LETTER

An experimental investigation of attraction between knots in a stretched DNA molecule

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An experimental investigation of attraction between knots in a stretched DNA molecule

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Abstract – It has been predicted through simulations that two knots in a stretched polymer will feel an attraction towards one another and will tend to be found in close proximity. Here, we examine data from experiments in which we stretched knotted DNA molecules and investigate a subset which contained two or more knots. In contrast to the expected behavior of an isolated knot in an elongational field, we observe that pairs of knots move towards each other from distant sections of the molecule. After meeting, pairs of knots fluctuate in and out of visual contact, and can remain in proximity for minutes at a time. Our experimental results suggest that knots on an extended polymer experience an attractive interaction.

Introduction. – For over two decades, genomic double-stranded DNA molecules have served as a model experimental system for single-polymer physics [1,2]. The scientific developments in single-polymer physics have emerged in step with the development of single-molecule genomics technologies such as nanochannel mapping and nanopore sequencing [3]. More recently, there has been interest in the physics of knots in polymers. Knots in single polymers represent a minimal system of polymer entanglement. Knots have been observed forming stochastically in DNA in single-molecule genomics tools [4], potentially leading to false positives in nanochannel assays, and are a potential source of blockages in nanopore sequencing [5]. The application of statistical mechanics and reptation theory to polymer knotting has led to the elegant concept of knot metastability, predicting the entropic self-tightening of a knot to limit the length of confined contour in the knot [6].

Most computational and experimental studies have focused on molecules with a single knot, "prime" in the language of knot theory. A sufficiently long polymer chain at thermal equilibrium is virtually guaranteed to be knotted [7]. While trefoil knots are the most common, simulations of knot formation in polymers parameterized to DNA find that with respect to contour length, the probability of multiple trefoil knots ("composite knots") increases faster than that of more complex prime knots [8].

The largest commercially available DNA molecules, from the bacteriophage T4, are predicted to contain knots 40% of the time at high ionic strength. This has been recently verified by two nanopore translocation experiments [5,9]. It was shown by Amin *et al.* [10] that a strongly compressed DNA molecule is more likely to form two knots than a single knot. We have reported that two different-sized knots in a relaxing DNA molecule will expand at the same rate [11], that a molecule with two knots relaxes faster than a molecule with one knot [12], and that each of two knots in a stretched molecule can undergo diffusive or convective motion [13].

There have been four simulation papers explicitly considering the interaction of two knots on a stretched chain. They each consider distributions of the knot-knot distance and argue that there is an entropic attraction between knots —the global maximum in entropy occurs when the smallest amount of contour is within the knot, which occurs when the two knots are intertwined within one-another. The first paper by Trefz *et al.* makes the intriguing prediction that two knots can pass through one-another [14]. The second and third papers by Najafi *et al.* examine the role of knot chirality and bending rigidity on the intraknot attraction [15,16]. The most recent, by Richard *et al.* from the same group as the first, parameterizes the chain to DNA and investigates the strength of the attraction potential [17]. Because these studies use

similar methodology to arrive at similar results, we will refer to them collectively.

The present manuscript is inspired by these simulation papers, and attempts to empirically investigate whether there is indeed an attraction between knots in stretched DNA. Our group has been investigating the physics of self-entangled and knotted DNA, using microfluidics to stretch and interrogate viral genomic DNA molecules that can be self-entangled with strong electric fields. Our experimental efforts have been supported by Brownian dynamics simulations and polymer theory. We have gained considerable insight into the physics of knots in DNA —we have found that entanglements make molecules more difficult to stretch [18], that knots affect the relaxation time of an individual molecule [11,12], that knots can be driven towards the ends of molecules with elongational fields [13], and that the untying process can occur in multiple stages and that it is coupled to the elasticity of the molecule [19,20]. For simplicity, we focused our past experiments and simulations on individual knots, but in many experiments the molecules were observed with two or more knots. Having made our conclusions from single-knot investigations, here we report on the possibility of interactions between knots in a DNA chain.

