Preparation and Characterization of a Set of Linear DNA Molecules for Polymer Physics and Rheology Studies

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ABSTRACT: Imaging of single DNA molecules has enabled detailed studies of dilute polymer dynamics and rigorous testing of assumptions and predictions of molecular theories. It is of interest to extend these methods to the study of entangled polymers and to correlate molecular dynamics with rheology measurements. Progress in this direction has been hampered, however, by a lack of available DNA samples in sufficient quantities and covering a wide range of lengths. Here we describe the preparation of a suitable set of molecules ranging in length from ∼3 to 300 kilobase pairs. These constructs are replicated as plasmids or as fosmids or bacterial artificial chromosomes fitted with an inducible high-copy number origin of replication. DNA sequences were chosen to allow molecules to be linearized by single-cutting restriction enzymes. We show that these molecules can be imaged and characterized by fluorescence microscopy and can be prepared in sufficient quantities for bulk rheology measurements.

Introduction

In addition to being of great biological importance, DNA is a valuable model compound for studying polymer physics and polymeric fluid rheology.[1] Indeed, some of the early pioneers in polymer physics research, such as Bruno Zimm, were motivated by this connection.[2−4] Polymer solutions of all compositions display intriguing non-Newtonian properties that are of wide interest and relevance in the physical and biological sciences and engineering. Investigation of the connection between the molecular dynamics and the macroscopic properties of polymeric fluids has been a long-standing area of research for more than half a century.[5−7]

Over the past decade, the development of a single molecule approach to studying polymer conformation and dynamics based on observation and manipulation of individual DNA molecules has resulted in many advances in understanding.[8] Most of the single molecule imaging experiments undertaken to date have examined the dynamics of dilute, isolated DNA molecules. Hydrodynamic deformation and entropic relaxation of single tethered molecules have been studied using optical tweezers,[9−12] free diffusion has been studied by single molecule tracking of Brownian motion,[13] and the dynamics of molecules in flow has been studied by imaging molecules in various types of miniature fluidic cells.[14−22] Only a few of these studies utilized molecules of varying lengths to investigate length dependence, and none required preparation of large quantities of homogeneous, monodisperse DNA samples. Heterogeneous samples, such as randomly ligated λ DNA concatemers, λ restriction fragments, or partly fragmented *E. coli* genomic DNA, were acceptable for use in these dilute solution studies because the lengths of isolated molecules could be determined during the measurements.[13,18] However, extension of such studies to entangled polymers is preferably done with monodisperse samples.[7]

Entangled DNA molecules have only been imaged in two previous studies. In the first, single fluorescent-labeled DNA molecules were entangled in a concentrated solution of unlabeled λ DNA (48.5 kilobase pairs (kbp) = 16.3 μm) and manipulated by optical tweezers. In this study we confirmed by visual inspection that entangled polymers undergo tubelike motion, as postulated in the widely applied reptation model of P. G. de Gennes.[9] In the second, diffusion of λ DNA and shorter fragments of λ DNA was imaged in an entangled solution of unlabeled λ DNA.[23] This study allowed for estimation of the time scale of entanglements and threshold concentration for reptation, but only for entangling DNA molecules of that one particular length.

Several extensions of these previous experiments, which demonstrated the feasibility of studying single entangled polymer dynamics with DNA, are of great interest. First, one would like to vary the length of the entangling chains to investigate scaling laws; second, to study the dynamics of entangled molecule in flows, as has been done with dilute solutions; and third, to characterize the same samples used in the single molecule experiments by bulk rheological measurements.[24,25]

A major obstacle standing in the way of this program is the lack of available DNA molecules in large quantities covering a range of lengths from below to well above the critical entanglement length (which we estimate to be ∼10 kbp, as discussed below). Currently, λ and T7 DNA, of ∼49 and ∼40 kbp, are the only purified samples that are commercially available in the USA in milligram quantities (from New England Biolabs, Invitrogen, and Boca Scientific). These samples are quite expensive (currently ∼$200−1400 (US) per mg), especially when one considers that quantities of ∼10 mg would typically be desired for entangled polymer dynamics experiments. T2 and T4 DNA, of ∼170 kbp, which have been used in a couple of earlier rheology studies[4,26] are no longer commercially available. Therefore, to overcome the problem of sample availability, we describe here the design and preparation of large quantities (up to tens of milligrams per preparation) of eight different double-stranded DNA constructs ranging in length from ∼3 to 300 kbp (∼1 to 100 μm in physical contour length). These samples can be prepared by DNA replication using a single consistent method, and any research group can produce exact copies of these samples by following the protocols described here.

