

Video Article

Synthesis of Cyclic Polymers and Characterization of Their Diffusive Motion in the Melt State at the Single Molecule Level

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Abstract

We demonstrate a method for the synthesis of cyclic polymers and a protocol for characterizing their diffusive motion in a melt state at the single molecule level. An electrostatic self-assembly and covalent fixation (ESA-CF) process is used for the synthesis of the cyclic poly(tetrahydrofuran) (poly(THF)). The diffusive motion of individual cyclic polymer chains in a melt state is visualized using single molecule fluorescence imaging by incorporating a fluorophore unit in the cyclic chains. The diffusive motion of the chains is quantitatively characterized by means of a combination of mean-squared displacement (MSD) analysis and a cumulative distribution function (CDF) analysis. The cyclic polymer exhibits multiple-mode diffusion which is distinct from its linear counterpart. The results demonstrate that the diffusional heterogeneity of polymers that is often hidden behind ensemble averaging can be revealed by the efficient synthesis of the cyclic polymers using the ESA-CF process and the quantitative analysis of the diffusive motion at the single molecule level using the MSD and CDF analyses.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54503/>

Introduction

Cyclic polymers are unique in that they do not have chain ends. They often exhibit unusual behaviors which is distinct from their linear counterpart, including increased thermal stability of polymer micelles by a linear-to-cyclic conversion,^{1,2} and spatial organization of DNA in bacterial cells by a loop formation.³ Topological interactions between the cyclic chains are believed to be the critical factor for such unusual behaviors.^{4,5} Therefore, characterizing the motion and relaxation of cyclic polymers under entangled conditions has been an important research topic in polymer science for decades.⁶

Cyclic polymer dynamics has been investigated using both synthetic and naturally occurring molecules by means of ensemble averaged experimental methods such as nuclear magnetic resonance (NMR), light scattering, and viscosity measurements.⁷⁻⁹ However, these studies often suffer from impurity molecules in the samples.¹⁰ Furthermore, spatiotemporal heterogeneities of the motion of individual molecules caused by inherent structural heterogeneity of entangled polymers are often hidden behind the ensemble averaging in these studies. In order to characterize molecular level dynamics of cyclic polymers, a synthesis method that provides high purity cyclic polymers and an experimental and analysis methods that allow for quantitative characterization of molecular motion at the single molecule level have to be developed. Here, we will show a method to synthesize high-purity cyclic and dicyclic poly(THF)s that incorporate a fluorophore unit using an electrostatic self-assembly and covalent fixation (ESA-CF) process¹¹⁻¹³ and a method to analyze the motion of the individual fluorophore-incorporated polymer chains using a combination of mean-squared displacement (MSD) and cumulative distribution function (CDF) analyses.

A proper data processing has been shown to be essential for the accurate characterization of the diffusive motion. With an adequate MSD and CDF analyses, a multiple-mode diffusion of the cyclic and dicyclic polymers in the melt and semi-dilute solution of the linear polymer chains has been revealed,¹⁴⁻¹⁶ suggesting the significant effects of the topological states of the polymers on the diffusive motion of the chains under entangled conditions.¹⁷ While the experimental and analytical approaches to characterize the motion of the cyclic polymers are described in this protocol, the same method can be used to quantitatively characterize the diffusive motion in many other heterogeneous systems. The approach would be especially suitable when multiple diffusion components existing in the samples are to be analyzed.

Protocol

1. Synthesis of Monofunctional and Bifunctional Poly(THF)

1. Monofunctional poly(THF)

1. Flame dry a 2-neck 100-ml round-bottom flask. Vacuum and fill the flask with nitrogen (3 cycles).
2. Add distilled tetrahydrofuran (THF) (50 ml) to the flask. Put the flask in a water bath at 20 °C and equilibrate the temperature.
3. Add methyl triflate (0.5 mmol) to the flask by a syringe. Stir the mixture for 5-10 min at 20 °C.
4. Add *N*-phenyl pyrrolidine (4-6 equiv.) to the flask by a syringe. Stir the mixture for 30-60 min.
5. Completely remove the solvent under reduced pressure (ca. 100 Torr). Dissolve the residue in 3-5 ml of acetone. Add the acetone solution into 300-500 ml of *n*-hexane. Filter the precipitate and dry it under reduced pressure.