Experiments. – Our experimental setup involved stretching DNA from the T4 bacteriophage (166 kbp, approximately 77 μm contour length when stained with YOYO-1 fluorescent dye [21]). Additional staining details may be found in the Supplementary Information `Supplementarymaterial.pdf` (SI). We used microfluidic devices cast in polydimethylsiloxane (PDMS) that contain two perpendicular channels 2 microns in height that meet in either a cross-slot or T-junction (fig. 1(a)). The corners at the junctions are smoothed to hyperbolae. An aqueous ionic buffer containing the DNA was pipetted into the reservoirs of the device to wet the channels. Platinum wires connected to a DC voltage supply were inserted into the reservoirs, with positive terminals connected to the horizontal arms of the device and grounds in the vertical or verticals. This circuit gives rise to a divergent electric field which stretches DNA molecules at its stagnation point (fig. 1(b)) in a manner akin to planar elongational flow —the velocity of a charged test particle diverges from the center at a speed proportional to its distance from the center. Similar devices have been used to stretch and interrogate DNA molecules with [11,12,18] and without knots [22]. A typical experiment involves electrophoretically translating a molecule to the stagnation point and then stretching it with a constant voltage, and either observing its steady-state dynamics or terminating the field to observe its relaxation. The strength of the elongational field is characterized by the Weissenberg number (Wi), which is the product of the molecule's longest relaxation time (2.2 seconds) and the strain rate of the electric field.

The method we use to form knots in DNA involves applying a strong (1 kV/cm) electric field which induces an
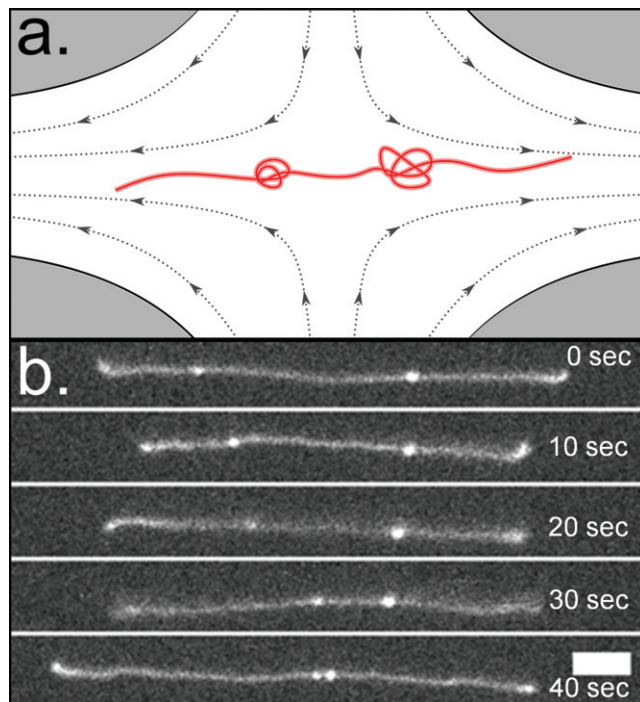

Fig. 1: Schematic diagram and images of a typical experiment. (a) A DNA molecule containing two knots is stretched in a microfluidic channel using a planar elongational electric field and held at the stagnation point. (b) Images from an experiment showing two knots moving towards each other from opposite ends of the molecule. Scale bar is 5 μm.

electrohydrodynamic instability that causes the molecule to collapse into a tight globule [18,23]. We apply the field as a square wave with a 10 Hz frequency for approximately 0.5 seconds. When the molecule is stretched from this globular state, the chain ends are pulled through the compressed molecule and are very likely to trap knots in the molecule's interior. The probability of knot formation is essentially 100%, forming knots that are significantly more complex than trefoils, with an order-of-magnitude more entangled contour [11].

The formation of knots through the electrohydrodynamic mechanism is highly stochastic and produces knots of widely varying sizes [12]. The complexity of the stretched knot depends not only on the initial collapse, but also on the elapsed time before the ends of the molecule can be pulled out of the tangle, during which diffusive disentanglement can simplify the knot. As part of the data collection for our previous studies [13,20], we recorded 334 stretched DNA molecules, of which 81 (24%) contained two knots, 15 (4.5%) contained three knots, and 2 (0.5%) contained four knots. Our observed fraction of composite knots is roughly twice that seen in equilibrium conditions by Sharma *et al.* [9], while Amin *et al.* found that molecules under extreme confinement become more likely to form two knots than one [10].

