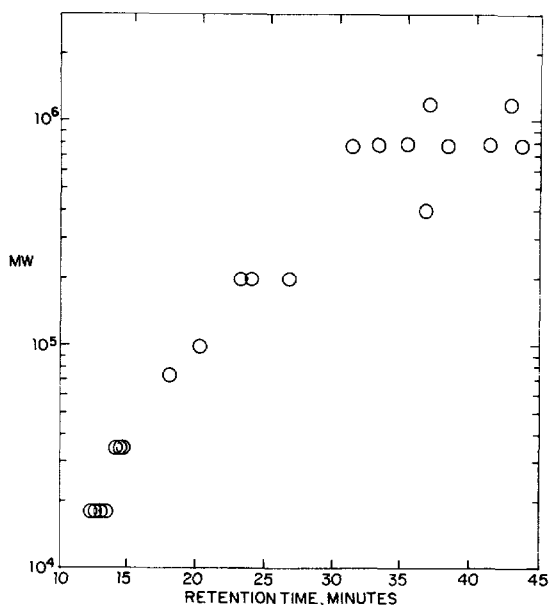


Fig. 7. Calibration plot of sodium polystyrenesulfonate. Conditions as for Fig. 1, except: mobile phase, 2 mM Tris-Na₂SO₄; detector, UV; sample, 25 μ l, 1 mg/ml each.

The general trend of the data in Fig. 7 fits the TDE-TFFF theory; the retention time vs. \ln MW relationship is approximately linear, except with low-MW standards that are eluted near V_0 . SPS standards fractionated in low-ionic-strength mobile phases also exhibit much broader peaks than those in higher-ionic-strength systems. This effect is in keeping with the very low diffusion coefficients of the unfolded molecules, resulting in significant limitations on mass transfer.

At higher buffer concentrations, the reproducibility of data for the SPS standards is greatly improved; the molecular unfolding process is much more reproduc-

ible. The TDE-TFFF data in Fig. 8, obtained with an aqueous mobile phase of 5 mM of both Tris and sodium sulfate, illustrate this phenomenon. Again, the calibration plot of $\ln MW$ vs. retention time is linear. As expected, retention and the slope of the calibration plot are lower at higher ionic strength, suggesting that these molecules are less "unfolded"—more tightly coiled—than at the 2 mM level.

Thus, as previously predicted¹³, TFFF of charged ionic molecules can be successful if enough salt is used in the mobile phase to swamp the charges and inhibit unwanted molecular repulsion. However, the concentration should be maintained at a minimum to keep the molecules in an "unfolded" state for maximum retention.

The data in Fig. 8 also show the effect of changing the time-delay/decay constant (τ) on the MW calibration plot. As anticipated from eqn. 2, the data for the smaller τ values rise more steeply, indicating less resolution with the shorter analysis time. Thus, resolution in TDE-TFFF is easily controlled by changing τ for the separation. Some of the deviation from a linear plot in Fig. 8 may be due to slightly inaccurate MW values for the SDS standards.

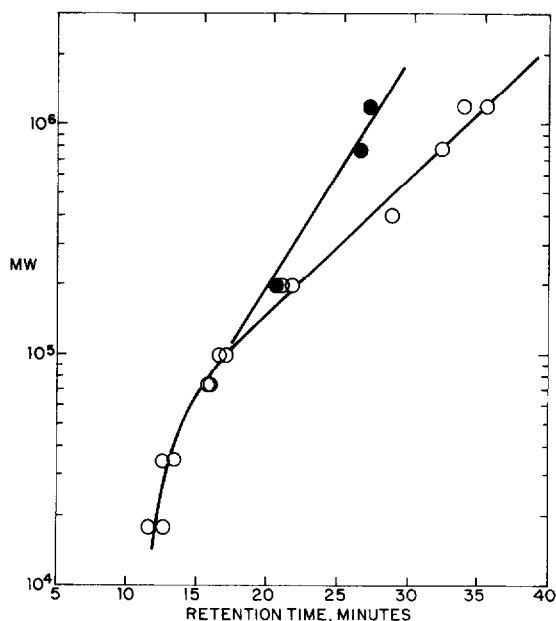


Fig. 8. Calibration plot of sodium polystyrenesulfonate. Conditions as for Fig. 7, except: mobile phase, 5 mM Tris- Na_2SO_4 ; open circles: time delay constant, 20 min; time decay constant, 40.0 min; solid circles: time delay constant, 15.0 min; time decay constant, 30 min.

