

# Biophysics of Knotting

Dario Meluzzi,<sup>1</sup> Douglas E. Smith,<sup>2</sup>  
and Gaurav Arya<sup>1</sup>

<sup>1</sup>Department of Nanoengineering and <sup>2</sup>Department of Physics, University of California at San Diego, La Jolla, California 92093; email: des@physics.ucsd.edu, garya@ucsd.edu

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## Key Words

polymer physics, entanglement, DNA, proteins, topoisomerase

## Abstract

Knots appear in a wide variety of biophysical systems, ranging from biopolymers, such as DNA and proteins, to macroscopic objects, such as umbilical cords and catheters. Although significant advancements have been made in the mathematical theory of knots and some progress has been made in the statistical mechanics of knots in idealized chains, the mechanisms and dynamics of knotting in biophysical systems remain far from fully understood. We report on recent progress in the biophysics of knotting—the formation, characterization, and dynamics of knots in various biophysical contexts.

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## INTRODUCTION

Knots are fascinating topological objects that have captured human imagination for centuries. They find a plethora of useful applications, from tying shoelaces to securing surgical sutures. But knots can also be a nuisance, cropping up in long hair, electrical cords, and other inconvenient places. Equally important, knots are interesting subjects for scientific inquiry and have attracted increasing attention from physicists and biophysicists: Various physically relevant systems have an undeniable capacity to become entangled. Notable examples include biopolymers such as DNA and proteins. An understanding of these knots beyond the confines of mathematical topology and theoretical physics is essential to bring about new discoveries and practical applications in biology and nanotechnology.

Here we describe some recent experimental and theoretical efforts in the biophysics of knotting. We begin with a brief introduction to knot classification. We then explore a

variety of topics related to the biophysics of knotting. The organization of these topics reflects our attempt to address the following general questions: Where and how do knots form? How likely are knots to form? What are some properties of knots and knotted systems? In what processes do knots play a role? When and how do knots disappear? In addressing these questions, we aim for a qualitative presentation of recent works, emphasizing the diversity of methods and results without delving extensively into technical details. A more comprehensive treatment of specific topics can be found in books (1, 19) and in the various cited reviews.

## TYPES OF KNOTS

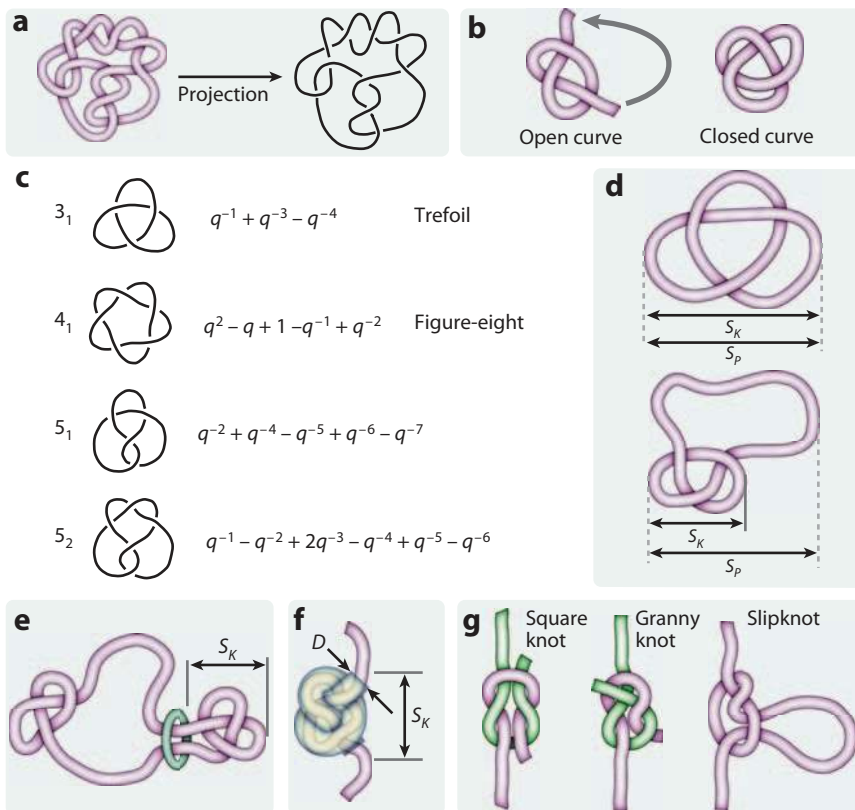
The ability to discern and classify different kinds of knots is an essential requirement for understanding biophysical processes involving knots. The mathematical field of Knot theory offers powerful tools for detecting and classifying different knots (1). A knot is a topological state of a closed, nonintersecting curve. Two closed curves contain knots of the same type if one of the curves can be deformed in space to match the other curve without temporarily opening either curve. In practice, a 3D knotted curve is mathematically analyzed by first projecting it onto a 2D plane and then examining the points, known as crossings, where the curve crosses itself in the 2D projection (**Figure 1a**). Note that when we talk about knots in open curves, such as a linear string or DNA molecule, we are imagining that the ends of those curves are connected using a sensible, well-defined procedure to yield corresponding closed curves (**Figure 1b**).

The absence of a knot is called the unknot or trivial knot. It can always be rearranged to yield a projection with zero crossings. Knots, in contrast, give rise to projections with nonzero numbers of crossings. The minimum number of crossings,  $C$ , is an invariant for any arrangement of a closed string with a given knot.  $C$  is often used to classify knots into different types. Specifically, each knot type is denoted as  $C_S$ , where  $S$  is a sequence number within

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**Knot:** a topological state of a closed 3D curve, also a knot-like conformation of an open chain

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**Figure 1**

Knot types and features. (a) Knots formally exist only in 3D curves (*left*). Knot projections are 2D representations of knots (*right*). (b) Knot-like conformations in open curves are often encountered in biophysics (*left*). To analyze such knots, their loose ends must be connected, according to some procedure, to obtain a closed curve (*right*). (c) Projections of the four simplest nontrivial knot types, with the corresponding  $C_S$  denominations and Jones polynomials (see text for definition of  $C_S$ ) (adapted from [http://katlas.math.toronto.edu/wiki/Main\\_Page](http://katlas.math.toronto.edu/wiki/Main_Page)). (d) The size of a knot,  $S_K$ , in a polymer may be less than the size of the polymer,  $S_P$ , containing the knot. (e) In a slip link arrangement, entropic competition between the knotted loops causes the ring to squeeze one of the knots. The size of the latter can be deduced from the position of the ring. Adapted from Reference 64. (f) The size of a tight knot can be estimated from the volume of the enclosing ideal knot representation:  $S_K \sim (D^2L)^{1/3}$ , where  $D$  and  $L$  are the diameter and length of the outer tube. Adapted from Reference 39. (g) Square and granny knots can tie ropes together but unravel easily at the molecular scale. Slipknots in proteins have been studied to assess the effects of knots on stability.