The elongational field used to stretch DNA molecules has two main effects on the behavior of knots that differ

from the isotension [24] or nanochannel [10] cases. In an elongational field, the tension profile in the chain is approximately quadratic, decreasing from the center of the molecule towards the end. Knots find themselves under lower tension towards the end of the molecules. There is additionally a motive force on the knots that drives knots towards the chain ends, although in the presence of thermal fluctuations the knot motion is by no means deterministic [13,25]. The non-uniform tension profile, the motive force on the knots, the propensity to untie, and the large topological complexity of the knots differentiate our experiments from many simulations, but comparisons can still be made.

In this manuscript, we report on the behavior of several dozen molecules containing two knots encountered in unpublished data gathered for previous studies [13,20]. Because some experimental protocols involved increasing or decreasing the Weissenberg number during observation to promote or hinder untying, not all previously gathered data are suitable for studying two-knot interaction. To further investigate the interaction between knots on a stretched chain, we performed an additional experiment in which we ignored single-knotted molecules and recorded 13 molecules containing two knots, stretching them at several different field strengths for minutes at a time.

Results and discussion. – We begin with a qualitative description of the behaviour of knot pairs. We cannot resolve the topology of a given knot within our experiments, but they may be distinguished from one another by their relative brightness, which serves as proxy for the amount of contour within a knot. When the knots' separation is less than the resolution limit of the microscope, they appear as a single brighter spot which is analogous to the "intertwined" case described in simulations. There were no instances in which two knots appeared to switch places after merging, preventing us from validating the predictions of Trefz et al. [14].

In many instances, we observe knots move towards each other from distant sections of the molecule, a behavior we refer to as "long-range" attraction (see Supplemental Videos `SI_vid-1.gif`, `SI_vid-2.gif` and `SI_vid-3.gif`). Three examples of this can be seen in the kymographs in fig. 2, with an additional twelve in fig. S1 in the SI. This can happen at timescales ranging from tens of seconds to several minutes, long compared to the 2.2 second relaxation time of the molecule. This is a visually striking effect, and is the opposite behavior expected for two non-interacting knots: the elongational field would tend to drive them in opposite directions towards each chain end [13,20]. Indeed, in the cases in which the knots do not interact, they typically start far apart on the molecule and quickly untie.

What is the possible mechanism of such long-range attraction? In the four simulation papers [14–17] predicting knot attraction, a long-range attractive free energy landscape was due to translational entropy —there are more
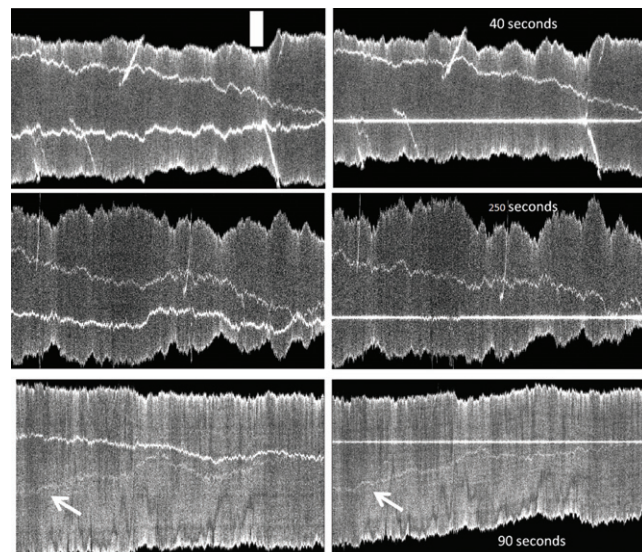

Fig. 2: Three kymographs of molecules with two knots in which they undergo "long-range" attraction. Images on the left are aligned with the center of the molecules, and images on the right are aligned to one of the knots. Bright streaks indicate other molecules passing through the field of view, which may lead to alignment errors. The duration of each kymograph is listed on the right. The vertical scale bar is $10\,\mu m$. Experimental details and additional kymographs can be found in the SI.

ways to position a pair of knots when they are close together than when they are far apart. However, such a mechanism may be an artifact of the simulation scheme; in those studies, the ends of the chain are fixed to walls to maintain a constant separation and topology. With the freedom to untie, allowing the knots to diffuse will lead to them untying, rather than exploring every possible pair of locations on the chain. Indeed, this contribution to the free energy is explicitly subtracted by Najafi et al. [16] to yield an effectively flat landscape with no long-range attraction. However, even after removing the topology-enforcing walls, Richard et al. [17] still observe a long-range attractive potential in free chains, although it is approximately flat at intermediate distances. Intriguingly, if the knots in some way anchor the fluctuations of a semiflexible chain, the restriction of fluctuation modes between the two knots may create a thermal Casimir effect that would induce mutual attraction. Such a mechanism is analogous to the attraction seen between simulated rings on a fluctuating polymer [26,27]. Any proposed mechanism for long-range knot attraction in our experiments is convoluted by the elongational field and its associated non-uniform tension profile. Experiments on two-knotted molecules in which the chain tension is uniform, such as those performed in nanochannels by Amin et al. [10], would provide further insight into the mechanism of knot attraction.