2. Bifunctional poly(THF)

1. Flame dry a 2-neck 100-ml round-bottom flask. Vacuum and fill the flask with nitrogen (3 cycles).
2. Add distilled THF (50 ml) to the flask. Put the flask in a water bath at 20 °C and equilibrate the temperature.
3. Add triflic anhydride (0.3 mmol) to the flask by a syringe. Stir the mixture for 5-10 min at 20 °C.
4. Add *N*-phenyl pyrrolidine (4-6 equiv.) to the flask by a syringe. Stir the mixture for 30-60 min.
5. Completely remove the solvent under reduced pressure (ca. 100 Torr). Dissolve the residue in 3-5 ml of acetone. Add the acetone solution to 300-500 ml of *n*-hexane. Filter the precipitate and dry it under reduced pressure.

2. Synthesis of Perylene Diimide-incorporated 4-armed Star and 8-shaped Dicyclic Poly(THF)

1. Armed star poly(THF)

1. Ion exchange

1. Dissolve perylene diimide tetracarboxylate sodium salt in water (10 mg/ml, 150 ml). Dissolve monofunctional poly(THF) in acetone (160 mg/ml, 4 ml). Add the acetone solution dropwise into the vigorously stirred aqueous solution. Collect the formed precipitate by filtration.
2. Repeat the above procedure with the recovered precipitate (2.1.1.1) four times.

2. Covalent fixation

1. Dissolve the obtained precipitate in toluene (5 mg/ml). Reflux the solution for 4 hr.
2. Completely remove the solvent under reduced pressure (ca. 100 Torr). Filter the residue through a plug of silica gel with *n*-hexane/acetone (2/1 vol/vol). Add the solution into ice-cooled water (300-500 ml) to precipitate the product. Collect the precipitate by filtration.

2. Dicyclic 8-shaped poly(THF)

1. Ion exchange

1. Dissolve perylene diimide tetracarboxylate sodium salt in water (6 mg/ml, 50 ml). Dissolve bifunctional poly(THF) (0.5 g) in 30-50 ml of acetone. Add the acetone solution dropwise into the vigorously stirred aqueous solution at 0 °C. Collect the formed precipitate by filtration.
2. Repeat the above procedure with the recovered precipitate (2.2.1.1).

2. Covalent fixation

1. Dissolve the obtained precipitate in toluene (0.05 g/L). Reflux the solution for 4 hr.
2. Completely remove the solvent under reduced pressure (ca. 100 Torr). Add toluene to partially dissolve the residue. Re-precipitate into 300-500 ml of *n*-hexane.
3. Filter the formed precipitate through a plug of silica gel with *n*-hexane/acetone (2/1 vol/vol). Re-precipitate into 300-500 ml of water.
4. Purify the formed precipitate by column chromatography¹⁸ using a polystyrene gel. Further purify the crude product by preparative gel permeation chromatography (GPC)¹⁹ with an eluent of CHCl₃ to remove byproducts by monitoring refractive index (RI) and UV detectors.

3. Single-molecule Fluorescence Imaging Experiment

1. Sample preparation

1. Cleaning of microscope cover slips

1. Place No. 1.5 24 x 24 mm microscope cover slips in a staining jar.
2. Add 1 M potassium hydroxide solution (100 ml) into the jar and sonicate for 15 min. Pour off the potassium hydroxide solution by decantation and rinse the cover slips with ultra-pure water for several times. Add spectroscopic grade ethanol (100 ml) into the jar and sonicate for 15 min.
3. Pour off the ethanol by decantation and rinse the cover slips with ultra-pure water for several times. After pouring off the ultra-pure water by decantation, repeat the step 3.1.1.2.

4. Add ultra-pure water to the jar and sonicate for 15 min. Rinse the cover slips with ultra-pure water for several times. Take out the cover slips from the jar by a plastic tweezers and dry them by either dry air or dry nitrogen.

