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Accepted Manuscript

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Critical Review

Single-molecule portrait of DNA and RNA double helices

Cite this: DOI: 10.1039/x0xx00000x

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The composition and geometry of the genetic information carriers was described as double-stranded right helices sixty years ago. The flexibility of their sugar-phosphate backbones and the chemistry of their nucleotide subunits, which give rise to the RNA and DNA polymers, were soon reported to generate two main structural duplex states with biological relevance: the so-called A and B forms. Double-stranded (ds) RNA adopts the former whereas dsDNA is stable in the latter. The presence of flexural and torsional stresses in combination with environmental conditions in the cell or in the event of specific sequences in the genome can, however, stabilize other conformations. Single-molecule manipulation, besides affording the investigation of the elastic response of these polymers, can test the stability of their structural states and transition models. This approach is uniquely suited to understanding the basic features of protein binding molecules, the dynamics of molecular motors and to shedding more light on the biological relevance of the information blocks of life. Here, we provide a comprehensive single-molecule analysis of DNA and RNA double helices in the context of their structural polymorphism to set a rigorous interpretation of their material response both inside and outside the cell. From early knowledge on static structures to current dynamic investigations, we review their phase transitions and mechanochemical behaviour and harness this fundamental knowledge not only through biological sciences, but also for Nanotechnology and Nanomedicine.

Received 00th January 2014,
Accepted 00th January 2014

DOI: 10.1039/x0xx00000x

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Insight, innovation, integration

Single-molecule Biophysics is reshaping our perspective of Molecular Biology because it is bringing the physical analysis of cell machinery into reality. Central to this analysis is the study of information processing and storage. Nucleic acids are no-longer merely viewed as biochemical molecules that react with proteins for their replication and transcription, but also as physical entities that are stretched, bent, twisted or unzipped by motor proteins. These studies are also bringing to the forefront of Chemistry the importance of the mechanical behaviour of macromolecules that exhibit transient interactions through mobile domains. Here, we review the mechanochemistry of double-stranded DNA and RNA to provide a comparative discussion between them and to integrate the single-molecule body of knowledge into former structural information.

Introduction

Double-stranded DNA secondary structure and its transitions among its different forms[1] have constituted one of the first problems in molecular and structural biology. In particular, the study of the transformation from physiological B-DNA to dehydrated A-DNA dates back to 1953[2] and thus far it has been studied for its possible consequences in transcription, recognition and protection.[3] Other forms of dsDNA also exist,[4] including those with reverse helicity.[4d] In contrast to DNA polymorphism, and although a left-handed RNA were soon demonstrated,[5] it was intriguing that dsRNA did not display such structural changes and remained in the A form.[3a, 6] This fact together with the existence of the A form in the RNA:DNA hybrids has led to suggest that the A conformation could be important from an evolutionary point of view to help DNA in substituting RNA as the carrier of the genetic information in a transition period from an RNA to a DNA world.[7] In this respect, it is possible that primordial phenomena such as transcription, organization and compaction of the genome (ordered condensation of dsDNA as opposed to a random coil collapse), or photodamage protection could be mediated by the B-A transformation.

The development of single-molecule techniques in the last two decades has allowed the mechanical characterization of individual DNA molecules, shedding light on their elastic properties and the physical constraints affecting the proteins that interact with them.[8] Detailed studies from the dynamic point of view on the activities of polymerases, topoisomerases, helicases and DNA-packaging systems, among others, have been made possible thanks to DNA mechanical manipulation.[8a, 8c, 9] Knowledge derived from these approaches has also enhanced the use of this polymer for nanotechnological purposes to the point that DNA Nanotechnology is now a well-established field.[10] Single-molecule studies have also contributed to a deeper understanding of the influence of base sequence and environmental conditions on DNA structure, which has in turn very recently entailed new insights into the existence of its transition from the B to the A,[11] Z,[12] P[13] or S forms,[14] and their relation with genome compaction.[11c, 15]