We can make more quantitative observations of knot interaction by investigating their short-range behavior. After two knots overlap for the first time, they typically

fluctuate in and out of contact for up to several minutes (fig. S3 in the SI), yielding a distribution of intraknot distances. This distribution can be interpreted as being due to a Boltzmann-weighted free energy landscape following the analysis of Trefz *et al.* [14], such that the free energy profile can be calculated from a normalized histogram of knot-knot separations (x_{kk}):

$$\frac{F(x_{kk})}{k_B T} = -\log(P(x_{kk})), \tag{1}$$

where $k_B T$ is the thermal energy and $P(x_{kk})$ is the probability of finding two knots at a separation distance x_{kk}.

We selected contiguous video sections of two-knotted molecules in which the knots are in proximity, such that the long-range component of the dynamics are removed and we can examine the steady-state behavior. To choose our contiguous video sample, we measured the farthest distance between the two knots that occurs between their initial and final contact, and selected all time-points in which the two knots were closer than that local maximum. The observation time for short-range interaction was at least one minute, corresponding to over 1000 time points at the typical recording rate of 17 frames per second. An example kymograph is seen in fig. 3(a), for a DNA molecule stretched at Wi = 1 with the two knots making up roughly 7.9% and 4.2% of the molecule's fluorescent intensity. We measured the positions of the two knots in each frame (the position taken as their brightest pixel), to get a time series of the intraknot distance, which is normalized by the extension of the molecule, such that if a molecule is stretched to $50\,\mu$m and one knot is located $10\,\mu$m from one end and the other is $25\,\mu$m from that end, the normalized knot separation is $15/50 = 0.3$ (fig. 3(b)). These were then sorted into $\sqrt{N}$ bins, where N is the number of frames analyzed. The histograms were normalized to have total area 1, and free energies were found according to eq. (1).

The short-range interaction distribution and free energy of two knots can be seen in fig. 3(c). The histograms and free energy landscapes have a common form —the most common separation is close to zero, corresponding to a global free energy minimum. At a short distance, there is a local minimum in the histogram corresponding to a local maximum in the free energy landscape. At farther distances, there is a wide and approximately normal distribution in separations corresponding to an approximately quadratic landscape, with no observations beyond the maximum distance. The free energy landscapes that we observe are qualitatively and quantitatively similar to those reported in the four simulation studies, which also feature a global minimum at zero distance, a short-range barrier of approximately 2 $k_B T$, a local minimum past the barrier, and a roughly quadratic rise in free energy to global maximum when the knots are far apart. The short-range landscape is theorized to be based on a balance of entropic forces contracting the pair into a single compositely knotted complex, and excluded volume interactions
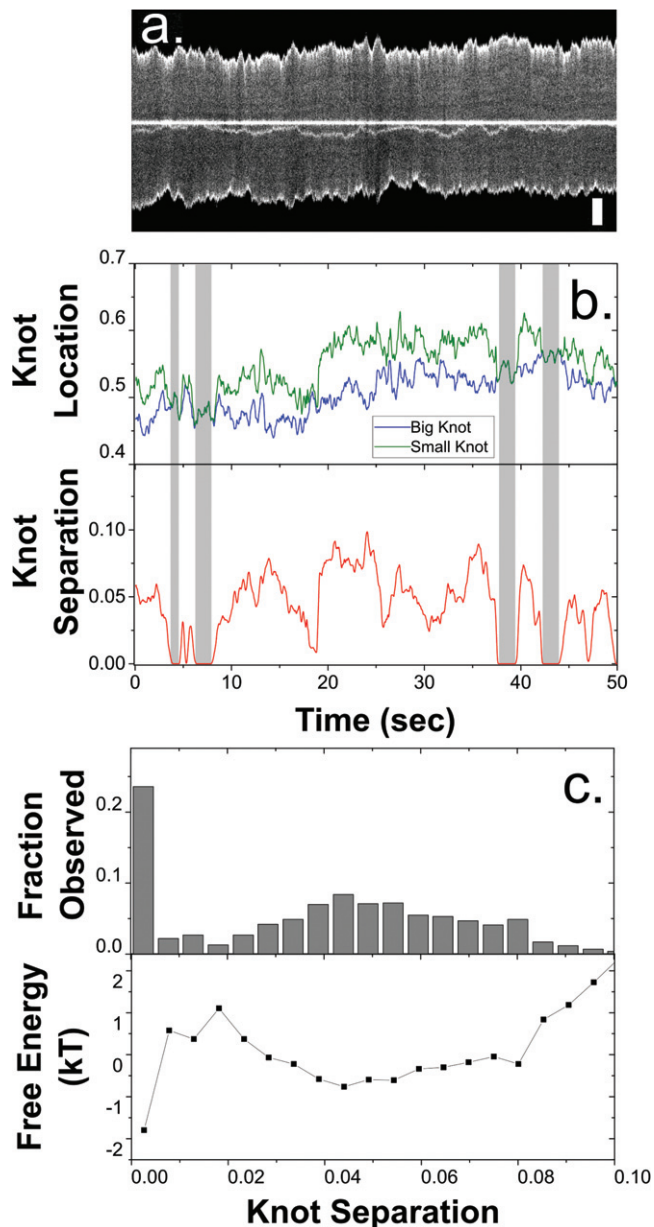