2. Preparation of polymer melt samples^{14,15}

1. Add 100 μ l of non-labeled linear poly(THF) in a glass bottle and heat it to a temperature above the melting point (approximately 25 °C) using a hair dryer.
2. Dissolve the fluorophore-incorporated polymer (linear, 4-armed star, cyclic, or 8-shaped dicyclic synthesized in 2.1 and 2.2) in chloroform (1 ml, 10^{-6} M). Add 1 μ l of the solution to the 100 μ l of the melt of the non-labeled linear poly(THF).
3. After thoroughly mixing the sample with a pipette tip, evaporate chloroform by heating the sample using a dryer.
NOTE: This provides a melt of the non-labeled linear poly(THF) containing 10^{-8} M of the fluorophore incorporated polymers.
4. Take 10 μ l of the sample using a micro-pipette and drop it on a cleaned cover slip. Put another cleaned cover slip on the sample and sandwich the sample between the two cover slips.
5. Press the sample gently using a plastic tweezers.

2. Wide-field fluorescence imaging setup¹⁵

1. Introduction of an excitation laser (488 nm) into the back port of the inverted microscope

1. Insert an excitation bandpass filter and polarizer into the beam path.
2. Expand the beam to approximately 1 cm in diameter by a beam expander.
3. Insert a quarter wave plate into the beam path. Set the optical axis of the wave plate at 45 degree with respect to that of the polarizer. Alternatively, insert a Berek compensator and set the optical delay to $\lambda/4$.
4. Insert a diaphragm in the excitation beam path to adjust the beam size.
5. Before introducing the laser beam into the back port of the inverted optical microscope, insert a focusing lens (plan-convex lens, focal length \approx 300 mm) at a position where the laser beam out of the objective lens is collimated.

2. After reflecting the laser beam using a dichroic mirror mounted on a filter cube, introduce the laser beam to the sample through a high numerical aperture (NA) objective lens (e.g., NA 1.3, 100X magnification, oil immersion).

3. Attach an objective heater to the objective lens and set the temperature to 30 °C.

4. Mounting the sample on the stage of the inverted microscope

1. Drop one drop of immersion oil on the objective lens and mount the sample on the microscope stage.
2. Ensure that the sample thickness of approximately 10 μ m is obtained by checking the axial position of the bottom and top surface of the sample.
3. Adjust the focus of the microscope to a few micrometers above the bottom surface of the sample.

5. Obtain circularly polarized excitation light under the objective lens

1. Insert a polarizer into the collimated laser beam out of the objective lens.
2. Record the intensity of the laser transmitted through the polarizer by inserting a power meter after the polarizer. Record the transmitted laser power at different polarization angles by rotating the polarizer.
3. If the transmitted laser power is not constant at all polarization angles, slightly rotate the quarter wave plate or Berek compensator inserted in the excitation beam path.
4. Repeat step 3.2.5.2 and 3.2.5.3 until the constant transmitted laser power is obtained at all polarization angles. Ensure that the circularly polarized light is obtained at the sample.

6. Setup the EM (electron multiplying)-charge coupled device (CCD) camera

1. Attach the EM-CCD camera to the side port of the microscope and connect it to the image acquisition software.
2. If necessary, synchronize the camera exposure to a mechanical shutter or acousto-optical tunable filter inserted in the excitation beam path by sending the transistor-transistor logic (TTL) signals generated by the EM-CCD camera to the devices. Alternatively, synchronize the camera exposure to the laser output by sending the TTL signals generated by the EM-CCD camera to the laser.

NOTE: The latter option is applicable only when a solid-state laser whose output power can be modulated by input transistor-transistor logic (TTL) signals is used for the experiment.

3. Apply an EM gain (typically approximately 300) to the CCD camera using software controlling the camera in order to obtain a high quality fluorescence image of the single fluorophore.
4. Set a region of interest (ROI) (typically 128 x 128 pixels at the center of the field of view) using software controlling the camera.
NOTE: This allows for the imaging experiments at the frame rates of 100 - 200 Hz in the frame transfer mode, which is required for visualizing the motion of the fluorophore-incorporated polymer chains in the melt sample.