We note that a similar effort, producing a collection of DNA samples for use as model compounds in physical studies, was

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Table 1. Collection of DNA Constructs

name	size (kbp)	1 cutter	antibiotic resistance
	Plasmids		
pUC19	2.7	BamHI[a]	ampicillin
pYES2	5.9	BamHI	ampicillin
pPIC9K⟨*TRL*5⟩	11.1	BamHI	ampicillin
	Fosmids		
pCC1FOS-25	25	ApaI[b]	chloramphenicol
pCC1FOS-45	45	ApaI	chloramphenicol
	BACs		
CTD-2342K16⟨oriV/KAN-2⟩	114.8	MluI[c]	chloramphenicol/ kanamycin
CTD-2609C22⟨oriV/KAN-2⟩	185.4	MluI	chloramphenicol/ kanamycin
CTD-2657L24⟨oriV/KAN-2⟩	289.0	MluI	chloramphenicol/ kanamycin

[a] Fisher#S66767. [b] Fisher #NC9841497. [c] Fisher#NC9256002.

recently published by Planken et al.; however, they focused on molecules of shorter lengths, ranging from 0.2 to 1.6 kbp.[27] Our constructs, which cover the higher range of ∼3−300 kbp, are relevant for studying entangled polymer dynamics and very nicely extend the range of molecular lengths developed in this previous work. Also, we have succeeded in obtaining an order of magnitude higher yield than was achieved in this previous work.

Double-stranded DNA (dsDNA) has a ∼2 nm diameter, and its elasticity is in excellent agreement with predictions of the wormlike chain model, indicating a persistence length of ∼50 nm and contour length of 0.34 nm/bp.[28,29] DNA in single-stranded form (ssDNA) has an order of magnitude smaller persistence length and can be produced by placing dsDNA in alkaline conditions. An additional advantage of DNA as a model system for polymer physics studies is that its length can be precisely controlled at the level of a single base pair (0.34 nm).

DNA Constructs

We have prepared samples by replication of cloned DNA in *E. coli* using plasmid, fosmid, and bacterial artificial chromosome (BAC) constructs (Table 1). Each construct consists of a vector, containing DNA sequences required for replication and conferring antibiotic resistance, and an inserted DNA segment containing cloned DNA sequences. As described below, special effort was required to choose or design these constructs to have certain properties desired for their intended use in polymer physics and rheology experiments. In particular, we sought to prepare a collection of constructs having desired molecular lengths, capability for high yield production, and sequences allowing for preparation of linear molecules.

Plasmids are supercoiled DNA molecules, ranging from ∼3 to 15 kbp, that can be replicated in bacteria at up to ∼500 copies per cell.[30] Plasmid vectors are typically ∼3−8 kbp, and stable plasmid inserts are typically less than 10 kbp, so these constructs are only useful for producing DNA molecules up to 5 μm (∼100 persistence lengths), which brackets the small end of what is desired for studies of entangled polymer physics. Commercially available plasmids are only sold in microgram quantities, which are insufficient for direct use in physical experiments. We have thus prepared three plasmid constructs ranging from 2.7 to 11.1 kbp for inclusion in our collection of DNA samples.

