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Determination of Molecular Weights in Polyelectrolyte Mixtures Using Polymer Translocation through a Protein Nanopore

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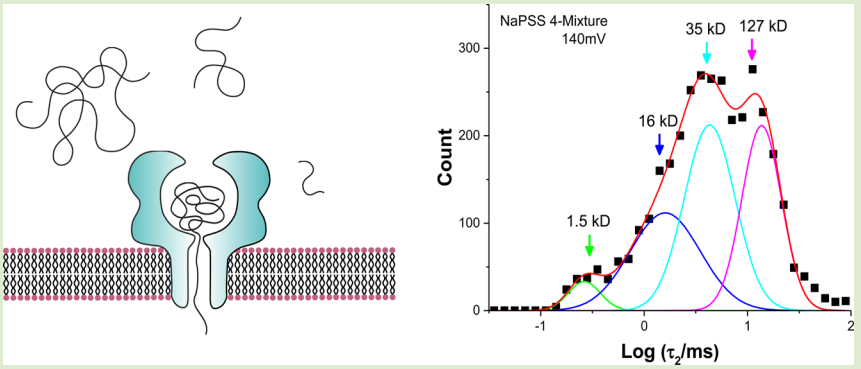
Supporting Information



ABSTRACT: We introduce a single molecular analysis technique for the evaluation of molecular weight distributions of polyelectrolyte solutions by measuring translocation times of sodium polystyrenesulfonate (NaPSS) chains in a mixture passing through an α-hemolysin protein nanopore. The ionic current through an α-hemolysin nanopore is partially blocked transiently when the pore is occupied by a polymer chain with an average residence time proportional to the molecular weight of the polymer chain. We have measured the translocation times for an equimolar mixture of four different molecular weight NaPSS standards and observed distinct translocation time distribution peaks, each of which corresponding to the different components in the mixture. Size exclusion chromatography analyses were performed on the equimolar and equiweight NaPSS mixtures of the same components and compared with the translocation time measurements. The experimental results demonstrate that measuring translocation times can be a competitive technique for estimating the broad molecular weight distributions of polyelectrolytes.

The molecular weight of synthetic polymers has distributions rather than being a single value. Among many techniques for determining molecular weights of macromolecules, size-exclusion chromatography (SEC) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry are the two most common. Application of these techniques for characterizing molecular weights of polyelectrolytes is faced with many difficulties, as well-documented in the literature.[1−8] For mixtures of polyelectrolytes, it has been reported that the determination of broad molecular weight distributions is challenging due to interference among molecules from different molecular weight populations. In addition, specific experimental conditions are required, depending specifically on the molecular weight of polymer chains in the sample.[1−8] For example, in the MALDI-TOF mass spectrometry, Martin et al. showed that optimal laser powers are required for desorption/ionization reactions, depending specifically on the molecular weights of polymer samples.[1] Also, in the case of SEC, Mori has pointed out that in aqueous SEC analyses of NaPSS the retention volume is not only governed by size-exclusion but also affected by ion-exclusion effects and hydrophobic interactions.[9] For aqueous SEC analysis with ionic polymers, optimal conditions of pH and ionic strength of the mobile phase ought to be sought out in order to minimize the ion-exclusion effect. In addition, selection of eluent should be carefully considered to overcome hydrophobic interactions between polymers and the column support materials.[10]

In this Letter, we present an additional method to determine the molecular weight distributions of polyelectrolyte mixtures based on single-molecule electrophoresis through a nanopore. Recently, biological and solid-state nanometer-scale transmembrane pores have been used to characterize diverse water-soluble analytes including single-stranded and double-stranded polynucleotides, proteins, synthetic ionic/nonionic polymers, and small organic molecules.[11−26] Upon externally applied electric potential across a nanopore-embedded membrane, ion flow through the nanopore is transiently blocked by analyte molecules. The blockage times and amplitudes of ionic current blockades are specific to the particular analyte, enabling the characterization of the analyte.

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There have been reports of developing a mass spectrometric method for poly(ethylene glycol) (PEG) using an α-hemolysin protein pore through a series of studies.[27−30] Using the fact that the amplitude of the PEG-induced current blockade of the α-hemolysin pore depends on the polymer molecular weight,[19] the studies clearly resolved the repeat unit of ethylene glycol from a polydisperse PEG sample. However, the technique has two limitations: (1) The application of this technique is limited for short PEG molecules, $n < 50$, where n is the number of repeat units, because of saturation of current blockades for larger PEG molecules. (2) It is an analyte-specific analysis in that the method is based on the PEG−protein pore interaction.