the family of knot types having the same  $C$  (**Figure 1c**). Some common knots are also referred to by name:  $3_1$  and  $4_1$  are called trefoil and figure-eight, respectively. The number of different knot types having the same  $C$  increases rapidly with  $C$ : There are only 3 knots with 6 crossings, but 1,388,705 knots with 16 crossings (42). The number  $C$  serves as a measure of knot complexity.

Simple knots can be distinguished visually by comparison with published tables, but extensively knotted systems require mathematical methods of knot classification. One ingenious strategy for classifying knots is to transform a knot projection into a special polynomial formula, which depends on the knot type but not on any particular projection. Comparing this polynomial with those enumerated in

**Jones polynomial:**

a mathematical expression that can be computed by analyzing the crossings in any particular 2D projection of a knot, and serves as fingerprint to uniquely identify the type of the knot

**dsDNA:**

double-stranded DNA

**DNA****topoisomerases:**

enzymes that allow single or double strands of DNA to pass through other single or double strands of DNA to change the topology of a closed dsDNA molecule

knot tables enables the identification of the knot type from a given projection. Examples of such polynomials include the Alexander, Jones, and HOMFLY polynomials (1). V.F.R. Jones was awarded the famous Fields Medal in mathematics in 1990 for his groundbreaking discovery of the Jones polynomial. These polynomials occasionally fail to distinguish different knots and become computationally prohibitive with projections of many crossings, but they are invaluable tools for analyzing the vast majority of simpler knots.

## KNOTTING IN BIOPHYSICAL SYSTEMS

Knots can form via two general mechanisms: threading of loose ends or breaking and rejoining of segments. Linear double-stranded DNA (dsDNA) molecules undergoing random cyclization in solution exemplify the first mechanism. Cyclization is possible when the ends of a linear dsDNA molecule have complementary single-stranded overhangs. A knotted molecule results whenever the molecule's ends pass through loops within the same molecule before joining (90, 95).

Knots can arise from cyclization of viral genomic DNA from tailless P2 and P4 phages (57, 58) and intact P4 deletion mutants (119) (**Figure 2a,b**). At least half of the knots form while the DNA is still in the capsid (6). Production of knotted DNA from P4 phages (45) is useful for assessing the activity and inhibition of enzymes such as DNA topoisomerases, which can change the topology of DNA. Mutant P4 phages generate knots even in nonnative DNA molecules. The genomic DNA of phage P4 is 11.2 kbp long, but these capsids produce knots in plasmids as short as 5 kbp (106). The yields of knotted DNA were >95%, much greater than yields from random cyclization of DNA in solution (95). Although the specific mechanism of knot formation in viruses remains unclear, both confinement and writhe bias seem to play an important role (5).

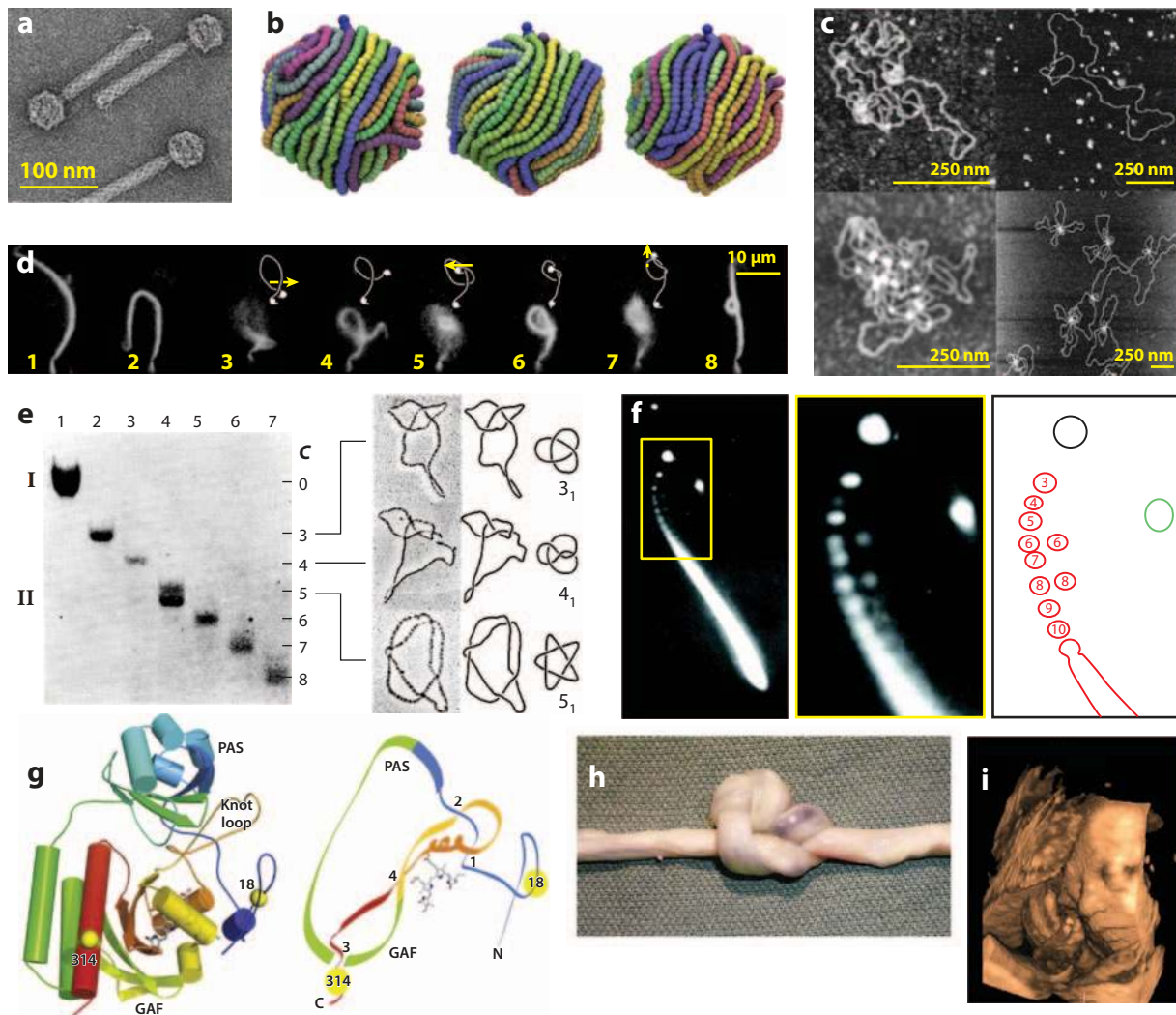
The second DNA knotting mechanism, which relies on the breaking and rejoining of