Fig. 3: Short-range interaction of two knots at Wi = 1. (a) Kymograph of a two-knotted molecule in the reference frame of the larger knot. At certain times, the two knots cannot be distinguished. Scale bar is $10\,\mu$m. (b) Plots of the two knots' position over time, and of the separation between them. Instances where the two knots are indistinguishable are marked in gray. (c) Histogram of the distances of the two knots, and a free energy landscape based on the logarithm of the distance distribution. The histogram shows a peak when the molecules are co-localized which corresponds to the minimum in the free energy plot.

between the two individual knots. We note that knot-knot excluded volume interactions were put forth as a mechanism by Amin *et al.* [10] to explain the observed distribution of knots formed by compression in nanochannels. The visual overlap we observe in experiments is likely not true topological intertwining, which would involve much

longer residence times and eventual pass-through, but may involve at least partial ingress of one knot into another.

Figure 4(a) shows landscapes from five different molecules stretched at Wi = 1.8, each with its own unique set of knot topologies. Each landscape has the same overall features: a minimum at zero distance, a local maximum at a short distance that is 2–3 k_BT above the minimum, and a broad shallow potential at larger distances. The exact details of the landscapes vary between molecules, particularly the "range" of the interaction, which is representative of how far knots will fluctuate from one another in between periods of contact. We note that although the longer-range sections of the landscape differ, in part due to differences in the knot topology between molecules, they are quantitatively similar at close range.

Figure 4(b) shows interaction landscapes obtained in an experiment where we hold a single molecule with two knots and vary the field strength (five different Wi from 1.4 to 3.0). In this experiment, the knot topologies in each curve are the same. Generally, the short-distance energy barrier increases with Wi, while longer-range deviations are more likely at low Wi. Richard *et al.* [17] found that with respect to the fixed distance between the chain ends, the interaction range decreases strongly with increasing separation, a finding consistent with our results. However, in both our experiments and in simulations, it is difficult to draw conclusions about the effect of tension or Wi on the depth of the attractive minimum. An additional seven landscapes can be found in fig. S4 in the SI.

We note that the landscapes are similar even when parameters dictate different behaviors for individual knots [13]. For example, the Wi = 3 landscape in fig. 4(b) is in the regime where jamming can occur, whereas the Wi = 1.4 observations occur in a regime where knot motion is likely. Indeed, knots have been seen moving towards one another even when they are very near the end of the molecule and would be expected to quickly untie (cf. fig. S1(k) in the SI), suggesting that the attraction between the knots is sufficient to overcome the opposing driving force of the elongational field. Overall, our experiments demonstrate that separate knots on a DNA chain have significant attraction that drives them into long-lived, close contact. The energy landscapes we extract from experiments bear a strong resemblance to those reported previously in simulations, despite differences in how the chain is stretched.