Also using the α-hemolysin pore, but with long synthetic polyelectrolytes and by measuring translocation times, we introduce here a technique of single-molecule-level analysis to determine molecular weight distributions of charged polymers. In order to determine the molecular weights of macromolecules with broad distributions, such as a multicomponent mixture of polymer standards with narrow polydispersities, a desirable method is to measure the length of a single polymer chain, one at a time. In nanopore−polymer translocation experiments, especially for the α-hemolysin protein pore, a long polyelectrolyte chain transports through the pore only in single file, and an average time taken for the chain to completely pass the pore is known to be proportional to the chain length.[12,31−33] In this work, molecular weight distribution of a mixture of sodium salts of polystyrenesulfonate (NaPSS) with different molecular weights was determined by measuring durations of NaPSS passing through an α-hemolysin pore. SEC measurements were performed on NaPSS mixtures with the same components used in translocation experiments, and the results are compared.

Samples of NaPSS mixtures for translocation experiments are prepared by mixing equal moles of NaPSS polymers with four different molecular weight distributions (Scientific Polymer Products, Inc., NY) in 10 mM HEPES buffer with 1 M KCl with pH 7.5. The weight-average molecular weights of NaPSS standards are 1.53, 16, 34.7, and 126.7 kg/mol with polydispersity indices with 1.12, 1.13, 1.16, and 1.17, respectively, as given by the manufacturer. The experimental details are illustrated in Figure 1a, and procedures for the preparation of the lipid bilayer and a single α-hemolysin pore can be found in our previous reports.[32,34] After forming a single α-hemolysin pore on the lipid bilayer membrane in 10 mM HEPES buffer with 1 M KCl at pH 7.5, 10 μL of a 0.1 mM NaPSS mixture sample was added to one side of the membrane (*cis*) and waited for about 10 min for mixing. The ionic current was recorded using pClamp software (Molecular Devices, LLC., MA) with a 3 μs interval and a 10 kHz low-pass Bessel filter, while constant electric potential was applied in the voltage clamp mode by a patch clamp amplifier (Axon Instruments, CA) across the pore. All translocation experiments were performed at room temperature. The recorded current traces were analyzed with MATLAB (Mathworks, Inc., MA) software customized in our laboratory. As seen in Figure 1b, two current blocking levels are detected for NaPSS passing through an α-hemolysin pore, where once a polymer chain enters into the pore mouth and occupies the vestibule region of the α-hemolysin pore the ionic current drops as 30−60% of the open-pore current, and when one of the chain ends finds the narrowest region of the pore and threads though the pore, the current is blocked about 80−95%. We set 70% of open pore current as a threshold for deep blocking events (threshold 2) and measured the successful translocation times, τ_2.