3. Running the experiment

1. Optimizing the experimental conditions

1. Adjust the illumination area of the sample to approximately 20 μ m in diameter using the diaphragm inserted in the excitation beam path.
2. Set the excitation laser power at the sample to 4 - 8 mW by manually selecting an appropriate neutral density (ND) filter inserted in the excitation beam path.
NOTE: This provides the mean laser power of 1 - 2 kW cm⁻² at the sample.
3. Record fluorescence images of the sample at the frame rates of 100 - 200 Hz. If the fluorescence intensity obtained from the individual fluorophore-incorporated polymers is too low, gradually increase the excitation power using the ND filter until reaching approximately 100 mW at the sample.
4. If the quality of the single-molecule fluorescence image is still not satisfactory, check the fluorescence impurities in the sample by recording fluorescence images of a pure melt of the non-labeled poly(THF). In case a high fluorescence background is observed, use different non-labeled poly(THF).

5. If the density of the fluorescence spot obtained from the fluorophore-incorporated polymers in the melt is too high to spatially isolate them (this causes errors in the analysis of the diffusive motion), decrease the concentration of the fluorophore-incorporated polymers in the sample until spatially isolated spots are observed.
 6. If the density of the fluorescence spot obtained from the fluorophore-incorporated polymers in the melt is too low (this causes a low throughput of the imaging experiment), increase the concentration of the fluorophore-incorporated polymers in the sample until an appropriate density of the fluorescence spot is reached.
 7. If the fluorescence images obtained from the fluorophore-incorporated polymers in the melt are blurred, increase the frame rates of the imaging acquisition.
- NOTE: This often requires a smaller ROI, typically 64 x 64 pixels.

4. Image acquisition

1. Once the experimental conditions are optimized, leave the mounted sample on the microscope stage for an hour so that the sample reaches equilibrium conditions.
2. Record 500 - 1,000 fluorescence image sequences of the fluorophore-incorporated polymers in the melt state at a 100 - 200 Hz frame rate. If the default file format is not TIFF, convert all the image sequences to the TIFF format.

4. Analysis of the Diffusive Motion

1. Mean-squared displacement (MSD) analysis

1. Crop the fluorescence image sequences in such a way that each image sequence contains a single and well-focused diffusing fluorophore-incorporated polymer using image processing software, such as ImageJ.
2. When the cropped image sequences contain more than 10 frames, split the image sequences into multiple sequences such that each sequence consists of 10 frames.
3. Determine the positions of the molecules in each image sequences accurately by two-dimensional Gaussian fitting of the images.
4. Determine the diffusion coefficient (D) of individual molecules by mean-squared displacement (MSD) analysis of the diffusion trajectories (*i.e.*, time-dependent positions of the molecule) using an equation²⁰

$$\text{MSD}(\Delta t) = \langle (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2 \rangle = 4D\Delta t$$

where x_i and y_i are the positions of the molecule in the image frame i , and n denotes the frame number with the time lapse Δt from frame i .

5. Plot the diffusion coefficients in a frequency histogram.
- NOTE: Typically, the histogram is constructed from more than 100 molecules.

2. Cumulative distribution function (CDF) analysis

NOTE: A CDF, $P(r^2, i\Delta t)$ corresponds to the cumulative probability of finding the diffusing molecules within a radius r from the origin after a certain time lag $i\Delta t$.

1. Calculate the squared-displacement occurring during time lags of $1\Delta t$, $2\Delta t$, ..., $i\Delta t$ for all the diffusion trajectories obtained in 4.1.3.
- NOTE: These calculations give total m_i squared-displacements for the time lags of $i\Delta t$.
2. Calculate numbers of the squared-displacements (l_i) within total m_i data set that are smaller than r^2 at different r^2 values ($0 < r^2 < \infty$). Normalized l_i vs r^2 plots correspond to the CDF, $P(r^2, i\Delta t)$.

3. Analysis of CDFs with distinct diffusion models

Note: The obtained CDFs are fitted by distinct diffusion models; homogeneous diffusion model, multiple diffusion modes in which the D distribution is described by a Gaussian (single Gaussian model), and multiple diffusion modes in which the D distribution is described by multiple Gaussian (multiple Gaussian model).

