Single Molecule Tracking of a Semiflexible Polyelectrolyte Chain in Solvent Under Uniform Electroosmotic Flows

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Direct single molecule tracking of a polyelectrolyte chain by using fluorescence microscopy has allowed both the assumptions and the predictions of relevant theories to be tested. The center-of-mass displacement is determined as a function of the time that elapses between images, where the radius of gyration can be estimated from a first moment of the image distribution. The translational self-diffusion for the molecule is an ensemble property of the mean square displacement (MSD) with lag time in each trajectory. Experimentally viable two-dimensional imaging of semiflexible polyelectrolyte was performed on a fluorescein-labeled xanthan chain in electroosmosis-driven uniform flow fields. The radius of gyration was almost constant under variations of the electroosmotic flow velocity determined by an externally applied electric field. We try to develop a correction of the MSD in flow field, taking into account the velocity fluctuations. Its advantage allows acquiring the linear fit for the MSD vs lag time, for a good estimate of the translational diffusion. Increasing behavior of the diffusion with increasing fluid velocity ensures a quadratic equation fit, which should connect with the convective effect. Our results exhibit a screening effect such that strong screening caused by a high ionic concentration leads to higher diffusion due to the compact chain conformation. Considering the uniform flow serves as a basis for understanding the behavior of individual polyelectrolyte chains under controlled fluidic flow in confined spaces.

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I. INTRODUCTION

For many decades, polymer dynamics was experimentally studied by using indirect methods such as bulk rheology, light scattering, and birefringence. Single molecule tracking, developed within the last 20 years, uses fluorescence microscopy, which permits a passive visualization of the position, conformation, and motion of single molecules. Investigation of single molecule properties and dynamics provides not only the average values of observables for ensembles of molecules but also reveals the characteristics of individual molecules with precision at the submicron level [1–3]. In particular, the conformational dynamics of a single molecule in confined spaces of micro/nanofluidic channels has attracted increasing attention because it is important in many biophysical applications, for instance, DNA separation, gene delivery, and gene mapping by using lab-on-chips [4–6]. These applications require controlled manipulations subject to stretching, condensing, moving, and packing of individual molecules.

A polyelectrolyte, a kind of complex fluid, represents an interesting and broad class of soft matter that should be considered as an additional complication arising from the Debye screening of the long-range electrostatic interaction by ions [7]. These characteristics prevent direct application of many reliable theories developed for neutral polymers, although polyelectrolytes have stimulated interest from a fundamental, as well as from a technological, point of view due to their fascinating conformational changes even in an aqueous medium that is a poor solvent for most synthetic polymers. It is usually necessary to consider more system-specific aspects represented by the surface charge, relaxation, and the condensation of counterions and salt ions. A precise understanding of charged soft matter has received great attention in life
science and molecular biology because polyelectrolytes include many proteins and other biopolymers, such as DNA, polysaccharides, and microtubules [8].

Properties of a semiflexible or wormlike polyelectrolyte chain in solvent have been investigated by employing either computations or experiments. The model polyelectrolytes in the previous studies were almost always confined to a DNA molecule with a contour length of above several micrometers. Mesoscale simulations were performed by using the Brownian dynamics (BD) method with a hydrodynamic interaction (HI), where the solvent was treated as a continuum, while the molecule was usually represented by a coarse-grained model, such as a bead-spring chain [9]. Many studies have contributed to an understanding of how a single polyelectrolyte chain behaves when exists in either unbounded bulk [10] or confined spaces [11–14]. As a study of the submicron sized polyelectrolyte chain, which had not been attempted till then, Jeon and Chun [15] developed coarse-grained models of polysaccharide xanthan in bulk solution as well as under confinement, taking into account both HIs and electrostatic interactions between pairs of beads. Subsequently, Chun et al. [16] explored experimental verifications on changes in the conformation and translational diffusion, by comparing the results obtained by BD simulations and single molecule tracking. It revealed that their BD simulations successfully represented the properties of a single xanthan chain, discussing in-depth the experimental restrictions.