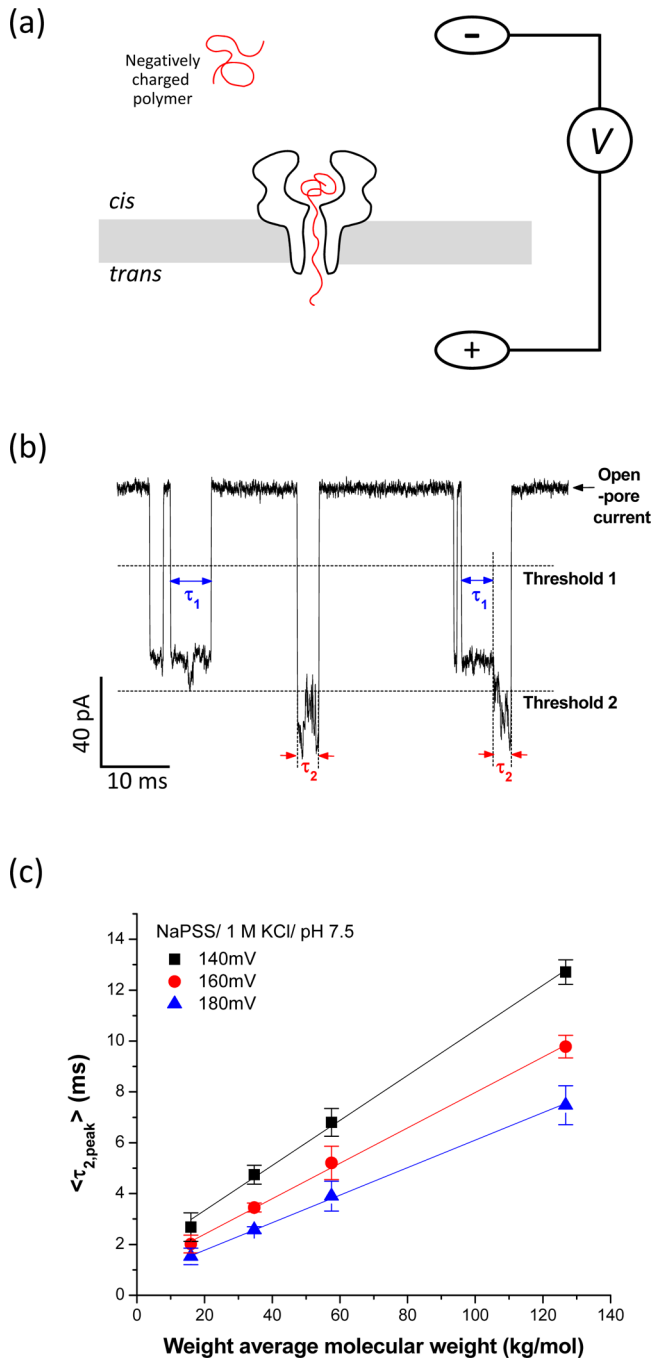

Figure 1. (a) Schematic description of polymer translocation through the α-hemolysin pore. (b) Ionic current through the α-hemolysin pore for translocation events of 16 kDa NaPSS at 140 mV. Threshold 1 and 2 are 75% and 30% of the open pore current, respectively. (c) Average successful translocation time is proportional to the molecular weight of NaPSS.

Polymer translocation is a stochastic phenomenon with a distribution of translocation times even for monodisperse polymers (see Figure 1 of Supporting Information (SI) for the precisely monodisperse poly(dT)$_{80}$). We have constructed histograms of log τ_2 for different molecular weight samples of NaPSS at different externally applied voltages (140, 160, and 180 mV). Such histograms are taken as the standards for the individual components in analyzing the data for mixtures. Typical histograms for the standards are given in Figure 2 of SI.

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We have plotted the peak positions ($\tau_{2,peak}$) of these standard histograms in Figure 1c as a function of the weight-average molecular weight of NaPSS standards for 140, 160, and 180 mV.

When the equimolar NaPSS four-mixture sample was added into the *cis* side, the historgram of log τ_2 shows multiple peaks as expected, corresponding to different NaPSS components in the mixture. At 180 mV, the peaks are highly overlapped, and only two peaks for 35 and 127 kDa NaPSS are observable. As applied voltage decreases, distributions of translocation times become broader, and differences between peaks for different NaPSS components increase. We observed that the translocation time distributions of the four different molecular weight NaPSS are most distinguishable at 140 mV. As shown in Figure 2, three distinct peaks and an inflection point were

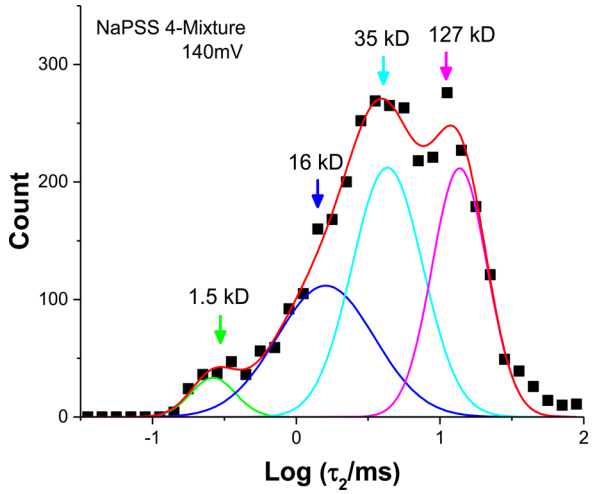

Figure 2. Histogram of translocation times for the equimolar NaPSS 4-mixture at 140 mV of applied voltage (black dots). The Gaussian fitting with fixed fwhm values for each NaPSS component allows deconvolution of the histogram into its components (purple, 127 kDa; aqua, 35 kDa; blue, 16 kDa; and green, 1.5 kDa). The sum of these components is the red curve.

observed, allowing us to estimate the molecular weights of the NaPSS components in the mixture using a $\tau_{2,peak}$ vs M_w plot (Figure 1c). All components in the mixture are readily identified at 140 mV.

