Notes

A New Synthesis of Fluorescein Isothiocyanate Labeled Poly(styrenesulfonate sodium salt)

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Introduction

There has been increasing interest surrounding the use of fluorescent probes to characterize macromolecules[1,2] and macromolecular complexes[3,4] with the fluorescence photobleaching recovery technique (FPR). The measured transport property, which is the optical tracer self-diffusion of the labeled molecules, can be derived from the recovery rate of the fluorescence signal after bleaching. For multicomponent systems, the high selectivity of FPR allows the observation of one tagged macromolecule in the presence of others. The most important prerequisites are the ease and permanence of photobleaching. Fluorescein and its derivatives are often chosen thanks to their high rate of photobleaching[5] and an excitation maximum at ∼494 nm that closely matches the 488 nm spectral line of the argon ion laser.

Pseudolinear and regularly branched water-soluble macromolecules are commercially available in fluorescently labeled form; these include dextrans and poly(amidoamine) dendrimers, respectively. The same is not true for linear, strong polyelectrolytes, the most commonly studied example being poly(styrenesulfonate sodium salt) (NaPSS). Controversy surrounds its reported "extraordinary behavior" at low ionic strength.[6−8] Scattering studies suggest the formation of "temporal aggregates". As an independent technique, FPR is expected to provide crucial information about transport behavior of the molecule and any aggregates on time and distance scales that differ from those probed by scattering. Other applications for fluorescent NaPSS may include permeability probes and formation of polymer multilayers through sequential deposition schemes. Layer-by-layer alteration of fluorescent and nonfluorescent materials may produce useful optical effects. The study of polyelectrolyte−colloid interactions is sometimes aided by fluorescent labeling.[1]

NaPSS lacks highly reactive functional groups that would permit the direct attachment of fluorescein or its derivatives. A two-step reaction, chlorination of sulfonate group in a $PCl_3/POCl_3$ mixture[9] or pure[1] $POCl_3$, followed by the attachment of fluoresceinamine, was developed in the 1990s. The harsh labeling conditions, 105 °C for more than 24 h, may lead to chain scission. Because of the poor solubility of NaPSS in $PCl_3/POCl_3$ and $POCl_3$, this heterogeneous reaction may result in uneven chlorination, which ultimately causes an uneven distribution of labeling. That may give rise to hydrophobic patches along the NaPSS chains, leading to unwanted associations in aqueous environments. A more important problem may be the incomplete degree of sulfonation in the starting NaPSS material.[10,11] The commercially available, narrowly distributed NaPSS samples, widely used as standards for aqueous GPC, are made via sulfonation of anionically polymerized polystyrene. The degree of sulfonation ranges from 85% to about 99%. It has been debated whether unsulfonated, hydrophobic patches contribute to the formation of "temporal aggregates" of NaPSS in solution.

We present an efficient synthesis of fluorescein isothiocyanate (FITC) labeled NaPSS under mild conditions. Starting from monomeric styrenesulfonate, this new approach takes advantage of the low reactivity of allylamine, giving a lightly labeled product well suited to FPR measurements. Low polydispersity is realized automatically through the use of a solvent/nonsolvent pair or through analytical scale gel permeation chromatography, depending on molecular weight. The amount of dye on the polymers is estimated from absorption measurements, and the effect of dye on polymer conformation is tested by GPC with online multiangle laser light scattering detection, GPC/MALLS. The fluorescence spectra of FITC labeled NaPSS are investigated at various salt concentrations. Rapid and simple preparation of a series of almost monodisperse, fluorescently labeled NaPSS lacking hydrophobic patches is demonstrated, as is the suitability of those polymers for FPR experiments.

Results

Fluorescein isothiocyanate (FITC) labeled poly(allylamine-*co*-styrenesulfonate sodium salt) was synthesized by the copolymerization of allylamine with 4-styrenesulfonic sodium salt, followed by attaching FITC to the amine group.