With regards to RNA, current investigation is refreshing our conception on the role of its duplex structure. Besides the long-known existence of dsRNA viral genomes, the discovery of RNA interference has shown that long dsRNA molecules can trigger sequence-specific gene silencing[16] and, as a consequence, play a key role in Nanomedicine.[17] From a chemical point of view, RNA differs from DNA in the sugar −ribose in RNA and deoxyribose, with one more hydroxyl group, in DNA−, and in the use of the uracil nucleobase instead of thymine, with an extra methyl group on its ring. These changes determine the relative spatial arrangement of the atoms in the sugars, a fact that gives rise to different structural double-helix types and consequently to different interactions with other molecules. Single-molecule experiments are also providing access to the link that exists between structure and mechanochemistry in these nucleic acid polymers.[18]

The aim of this review is to integrate the single-molecule body of results that has been obtained in the last two decades into the more established structural and biochemical knowledge. The single-molecule approach is unique in testing the stability of the different structural types and their intrinsic transitions because the molecules are not constrained by the multiple interactions that take place in an ensemble of equivalent molecules. Moreover, force and torque spectroscopies at the single-molecule level have provided the mechanochemical perspective that is needed to interpret their functional activities in relevant physiological contexts and to test their use from a materials science perspective. This review is structured as follows: in the next section, we will present a summary of the current structural knowledge on DNA and RNA double-helices. Next, we will introduce the single-molecule approach, first by overviewing the instrumentation and second by presenting the new structural information on duplex nucleic acids. Then, we will analyse the elastic behaviour of DNA and RNA helices. In the discussion section, we will explain the mechanochemical behaviours of dsRNA and dsDNA by relating them to their different double-helix types and to the stereochemical identities of their nucleobases. Finally, we will address perspectives on molecular motors, discuss other structural arrangements and show applications of DNA and RNA to other fields.

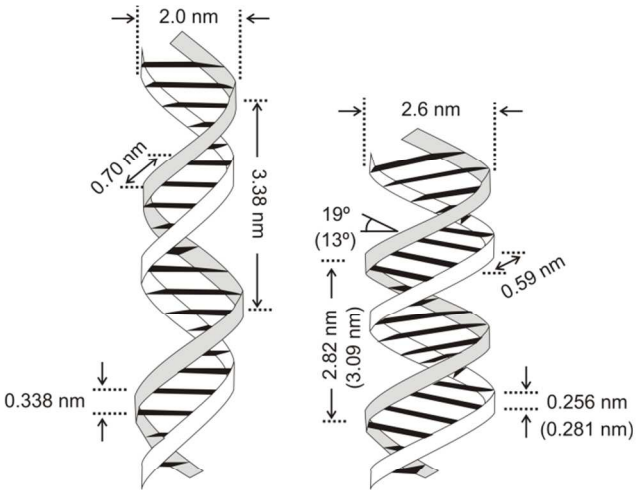

Fig. 1 Schematic view of the B (left) and A (right) double-helix forms. Structural parameters are approximate, based on sequence averages and in the absence of force. Some of them are slightly different for the A-form in dsRNA (in brackets) with respect to that in dsDNA. Helix diameters have been estimated from the dislocation data in [6]. Base-pair tilts are negative and much smaller for B-DNA than for both A-DNA and dsRNA and therefore have not been represented in the left diagram.

Structural studies of DNA and RNA

Structural families

The DNA double helix is known to adopt the B form in physiological conditions, but this conformation is not unalterable. In reality, dsDNA has a significant ability to change its form.[6, 19] This structural variability has been found to take place through changes in its environmental conditions, such as humidity level,[4b, c, 20] ion activity[4c, 21] and temperature,[22] in the sequence of nucleotide bases[4a, 23] or by interaction with some ligands,[24] all of which induce transitions from the B to the A form[1] and some of which induce transformation to the Z form.[4d] In contrast, the RNA double helix adopts the A form and is almost structurally conservative.[3a, 6, 25] RNA:DNA hybrids, which are formed during transcription, have been reported to adopt the A form,[26] although certain base-pair sequences can also lead to structures very similar to the B form.[27]