Note that, despite the many practical applications have yielded, a complete theoretical explanation underlying polymer physics and micro-rheology has not been developed. Thus, a final goal of the present study is to provide a connection between the microscopic and the macroscopic properties of a single polyelectrolyte chain. We examine the conformation and translational diffusion of a single xanthan chain under an external flow field, which can serve as an extended experimental work for further understanding the behavior of submicron sized semiflexible or wormlike polyelectrolyte chains in solvent. In view of the initial stage, we consider here the uniform flow driven by an electric field, so-called the electroosmotic flow (EOF).

II. SINGLE MOLECULE TRACKING

1. Epi-fluorescence Microscopy

Recently, observations at the level of individual atoms and molecules have become possible with microscopy and spectroscopy. Imaging of a single fluorescence molecule has been achieved to find non-uniform molecule distribution and hidden mechanism from the averaging process. The direct observation of molecular motion by using epi-fluorescence microscopy has some essential advantages in accessing single molecule trajectories, which cannot be realized in conventional methods [16]. Single molecule imaging has some challenges. First, optical imaging systems have an inherent limitation on their spatial resolution due to the diffraction of light. The resolution $R$ of an optical microscope is defined as the shortest distance between two points on a specimen that can still be distinguished by the observer or camera system as separate entities. Shorter wavelengths $\lambda$ and higher numerical aperture (NA) number yields higher resolution, because $R = \lambda/NA$. Second, real-time imaging of fast changeable phenomena is difficult to detect due to the limitation on the temporal resolution. The speed at which one complete image is acquired determines the temporal resolution of camera, combining with readout speed and the time of signal integration.

In this study, fluorescein-labeled xanthan molecules in a microfluidic channel were monitored using an inverted epi-fluorescence microscope (Nikon, Eclipse Ti-E, Japan) with a 100× [NA of 1.3, resolution of 0.2 µm, focal depth of 0.41 µm, working distance of 200 µm] oil immersed objective, as depicted in Fig. 1. With the excitation filter, only 450 ∼ 500 nm wavelengths from the mercury laser can pass through the fluorophore. The emitted light, wavelength of 520 nm or above, can pass through both the dichroic mirror and the emission filters. Images were taken by a digital 12-bit charge-
coupled device (CCD) camera (Nikon, Digital Sight DS-Q1, monochrome cooled, Japan). The exposure time of the CCD camera was set in the range of 4 – 20 ms, recording 1280 × 1024 (for 1 × 1 binning) and 640 × 480 (for 2 × 2 binning) pixels. Under 100× magnifications, images were recorded at 10 frames per second (fps), in which each image pixel corresponds to 91 nm. The use of 40× magnification was able to improve the frame rate up to 32 fps, but the resolution of the image pixel was reduced (i.e., 450 nm). The data acquisition was performed using the NIS-Elements software.

2. Uniform Flow Driven by an Electric Field: EOF

Due to the fixed surface charge at the solid interface, an oppositely charged region of counterions develops in the liquid to maintain the electroneutrality of the solid-liquid interface. This screening region is called an electric double layer (EDL), which is divided into two parts: an inner layer (namely, the Stern layer) and an outer diffusive layer. The EDL thickness increases with decreasing electrolyte concentration. As the surface is negatively charged, the viscous interaction between net excess of positive ions in the EDL and the liquid causes flow toward the cathode along the channel. The liquid velocity is zero at the wall and increases to a maximum value with the EOF velocity at some distance from the wall, after which it remains constant as a plug type. When the EDL thickness decreases, the flow rate dependence on the zeta potential becomes more linear. In the thin EDL limit, one obtains a straight line in accordance with the Helmholtz-Smoluchowski (H-S) equation for the EOF velocity inside a channel of arbitrary shape [17]:

\[ V_{EOF} = -\frac{\varepsilon \zeta E_x}{\eta}. \]  

Here, \( E_x \) is the applied electric field (voltage per unit length) in the \( x \) direction, \( \eta \) the viscosity of fluid, \( \zeta \) the zeta potential, and the dielectric constant \( \varepsilon = \varepsilon_0 \varepsilon_r \) is defined as dielectric constant at vacuum \( \varepsilon_0 \) and relative permittivity \( \varepsilon_r \).