Figure 1 shows the ^1H NMR spectrum of FITC labeled poly(allylamine-*co*-styrenesulfonate sodium salt) in D_2O at 25 °C. The peaks around 1−2 and 6−8 ppm correspond to the backbone and aromatic ring of NaPSS, respectively. The other four sharp signals correspond to residual HDO, DMSO, methanol and acetone, as confirmed in the lower trace of Figure 1. The integral ratio of the peak at 1−2 ppm to the peak at 6−8 ppm gives 0.76, which is in good agreement with the expectation value for NaPSS, 0.75. The amount of allylamine incorporated is so low as to defy precise characterization, despite the use of significant amounts during synthesis (Table 1). The reason for the large amount of volatile allylamine in the feed was to provide enough for the

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Table 1. Synthesis Conditions for Poly(allylamine-*co*-styrenesulfonate sodium salt) and Characterization by GPC/MALLS and FPR

product (L = labeled)	allylamine (mL)	4-styrenesulfonate sodium salt(g)	methanol/ DMSO (g/g)	AIBN (mg)	T (°C)	M_w[a]	PDI M_w/M_n	$D/10^{-7}$ cm^2 s^{-1} [a]
1	5.0	4.0	20/45	10	75	39 000 ± 3000	1.2	
2	5.0	4.0	20/45	9.4	60	37 000 ± 1000	1.2	
L3	5.0	4.2	30/50	9.2	55	28 000 ± 2000	1.2	5.78 ± 0.02
4	15	12.1	15/130	2.6	55	185 000 ± 9000	1.5	
L4						228 000 ± 5500	1.45	
L4-GPC fraction 2						320 000[b]	1.04 ± 0.03	1.83 ± 0.16
L4-GPC fraction 3						178 000[b]	1.03 ± 0.02	2.56 ± 0.23
L4-GPC fraction 4						114 000[b]	1.01 ± 0.03	3.65 ± 0.43
L4-GPC fraction 5						89 900[b]	1.00 ± 0.03	4.06 ± 0.48
L4-GPC fraction 6						72 100[b]	1.01 ± 0.03	4.41 ± 0.083
L4-GPC fraction 7						53 500[b]	1.00 ± 0.04	5.20 ± 0.41

[a] Standard deviation was estimated from three repeat measurements. [b] Error estimated as ±5%.

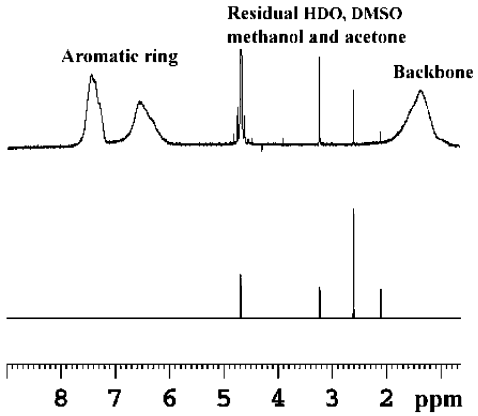

Figure 1. Top: ^{1}H NMR spectrum of FITC labeled poly(allylamine-*co*-styrenesulfonate sodium salt) in D_2O. Bottom: ^{1}H NMR spectrum of mixed DMSO, acetone, and methanol in D_2O.

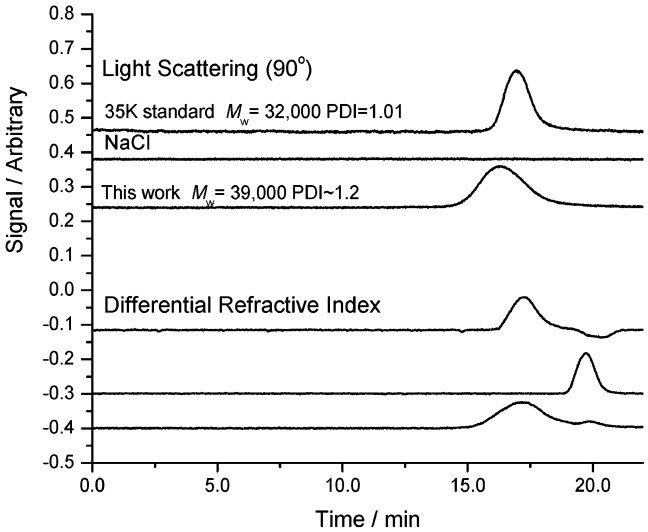

Figure 2. GPC/MALLS traces, offset vertically for clarity. Positive signals show scattering intensities at 90° scattering angle. From top to bottom: commercially available NaPSS standard, 24 μM NaCl, and product 1. Negative signals show the respective differential refractive index detector responses.