Conformations of dsDNA are classified in families:[6, 28] The "B" family is the most common and its main member is the canonical B-DNA, which is stable in aqueous solution, see Fig. 1 (left). One of its most frequent allomorphs is C-DNA. It can be found in water-alcohol solutions, provided that the final mixture is not extremely non-polar (methanol or low concentration of ethanol are typically used as co-solvents), and at high-salt solutions.[4b, 29] Beyond considerations at the nucleotide base-pair level (i.e., *sugar-puckering* configurations and *base-stacking* interactions, see below), DNA conformation can be globally characterized by geometrical parameters of the double-stranded helix. In the "B" family, the rise per residue is in the range 0.30 to 0.34 nm and the rotation per residue, from 36º to 45º. The helix diameter of canonical B-DNA is ~2.0 nm and the number of base pairs per turn, ~10 (for C-DNA, this number is between 9 and 10).[6]

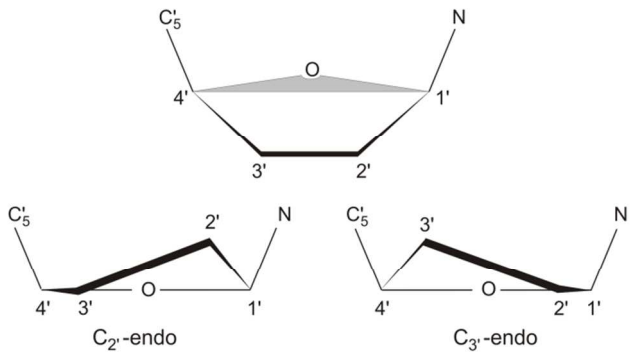

Fig. 2 Geometrical disposition of the atoms in the five-membered sugar into puckering modes. Top, starting position with flat conformation. Bottom left, $C_{2'}$-endo sugar-puckering mode in which the 2' carbon, whose bonds are not compromised to the backbone units, is raised north. This mode is observed in B-DNA. Bottom right, $C_{3'}$-endo mode in which the 3' carbon atom, the one that links adjacent bases through a phosphate group in the strand backbone, is raised north. This mode is observed in dsRNA and in A-DNA. In reality, $C_{2'}$-endo and $C_{3'}$-endo modes consist of two each families of closely related puckering modes.[6] Mechanical tension may cause continuous transitions within a puckering family because their states lack of substantial potential energy difference. Transitions $C_{2'} \leftrightarrow C_{3'}$ are however energetically less probable.

"A"-family structures in DNA, Fig. 1 (right), can be also stabilized in water-alcohol solutions, but in this case, a highly non-polar alcohol (ethanol, typically) in a high concentration (normally, above 70%) is needed. DsRNA right-handed helix is confined to the "A" family and its most common allomorph, known as A', is induced at high salt concentration.[3a, 6] Rise per residue is more variable in "A" family than in "B" family: from 0.26 to 0.33 nm, in contrast to the rotation per residue, which is rather constant and changes only from 30º to 33º. A-DNA is broader than B-DNA –helix diameter, ~2.6 nm, due to the dislocation of base-pairs away from the helix axis– and holds ~11 base pairs per turn (~12 for A').[6]

The third and least common DNA family is "Z", whose main characteristic is that its double helices are left-handed. The main inductor of Z-DNA is a stringent sequence of nucleotides −majorly, alternating G•C and C•G base pairs–,[4d, 6] although the alcohol co-solvent (trifluoroethanol, typically, concentration above 60%)[30] and considerable ion concentrations[31] also contribute. The periodic sequence that produces a zig-zag in the course of the line that links alternating phosphates made this configuration be named Z. Both Z-DNA[32] and Z-RNA[5] along with their transitions from B and A forms, respectively, were found during the 1980s. Z-DNA, which comprises several allomorphs, holds ~12 base pairs per turn, which results from a rise per residue of 0.38 nm combined with a -60º rotation per dinucleotide repeat.[6] Z-RNA possesses very similar helical parameters (12.4 base-pairs per turn, rise per residue of 0.30-0.36 nm and a -61º rotation in GpC steps combined with a +4-7º rotation in CpG) despite the base-stacking and base-pair positions relative to the helix axis differ from those in Z-DNA.[33] Although the B-Z transition in DNA is temperature-independent, high temperatures have been demonstrated to favour the A-Z transition in RNA. Likewise, even higher ionic strengths are needed to favour Z-RNA compared to Z-DNA, these conditions overall revealing a higher energetic barrier of the A-Z transition in RNA than that of the B-Z transition in DNA.[34]