In order to have xanthan molecule be injected into and moved with uniform flow fields in the microchannel, we used a high-voltage power supply (HVS448, LabSmith, CA) capable of providing from 0 to 3 kV. Channel reservoirs were connected to the electrodes as Ag wire. To provide the microchannel, a microfluidic chip was fabricated by O2 plasma bonding with a 1-mm thick PDM (polydimethylsiloxane) replica and 100-\( \mu \)mated by O\( \text{2} \) provide the microchannel, a microfluidic chip was fabri-
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3. Model Polyelectrolyte Xanthan

Xanthan, a polyelectrolyte, is an extracellular polysaccharide produced by bacterium. Its primary structure consists of a main chain with charged trisaccharide sidechains on every second residue and the side chain. Its secondary structure is known to undergo an order → disorder (i.e., helix → coil) transition, depending on conditions of temperature and salinity. Native xanthan is generally believed to be in a double strand [18,19]. For low ionic strength at room temperature (RT, i.e., 25 °C), the extension of xanthan and the corresponding increase in the solution viscosity are likely to involve a transition of the secondary structure from a double strand to a disordered form with lower strandedness. This denatured xanthan can undergo a transition from the double to the single helical state when sufficient salt is present. Xanthan can also be ordered if it is gradually cooled after heating.

The powdered xanthan was purchased from Sigma Chemical Co. (St. Louis, MO). Following the procedure described in detail in a previous study, native xanthan was tagged by attaching 5-amino fluorescein to the carboxyl groups of the side chain, which was first outlined by Holzwarth [20]. After purifications, fluorescein-labeled xanthan was stored in a refrigerator before being used with a sufficiently dilute concentration in the range from 0.1 to 1 ppm. The degree of fluorescein labeling was found to be 0.5 ~ 1.0 wt%, as determined by UV-Visible absorption spectrophotometry (S-3100, Scinco, Seoul). When the polyelectrolytes are dissociated in aqueous solutions, the chain is extended or coiled due to the electrostatic repulsion between monomers. Adding the salt results in the polyelectrolyte chain being screened by oppositely charged salt ions.

The shear viscosity was measured at different xanthan concentrations ranging from 50 to 1000 ppm at 0.1-mM NaCl using a rheometer (AR2000ex with bob and cup fixtures, TA Instrument, DE). Figure 2 shows the typical non-Newtonian character of the pseudoplasticity. Here, shear thinning behaviors over a wider shear rate are known to be described by the Carreau A constitutive model [21,22]:

\[ \eta(\gamma) = \frac{\eta_0}{1 + (\lambda \gamma)^2(1-n)/2}, \]  

where \( \eta_0 \) is the zero-shear-rate viscosity, \( \gamma \) the shear rate, \( \lambda \) the empirical time constant, and \( n \) is the power-law exponent. It possesses two asymptotes: the xanthan solution shows a constant viscosity of \( \eta_0 \) (Newtonian region) as \( \gamma \to 0 \); on the other hand, the viscosity decreases linearly in a log-log plot (shear thinning region) as \( \gamma \to \)
From the best fit of each data for different xanthan concentrations $C$ up to 1000 ppm, the parameters in Eq. (2), $\eta_0$, $\lambda_T$, and $n$ can be empirically expressed as a function of $C$ in percent by weight:

$$\eta_0 = -1.67 \times 10^{-2} + (1.65 \times 10^{-2}) \exp(25.37C),$$

$$\lambda_T = 4.01 \times 10^{-1} + 10.88C + (2.21 \times 10^{2})C^2,$$

$$n = 9.28 \times 10^{-1} - 1.29C^{4.83 \times 10^{-1}}. \quad (5)$$

III. RESULTS

1. Correct Analysis of Image Tracking

Various distinction of the real image of fluorescein-labeled xanthan (Fig. 3(a)) can be analyzed using the image processing algorithm based on Crocker and Grier's Interactive Data Language (IDL) code (RSI, Boulder, CO) [23]. At first, each pixel intensity of the real image was obtained (Fig. 3(b)) by suitable IDL-based software (MatLab Particle Tracking Code provided by D. Blair and E. Dufresne). Pixel images were spatially filtered. Then, white noises could be minimized, and unnecessary blobs could be removed in the image (Figs. 3(c) and 3(d)). The next step should be to identify the blobs locating all peaks that are above a given threshold value and can estimate the brightest feature. According to the post-processor developed in our laboratory, the total intensity, the center-of-mass, and the radius of gyration were finally computed. Note that determining the boundary of the pixel intensity of images was guaranteed by accomplishing the same procedure with latex spheres of 1-µm in diameter.

The zeroth moment accounts for the total intensity $I(t)$ of a single molecule, which can be calculated from the intensity distribution function $I(r, t)$ of a pixel with a set of coordinates $r$ [24,25]:

$$I(t) = \int I(r, t) dr \approx \sum_{p,q} I_{pq}(t). \quad (6)$$

We assumed that the fluorescent label was uniformly distributed within a molecule and that the camera gain was a linear function of the fluorescent signal with an intensity $I_{pq}$ for the pixel $\{p,q\}$. The first moment of the intensity distribution denotes the position of the center-of-mass:

$$R_{CM}(t) = \frac{1}{I(t)} \int r I(r, t) dr \approx \frac{1}{I(t)} \sum_{p,q} I_{pq}(t)r_{pq}(t). \quad (7)$$

The second moment determines the radius of gyration tensor [26]:

$$G_{ij}(t) = \frac{1}{I(t)} \int (r_i - R_{CM,i})(r_j - R_{CM,j}) I(r, t) dr. \quad (8)$$
Fig. 4. (a) Time evolution of the total intensity of xanthan molecules under an electric field of 100 V/cm in 10^{-4} mM KCl solution, (b) probability distribution of the radius of gyration for one subset with an average $R_G$ of 386 nm, and (c) distribution of the displacement length for one subset with an average value of 2 µm.

For $x$ and $y$ directions, Eq. (8) is expressed as

$$G_{xy}(t) \approx \frac{1}{I(t)} \sum \left[ (r_{pq,x}(t) - R_{CM,x}(t)) \times (r_{pq,y}(t) - R_{CM,y}(t)) I_{pq}(t) \right].$$

Then, note that the common radius of gyration can be obtained as $R_G^2(t) = \text{trace}(G_{ij})$. The $R_G$ of a trajectory describes the amount of space that the molecule explores during its movement.

As the instantaneous evolution of each moment, Fig. 4(a) shows that the zeroth moment giving $I(t)$ of a single xanthan molecule appears to be almost constant over time with small fluctuations. Once photobleaching begins, the intensity of fluorescence emission may decay over the observation time. As displayed in Figs. 4(b) and 4(c), both the radius of gyration and the displacement length follow Gaussian distributions. In this, the displacement direction in each step is a random process exhibiting Brownian motion. Figure 5 shows an illustrative example for trajectories of the center-of-mass in streamwise ($x$) and transverse ($y$) directions under electroosmosis-driven uniform flows. We recognize that $y$-displacements are really small compared to $x$-displacements and that most of the displacements have approximately the same length.

For an in-depth interpretation, the molecular trajectories in the $x$ and $y$ directions were plotted as a function of time, as shown in Fig. 6(a). The linear increase in the $x$ directional displacement over time indicates a well-defined average EOF velocity. A net velocity is observed in the $y$ direction. The $x$ and the $y$ components of the instantaneous molecular velocity are provided in Fig. 6(b) as a function of time. The $y$-directional displacements are independent of the applied EOF velocity and reflect thermal Brownian diffusion alone.