To prepare longer molecules, we chose to use fosmid and BAC clones, which were originally developed for use in DNA cloning in genome sequencing projects. Fosmids are usually ∼30−50 kbp while BACs allow for an upper limit as high as ∼300 kbp and are designed to replicate at only one or two copies per cell for many generations with high stability.[31] While the low copy number confers long-term stability in stock cultures, it also results in much lower yield of DNA. To overcome this problem, we integrated an inducible high-copy number origin of replication (oriV) into the fosmid constructs during cloning and into the BAC constructs by means of DNA transposition.[32] The cloned DNA is then replicated at low copy number, which allows for stable maintenance of the clone in stock cultures. However, addition of an inducer (L-arabinose) during DNA preparation boosts replication, which results in significantly higher yields.[33]

DNA Sequences

To convert the DNA to linear form, we sought to use restriction endonucleases that would cut only once in the DNA template. Thus, part of the challenge of this project was to choose or design such constructs. Satisfying this requirement proved to be relatively easy in the case of plasmids due to their short length. We obtained three plasmid constructs from ∼3 to 11 kbp that are cut once by BamHI, which is an inexpensive and widely available restriction endonuclease. Two constructs were simply standard cloning vectors, while the third, pPIC-⟨TRL5⟩, contained an insert (gene sequence coding for TRL5).

We prepared fosmid constructs by cloning because we were unable to locate any existing constructs that were publicly available, sequenced, and contained a high-copy origin. We therefore cloned fragments of λ DNA using the pCC1FOS vector,[34] which contains the oriV origin. Two clones of ∼25 and 45 kbp (termed pCC1FOS25 and pCC1FOS45) were identified to have a single ApaI cut site following screening of clones with a battery of restriction enzymes.

While many BAC clones are publicly available due to their use in the human genome-sequencing project, we found that most randomly tested BAC sequences did not contain a single-cutting restriction site, or at least one corresponding to an available, suitably inexpensive restriction endonuclease. This situation is easily understood as being due to the long lengths of BACs. Therefore, we screened BAC sequences from a large library at Caltech (CTD library) for suitability by using bioinformatics techniques. In particular, we used sequence alignment software[34] to map sequenced BAC end pairs onto the human genome sequence, which then allowed us to identify several clones that could be cut once by MluI, which is reasonably inexpensive. We then retrofitted these BAC clones with an inducible oriV origin, as described above.

DNA Purification

Detailed, step-by-step protocols for preparing these DNA constructs are given in the Supporting Information. We sought to devise the simplest protocol that would be sufficient for the intended use of the samples. A commercial kit for purifying fosmid and BAC DNAs is available from Qiagen; however, this kit is prohibitively expensive for producing large quantities of DNA (cost of ∼$300 for a kit that can purify only up to 1.5 mg of BAC DNA, which does not include the cost of the restriction endonuclease needed to linearize the DNA). Our method was modified from various standard methods in molecular biology. Briefly, cultures of *E. coli* containing the desired DNA clones are grown from frozen stocks, and the cells are lysed via treatment with an alkaline solution. The cloned DNA is renatured by an acidic detergent solution in which genomic DNA and cellular debris precipitate and are removed by centrifugation. The DNA is then precipitated in cold 2-propanol, washed in 70% ethanol, and redissolved in aqueous