entire course of polymerization at elevated temperatures. An upper bound can be estimated on the basis of the integral ratio just given: the amount of allylamine in poly(allylamine-*co*-styrenesulfonate sodium salt) is estimated to be less than 2 mol %. No ^{1}H peaks from allylamine or FITC appeared in the ^{1}H NMR spectrum because of the low content of these groups. The percentage of sulfonated styrene monomers in FITC labeled poly(allyamine-*co*-styrenesulfonate sodium salt) must be at least 98%, which exceeds the degree of sulfonation achieved in most commercially available standard NaPSS samples even before the chlorination that precedes conventional labeling. The low reactivity of allylamine in free radical polymerization is well-known. Only two papers discuss copolymerization of allylamine with ethylenically unsaturated hydrocarbons.[12,13] The reason is that allylamine radicals participate in chain transfer rather than free radical propagation. The low content of allylamine in poly(allylamine-*co*-styrenesulfonate) is beneficial for fluorescent labeling, as it minimizes perturbations to the chain conformation.

Table 1 lists the characterization results for FITC labeled poly(allylamine-*co*-styrenesulfonate sodium salt). The first two entries, which are repeat syntheses, demonstrate good reproducibility. At these low M's, the polydispersity index M_w/M_n was 1.1−1.2, much lower than theoretically expected for free radical polymerization (≤2.0) but worse than anionically polymerized polystyrene samples,[14] as demonstrated in the GPC/MALLS traces of Figure 2. (An injection of 24 μM NaCl is also included to show that the small "blips" in the differential refractive index responses trace to salt.)

Our experimental condition for the synthesis specifically combines polymerization with fractionation. Methanol is a poor solvent for NaPSS, while DMSO is a good solvent. In methanol/DMSO mixed solvent, the polymer chains that have grown to a certain length will precipitate, terminating the polymerization. Short chains remain in the supernatant. By varying temperature and the MeOH:DMSO mixing ratio, the molecular weight of poly(allylamine-*co*-styrenesulfonate sodium salt) was found to be controllable (compare entries 1 and 2 with entry 3 in Table 1). Conditions that favor solubility can result in high mass and increased polydispersity, as demonstrated by products 4 and L4 (the latter being somewhat higher in average molar mass because rinsing steps to remove unreacted dye removed some low-M polymers, not because of the few dye groups attached).

The successful attachment of FITC to poly(allylamine-*co*-styrenesulfonate sodium salt) was obvious; no amount of rinsing would remove the yellowish color. Additionally, it was readily apparent in the FPR device that the diffusion was substantially less than that of free dye;[15] indeed, FPR proved useful for following the removal of unattached dye (and associated fast recovery mode) with chromatographic or centrifugal dialysis operations. Figure 3 shows the fluorescence spectra of the collected effluent from Sephadex G-25M PD-10 column (Amer-

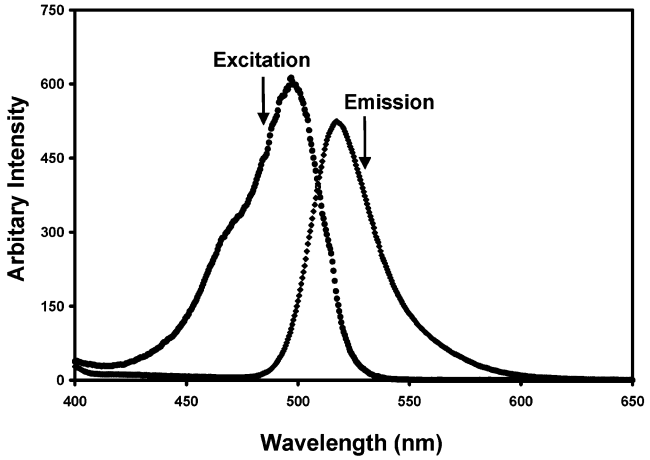

Figure 3. Fluorescence spectra for FITC labeled poly(styrenesulfonate sodium salt). The sample was collected from the effluent of PD-10 column in 50 mM NaCl. Emission wavelength: 515 nm; excitation wavelength: 350 nm.