1. In the homogeneous diffusion model, determine a mean D by fitting the CDF using an equation²¹

$$1 - P(r^2, i\Delta t) = 1 - A \exp[-r^2/4D(i\Delta t)]$$

NOTE: Any deviation from the equation suggests the heterogeneous diffusion of the molecule.

2. In the single Gaussian model, determine the probability distribution of D described by a Gaussian ($f(D)$) by fitting the CDF using¹⁵

$$1 - P(r^2, i\Delta t) = \int_0^\infty f(D) D^{-1} \exp[-r^2/4D(i\Delta t)] dD$$

$$f(D) = A \exp\{-(D - D_0)^2/2w^2\}$$

where A , w , and D_0 are the amplitude, width, and center of the Gaussian.

3. In the double Gaussian model, determine the probability distribution of f^{th} component of D described by a Gaussian ($f(D)$) by fitting the CDF using¹⁴

$$1 - P(r^2, i\Delta t) = \sum_j A_j \int_0^\infty f(D_j) D_j^{-1} \exp[-r^2/4D_j(i\Delta t)] dD_j$$

$$f(D_j) = \alpha_j \exp\{-(D_j - D_{0j})^2/2w_j^2\}$$

where A_j is the fraction of each diffusion component, and α_j , w_j , and D_{0j} are the amplitude, width, and center of the j^{th} component of the Gaussian.

4. Calculation of the theoretical probability distribution of diffusion coefficient

NOTE: The probability distributions of D occurring due to the statistical errors ($p(D)dD$) are calculated for the different diffusion models; homogeneous diffusion model, multiple diffusion modes in which the D distribution is described by a Gaussian (single Gaussian model), and multiple diffusion modes in which the D distribution is described by multiple Gaussian (multiple Gaussian model).²²

1. In the homogeneous diffusion model, calculate the statistical probability distribution of D using an equation²²

$$p(D)dD = \{1/(N-1)!\}(N/D_0)^N D^{N-1} \exp(-ND/D_0) dD$$

where N is the number of the data points in a diffusion trajectory ($N = 10$, see 4.1.2), D_0 is the mean diffusion coefficient (determined by the CDF analysis, see 4.2.3.1), and D is the experimentally obtained diffusion coefficient for an individual trajectory.

2. In the single Gaussian diffusion model, calculate the statistical probability distribution of D using an equation¹⁵

$$p(D)dD = \int_0^\infty f(D) \{1/(N-1)!\}(N/D_0)^N D^{N-1} \exp(-ND/D_0) dD$$

where $f(D)$ denotes probability distribution of D determined by the CDF analysis (see 4.2.3.2), and D_0 is the mean diffusion coefficient (determined by the CDF analysis, see 4.2.3.2).

3. In the double Gaussian diffusion model, calculate the statistical probability distribution of D using an equation¹⁴

$$p(D)dD = \sum \int_0^\infty f(D_j) \{1/(N-1)!\}(N/D_{0j})^N D_j^{N-1} \exp(-ND_j/D_{0j}) dD_j$$

where $f(D_j)$ denotes probability distribution of the j^{th} component of D (D_j) determined by the CDF analysis (see 4.2.3.3), and D_{0j} is the mean diffusion coefficient of the j^{th} component (determined by the CDF analysis, see 4.2.3.3).