chain segments, is facilitated by enzymes such as topoisomerases and recombinases. Fundamental insights into the mechanisms of these and other enzymes have resulted from detailed analyses of knots in DNA (13, 59, 98, 115).

DNA topoisomerases are classified as type I or type II (93). Type I DNA topoisomerases temporarily break a single DNA strand and allow it to pass through the complementary strand (7). Knotted dsDNA results when circular dsDNA is nicked or gapped and the enzyme breaks a strand at a location opposite the nick (23). In contrast, type II topoisomerases temporarily break both strands in one segment of dsDNA, allowing one segment to pass through another intact segment before the strands are chemically rejoined (72, 93). Type II DNA topoisomerases introduce knots into supercoiled circular DNA *in vitro* (114), providing a way to assess the DNA supercoiling activity of other enzymes, such as condensins (79). *In vivo*, type II DNA topoisomerases remove knots from DNA. Such knots arise naturally during replication, as evidenced by the presence of knots in partially replicated plasmids (73, 96).

Recombinases are responsible for site-specific genetic recombination of DNA. Like topoisomerases, they operate by breaking and rejoining single or double strands. Their function, however, is to insert, excise, or invert a segment delimited by appropriate recombination sites (37). When the substrate is supercoiled DNA, recombinases yield knotted DNA (13). The latter was used to assay the unknotting activity of *Escherichia coli* topoisomerase IV (26).

Besides DNA, long peptides may also become knotted. Several proteins exhibit a knotted conformation in their native state (**Figure 2g**), which only becomes evident when the backbone is closed and smoothed by numerical methods (103). Presumably, these proteins become entangled while they fold into their native structures (62). Thus, the ability of protein backbones to form knots complicates the already difficult problem of explaining how proteins fold (62, 104, 120). Nevertheless, recent studies on knotted proteins are rapidly



**Figure 2**

Knotted biophysical systems. (a) Negative stain electron micrograph of P2 virions. Adapted with permission from Reference 21. (b) Conformations of packed P4 genome as determined by coarse-grained molecular dynamics simulations. Reprinted with permission from Reference 89. (c) Atomic force microscopy images of knotted DNA, isolated from P4 phage capsids and strongly (*left column*) or weakly (*right column*) adsorbed on mica surface. Reprinted with permission from Reference 34. (d) Optical tweezers tying a trefoil knot in a fluorescently labeled actin filament. Adapted with permission from Reference 3. (e) Left panel: electrophoretic mobility of knotted DNA plasmids in agarose gel increases with minimum number of crossings, *C*. Lane 1: unknotted DNA; lanes 2–7: individual knotted DNA species isolated by prior gel electrophoresis. I and II are the positions of markers for circular and linear DNA, respectively. Right panel: electron micrographs of knotted DNA molecules isolated from gel bands (*left column*), interpretation of crossings (*middle column*), and deduced knot types (*right column*). The molecules were coated with *Escherichia coli* RecA protein to enhance visualization of DNA crossings. Adapted with permission from Reference 23. (f) Knotted DNA from bacteriophage P4 capsids separated by agarose gel electrophoresis at 25V for 40 h (dimension I) and at 100V for 4 h (dimension II). Adapted with permission from Reference 105. (g) Structure of the chromophore-binding domain of the phytochrome from *Deimococcus radiodurans* (*left*) containing a figure-eight knot (*right*). Reprinted with permission from Reference 12. (h) An umbilical cord (diameter ~2 cm) with a composite knot. Reproduced with permission from Reference 20. (i) 3D image, obtained by 4D ultrasonography, of a knotted umbilical cord next to the fetal face. Adapted with permission from Reference 18.

**Molecular dynamics**

**(MD):** a simulation technique in which the Newtonian equations of motion for a system of many particles are approximately, but efficiently, integrated over time to observe the evolution of the system and to determine its statistical mechanical properties

**Minimum crossing**

**number:** the minimum number of points where a knotted curve crosses over itself when viewed in any 2D projection

gathering new clues. For example, a  $5_2$  knot is present in the human protein ubiquitin C-terminal hydrolase UCH-L3, which is involved in the recycling of ubiquitin. After denaturation, this protein folds back into its native knotted conformation without any help from chaperones, suggesting that knot formation in UCH-L3 is encoded by the amino acid sequence (2). Molecular dynamics (MD) simulations of the homodimeric  $\alpha/\beta$ -knot methyltransferases YibK and YbeA, both of which feature a trefoil knot, and of the proteins AFV3-109 and thymidine kinase, both of which feature a slipknot (100), have suggested that knots form through a slipknot intermediate, rather than by threading one terminus through a backbone loop.

Although they arise naturally, nanoscale knots can also be tied directly by humans. In particular, polystyrene beads attached to the ends of actin filaments or dsDNA molecules were maneuvered with optical tweezers to construct trefoil knots (**Figure 2d**) (3). Using similar techniques, Bao et al. (8) tied the more complex knots  $4_1$ ,  $5_1$ ,  $5_2$ , and  $7_1$  in dsDNA. Trefoil and figure-eight knots can be created also in single-stranded DNA and RNA by exploiting self-assembly of nucleic acids (94). A refined approach, based on annealing and ligation of DNA oligonucleotides with stem and loop regions, yielded knots with three, five, and seven crossings (15).