Handedness represents a qualitative jump when comparing A with Z or B with Z forms, radically different than the comparison between A and B forms, which involve only quantitative changes in the helix parameters. This fact brought as a consequence that the biological relevance of Z-form and its *in vivo* presence, though marginal due to the low occurrence of alternating G•C and C•G tracts in genomes, had already been shown. Topological stress in DNA plasmids, Chromosomes, viral DNA and in transcription, in combination with low humidity conditions in the cell, can favour a Z-like conformation.[6, 35] In particular, the Z-conformation may variably regulate supercoiling.[6, 36] Furthermore, the Z-form has demonstrated distinctive interactions with drugs and proteins relative to its right-handed analogues, including recognition of Z-RNA with respect to Z-DNA.[6, 37]

Transition mechanisms in the absence of mechanical stress

Except for sequence-dependent induced structures, from a physicochemical point of view, water and counterion activity can account for the transitions between families. The conformational transitions are reversible and cooperative between families (dependent on the degree of non-polarity and concentration of co-solvents) and of non-cooperative character within a family (dependent on ionic strength and nature of counterions).[4c] A decade ago, however, mechanical manipulation assays revealed new secondary structures of dsDNA induced by tension and torque and additional insight into known ones, as we will analyse in later sections.[8a]

The major distinction between the A and B structures at the base-pair level stems from the sugar puckering in the nucleotides, $C_{2'}$-endo for B form and $C_{3'}$-endo for A form,[6, 38] as represented in Fig. 2. The fact that this variation provides a different distance between adjacent phosphates in the same strand, thus cooperatively enabling a specific, "macroscopic" helical arrangement,[1, 4c, 39] has been under debate.[7, 40] In a different model, this variation in macroscopic arrangement has been described as a consequence of the sequence-dependent base-stacking interactions. The latter model is known as the *base-centred* explanation of the B-A transition,[19, 23] which contrary to the preceding *backbone-centred* view,[6, 39] proposes that certain base-pair steps, mainly those of the purine-pyrimidine variety, can stack bistably, and that such local bistability is cooperatively promoted to the passive, elastic sugar-phosphate backbone to globally produce a specific helical structure, either B or A.

Environmental inductors of conformations

The role of water activity has also been under discussion. Hydration is considered to drive the B-A transition in the backbone-centred explanation: at a threshold of ~ 20 water molecules per nucleotide, a sharp transformation takes place implying a cooperative change in the sugar-puckering modes.[6] In fact, the economics in hydrating phosphate groups by bridging contiguous base-pair steps has been

suggested to stabilize A-DNA, unlike B-DNA with individual hydration of phosphate groups.[41] In the base-centred explanation, hydration plays a secondary role and, different from the previous coupling scheme, this model suggests that water bridges within the minor-groove of neutral (i.e., non-bistable) base-pair steps stabilize B-DNA.[23] Irrespectively of the water bridging scheme, it is clear that water activity also mediates DNA secondary structure by regulating both the hydrophobic effect in the base-stacking interactions and the strength of the hydrogen bonds.[4c, 28b, 42]

Beyond the molecular basis, experimental and numerical results have shown evidence that guanine-cytosine (G•C) -rich regions of a dsDNA are more A-philic than adenine-thymine (A•T) -rich regions. The latter regions have also been found in A-form,[43] but they are generally resistant to adopting this conformation even at very low humidities.[4a, 22b, 44] Also, beyond considerations on the molecular mechanism,[45] the addition of multivalent cations, such as organic polyamines and inorganic Cobalt hexaammine (III) ion, $[Co(NH_3)_6]^{3+}$, stabilizes A-DNA at lower alcohol concentrations,[21a, 46] as well as higher-order compact structures,[47] due to their ability to reduce water activity in the vicinity of the DNA surface. For appropriate DNA sequences, this A-stabilizing effect has been observed even in the absence of the non-polar component of the solution.[11a, 48] By contrast, monovalent and divalent cations are more diverse in favouring a specific DNA conformation: their activity depends on the specific ion, the non-polar solution component and the overall ionic strength.[1, 4c, 21b]