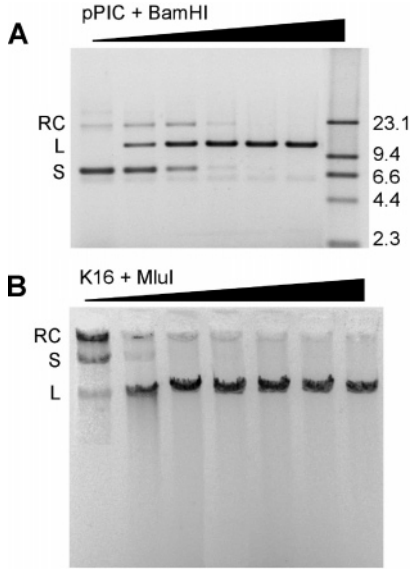

Figure 1. Agarose gel electrophoresis showing conversion of DNA constructs to linear form. Bands corresponding to molecules in the supercoiled circular, relaxed circular, and linear forms are indicated by the labels S, RC, and L, respectively. (A) Cleavage of the plasmid clone pPIC9⟨TLR5⟩ at one recognition site by BamHI. Gel lanes (from left to right) show DNA incubated with increasing amounts of enzyme (0, 0.02, 0.06, 0.17, 0.5, and 1.5 units of BamHI per μg of DNA) at 37 °C for 6 h. (B) Cleavage of the BAC clone K16 at one recognition site by MluI. Gel lanes (from left to right) show DNA incubated with increasing amounts of enzyme (0, 0.03, 0.1, 0.3, 1, 3, and 10 units of MluI per μg of DNA) at 37 °C for 6 h. Samples were run on a 0.8% agarose gel in 1X TAE buffer at 3 V/cm (dc) for 3 h and stained with ethidium bromide.

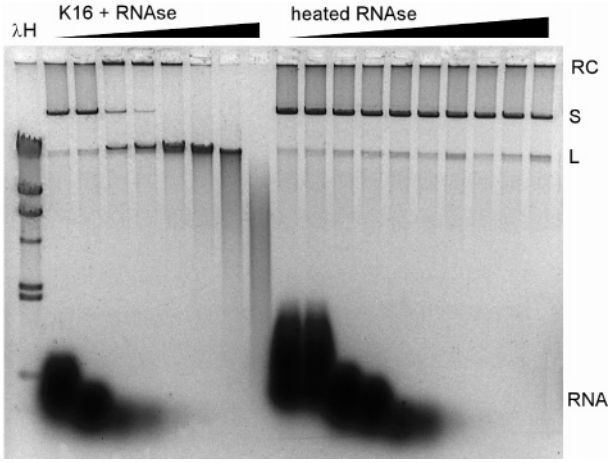

Figure 2. Removal of contaminating RNA from the K16 BAC DNA sample as determined by gel electrophoresis. Bands corresponding to molecules in the supercoiled circular, relaxed circular, and linear forms are indicated by the labels S, RC, and L, respectively. Far left lane: λ DNA HindIII digest used as a molecular weight standard. Lanes 2−9: samples following incubation with increasing amounts of RNase A (0.3, 1, 3, 10, 30, 100, 300, 1000 ng of RNase per μg of DNA) for 1 h at 37 °C. Lanes 10−19: samples following incubation with increasing amounts of RNase A (0.3, 1, 3, 10, 30, 100, 300, 1000, 3000, and 10000 ng of RNase per μg of DNA) after the enzyme was preheated to 80 °C for 20 min. Note the suppression of DNA degradation obtained with the preheated RNase. The gel was run as in Figure 1.

solution at pH 8. The molecules are then converted into linear form by treatment with the appropriate restriction enzyme (Table 1). To remove contaminating RNA the sample is treated with RNase A, and protein is removed by phenol−chloroform extraction followed by dialysis. Finally, the samples are concentrated by a second 2-propanol precipitation.

Sample Characterization

We characterized the purified DNA samples by agarose gel electrophoresis, UV absorption spectroscopy, colorimetric protein-dye binding assays, and single molecule fluorescence imaging. We found that purifying DNA from 3 L of bacterial culture is a quite feasible scale for a small laboratory and typically yields ∼10−30 mg of DNA, with ∼70−90% recovery of that obtained in the initial lysis step. Following the final 2-propanol precipitation, we redissolved the DNA at a concentrations ranging from ∼1 to 10 mg/mL. We determined the concentration of our samples by gel electrophoresis with ethidium bromide staining and comparison against a standard of known concentration (λ DNA from New England Biolabs). As ∼0.5 mg/mL of λ DNA is the threshold for observing entanglement effects,[23] a yield of tens of milligrams is a sufficient quantity for many types of rheology measurements. While our present focus is on single molecule imaging, we expect that researchers with the expertise and equipment needed for carrying out various types of rheology measurements will be interested in studying these samples.