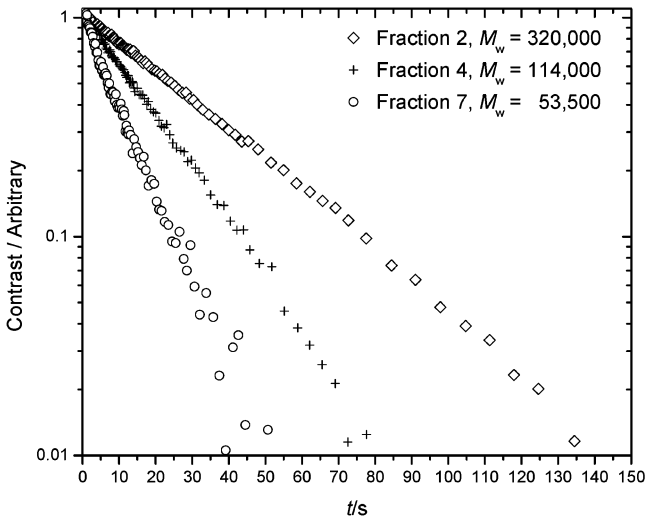

Figure 4. Semilogarithmic FPR traces for three fractions collected following a single injection of 1.2 mg of the FITC labeled product L4 into an analytical scale GPC/MALLS (running pH 7.5 buffer as mobile phase). Fractions were adjusted to mildly alkaline pH prior to FPR. Concentrations were essentially as eluted (50−150 μg/mL).

sham Pharmacia Inc.) at pH 9.5. They resemble those recorded[16] for fluorescein at pH 9, with the maximum excitation and emission wavelength at 499 and 520 nm, respectively. The fluorescence of FITC labeled poly(allylamine-*co*-styrenesulfonate sodium salt) depends on the amount of added salt and pH, which is also true for fluoresceinamine labeled NaPSS (work in progress). A hypsochromic shift and spectral broadening were observed with decreased ionic strength. A small ground-state interaction between the aromatic rings of the dye and styrenesulfonate groups is proposed. Although this hypothesis requires further study, the putative interaction does not prevent FPR measurements.

The level of dye labeling was estimated by comparing the absorbance of polymer solutions to solutions of fluorescein in water at pH 9.6 (where fluorescein is sparingly soluble). The comparison suggests about one FITC group attached per thousand styrenesulfonate monomers. For most molecular weights, most of the polymers will be singly tagged or untagged; thus, intramolecular associations between the dye units are unlikely to alter the conformation. To test this, the radius of gyration, R_g, was measured as a function of M from GPC/MALLS runs on unlabeled and labeled NaPSS from the polydisperse, high-M products 4 and L4. As the GPC/MALLS instrument uses a long wavelength (632.8 nm) laser, only large molecules from the high-M side of the distribution could be sized, and then with less precision than desired. For these small polymers, R_g vs M results may depend sensitively on peak selection and interdetector delay alignment. The impression from several repeat runs is that the labeled and unlabeled materials adopt very similar molecular conformations under the conditions in the GPC during these runs (pH 7.5 buffer).

Product L4 was separated by analytical scale GPC. Eleven fractions were collected at 1 mL intervals after a single injection of 1.2 mg (1.2% solution × 0.1 mL). Dilution in the GPC lowered the concentration to the point where no fraction appeared colored, although each was faintly fluorescent when illuminated with blue laser light. This confirms that reactive allylamine groups are present across the entire molecular weight range (ca. 40 000−600 000, although not all fractions appear in Table 1).