As interesting as the knots found in biomolecules are those encountered in biomedical contexts. For example, following a ventriculoperitoneal shunt operation to relieve excessive buildup of spinocerebral fluid, the surgically implanted catheter tube has been found in some cases to become spontaneously knotted, thus blocking drainage (33). Also notable is the knotting of umbilical cords during human pregnancy, a phenomenon reported in about one percent of live births (35) (**Figure 2b**). Although these knots are not always harmful (20, 61), they can sometimes be fatal (22, 97). Recent advances in understanding the dynamics of knotting in agitated strings (83) as well as technological advances in

ultrasound imaging (18) (**Figure 2i**) promise to facilitate the study and diagnosis of umbilical knots.

To understand the mechanisms of knotting, physicists have studied macroscopic model systems that are easier to implement and control than their molecular counterparts. For instance, a hanging bead chain shaken up and down at constant frequency occasionally produces trefoil and figure-eight knots (10). Recently, our group investigated tumbling a string in a rotating cubic box, which rapidly produced knots (83) (**Figure 3a**). Determination of the Jones polynomial for the string after only ten 1-Hz revolutions of the box revealed a variety of complex knots with a minimum crossing number  $C$  as high as 10. The resulting knot distribution was well explained by a model that assumed random braid moves of the ends of a coiled string (**Figure 3c**).

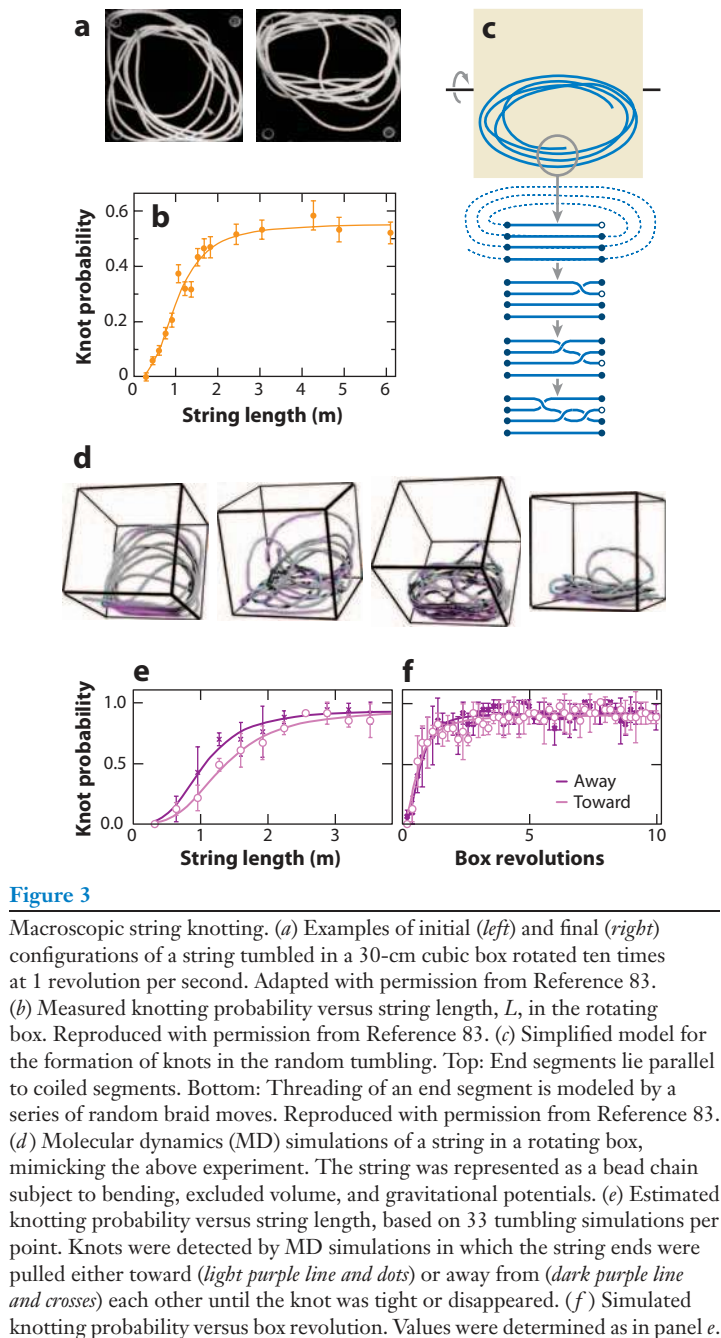
## PROBABILITIES OF KNOTTING

As knots arise in several biophysical systems, one may wonder how likely are such knots to form. This basic question was posed in 1962 by the famous biophysicist Max Delbrück (27) and since then has been frequently investigated by polymer physicists. Grosberg (38) recently reviewed some key results on the probability of knotting in polymers. Most notably, the probability of finding a knot of any type  $K$ , including the unknot, in an  $N$ -step self-avoiding random walk is predicted to be  $P_K \sim e^{-N/N_0}$ , where the constant  $N_0$  is model dependent, and the prefactor depends on the knot type. The overall probability of finding a nontrivial knot and the average complexity of knots are thus predicted to increase with increasing polymer length, and the probability of finding the unknot is predicted to approach zero as  $N \rightarrow \infty$ . Besides  $N$ , other parameters, such as solvent quality, temperature, and confinement, affect knotting probability. These nontrivial effects have been investigated theoretically or through computer simulations and are summarized in several excellent reviews (46, 75, 102, 118).

The knotting probability depends strongly on the space available to the polymer. Early numerical studies of self-avoiding random walks found the knotting probability of ring polymers to increase with increasing confinement by a sphere (70). More recent Monte Carlo (MC) simulations of phantom polymer rings, which are free from topological constraints, found that knot formation is inhibited when the radius of the confining sphere becomes too small (68). Also, in the case of umbilical cords, confinement of the growing fetus in the amniotic sac was theorized to hinder knot formation (35). Thus, effects of confinement depend on the specific physical context or theoretical assumptions.

Spatial confinement also affects knotting of DNA in phage capsids. MC simulations of P4 phage DNA, modeled as a semiflexible circular self-avoiding random walk in a confining sphere, reproduced the experimentally observed prevalence of chiral knots over achiral knots (69). However, contrary to experimental results,  $5_2$  knots outnumbered  $5_1$  knots, possibly owing to insufficient confinement or to inaccurate modeling of DNA dynamics within the capsid. In another study, the packaging of DNA in viral capsids, which has been studied experimentally (84), was modeled using random spooling polygons without excluded volume or electrostatic interactions (4). This work reproduced qualitatively both the chiral bias and the distribution of knot types observed with tailless mutants of P4 bacteriophages.