The action of a particular cation is not, in general, electrostatically sequence-specific and a DNA interaction site can be occupied by different ionic species, besides water molecules,[49] although there are proteins and other ligands with selective binding and conformational recognition.[24a, 44h, 50] Likewise, the binding of proteins and other ligands to DNA and RNA:DNA hybrids have been reported, or at least suggested, to convert the length of the double-stranded helical polymer that they cover into different conformations,[24a, 26b, 50c, 51] thus relating the specific protein function to the DNA-induced or DNA-sensed conformational change. In particular, A-DNA was formerly suggested to be tied to an RNA transcription role,[3, 52] although, in general, other non-B DNA conformations have been similarly described in transcription complexes.[53] B and A forms, and structures in between, have been found in replication.[54] A-DNA and its induction by spore proteins have been reported to be involved in UV-photodamage protection.[24b, 44a, 55] Binding of carbon nanotubes[56] and drugs[57] have also been found to induce the B-A transition.

Condensation and aggregation

The conditions that induce the B-A transition are paradigmatically within the ranges that also strongly induce both DNA aggregation and condensation.[47, 58, †] In fact, it has been suggested that DNA electrostatic aggregation/condensation stabilizes the A form by establishing lateral, intermolecular and intramolecular associations, respectively, between the DNA helices,[44c, 44f, 58d, 59] a synergistic effect which is further enhanced in the presence of multivalent cations (as described and referenced above) and which may subsequently help packaging processes.[60] Nonetheless, some literature reports that the B-A transition may proceed with no need of aggregation/condensation.[61] The possibility that DNA aggregation/condensation may preclude the observation of the A form by circular dichroism (CD),[4b] which is the technique that has been majorly used to identify DNA secondary structure,[62] was later on discarded by contrasting CD assays to Raman spectroscopy,[59a] X-ray diffraction[59b] or sedimentation[61b] assays in the same study. CD spectra from DNA in high alcohol concentration had been previously identified as from A-form due to their qualitative similarity with those from RNA.[20]

Due to the fluctuating nature of the system, the interaction of the multivalent cations with nucleic acids and subsequent screening, condensing and site binding effects –depending on the distance to the electrolyte– are mainly dynamic in nature: cations majorly form transient and non-sequence-specific contacts with the electronegative binding sites of the DNA. In this picture, DNA condensation requires ionic shielding from Coulombic, repulsive interactions, with ensuing DNA charge neutralization (89% to 90% of the phosphates),[63] and eventually, attractive forces (London dispersion and dipole-induced dipole interactions, mainly),[64] which may involve charge inversion.[65]

Single-molecule instrumentation

What primarily determines the utility of an instrument for the mechanical manipulation of a single polymer is its capacity to generate appropriate tension and torsion. Specifically, force and torque must be high enough to produce structural changes but low enough to not irreversibly break the sample molecule. Secondly, for quantitative characterizations, the instrument should be capable of measuring either force or torque or both in real time in suitable ranges; and thirdly, it is imperative an adequate resolution for such measurements. The elasticity and tensile structural transitions of nucleic acids are in the range of a few to tens of pico-Newtons, as well as the forces developed by DNA-binding molecular motors, with temporal frames of a few seconds. The torque-induced phase transitions are measured in pN·nm with similar temporal frames. Typical force and torque resolutions for the detection of DNA and RNA transitions are of 0.1 pN and 1 pN·nm, respectively, with temporal precisions of milliseconds.