Representative Results

The perylene diimide-incorporated 4-armed star and 8-shaped dicyclic poly(THF)s were synthesized using the electrostatic self-assembly and covalent fixation (ESA-CF) process (Figure 1, Figure 2). Time-lapse single-molecule fluorescence images were measured for the 4-armed (Figure 3a) and 8-shaped (Figure 3b) polymers. The time-lapse fluorescence images (Figure 3) show spatially isolated bright and sharp spots due to the incorporation of the highly fluorescent perylene diimide fluorophore²³ into the chains. Frequency histograms of the diffusion coefficient were calculated for the 4-armed (Figure 4a) and 8-shaped (Figure 4b) polymers by the mean-squared displacement (MSD) analysis of the time-lapse images. The calculations of MSD plots and CDFs are conducted using routines written in MATLAB. The fitting of the CDFs obtained from the experiments is conducted using data processing software such as Origin Pro. The frequency histograms of the diffusion coefficient determined by the MSD analysis display broad distributions (Figure 4) resulting from both the statistical error of the analysis and heterogeneity of the diffusion. The frequency histograms show clear deviations from the homogeneous diffusion model (green line in Figure 4), which demonstrates heterogeneous diffusion of the polymer molecules.¹⁴ Cumulative distribution functions (CDFs) were calculated for the 4-armed (Figure 5a) and 8-shaped (Figure 5b) polymers and fitted by the single Gaussian (Figure 5a) and double Gaussian (Figure 5b) models. The statistical probability distributions of the diffusion coefficient were calculated for the 4-armed (Figure 4a) and 8-shaped (Figure 4b) polymers by the single Gaussian, or double Gaussian models. The single (Figure 5a) and double (Figure 5b) Gaussian models fit the experimentally obtained CDFs well. These results demonstrate that the diffusion of the 4-armed polymer is described by the broad distribution of the diffusion coefficient, whereas the 8-shaped polymer displays two distinct diffusion modes.

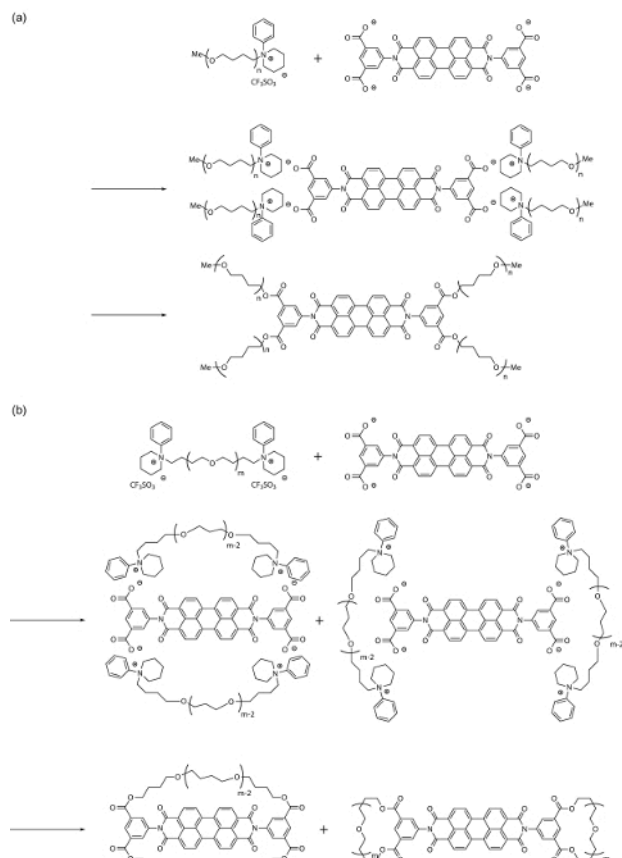


Figure 1. Synthesis route of perylene diimide-incorporated poly(THF)s. Synthesis route of (a) 4-armed star polymers and (b) 8-shaped dicyclic polymers. [Please click here to view a larger version of this figure.](#)