Following the initial extraction and precipitation of the cloned DNA we find, as anticipated, that most of the molecules are in the supercoiled form or in a combination of the relaxed circular and supercoiled forms. As illustrated in Figure 1, the molecules may be converted into the linear form by digestion with the appropriate restriction endonuclease. We have carried out titrations in order to estimate the minimum amount of enzyme required, rather than simply using an excess, because the restriction enzyme is the most expensive reagent needed in the preparation. Although an often stated rule of thumb in molecular biology is that ∼1 unit of enzyme is recommended for digestion of ∼1 μg of DNA in a typical reaction, we found that it was possible to use significantly less enzyme. By using siliconized tubes, keeping the DNA concentrated, avoiding serial dilution of the enzyme, and extending the reaction time to 6 h, we found that complete cutting could be obtained with as little as 0.1 units of enzyme per microgram of DNA. This approach further lowers the cost of each preparation.

As the DNA was extracted from bacteria, the purity of the final sample is an important issue. UV spectroscopy indicated an absorbance peak at 260 nm and $A_{260}/A_{280} \cong 2$, which are characteristic of pure double-stranded DNA.[30] Successful removal of contaminating proteins was also assessed more sensitively by using a colorimetric protein-dye binding assay (Micro BCA protein assay, Pierce Biotechnology). This assay indicated a low residual protein contamination of only ∼2 μg of protein per mg of DNA. Successful removal of contaminating RNA is shown in Figure 2. We titrated the RNase to determine the amount needed to fully digest the RNA. Highly purified RNase A, certified to be free of DNase activity, is available but is prohibitively expensive given the scale of our preparation. We therefore chose to use an inexpensive preparation of RNase A from bovine pancreas (Fisher Scientific). While we found that this RNase preparation causes degradation of the DNA, we found that this problem was eliminated by preheating the RNase A to 80 °C for 20 min.

We also tested the amplification in yield obtained by induction of the oriV origin of replication that we included in our constructs. As shown in Figure 3E, we found that cultures grown with 0.1 to 0.01% (w/v) of L-arabinose produce a gain of at least 10-fold in the DNA yield. Based on gel electrophoresis and fluorescence microscopy, there was no effect on the quality of the DNA prepared following induction with L-arabinose. This amplification method is thus valuable for preparing the desired large quantities of DNA. The cost of the reagents needed to