The peak widths are seen to depend on the peak position even though the NaPSS standards have similarly narrow polydispersity indices. This is because we have plotted the logarithm of τ_2 on the *x*-axis. To confirm the individual peaks in the histogram for the mixture, we have calculated the average full width at half-maximum (fwhm) values for log τ_2 distributions of 16, 35, and 127 kDa NaPSS standards from multiple experiments. Using OriginPro 8.5 software, multipeak fitting was performed with fixed average fwhm values, 0.8, 0.58, and 0.45 for 16, 35, and 126 kDa, respectively (Figure 2). Using this deconvolution procedure, the integrated areas for the individual components are 95.2, 131.0, and 101.4, respectively, for 16, 35, and 126 kDa. These areas are roughly the same, as expected for an equimolar mixture. It is remarkable that even the 1.5 kDa component in the mixture is identifiable. The area for this low molar mass component is 13.0, as not all translocation events are captured for such fast-moving molecules (see Figures 2(a) and 3 of SI). The equivalence of equal areas for the components of the equimolar mixture is seen in our experiments due to the fact that the capture rate and the probability of successful translocation are independent of molecular weight under the experimental conditions reported here.

The SEC system consisted of an Agilent 1100 Series Isocratic Pump (Agilent Technologies, Inc., CA), Optilab rEX refractive index (RI) detector (Wyatt Technology, Inc., CA), and DAWN EOS light scattering detector (Wyatt Technology, Inc., CA). For all experiments a Waters Ultrahydrogel Linear Column (Waters Corp., MA) was used, and the elution profiles were recorded using Wyatt ASTRA software. The aqueous solution of 0.1 M $NaNO_3$ and 0.02% NaN_3 was mixed with acetonitrile with a volume ratio of 80:20 and used as a mobile phase and solvent in all SEC experiments. The amounts of samples injected were 100 μL each, and the flow rate was fixed at 0.5 mL/min.

Figure 3a illustrates, from top to bottom, the SEC mass spectra from the RI detector for 0.1 mM of 1.5, 16, and 35 kDa NaPSS and 0.025 mM of 127 kDa NaPSS solution. The last one is for the equimolar NaPSS 4-mixture sample (0.025 mM for each component) with the same composition as the sample used in the translocation experiments. The detailed view of the SEC profile for the 1.5 kDa NaPSS lets us observe a very small peak which comes from the NaPSS molecules. This peak is overlapped with a later elution peak for small ions, resulting in a peak tailing as shown in the inset of the plot. In the next three plots, a single large peak appears each for 16, 35, and 127 kDa of NaPSS standards. In the last elution profile of Figure 3a, for the equimolar 4-mixture, the RI detector illustrates two distinguished peaks (35 and 127 kDa) and a very small curved slope (1.5 k, first small hill in the inset), which is followed by a small peak for the small ions. The signal for the 16 kDa NaPSS is overlapped with that for the 35 kDa NaPSS and only slightly bends the curve (around 32 min of elution time). A light scattering detector was also used simultaneously, but discrimination of peaks for the NaPSS 4-mixture was even worse (data not shown) than RI results.

RI detector in SEC analyses records elution profiles based on the concentration of the polymer eluted (weight-based detection), while measuring τ_2 is a number-based analysis where we count the number of polymer chains passing through a nanopore. For example, in the elution profile of the equimolar NaPSS 4-mixture, the elution peaks for 1.5, 16, and 35 kDa NaPSS are much smaller than that for 127 kDa because the total weight of molecules is much less. Therefore, we wondered whether one could observe distinct elution peaks if the weights of the four NaPSS components in the mixture are comparable. In view of this, the equiweight NaPSS 4-mixture was prepared by mixing an equal volume of 3 mg/mL of 1.5, 16, 35, and 127 kDa NaPSS standards and introduced to the SEC column. As shown in the last plot of Figure 3b, four distinct peaks were observed in the elution profile of the NaPSS equiweight mixture, with each peak representing the four NaPSS components in the mixture. Although this result is different from the equimolar mixture result, however, we noticed that the elution times of each component in the equiweight NaPSS mixture are not consistent with those of single-component NaPSS standards. 1.5, 16, and 127 kDa NaPSS chains in the equiweight mixture were eluted earlier than those in the single-component standards as much as 0.11, 0.18, and 0.5 min, respectively (0.06, 0.09, and 0.25 mL differences in elution volume). These difficulties in inferring the correct molecular weights of the components in the mixture might be attributed