Three physical concepts have largely been evolved into techniques that fulfil these criteria (Fig. 3): the atomic force microscope (AFM), optical tweezers (OT) and magnetic tweezers (MT). Laminar flow fields and microfluidics are often combined with these devices to orient single molecules or particles with pico-Newton forces and to control the liquid environment in small chambers.[66] Labelling the sample molecule with fluorescent probes has additionally afforded the dynamic localization of the sample molecule while subject to mechanical operations.[67]

AFM is essentially an imaging instrument consisting of a tip at the end of a cantilever with which to scan a molecule on a surface (see Fig. 3A). This technique can be used to map individual molecules and their dynamics to a high temporal resolutions.[68] It can also be applied to manipulate and measure forces in real time; in

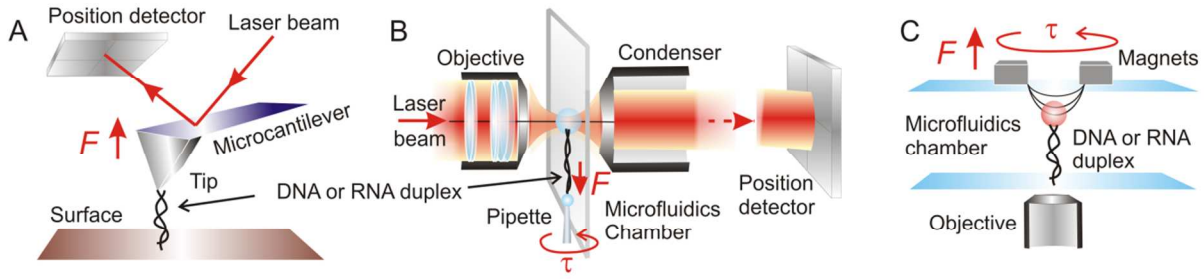

Fig. 3 Basic elements of the most used single-molecule mechanical manipulators. (A) AFM, a type of scanning probe microscopy that consists of a micro-cantilever ending in a nanometre-scale tip. A polymer can be stretched between the tip and a surface and the forces measured by following the cantilever displacement via laser-beam deflection on a photodiode. (B) In optical tweezers, laser light is highly focused by a microscope objective and collected by a condenser lens. A microbead can be trapped in the focal region, which is produced inside a fluidics chamber. A micropipette inserted in the chamber can hold a second bead by suction. By translating and rotating the micropipette relative to the trap, a polymer can be stretched and twisted, respectively. The collected exiting rays are registered by a position-sensitive photodetector. (C) Conventional magnetic tweezers uses two small magnets to pull and rotate a magnetic microsphere inside a fluidics chamber. Here, the polymer is held between the microsphere and the bottom coverslip. A microscope objective is used to monitor the experiment.

particular, it has been used to measure the force-extension curve of dsDNA, but due to the relatively high stiffness of the cantilevers, AFM is more suited for the detection of events at higher forces (e.g., bond strengths). Its design also makes it less convenient to produce torque.

OT and MT setups made possible both the extension and torsion of duplex nucleic acids for the first time and revolutionized the dynamic study of DNA-binding motor proteins. In the OT experiments, Fig. 3B, a single molecule is attached between two dielectric beads, one optically trapped and the other held by suction on a micropipette or by another optical trap.[69] By moving the micropipette relative to the optical trap, or one of the traps relative to the other, the polymer can be stretched or relaxed. This experimental procedure is also utilized in MT (Fig. 3C),[70] a similar instrument from a practical point of view in which the forces are produced by magnetic –instead of optical– gradients. In conventional MT, a magnetic microparticle and a coverslip are used for the attachment of the polymer. The two magnets can be translated to produce force or rotated to produce torque on the magnetic particle, thus stretching or twisting the tethered polymer. The generation of torque is trickier in OT. Initially, rotating micropipettes were implemented to add turns to the attached polymer;[71] more recently, the angular momentum of light has been used to provide an all-optical control on anisotropic and/or aspheric trapped microparticles. This advance is known as the optical wrench or the angular optical trap (AOT).[8b, 72]

These and other advances (including hybrid instruments) majorly based on the conventional optical and magnetic designs shown in Fig. 3 have prospered in recent years to not only produce both force and torque, but also to measure them to a high precision. Important to single-molecule measurements is the reduction of instrumental and environmental noises, which are not clearly distinguishable from the naturally-occurring thermal fluctuations. Extrinsic noise, such as acoustic and mechanical room vibrations, reduces the accuracy of the measurements and might lead to false signals.[8b, 69a]

Force and torque measurements in these instruments are most often performed by using Hooke's law, whose calibration constants are obtained, (i), by analysing the Brownian motion of the probe subjected to the thermal fluctuations, (ii), by using the Stokes's law or, (iii), by using the critical value of a known DNA structural transition. Particular to OT is the use of the principles of linear and angular momentum conservation for force and torque calibration, respectively.