Effects of spatial confinement on knotting probability were evident in our experiments with macroscopic strings in a rotating box (83). As the string length was increased, the knotting probability did not approach the theoretical limit of 1 expected for self-avoiding random walks (Figure 3*b*). The lower probability observed was due to finite agitation time and to the restricted motion experienced by long strings of nonzero stiffness within a box of finite size. In preliminary work (D. Meluzzi & G. Arya, unpublished data), we reproduced and further quantitatively studied these effects using MD simulations of macroscopic bead chains



**Figure 3**

Macroscopic string knotting. (a) Examples of initial (left) and final (right) configurations of a string tumbled in a 30-cm cubic box rotated ten times at 1 revolution per second. Adapted with permission from Reference 83. (b) Measured knotting probability versus string length,  $L$ , in the rotating box. Reproduced with permission from Reference 83. (c) Simplified model for the formation of knots in the random tumbling. Top: End segments lie parallel to coiled segments. Bottom: Threading of an end segment is modeled by a series of random braid moves. Reproduced with permission from Reference 83. (d) Molecular dynamics (MD) simulations of a string in a rotating box, mimicking the above experiment. The string was represented as a bead chain subject to bending, excluded volume, and gravitational potentials. (e) Estimated knotting probability versus string length, based on 33 tumbling simulations per point. Knots were detected by MD simulations in which the string ends were pulled either toward (light purple line and dots) or away from (dark purple line and crosses) each other until the knot was tight or disappeared. (f) Simulated knotting probability versus box revolution. Values were determined as in panel e.

in a rotating box (Figure 3*d,e*). We have also calculated the probability of knot formation as a function of box revolutions, predicting a rapid formation of knots: 80% of the simulated trials produced a knot after only two revolutions

MC: Monte Carlo

(Figure 3f). Such simulations may offer a convenient route for dissecting the mechanisms of knot formation.

## FEATURES OF KNOTTED SYSTEMS

Knotted systems can be studied in greater depth by analyzing a variety of static properties. Here we give a few examples of these properties and describe recent progress in studying biophysically relevant systems.

### Size of Knots and Knotted Systems

Several knot size measures have been investigated experimentally, theoretically, and computationally (74). In polymers, knot size may differ from the size of the polymer (Figure 1d). Polymer size is typically characterized by the radius of gyration,  $R_g$ , i.e., the average root mean square distance between each segment and the center of mass. For linear polymers,  $R_g \sim N^\nu$ , where  $\nu = 0.5$  for pure random walk chains and  $\nu \approx 0.588$  for self-avoiding random walk chains (56) or chains with excluded volume (24, 29). The same self-avoiding random walk scaling exponent has been observed for knotted and unknotted circular polymers in the limit of  $N \rightarrow \infty$ , as determined by MC simulations (38, 75).

The scaling in  $R_g$  was investigated experimentally via fractal dimensional analysis of atomic force microscope (AFM) images of circular DNA molecules strongly and weakly adsorbed on a mica surface (34) (Figure 2c). Strong adsorption gave  $\nu \approx 0.60$ , close to  $\nu \approx 0.588$  for 3D polymers, suggesting that it projects 3D conformations onto the surface. In contrast, weak adsorption yielded  $\nu \approx 0.66$ , intermediate between  $\nu \approx 0.588$  for 3D polymers and  $\nu = 0.75$  for 2D polymers, suggesting a partial relaxation of 3D conformations into a quasi-2D state (34). A similar intermediate scaling exponent was predicted by MC simulations of dilute lattice homopolymers confined in a quasi-2D geometry (41).

As knots shrink, their size or length can be investigated separately from the size of the

overall chain (Figure 1d). In ring polygons, knot size can be determined from the shortest portion of the polygon that, upon appropriate closure, preserves the topology of the chain (50, 64, 65). Another computational method involves introducing a slip link that separates two knotted loops within the same ring polygon. Entropic effects expand one loop at the expense of the other, and the average position of the slip link defines the length of the smaller knot (64) (Figure 1e).

The size of tight knots in open chains has also been studied (81). Open chains cannot be knotted in a strict mathematical sense. For theoretical arguments, knot size can be deduced from the volume of a maximally inflated tube containing the knot (39) (Figure 1f). Accordingly, it was predicted that the size of sufficiently tight and complex knots in an open polymer should depend on a balance between the entropy of the chain outside the knot and the bending energy of the chain inside the knot. If the chain tails are sufficiently long, the knot should neither shrink nor grow on average (39). In one study, the size of tight knots in stretched polyethylene was predicted from the distribution of bond lengths, bond angles, and torsion angles along the chain, suggesting that trefoil knots involve a minimum of 16 bonds (121). For comparison, ab initio calculations predicted a minimum of 23 bonds (92). Furthermore, the extent of tight knots has been determined experimentally. Fluorescence measurements indicated that trefoil knots in actin filaments can be as small as  $\sim 0.36 \mu\text{m}$  (3). Similar measurements on  $3_1$ ,  $4_1$ ,  $5_1$ ,  $5_2$ , and  $7_1$  knots in linear dsDNA yielded knot lengths of 250–550 nm for molecules stretched by a tension of  $\sim 1$  pN (8).

Knots can be tightened on proteins as well. The figure-eight knot present in the chromophore-binding domain (CBD) of the phytochrome from *Deimococcus radiodurans* (Figure 2g) was tightened with an AFM to a final length of 17 amino acids (12). Similarly, simulations of the  $5_2$  knot in ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) using a Go-like model suggested minimum lengths of either 17 or 19 residues, depending on the final



location of the tight knot along the backbone (101). More accurate all-atom MD simulations with explicit water found tight  $3_1$  and  $4_1$  knots in stretched model peptides to be about 13 and 19 amino acids long, respectively, in good agreement with the experiments (32). Curiously, in these simulations, a tight  $4_1$  knot in poly-leucine was found to trap a single water molecule, which escaped upon further tightening.

Assessing the size of tight protein knots is important for understanding their biological roles. Bulky knots could hamper the threading of polypeptides through the narrow pore of the proteasome, possibly protecting certain knotted proteins from rapid degradation (109). This hypothesis was supported by Langevin dynamics simulations of the translocation of a test peptide through a narrow channel (radius  $\sim 6.5$  Å). The presence of a  $5_2$  knot in the peptide reduced the translocation rate by two orders of magnitude, suggesting that knots may indeed hinder protein degradation by the proteasome (44).