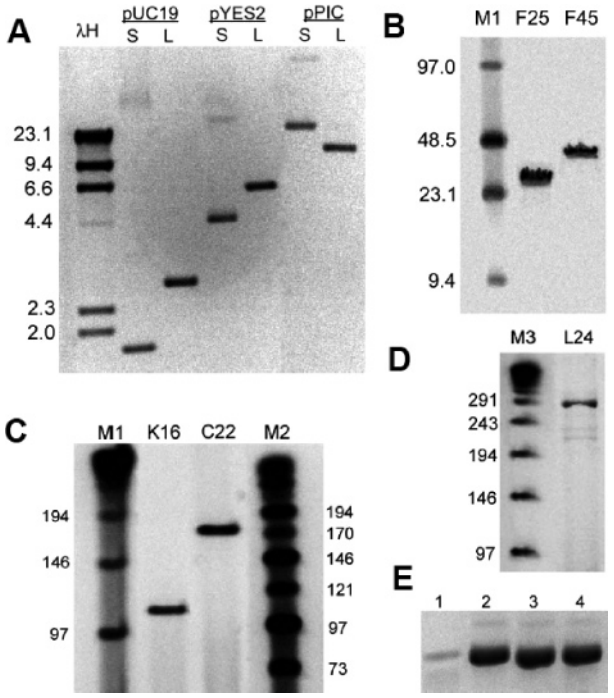

Figure 3. Analysis of the linearized DNA by agarose gel electrophoresis. (A) Plasmid DNA samples. Far left lane: λ DNA HindIII digest used as a molecular weight standard (λH). Subsequent lanes show indicated plasmid DNA samples in supercoiled form (S) and in linear form (L) following digestion with BamHI. The gel was run as in Figure 1. (B) Fosmid DNA samples. Far left lane (M1): low range pulsed field gel electrophoresis marker (New England Biolabs), with DNA sizes listed in kilobase pairs. Subsequent lanes show Fos25 and Fos45 fosmid DNA samples after linearization with ApaI. Samples were run on a 1% agarose gel in a cold room (5 °C) in 0.5X TBE buffer in a pulsed field gel system with clamped homogeneous electric fields (Biorad) at 200 V for 15 h. Field switch times were ramped from 1 to 15 s, and the gel was stained with ethidium bromide. (C) BAC DNA samples. Far left lane: low range marker (M1) (New England Biolabs), with DNA sizes listed in kilobase pairs. Lanes 2 and 3: indicated BAC clones retrofitted with the ⟨oriV/KAN2⟩ transposon. Far right lane: midrange II pulsed field marker (M2) (New England Biolabs). The gel was run as in (B). (D) L24 BAC DNA sample (the largest construct in our collection). Far left lane: λ ladder PFG marker (M3). The gel was run as in (B), but for 24 h with switch times ramped from 1 to 25 s. (E) Electrophoresis gel of the Fos45 construct showing the gain in sample yield upon induction of the oriV origin of repliction. Lane 1 is a control culture with no inducer added; lane 2 contains 1/1000 volume of Copy Control reagent (Epicenter); lanes 3 and 4 contain 0.1% and 0.01% (w/v) L-Arabinose. The gel was run as in (A).

prepare one batch of DNA by our protocol is ~$20−80 (US), with the greatest expense being for MluI. By comparison, purchase of an equal amount of λ DNA is ~10−50 times more expensive and limits one to studying only one particular length molecule.

Gel electrophoresis measurements were done to confirm that the sizes of the constructs corresponded to the expected DNA sequence lengths. As shown in Figure 3A, the linearized plasmid DNA constructs were clearly resolved. While the linear form of fosmids and BACs can be easily distinguished from the supercoiled or relaxed circular forms by DC agarose gel electrophoresis, it is not possible to size linear DNA constructs by this method because molecules longer than ~30 kbp have nearly the same mobility in constant voltage (dc) electrophoresis. Therefore, we used pulsed field electrophoresis with contour-clamped homogeneous electric fields[35] to confirm the sizes of the fosmid and BAC constructs (Figure 3B−D).

To substantiate our gel electrophoresis results for the sizes of our constructs and to test that the prepared DNA molecules could be used in the same manner that λ DNA has been used in previous single molecule experiments, we labeled the DNA samples with the dye YOYO-1 (Molecular Probes) and imaged them at high magnification in an epifluorescence microscope. Protocols used for labeling and imaging are given in the Supporting Information. As shown in Figure 4, single molecules of the plasmid, fosmid, and BAC constructs could be easily visualized, and individual samples of molecules appeared to be homogeneous. We observed that the areas of the images monotonically increased with expected size, pCC1FOS-45 (expected to be ~45 kbp) was roughly the same size as λ DNA (48.5 kpb), as expected, and no difference in the quality of our samples was detected compared to the commercially prepared λ DNA. We did not quantify these image sizes because measuring sizes of such bright, compact moving objects imaged with an intensified video camera is known to be inaccurate because of blooming artifacts in the camera, diffraction effects, and out-of-focus effects which have been discussed previously.[35]

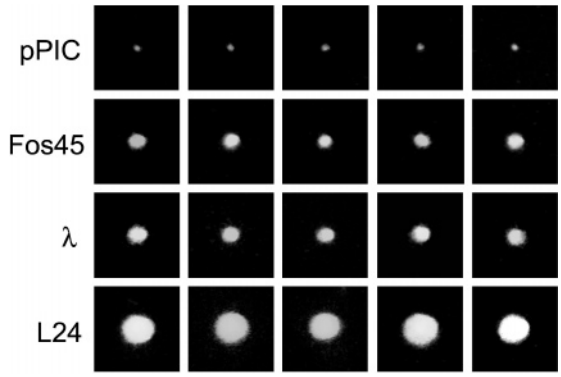

Figure 4. Fluorescence microscopy images of YOYO-1-labeled DNA molecules. Snapshots of five randomly chosen molecules are shown, including examples of a linearized plasmid (pPIC), fosmid (Fos45), and BAC (L24). Images of λ DNA purchased from New England Biolabs are shown for comparison. Each image covers a 12 μm by 12 μm field of view.