An attribute of FPR is that a useful number of experiments can be conducted on minute quantities of fluorescent polymer. To demonstrate this in a preliminary way for FITC labeled poly(allylamine-*co*-styrenesulfonate sodium salt), each fraction prepared by GPC/MALLS was adjusted to mildly alkaline pH by addition of trace NaOH. This facilitates photobleaching (Cong and Russo, work in progress) and eliminates any possibility that allylamine groups not covered with dye could acquire a positive charge and backbite negatively charged regions of the chain. Acceptable FPR results were obtained, despite the light labeling, very low concentrations (50−150 μg/mL according to the differential refractive index detector of the GPC/MALLS), and small number of dye groups photobleached to effect measurement (5%−10%). Each semilogarithmic FPR trace appearing in Figure 4 displays the nearly single-exponential behavior expected of a monodisperse preparation. Hundreds of such measurements would have been possible from the 1 mL fractions collected. The fractions eluting first exhibited the slowest diffusion, as expected. It is straightforward in the GPC/MALLS software to subdivide the one, broad peak into regions that correspond to the fractions collected. The molecular weight analysis of each region is reliable, even though the R_g results are less so, as already discussed. In the wings of the distribution, low concentration, diminished column resolution, and the "salt peak" seen at about 20 min in Figure 2 corrupt the molecular weight determinations; these fractions were not used. Fractions taken from the middle part of the broad distribution had M_w/M_n values about as low as found for anionically polymerized materials. Diffusion is plotted against molecular weight in Figure 5.

The uncertainty in D (8% on average) is higher than the several percent we have come to expect of FPR in aqueous systems due to the very low concentrations. The range of M values explored is not very wide, but the observed power law slope of about −3/5 does meet the expectation for a screened polyelectrolyte chain; i.e., the polymer behaves as a random coil in a good solvent in this buffer (200 mM $NaNO_3$ + 10 mM NaH_2PO_4 + 2 mM NaN_3 adjusted to pH 7.5).

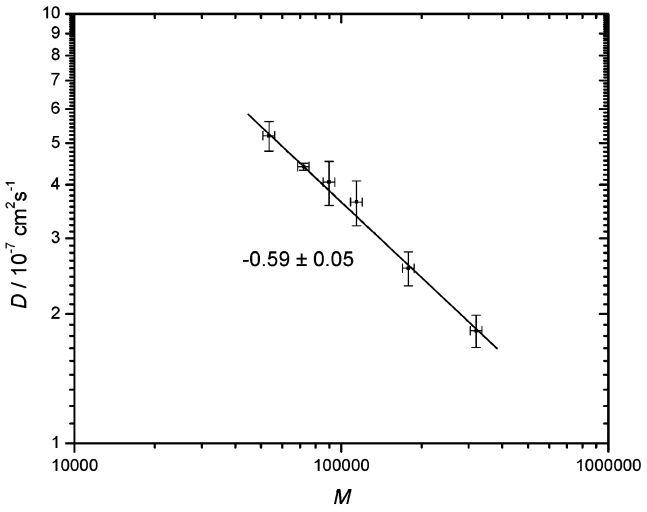

Figure 5. Diffusion of LNaPSS fractions obtained from a single analytical scale GPC injection. Error estimates for D were taken from three FPR measurements; the M errors are estimated as ±5%. The power law slope over the limited range of M available is indicated.

Conclusions

A convenient synthesis of FITC labeled poly(allylamine-*co*-styrenesulfonate sodium salt) has been demonstrated. The interplay between polymerization and fractionation in mixtures of good and poor solvents results, at sufficiently low molecular weights, in polydispersities only slightly worse than anionically polymerized samples and certainly acceptable for many studies. High-M material was more polydisperse but easily separated into narrowly distributed fractions by analytical scale GPC in quantities sufficient for FPR investigations and other fluorescence techniques. The approach developed takes advantage of the low reactivity of allylamine in free radical polymerization and starts with sulfonated monomer, yielding a labeled product that is more than 98% sulfonated. Any remaining groups are allylamine or FITC labeled allylamine. The dye content cannot exceed 0.2 mol % of monomer and is likely less than that judging from the large amount of unreacted dye removed and direct estimation by visible absorption spectroscopy. GPC/MALLS measurements before and after dye substitution show no strong perturbation of the structure. The dye content is sufficient for FPR studies, even at very low concentrations, and the polymers exhibit the expected scaling exponent for diffusion vs mass. A word of caution is in order. Allylamine sites not covered by FITC are less hydrophobic than the unsulfonated monomer repeat units always present in conventionally produced NaPSS, but they could acquire a positive charge at low pH. If the pH is still high enough that the styrenesulfonate residues are negatively charged, intrachain interactions could result. The consequences are beyond the scope of this article, but physicochemical investigations using materials prepared as described herein should probably be pursued only in alkaline solution.