### Knot Localization

Several studies have addressed the localization of knots in a polymer (**Figure 1d**), and various aspects of knot localization, including the role of entropic and electrostatic effects, have been reviewed (38, 48, 75). Knot localization within a closed knotted chain results from the gain in entropy by a long unentangled loop, which causes the knotted portion of the chain to shrink (38). This effect could be mimicked by vibrating a twisted bead chain on a horizontal plate (40). The same phenomenon was inferred from the size distributions of simple knots in random closed chains of zero thickness (50).

Numerically, knots are localized when their average size  $\langle \ell \rangle$  grows slower than the length  $N$  of the chain, or  $\lim_{N \rightarrow \infty} \langle \ell \rangle / N = 0$ . When  $\langle \ell \rangle \sim N^t$ , with  $t < 1$ , the knot is weakly localized (63). The value of  $t$  depends on solvent quality. MC simulations of trefoil knots in circular self-avoiding polygons on a cubic lattice (64) yielded  $t \approx 0.75$  in good solvent and  $t \approx 1$  in poor solvent, indicating that knots

are weakly localized in the swollen phase but are delocalized in the collapsed phase. Similar scaling exponents have been obtained for linear polyethylene in good and poor solvent via MC simulations (108). These exponents have been confirmed by analyzing the moments of the probability distributions of knot lengths for different types of knots (65).

Knot localization was observed in AFM images of circular DNA weakly adsorbed on a mica surface (34). Moreover, MC simulations of ring polymers adsorbed on an impenetrable attractive plane have predicted that lowering the temperature leads to strong knot localization, i.e.,  $\langle \ell \rangle$  becomes independent of  $N$  (63). Knot localization in DNA is important because it may facilitate the creation of segment juxtapositions and thereby may enhance the unknotting activity of type II DNA topoisomerases (59).

### Strength and Stability of Knotted Systems

Rock climbers are well aware that knots weaken the tensile strength of ropes. Similar effects hold for knotted molecules. Using Car-Parrinello MD simulations, it was shown that a linear polyethylene molecule with a trefoil knot breaks at a bond just outside the entrance of the knot, where the strain energy is highest, but is still only 78% of the strain energy needed to break an unknotted chain (92). Hence, the knot significantly weakened the molecule. Similarly, when the ends of single actin filaments containing a trefoil knot were pulled with optical tweezers, the filaments were found to break at the knot with pulling forces of  $\sim 1$  pN, indicating a decrease in tensile strength by a factor of 600 (3). On a macroscopic scale, experiments with fishing lines and cooked spaghetti confirmed that rupture occurs at the knot entrance, where the curvature was predicted to be the highest, causing local stresses that favor crack propagation (80).

Ordinary strings can be tied strongly with a square or granny knot (**Figure 1g**), but if two polymer chains were tied in this fashion and then pulled apart, the knot would invariably

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**Langevin dynamics:** a computationally efficient MD refinement that approximately accounts for the effects of random collisions of solvent molecules with the system

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**Brownian dynamics:**

Langevin dynamics with zero average acceleration, typically used to simulate overdamped systems

**Wormlike chain:** a semiflexible polymer chain

slip. However, Langevin dynamics simulations found that, when pulled strongly, smooth polymers untie more quickly than bumpy polymers (53). Increasing the pulling force makes the energy landscape of bumpy polymers more corrugated, thus hindering the thermally activated slippage of the strands.

Although they weaken tensioned strings, knots may actually increase the stability of certain systems. Increased stability could explain the presence of knots in some proteins (120). To test this effect, the deep slipknot (**Figure 1g**) in the homodimeric protein alkaline phosphatase from *E. coli* was cross-linked via a disulfide bridge between monomers, effectively increasing the knotted character of the overall dimer (52). A  $\sim 10^\circ\text{C}$  increase in melting temperature of this cross-linked dimer, relative to a control dimer cross-linked outside the slipknot loops, suggested that knots can increase the thermal stability of proteins. Yet, unfolding experiments with the  $4_1$ -knotted CBD of the phytochrome from *D. radiodurans* found that the knot did not significantly enhance mechanical stability (12). It was suggested, however, that this knot might serve to limit the possible motions induced by the chromophore on the CBD upon light absorption.

## DYNAMIC PROCESSES INVOLVING KNOTS

Finding and characterizing knots in biophysical systems naturally lead to an investigation of dynamic processes involving knots. We focus on three prominent examples: diffusion, electrophoresis, and unknotting.

### Knot Diffusion

As discussed above, knots may become localized. Once localized, a knot can diffuse along the chain. The resulting motion is governed by the inability of intrachain segments to pass through one another. The same constraints exist for intermolecular entanglements and thus dominate the dynamics of concentrated polymer solutions and melts. Such systems are well

described by the reptation model (24, 29), for which P.G. de Gennes was awarded the Nobel Prize in Physics in 1991. This model assumes that each polymer molecule slides within an imaginary tube tracing the molecule's contour. In agreement with this model, experiments have shown that linear DNA molecules larger than  $\sim 50$  kbp, in solutions more concentrated than  $\sim 0.5$  mg ml $^{-1}$ , exhibit tube-like motion, experience tube-like confining forces, and diffuse as predicted by reptation theory (77, 85, 86).

The notion that reptation may also govern knot diffusion was supported experimentally by Bao et al., with  $3_1$ ,  $4_1$ ,  $5_1$ ,  $5_2$ , and  $7_1$  knots in single, fluorescently stained DNA molecules (8). The knots were seen as bright blobs diffusing along the host DNA. The diffusion constants,  $D$ , of the knots were strongly dependent on knot type, and the drag coefficients deduced from  $D$  were consistent with a self-reptation model of knot diffusion (8). Brownian dynamics simulations of a discrete wormlike chain model of DNA yielded  $D$  values of the same magnitude as the values measured experimentally (110). Moreover, Langevin dynamics simulations of knot diffusion in tensioned polymer chains found  $D$  values consistent with a sliding knot model in which the friction between the solvent and the knot dominates knot dynamics at low tensions, whereas internal friction of the chain dominates the dynamics at high tensions (43). In the absence of tension, knot diffusion was proposed to consist of two reptation modes, one due to asymmetric self-reptation of the chain outside the knot, the other due to breathing of the knot region. The latter motion allows the knot to diffuse in long chains (67).