The 5 mM Tris-sodium sulfate mobile phase could be used to obtain quantitative information on SPS, if desired. Fig. 9 shows the separation of a two-component mixture of SPS standards. Under the separating conditions used here, the 18 000-MW standard overlaps V_0 , but the 400 000-MW SPS standard is well resolved. This higher-MW SPS standard displays a fairly broad peak. It is not known whether this is a function of MWD or a reflection of the relatively poor mass transfer associated with the extended, rod-like molecule.

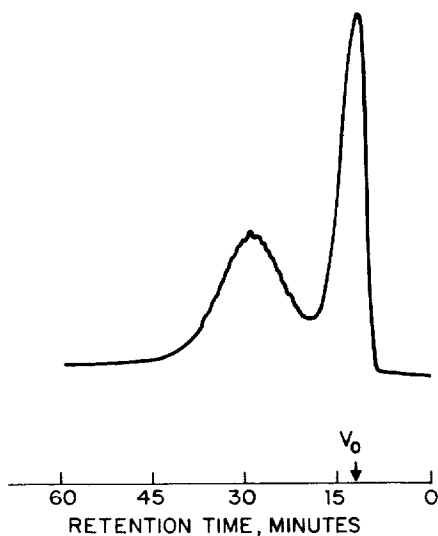


Fig. 9. Separation of sodium polystyrenesulfonate standards. Conditions as for Fig. 8, except: time decay constant, 40.0 min; sample, 25 μ l, 2 mg/ml of 18 000-MW standard and 4 mg/ml of 400 000-MW standard.

CONCLUSIONS

Certain water-soluble macromolecules can be fractionated in totally aqueous systems; others show little or no retention. Limited data suggest that TFFF retention in aqueous mobile phases is greater when the macromolecule has limited or poor solubility in water. Highly-water-soluble compounds, such as polysaccharides and high-MW proteins, show no retention under the conditions studied. Certain water-soluble ionic polymers exhibit sufficient retention for practical characterization when the conformation of the molecule can be adjusted to an open or rod-like structure by working at relatively low ionic strength. The addition of organic solvents to the aqueous mobile phase may also increase TFFF retention of certain macromolecules.

The influence of TFFF operating parameters appears to be the same in both organic and aqueous mobile phases. However, retention in aqueous systems is generally poorer, presumably because of the poorer thermal diffusion coefficients of compounds. Therefore, for useful separations in aqueous mobile phases, equipment should generate relatively large temperature differences between the channel faces to enable sufficient retention. In fortunate situations, aqueous TFFF fractionations can be successfully performed without significant changes in equipment or operating conditions. The opportunity for useful fractionations of certain water-soluble polymers in totally aqueous systems significantly expands the utility of TFFF.

REFERENCES

- 1 G. H. Thompson, M. N. Myers and J. C. Giddings, *Sep. Sci.*, 2 (1967) 797.
- 2 J. C. Giddings, M. Martin and M. N. Myers, *J. Chromatogr.*, 158 (1978) 419.
- 3 J. C. Giddings, M. N. Myers and J. Janča, *J. Chromatogr.*, 186 (1979) 37.

- 4 M. Martin and R. Reynand, *Anal. Chem.*, 52 (1980) 2293.
- 5 J. Janca and K. Kleparnik, *Sep. Sci. Technol.*, 16 (1981) 657.
- 6 G. H. Thompson, M. N. Myers and J. C. Giddings, *Anal. Chem.*, 41 (1969) 1219.
- 7 M. N. Myers, K. D. Caldwell and J. C. Giddings, *Sep. Sci.*, 9 (1974) 47.
- 8 M. E. Hovingh, G. H. Thompson and J. C. Giddings, *Anal. Chem.*, 42 (1970) 195.
- 9 J. C. Giddings, M. N. Myers, K. D. Caldwell and S. R. Fisher, *Methods Biochem. Anal.*, 26 (1980) 79.
- 10 G. Westermann-Clark, *Sep. Sci. Technol.*, 13 (1978) 819.
- 11 J. J. Kirkland and W. W. Yau, *Macromolecules*, (1986) in press.
- 12 A. Takahashi, T. Kato and M. Negasawa, *J. Phys. Chem.*, 71 (1967) 2001.
- 13 F. Brochard-Wyart, *Macromolecules*, 16 (1983) 149.