Experimental Section

Materials. Allylamine, fluorescein isothiocyanate isomer I (FITC), 2,2′-azobis(isobutyronitrile) (AIBN), D_2O, and 4-styrenesulfonic sodium salt were bought from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) and methanol were bought from Fisher Scientific. All chemicals were used without further purification.

Synthesis of Poly(allylamine-*co*-styrenesulfonate sodium salt). 4-Styrenesulfonic sodium salt, allylamine, and AIBN were directly added into a mixture of DMSO/methanol. The mixture was stirred under argon for 30 min until 4-styrenesulfonic sodium salt completely dissolved. The temperature was gradually raised to start polymerization. The reaction was terminated by leaking air into the system. Poly(allylamine-*co*-styrenesulfonate sodium salt) did not dissolve in the mixed DMSO/methanol, forming a white precipitate, which was easily separated from the supernatant by decanting. The product was purified at least three times by dissolving in DMSO and then precipitating with methanol. Eliminating as much residual allylamine as possible facilitated subsequent separation in labeling. The product was further washed with methanol and acetone and then oven-dried under vacuum. The recipes tried are shown in Table 1. A conversion of ∼50%, calculated from 4-styrenesulfonic sodium salt, was obtained due to an early termination of polymerization.

Covalent Attachment of FITC onto Poly(allylamine-*co*-styrenesulfonate sodium salt). 0.2 g of the product was dissolved in 4.2 g of Nanopure water (Barnstead, Inc.). 0.9 mg of FITC was dissolved in 2 mL of methanol. The ratio of FITC: styrenesulfonate monomeric unit was 2:1000 (mole:mole); the dye content cannot exceed 0.2%. The two solutions were mixed and kept in the dark for 24 h. After labeling, most of methanol was evaporated by purging filtered N_2. Unreacted dye was separated from labeled polymer by passing through a prepacked PD-10 column containing Sephadex G-25M (Amersham Pharmacia). The washing solution was 50 mM NaCl at pH 9.5. The effluent from the column is suitable for fluorescence and FPR measurements. The samples can also be cleaned from unreacted dye by precipitation in acetone or by using centrifugal concentrator units, such as Millipore Centricon YM-3 with a 3000 molecular weight cutoff (for globular proteins). In the latter procedure, several milliliters of solution was concentrated and then rinsed with NaOH (pH 9.6). The filtrate collected in the lower chamber of the concentrator was at first vividly fluorescent but after several passes became clear. The rententate initially had a fast FPR decay process with a diffusion coefficient similar to that of free dye. This disappeared after several washes.

Characterization. Molecular Weight Distribution. The molecular weight was obtained by GPC/MALLS using a Waters 401 differential refractive index detector and a Wyatt DSP light scattering detector (up to 16 different angles, wavelength 632.8 nm). The specific refractive index increment, dn/dc, was taken as 0.198 mL/mg.[17] The GPC columns were PL Aquagel-OH mixed 8 μm and PL Aquagel-OH-50 8 μm (Polymer Laboratories). Samples were dissolved in the mobile phase, either unbuffered 50 mM NaCl (products 1, 2, and L3) or 200 mM $NaNO_3$ + 10 mM NaH_2PO_4 + 2 mM NaN_3, adjusted to pH 7.5 (products 4 and L4). The injection volume was 0.10 mL, and the flow rate was 1.0 mL/min. The weight-average molecular weight and its standard deviation were calculated from three or more repeat measurements. In estimating R_g, a random coil model was selected in the Wyatt Astra software.

^{1}H NMR. ^{1}H NMR spectra were acquired on a Bruker ARX300 spectrometer at 25 °C with a 90° pulse of 6.95 μs. The amount of 8.57 mg product was dissolved in D_2O 48 h before NMR measurement.