In addition to diffusing along polymers, knots can affect the diffusion of the polymers themselves. Brownian dynamics simulations of ring polymers with knots of up to seven crossings found that the ratio of diffusion coefficients for knotted and linear polymers,  $D_K/D_L$ , grows linearly with average crossing number  $N_{AC}$  of ideal knot representations (47). Thus, intramolecular entanglement seems to speed up polymer diffusion. Nevertheless, diffusion

of knotted polymers may be complicated by intermolecular topological constraints. For example, we have found that circular DNA can diffuse up to two orders of magnitude slower when surrounded by linear DNA than when surrounded by circular DNA of the same concentration and length (87). Current reptation models fail to fully describe these findings, but qualitatively we believe that unknotted circular molecules are easily pinned by threading of linear molecules. Such pinning mechanisms are likely to affect the diffusion of knotted polymers as well.

## Electrophoresis

The strong negative charge on DNA molecules at sufficiently high pH is exploited in agarose gel electrophoresis to separate DNA molecules according to size and supercoiling state. For over two decades, the same technique has proven invaluable for analyzing knots in relaxed circular DNA (31, 55). In seminal experiments with *E. coli* topoisomerase I, electron microscopy revealed the topology of knotted DNA molecules from distinct gel bands (23) (**Figure 2e**). Remarkably, each band contained DNA knots with the same minimum number of crossings,  $C$ , which seemed to control the electrophoretic mobility of knotted DNA.

A follow-up study (99) uncovered a surprisingly linear relationship between the previously reported electrophoretic migration distances of DNA knots and the average number of crossings,  $N_{AC}$ , in the ideal geometric representations (49) of those knots. Because  $N_{AC}$  is linearly related to the sedimentation coefficient, which provides a measure of molecular compactness, it was concluded that DNA knots with many crossings are more compact and therefore migrate faster through the gel than DNA knots with fewer crossings (112). At high electric fields, however, the linear relationship between migration rate and  $N_{AC}$  no longer holds. This change in behavior has been exploited in 2D gel electrophoresis to improve the separation of knotted DNA (105) (**Figure 2f**) and has been reproduced in MC simulations of closed

self-avoiding random walks (117). Such change was attributed to increased trapping of knotted DNA by gel fibers at high electric fields. The distribution of trapping times obeyed a power law behavior consistent with the dynamics of a simple Arrhenius model (116), thus enabling the estimation of the critical electric field associated with the inversion of gel mobility of knotted DNA.

Despite considerable modeling efforts and extensive use of DNA electrophoresis, a complete theory that accurately predicts DNA mobility as a function of electric field and polymer properties is still lacking. Novel separation techniques provide additional motivation for understanding the dynamics of knotted polymers in electric fields (76, 54).

## Unknotting

Knot removal can occur via two main mechanisms: unraveling and intersegmental passage. Unraveling is the reverse of the threading-of-loose-ends mechanism that allows knots to form in open chains. A clear example of unraveling involved the agitation of macroscopic granular chains on a vibrating plate. A tight trefoil knot unraveled with an average unknotting time that scaled quadratically with chain length (11). This scaling behavior is reminiscent of knot diffusion in linear polymers predicted by a mechanism of “knot region breathing” (67).

As with diffusion, the unraveling of knots in polymers is affected by external constraints. MD simulations of polyethylene melts found that macromolecular crowding causes trefoil knots to unravel through a slithering motion with alternating hairpin growth and shrinkage, resulting in a scaling exponent of 2.5 for the average unknotting time (51). Similarly, a tight trefoil knot in a polymer constrained within a narrow channel was predicted to unravel through simultaneous changes in size and position, with a cubic dependency of mean knot lifetime on the polymer length (71).

A situation in which knots must unravel rapidly is during the ejection of DNA from viral capsids upon cell infection. The electrostatic

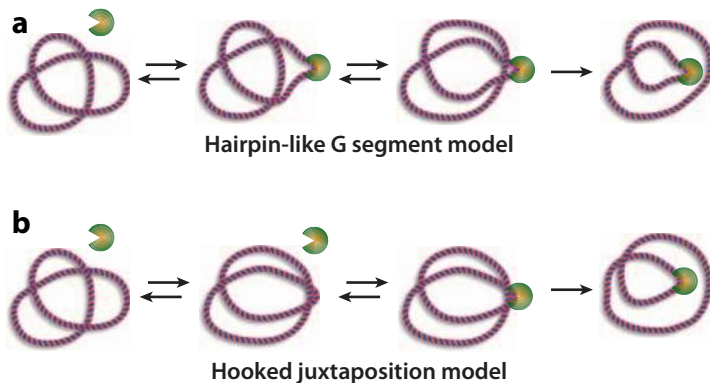
repulsions and entropic penalty experienced by DNA molecules confined within phage capsids result in high internal forces (78) of up to  $\sim 100$  pN, according to measurements by optical tweezers (84). Such forces are capable of removing DNA knots in some viruses upon exit from the capsid through a narrow opening, as confirmed by MD simulations of a coarse-grained polymer chain initially confined within a sphere (66). In this system, the ejection dynamics were controlled primarily by the reptation of the polymer through the knot (66), a process presumably similar to the knot diffusion observed experimentally by Bao et al. (8).

The second general mechanism of unknotting is intersegmental passage, which can also lead to knot formation. This mechanism consists of passing chain segments through temporary cuts on other segments of the same chain. This procedure is carried out at the cellular level by type II DNA topoisomerases, which use ATP to lower the fraction of knotted DNA below the levels observed in random cyclization (91). Knotting and catenation of DNA interfere with vital cellular processes (59), including replication (9), transcription (25), and chromatin

remodeling (88). Hence, type II DNA topoisomerases have been an attractive target for anticancer drugs (28) and antibiotics (107). The molecular mechanism by which type II DNA topoisomerases break, pass, and rejoin dsDNA is fairly well understood (36, 72, 93), but the higher-level mechanism that leads to a global topological simplification of DNA is a subject of continuing debate (59, 111).