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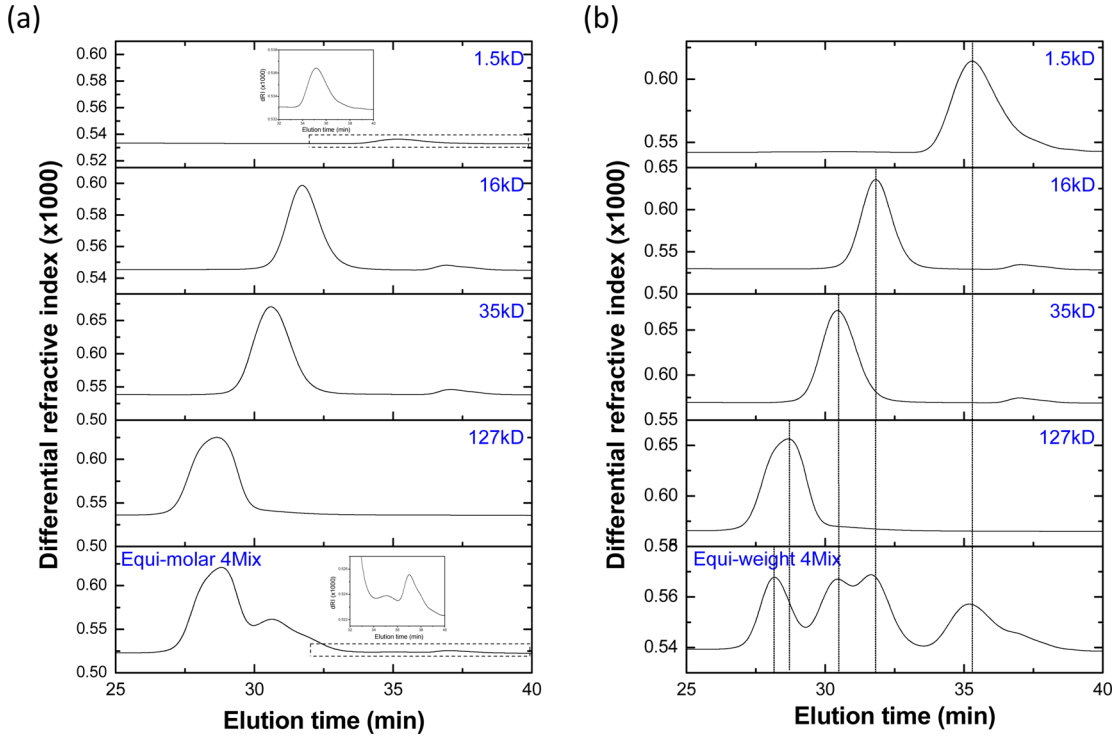

Figure 3. SEC elution profiles (a) for 0.1 mM NaPSS samples and the equimolar NaPSS 4-mixture and (b) for 3 mg/mL of NaPSS samples and the equiweight NaPSS 4-mixture.

to sequential size exclusion among the components and local concentration gradients.[3−8]

We have introduced a technique to evaluate molecular weights of polymer chains in polyelectrolyte mixtures by measuring the translocation times when the polyelectrolyte chains pass through an α-hemolysin nanopore. An equimolar mixture of four different standards of NaPSS (M_w = 1.5, 16, 35, and 127 kDa) was used as a model polyelectrolyte mixture sample. The histogram of translocation times at 140 mV shows three distinct peaks and one shoulder-like feature, and each peak position is in good agreement with its standard curve of translocation time vs molecular weight of NaPSS. This is because the NaPSS chains cannot transport through the α-hemolysin pore simultaneously, but only one at a time, unlike in the SEC where polymer molecules choose different paths with random motion and affect each other while being analyzed.

There are still several improvements to be made in fine-tuning this method into a routine technique for determining molecular weights of polyelectrolytes. There is an intrinsic stochasticity in the translocation time distribution even for very narrow molecular weight samples. In its own right, this is an intriguing problem, and substantial effort is being mounted in many laboratories worldwide to reduce this stochasticity. There are immediate avenues to explore in terms of different solvents, identity and amount of the low mass electrolyte which is primarily responsible for the ionic current, temperature, gradients in pH and ionic strength across the pore, and different kinds of nanopores, in order to transform the single-molecule electrophoresis into a more robust technique for characterizing and separating large polyelectrolytes. It is also highly desirable to establish a universal calibration for the translocation experiment.

■ ASSOCIATED CONTENT

Supporting Information

Additional data for histograms of translocation time for the individual components of the NaPSS mixtures and the perfectly monodisperse poly(dT)$_{80}$ are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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