A diffusive motion of the Brownian particle is parametrized by its translational self-diffusion coefficient through the Einstein-Smoluchowsky relation within the framework of Kirkwood theory in Cartesian coordinates [26]:

$$D_{T,\beta} = \lim_{\delta t \to \infty} \frac{1}{2 \delta t} \left( |R_{CM,\beta}(t) - R_{CM,\beta}(t - \delta t)|^2 \right)$$

for $\beta = x, y, z$. (10)
Here, \( \delta t \) is the lag time and the average \( \langle \cdots \rangle \) is the mean square displacement (MSD) carried out over the entire trajectory. In order to better approach the experimental situation, we adopt the 2-dimensional (2D) diffusion coefficient \( D_T^{2D} = \frac{\langle \sum_{\beta=x,y} R_{T,\beta}^2 \rangle}{2} \) with sufficient accuracy compared to the 3D one, as noted in the previous study [27]. For the case of no-flow field, the self-diffusion of the xanthan chain is determined from the MSD with the \( \delta t \) in each trajectory:

\[
D_T^{2D} = \lim_{\delta t \to \infty} \frac{\langle R_{CM}^2(0) \rangle}{4\delta t},
\]

where the MSD is given as

\[
\langle R_{CM}^2(t) \rangle = \langle [R_{CM,x}(t) - R_{CM,x}(t-\delta t)]^2 \rangle + \langle [R_{CM,y}(t) - R_{CM,y}(t-\delta t)]^2 \rangle.
\]

Binning the trajectory into several segments is needed; then, the \( R_{CM} \) displacement for each individual molecule within each segment is calculated as a function of the time elapsed between frames. In general, the MSD without an external field increases linearly with lag time, due to the isotropic random walk of the chain. For somewhat different chains of a stiff semiflexible filament, detailed results for the kinetics and dynamic properties in 2D confined space were reported by using the transverse and the longitudinal components of the MSD [28,29].

In the case of flow situations, the correct MSD should take into account the velocity fluctuations (i.e., mean-velocity-shifted \( R_{CM}(t) \)) in the time interval, expressed as

\[
\langle R_{CM}^2(t) \rangle = \langle [R_{CM,x}(t) - R_{CM,x}(t-\delta t) - V_{CM}(t)]^2 \rangle + \langle [R_{CM,y}(t) - R_{CM,y}(t-\delta t)]^2 \rangle.
\]

In Eq. (13), the average molecular velocity of the ensemble trajectory along the flow direction, \( V_{CM} \), is estimated by averaging instantaneous center-of-mass velocities for each electric field, as provided in Fig. 6(b). Here, the EOF does not affect the \( y \) directional displacement of a molecule [30]. Figure 7 shows that adopting the correct MSD changes the characteristic form of the plot between the MSD and the lag time, providing a fairly linear fit.

Let us provide further considerations in no-flow case. The translational self-diffusion of xanthan can be evaluated in the short-time Kirkwood value \( D_{T,short} \) when the chain does not involve the HI in wide channels. In this case, a scaling prediction for the diffusivity is in agreement with the Zimm model. While the HI gets to be involved in actually confined channels, the self-diffusion coefficient shows an asymptotic long-time value \( D_{T,long} \) as represented by Eq. (10). Then, the dependence of the diffusivity on the chain length agrees well with the scaling prediction of Rouse dynamics [31], suggesting that HIs are at least partially screened. According to Fixman’s Green-Kubo formula, \( D_{T,long} \) is lower than \( D_{T,short} \), however, it turns out that its difference in a single chain system is very small with a few percent. Details of the crossover between the short-time and the long-time behaviors can be found in the literature [31].

2. Conformational Dynamics of a Xanthan Chain in Uniform Flows

We changed the magnitude of plug-type EOF velocity \( (V_{EOF}) \) by applying an external electric field \( E_z \) ranging from 0 to 200 V/cm. The screening effect represented by the EDL thickness \( \kappa^{-1} \) was also considered with variations of the medium ionic strengths of xanthan dispersion, which was adjusted by using the KCl electrolyte concentration at a fixed pH of 6.2. The change over an experimental time course was quite small not to affect the medium ionic strength. The concentration of a symmetric monovalent electrolyte (e.g., KCl) equals the medium ionic strength IS. From the electrostatic principle with the Poisson-Boltzmann equation, the EDL thickness \( \kappa^{-1} \) is given by \([\text{IS (M)}]^{-1/2}/3.278\) at RT [16,17].