Fluorescence Spectroscopy. Fluorescence spectra were recorded with Perkin-Elmer luminescence spectrophotometer LIS 50B. A quartz cuvette with 1 cm path length was used. 5.8 mg/mL FITC labeled poly(allylamine-*co*-styrenesulfonate sodium salt) in 50 mM NaCl was run through a PD-10 column. The first bright yellow effluent was collected for fluorescence spectroscopy.

Absorption Spectroscopy. Visible absorption measurements were made on a Hewlett-Packard HP 845x UV−vis spectrograph in 1 cm plastic cells. A Beer's law calibration was established for fluorescein in pH 9.6 NaOH at 496 nm and used to estimate the dye content in labeled NaPSS.

FPR Measurement. The FPR apparatus has been described.[18] A striped pattern was created by illuminating a coarse diffraction grating (Ronchi ruling) held in the rear

image plane of a standard epifluorescence microscope with an intense, brief laser flash. With the laser intensity reduced by a factor of about 2000, an electromechanical modulation detector system monitors the subsequent disappearance of the pattern due to exchange of molecules that were bleached (i.e., in the bright regions during the flash) and those that were not (i.e., molecules that were not illuminated during the flash). The contrast signal (ac amplitude) from the modulation detector decays exponentially:

$$C(t) = \exp(-K^2Dt) \quad (1)$$

where the spatial frequency of the grating is $K = 2\pi/L$, with L representing the distance between stripes in the Ronchi ruling, and D is the optical tracer self-diffusion coefficient. Measurements were conducted at five different K values to ensure the absence of nondiffusive signal recovery, which would result in finite recovery rates even at $K = 0$. Subsequently, most runs were performed in triplicate at a single K value, 405.3 cm^{-1}.

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References and Notes

(1) Sohn, S.; Russo, P.; Davila, A.; Poche, D. S.; McLaughlin, M. L. *J. Colloid Interface Sci.* **1996**, *177*, 31.
(2) Zero, K.; Ware, B. R. *J. Chem. Phys.* **1984**, *80*, 1610.
(3) Cheng, Y.; Prud'homme, R. K. *Macromolecules* **2002**, *35*, 8111.
(4) Cong, R.; Pelton, R.; Russo, P.; Doucet, G. *Macromolecules* **2003**, *36*, 204.
(5) Song, L.; Hennink, E. J.; Young, I. T.; Tanke, H. J. *Biophys. J.* **1995**, *68*, 2588.
(6) Sedlak, M. *Langmuir* **1999**, *15*, 4045.
(7) Sehgal, A.; Seery, T. A. *Macromolecules* **1998**, *31*, 7340.
(8) Schmitz, K. S. *Macro-ion Characterization form Dilute Solutions to Complex Fluids*; ACS Symposium Series; American Chemical Society: Washington, DC, 1993.
(9) Sehgal, A. Ph.D. Dissertation, University of Connecticut, 2000.
(10) Brighton, C. A. *Styrene Polymers: Technology and Environmental Aspects*; Applied Science Publishers: London, 1979.
(11) Brown, D. W.; Lowry, R. E. *J. Polym. Sci., Polym. Chem. Ed.* **1979**, *17*, 1039.
(12) Declin, P. A. US Patent 3057833, 1962.
(13) Shcherbina, F. F.; Fodorova, I. P.; Goriov, Y. I. *Vysokomol. Soedin., Ser. A* **1970**, *12*, 2042.
(14) Lee, W.; Lee, H.; Cha, J.; Chang, T.; Hanley, K. J.; Lodge, T. P. *Macromolecules* **2000**, *33*, 5111.
(15) Bu, Z.; Russo, P. *Macromolecules* **1994**, *27*, 1187.
(16) Molecular Probe, Inc. *Handbook of Fluorescent Probes and Research Products*; Eugene, OR, Chapter 6.
(17) Borochov, N.; Eisenberg, H. *Macromolecules* **1994**, *27*, 1440.
(18) Fong, B.; Stryjewski, W.; Russo, P. *J. Colloid Interface Sci.* **2001**, *239*, 374.

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