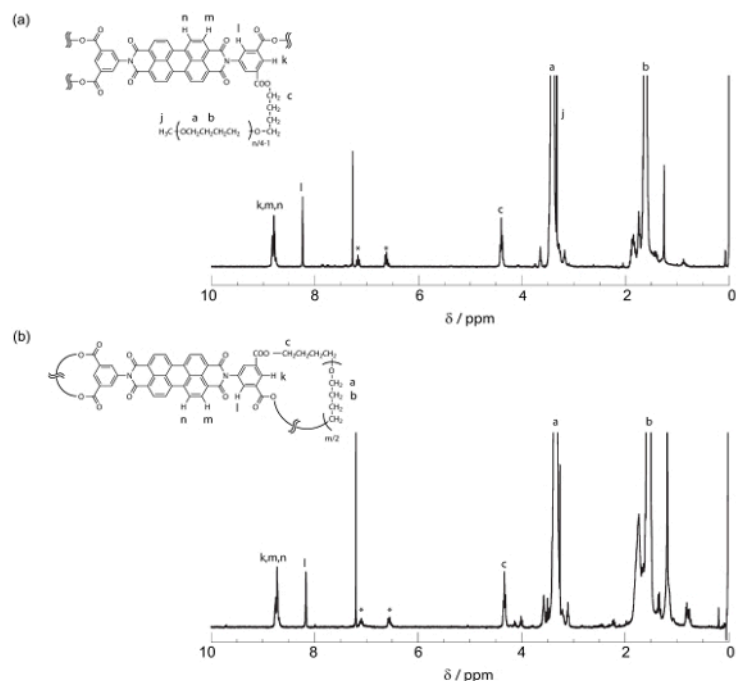


Figure 2. Characterization of the synthesized polymers. NMR spectra of (a) 4-armed star polymers and (b) 8-shaped dicyclic polymers. [Please click here to view a larger version of this figure.](#)

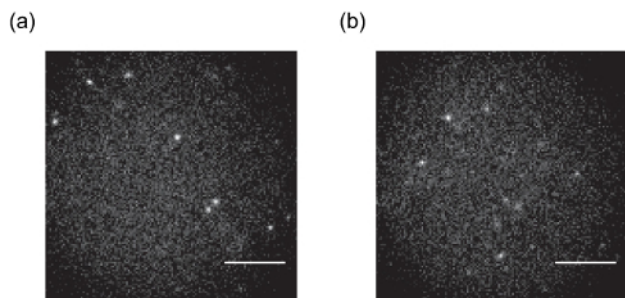


Figure 3 (movies). Single molecule fluorescence imaging of the perylene diimide-incorporated poly(THF)s. Time-lapse fluorescence images of (a) 4-armed star polymers and (b) 8-shaped dicyclic polymers in the melt of non-labeled linear poly(THF). Scale bar = 5 μm . Please click here to view movies (a) and (b).

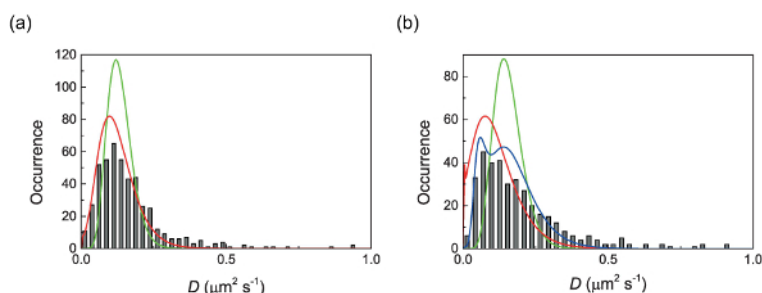


Figure 4. MSD analysis of the perylene diimide-incorporated poly(THF)s diffusing in the melt. Frequency histograms of the diffusion coefficient determined for individual (a) 4-armed star polymers and (b) 8-shaped dicyclic polymers in the melt of non-labeled linear poly(THF). The solid lines show theoretically calculated probability distributions of the diffusion coefficient based on the three different diffusion models; homogeneous diffusion model (green lines, see 4.3.1), single Gaussian model (redlines, see 4.3.2), and double Gaussian model (blue line, see 4.3.3).¹⁴ Please click here to view a larger version of this figure.

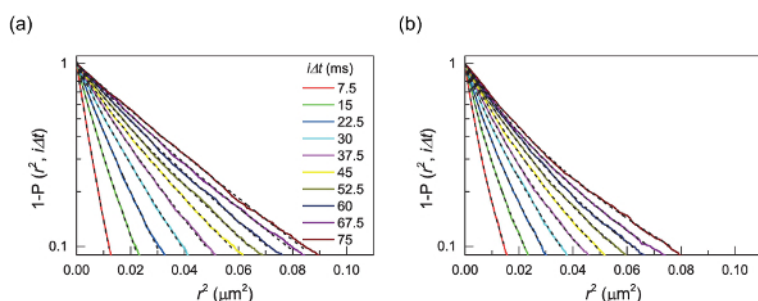


Figure 5. CDF analysis of the perylene diimide-incorporated poly(THF)s diffusing in the melt. Experimentally obtained cumulative distribution functions ($\Delta t = 7.5 - 75$ msec) in the form of $1-P$ for (a) 4-armed star polymers and (b) 8-shaped dicyclic polymers in the melt of non-labeled linear poly(THF). Dashed lines show fittings with (a) equations in 4.2.3.2 and (b) equations in 4.2.3.3.¹⁴ Please click here to view a larger version of this figure.