Much of the complexity arising in our analysis is due to the elongational field in our apparatus. In the experimental system used by Amin *et al.* [10], the knotted molecules are confined in nanochannels and are under effectively uniform tension after they have expanded from their initial compression. They report the observation of dozens of two-knotted molecules, and examining archival or future experimental data may reveal information about knot interaction on a uniformly tensioned chain. Indeed, in a related thesis [28], Amin presents kymographs of
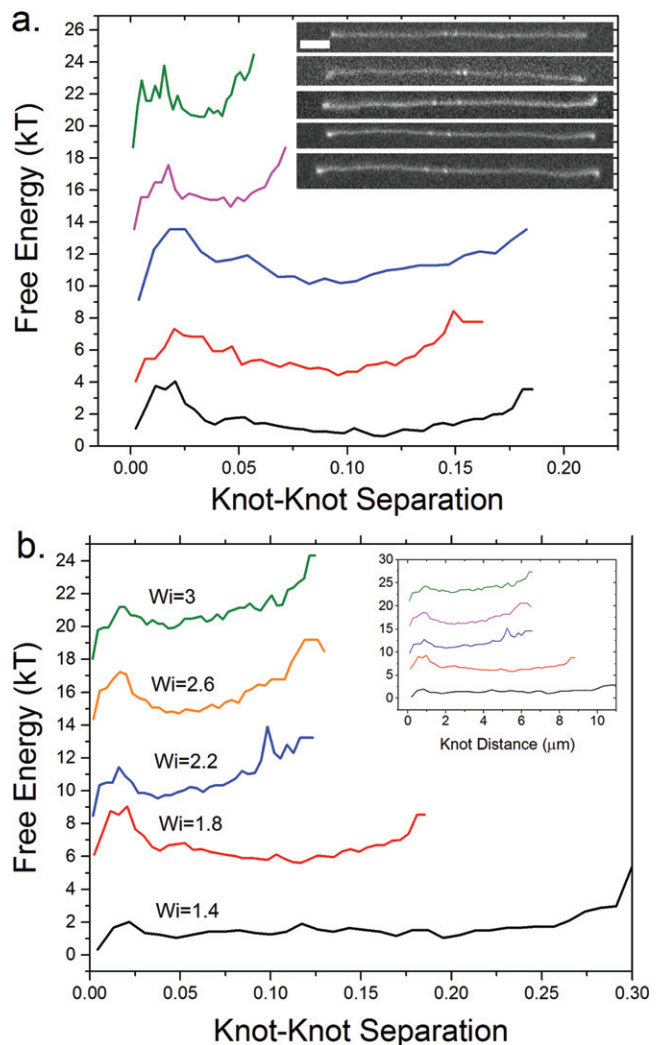

Fig. 4: (a) Interaction landscapes for five different molecules stretched at Wi = 1.8 on a staggered axis with images of each molecule. Scale bar is $5\,\mu$m. While there are differences in the details of each curve, the overall shape trends are similar. (b) Interaction landscapes for a molecule stretched at five different Wi numbers. Inset shows the same data with a dimensional x-axis.

two-knotted molecules that display a behavior that can be interpreted as knot attraction. The existing simulations of knot-knot interaction are somewhat limited by their use of a fixed-wall ensemble. Simulations based on a constant-force ensemble [20] or nanochannel confinement [29] may be useful in clarifying the mechanisms of knot attraction, although challenges arise due to the finite simulation window imposed by untying. Periodic boundary conditions, although unphysical, have been used in the simulation of stretched single knots [30], and a circular nanochannel, as demonstrated by Berard *et al.* [31], may be used to investigate the attraction of knots in stretched ring polymers with a physically realistic ensemble.

In our many experimental studies of polymer knots, we have stretched hundreds of entangled molecules and observed dozens of molecules containing two knots. In

many cases, we see the knots move towards each other in contrast to their expected behavior in an elongational field if they were not interacting. The knots also remain close to one another for minutes at a time. Meanwhile, computational studies have observed that pairs of knots in a stretched chain should attract. While there are differences in the computational studies compared to our experiments in terms of how the chains are stretched and boundary conditions, the semi-quantitative similarity of the effective interaction potential of the knots is striking. Our studies provide experimental support for the notion of knot-knot attraction and will hopefully motivate more experimental studies of this complex phenomenon.

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