Quantitative evidence that the molecules were behaving in accord with their expected lengths was obtained by using single molecule tracking to measure the diffusion coefficients (D) of the molecules in the limit of infinite dilution. Specifically, for pYES2 (5.9 kbp), pPIC9K⟨TRL5⟩ (11.1 kbp), pCC1FOS-25 (25 kbp), pCC1FOS-45 (45 kbp), CTD-2342K16⟨oriV/KAN-2⟩ (114.8 kbp), CTD-2609C22⟨oriV/KAN-2⟩ (185.4 kbp), and CTD-2657L24⟨oriV/KAN-2⟩ (289.0 kbp) we obtained D values of 1.282, 0.976, 0.605, 0.437, 0.260, 0.197, and 0.139 $\mu m^2/s$, respectively. These diffusion coefficients scale as $D \sim 1/R_G \sim (\text{length})^{-0.57\pm0.01}$, where R_G is the predicted radius of gyration, in agreement with theoretical predictions for long, flexible polymers in good solvent conditions.[6]

We also sought to establish that the length range of constructs chosen was appropriate for thorough investigation of entangled polymer dynamics. As a part of our continuing single molecule imaging studies, we made diffusion measurements by single-molecule tracking that allowed us to probe the behavior of our constructs in the dilute, semidilute, and concentrated regimes. First we measured the diffusion coefficients (D) for our constructs in the limit of infinite dilution, as described above. Using the relationship $D = 0.0829 k_B T/R_G^6$ along with an equation for the overlap concentration $C^* \cong (4/3)M/N_A R_G^3$,[6] where M is molecular weight and N_A is Avogadro's number, we calculated C^* for our constructs. For pYES2 (5.9 kb), pPIC9K⟨TRL5⟩ (11.1 kbp), pCC1FOS-25 (25 kbp), pCC1FOS-45 (45 kbp), CTD-2342K16⟨oriV/KAN-2⟩ (114.8 kbp), CTD-2609C22⟨oriV/KAN-2⟩ (185.4 kbp), and CTD-2657L24⟨oriV/

KAN-2⟩ (289.0 kbp), we obtained C^* values of 0.160, 0.133, 0.071, 0.047, 0.025, 0.018, and 0.010 mg/mL, respectively. Two previous studies have estimated the critical entanglement concentration (C_e) for DNA and reported values of $C_e \sim 10C^*$ for T2 DNA (164 kbp)[36] and $C_e \sim 15C^*$ for polydisperse calf thymus DNA (~13 kbp).[37] For our 45 kbp construct, which is similar in length to λ DNA, we observed that the diffusion coefficient decreased rapidly with concentration from the dilute D value, by a factor of 10 at ~6C^* and a factor of 40 at ~10C^*. The molecules shorter than 11.1 kbp exhibited a much weaker decrease with concentration, suggesting that they do not become fully entangled. For example, the diffusion coefficient for the 6 kbp construct decreased only ~4-fold at 6C^*. Therefore, the length range of constructs we have prepared is an ideal range that spans the entanglement molecular weight of DNA and allows for ample investigation of the length and concentration dependence of DNA dynamics in the dilute, semidilute, and concentrated regimes.

Conclusion

The samples described and characterized here will facilitate the extension of single polymer dynamics experiments to the case of entangled polymers and comparison with bulk rheology measurements. Having a wide series of molecular lengths will allow for examination of scaling laws and how polymer solution dynamics change during the crossover from the dilute to the entangled regimes.

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Supporting Information Available: Detailed, step-by-step protocols for preparing DNA constructs. This material is available free of charge via the Internet at http://pubs.acs.org.

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