Discussion

The 4-armed and 8-shaped polymers were prepared via the ESA-CF protocol (Figure 1), which is a critical step for the synthesis.^{12,24} Monofunctional and bifunctional linear poly(THF)s with *N*-phenylpiperidinium end groups were synthesized according to the previous procedure.¹¹ The ion exchange was carried out by reprecipitation of an acetone solution of a polymer precursor with triflate counteranions into an aqueous solution containing an excess amount of carboxylate.

The covalent conversion of the ion-exchange product for the 4-armed star polymers was performed in toluene (4.9 g/L) by reflux for 4 hr. If the conversion is not sufficient, extend the reaction time. The covalently bonded product was obtained by silica gel column chromatography with acetone/*n*-hexane and reprecipitation in water. The ¹H NMR of the 4-armed polymers is shown in Figure 2a. This ESA-CF procedure allows for the effective synthesis of cyclic polymers. However, this protocol is limited to polymers that have cyclic onium end groups.

For the 8-shaped polymer product, the reaction was carried out in toluene in dilution (0.2 g/L) under reflux for 4 hr and resulted in the formation of a large portion of an insoluble fraction, presumably due to the intermolecular reacted products. A soluble portion was reprecipitated in *n*-hexane, subjected to silica gel column chromatography with acetone/*n*-hexane, and reprecipitated in water. The obtained crude product was subjected to column chromatography with size exclusion beads and recycle GPC to allow isolation of the 8-shaped polymer product. The ¹H NMR of the 8-shaped polymers is shown in Figure 2b. This ESA-CF protocol can be applicable to further complex topological polymers.

The high quality fluorescence images are essential for the accurate analysis of the diffusive motion of the molecules. The fluorescence images are significantly deteriorated when 1) fluorescence impurities are present in the sample, 2) the fluorescence quantum yield of an incorporated fluorophore is low, and 3) the frame rate of the imaging is slower than the diffusive motion of the polymer molecules. Setting the temperature below room temperature (20 °C) or above 37 °C will cause a refractive index mismatch, which will also deteriorate the quality of recorded fluorescence images. Using a narrower band emission bandpass filter mounted on a filter cube sometime improves the quality of the fluorescence image. Since the exposure time of the EM-CCD camera used in the imaging experiment is usually limited to milliseconds, the diffusive motion faster than this time scale cannot be captured by this method.

The evaluation of the effect of the statistical error in the MSD analysis is the critical step for the characterization of the heterogeneous diffusion. The statistical error should be carefully evaluated by calculating the probability distribution of the diffusion coefficient using the homogeneous diffusion model²² before discussing the heterogeneous diffusion. The heterogeneous diffusion should also be carefully evaluated by the CDF analysis. When the CDFs show clear deviations from the homogeneous diffusion model (i.e., single-exponential decaying curve), this suggests the presence of multiple diffusing components. The quantitative characterization of heterogeneous diffusion requires combined MSD, CDF, and probability distribution analyses.^{14,15}

Polymer dynamics, including diffusive motion, has been described as ensemble-averaged values in the conventional methods such as NMR,⁷ light scattering,⁸ and viscosity measurements.⁹ Indeed, the heterogeneous diffusive motion revealed by the single-molecule imaging¹⁶ is often very difficult to detect in the ensemble-averaged methods. Considering the inherent heterogeneous nature of polymers,²⁵⁻²⁷ the method reported in this protocol is not restricted to the characterization of topological polymers, but is applicable to all kinds of polymers under entangled conditions.²⁸ Furthermore, the approach reported in this protocol will find a wide application in the analysis of heterogeneous diffusion in complex systems, such as molecular diffusion through mesoporous materials.²⁹

Disclosures

The authors have nothing to disclose.

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