Figure 8 shows that the radius of gyration \( R_G \) is almost independent of variations in the linear EOF velocity \( V_{EOF} \), but certainly depends on \( \kappa^{-1} \). Independence of \( V_{EOF} \) is attributed to the nature of its uniform flow field, where the isotropic stretching plays a role due to the nonexistence of the velocity gradient in transverse direction of the channel. We also plot as a function of the Peclet number \( \text{Pe} = V_{EOF}lp/D_0 \) (i.e., ratio of convection to diffusion). Here, the persistence length of the xanthan chain, \( lp \), which is a statistical concept on the chain flexibility, increases with decreasing IS, and \( D_0 = (kT/6\pi\eta a) \) is the Stokes-Einstein diffusion coefficient of the coarse-graining modeled xanthan molecule.
Single Molecule Tracking of a Semiflexible Polyelectrolyte Chain

Sunghun Choung et al.

Fig. 8. Variations in the radius of gyration of a single xanthan with EOF velocity and Pe for different screening effects with IS = 1.0, $10^{-2}$, and $10^{-4}$ mM KCl. IS = 1.0, $10^{-2}$, and $10^{-4}$ mM corresponds to $\kappa^{-1} = 9.7$, 97, and 970 nm and to $l_P = 160$, 460, and 600 nm, respectively. The error bars represent standard deviations.

With the Boltzmann thermal energy $kT$ and the hydrodynamic radius of coarse-grained bead $a$ taken as 5 nm, $D_0$ corresponds to $4.91 \times 10^{-11}$ m$^2$/s [15]. The extended $R_G$ with decreasing screening effect (i.e., decreasing IS or dimensionless inverse EDL thickness $\kappa l_P$) is accompanied by an increase in chain stiffness, which can be found in the further results of previous studies [15,16].

The in-plane diffusion coefficient was obtained from the initial slope of the correct MSD of the ensemble average vs lag time using Eqs. (11) and (13), in which error estimates were made using the bootstrap method for lots of subensembles of molecule paths. In Fig. 9, we see the dependence of $V_{EOF}$ on the translational self-diffusion $D_T$, ensuring quadratic equation fits. Increasing $D_T$ with increasing external flow results from the convective effect on the xanthan chain. The self-diffusion increases with increasing screening effect (i.e., increasing IS or $\kappa l_P$) since the xanthan chain becomes more compact, which is attributed to the decreased electrostatic repulsion by the high screening effect. During the image analysis, we set up the desirable range of fluorescence intensity and ignored xanthan molecules that appeared too dim due to an escape from the focal plane, which conflicts with 2D translational diffusion. As a result, the microscope allowed the motion of a xanthan chain in the focal plane of the channel to be observed in the $x$-$y$ direction. The velocity profile also develops at the edge of the EDL, but we adjusted the focal plane toward the channel center by means of a precise $z$-axis focus module.

IV. CONCLUSION

By using an inverted epi-fluorescence microscope, single molecule tracking of semiflexible polyelectrolyte was performed on fluorescein-labeled xanthan in electroosmosis-driven uniform flow fields. Considering this flow attempts to understand the behavior of individual chains under controlled fluidic flows. We measured the average behavior of ensemble trajectories and subsequently ignored uncorrelated fluctuations in the dynamics of single molecules, enabling a valid analysis of the properties in real time.

The radius of gyration was almost constant under variations of EOF velocity determined by the H-S equation for a given electric field. The self-diffusion coefficient was evaluated from the variation in the MSD with the lag time for each Brownian trajectory, in which the velocity along the flow direction was estimated by averaging the instantaneous center-of-mass velocity for each field. The behavior of EOF velocity dependence on the translational self-diffusion ensures a quadratic equation fit, which should be related to the convective effect. Our results show clearly the screening effect caused by the EDL, where strong screening caused by high salt concentration leads to higher diffusion. Experimental results...
on the conformation and the translational diffusion behavior qualitatively agree with the previous simulations, although there was a spatial limitation due to incomplete 2D image tracking.

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