A few interesting models of type II DNA topoisomerases action have been proposed (59, 111). Two of these models seem consistent with the structure of yeast topoisomerase II (30). In the first model (113) (**Figure 4a**), the enzyme binds to a DNA segment, known as the G segment, and bends it sharply into a hairpin-like structure. Next, the enzyme waits for another DNA segment, called the T segment, to fall into the sharp bend. Then, the enzyme passes the T segment through a break in the G segment, from the inside to the outside of the hairpin. Indeed, MC simulations of this model using a discrete wormlike chain found the presence of hairpin G segments to lower the steady-state fraction of knots by a factor of 14. This value, however, is less than the maximum of 90 observed in experiments with type II DNA topoisomerases (91).

The other model of topoisomerase action (**Figure 4b**) is based on two assumptions (14). First, hooked juxtapositions, or locations where two DNA segments touch and bend around each other, occur more frequently in globally linked DNA than in unlinked DNA. Second, the enzyme binds preferentially to DNA at hooked juxtapositions. Once bound, the enzyme passes one segment through the other. Hence, type II DNA topoisomerases disentangle DNA by selectively removing hooked juxtapositions. This model's ability to predict a significant steady-state reduction of knots and catenanes below topological equilibrium was supported by MC simulations with lattice polygons (60) and freely jointed equilateral chains (17). Nonetheless, these models of DNA may not be sufficiently accurate (111). Additional simulations with wormlike chains may clarify the significance of hooked juxtapositions (59).



**Figure 4**

Models of unknotting by type II DNA topoisomerases. (*a*) In the hairpin-like G segment model (111), the enzyme binds to the G segment and sharply bends it into a hairpin-like structure; the T segment is then allowed to pass only from the inside to the outside of the hairpin. Adapted from Reference 111. (*b*) The hooked juxtapositions model (59) assumes that hooked juxtapositions form frequently in knotted DNA and that the enzyme binds to DNA only at these juxtapositions. Once bound, the enzyme catalyzes the intersegmental passage. Adapted from Reference 59.

The negative supercoiling state of DNA also seems to affect the results of topoisomerase action. Early MC simulations of a wormlike chain model of circular DNA suggested that supercoiling reduces the free energy of highly chiral knots below that of unknotted DNA, effectively favoring knot formation in the presence of type II DNA topoisomerases (82). A more recent study explicitly accounted for the changes in linking number introduced by DNA gyrase after each intersegmental passage to maintain a constant level of torsional tension in DNA (16). The resulting knot probability distributions suggested that negative supercoiling opposes segment passages in directions that lead to knotting. Thus, the supercoiling action of DNA gyrase may be the principal driver toward low levels of DNA knotting *in vivo* (16).

## CONCLUSION

Knots have been discovered in a wide range of systems, from DNA and proteins to catheters and umbilical cords, and have thus attracted much attention from biophysicists. In this review we have explored a variety of topics in the biophysics of knotting. Despite the tremendous progress made in this field by theoretical and experimental studies, many open questions remain, which are summarized below. These questions could inspire new research efforts. In particular, computer simulations and single-molecule experiments hold great promise in clarifying knotting mechanisms, while emerging techniques for high-resolution molecular imaging should facilitate the study of knotting processes inside the cell.

### SUMMARY POINTS

1. The Jones, Alexander, and HOMFLY polynomials from knot theory are powerful tools for analyzing and classifying physical knots.
2. An agitated string forms knots within seconds. The probability of knotting and the knot complexity increase with increasing string length, flexibility, and agitation time. A simple model assuming random braid moves of a string end reproduces the experimental trends.
3. Knots are common in DNA and the different knot types can be separated by using electrophoresis techniques, which exploit the varying mobility of knotted DNA in entangled media in response to electric fields.
4. Knots have recently been discovered in proteins. The formation mechanisms and the biological function of these knots are just beginning to be studied.
5. Knots can be generated artificially in nanoscale systems and used to study fundamentals of knot dynamics. Localized knots in DNA diffuse via a random-walk process that exhibits interesting trends with respect to tension applied across the molecule.
6. Confinement and solvent conditions not only play an important role in determining the types and sizes of knots that appear in biophysical systems, but also affect the diffusion and localization of knots.
7. Knots appear to weaken strings under tension but can have a stabilizing effect on knotted systems such as proteins.
8. DNA topoisomerases are enzymes that play an important role in the disentanglement of DNA, and their mechanism of topological simplification is only now beginning to be understood.

## FUTURE ISSUES

1. The function of knotted structures within proteins and the mechanism by which these knots form remain mysterious. How do knots form in proteins? Are chaperones needed to fold knotted proteins? How do proteins benefit from having knotted backbones?
2. The effect of macromolecular crowding on the knotting dynamics of different biopolymers within the cell has not been examined so far. This effect could be important for understanding knotting in vivo.
3. The transitions of knots from one type to another in both open and closed chains are far from fully understood. Do these transitions follow thermodynamic probabilities and patterns or is the process chaotic? What are the dynamics of these transitions? How do they depend on the type of agitation and chain (open versus closed)?
4. The formation of knots in human umbilical cord and surgically implanted shunt tubes is undesirable, but the underlying causes are unclear. Can such processes be accurately studied and modeled? Can such knots then be avoided?
5. Improved imaging approaches for the visualization of knots, both molecular and macroscopic, and both in vitro and in vivo, are needed to facilitate the experimental investigation of knot dynamics.
6. Are there any useful applications for molecular knots in biotechnology, nanotechnology, and nanomedicine?

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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8. Reports the remarkable feat of tying a knot in a single DNA molecule with optical tweezers and imaging the diffusion of the knot within the molecule by fluorescence microscopy.

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23. Provides beautiful images of single knotted DNA molecules formed by *E. coli* topoisomerase I action, and demonstrates that different knot types can be resolved by gel electrophoresis.

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38. Reviews knots in polymers, including many fundamental results, key references, and a fine historical account on the role of knots in physics.

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49. Demonstrates localization of compact knots in simulated random walk polymer chains in thermodynamic equilibrium.

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52. Discusses the observation of knots in proteins and demonstrates experimentally that they can increase stability.

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**83. Reports a systematic experimental study of knot formation in a tumbling string and a theoretical model for knot formation via random braid moves.**

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**93. Discusses the properties, mechanisms, and structures of topoisomerase enzymes that knot and unknot DNA in vital cell processes and are the target of anticancer and antibacterial drugs.**

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97. Demonstrates that knotting of the human umbilical cord can cause fetal death.

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**Errata**

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