Torque detection is more challenging than that of force since rotation unlike translation fluctuations of the trapped bead are more difficult to track experimentally. An extra nanometre-sized bead attached to a DNA strand can be used to measure the torque imposed by the trapping field on the molecule as the nanoparticle is observed revolving about the long axis of the DNA strand; it is the so-called rotor bead tracking (RBT) method, which has been used in both OT and MT instruments and whose resolution depends on the rotor bead size.[71, 73]

In OT, twist angles can be independently tracked by analysing the polarization changes in the light scattered from an anisotropic bead, a strategy that is employed in the above mentioned AOT configuration. In MT instruments, torque measurements have been improved in recent years by decoupling the stretching force from the twisting confinement that the magnetic field exerts on the magnetic bead in the configuration of Fig. 3C. To that end, magnetic fields have been oriented axially, instead of transversely to the DNA molecule, by using a hollow, cylindrical magnet, as developed by Celedon et al.,[74] Kauert et al.,[75] as well as by Lipfert et al. in the so-called magnetic torque tweezers (MTT)[76] and freely orbiting magnetic tweezers (FOMT)[77] configurations. Recently, Janssen et al.[78] included two pairs of Helmhotz coils in the MTT setup to achieve full control over the transverse magnetic field. Other ideas include that of Mosconi et al.[79], who used a six-pole electromagnet to tune the magnetic field intensity and direction. These technical advances in force and torque measurement are detailed in recently appeared compendia.[8b, c, 73a, 80]

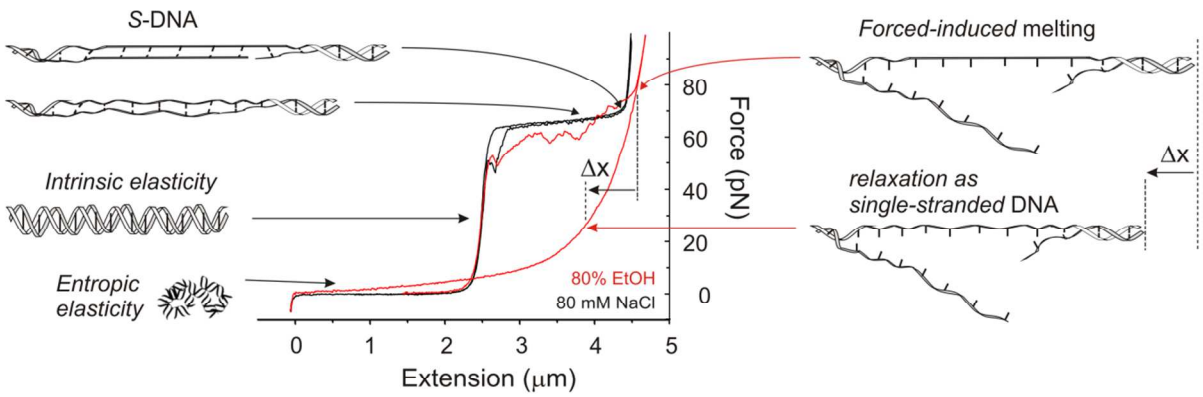

Fig. 4 Force-extension characterization of dsDNA. The graph shows an individual DNA molecule from pBACgus11 plasmid (8022 bp, 48% G•C), first stretched and relaxed in 80 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (black line), and subsequently stretched and relaxed in a mixture of the same buffer with 80% ethanol (red line). Pulling speed, 500 nm/s. The lateral schemes illustrate the strands arrangement as a function of the pulling/relaxing force according to the literature (see text for details).

Single-molecule structural analysis of DNA and RNA

Intrinsic structure of DNA under tension

Direct structural techniques such as X-ray diffraction, NMR and electron microscopy are used to characterize the different conformations of dsDNA with "ensemble-averaged" single-nucleotide resolution. In contrast, indirect techniques, such as CD and other light spectroscopies (UV, IR, Raman and light scattering) are very convenient to identify the secondary structure of macromolecules, although they do not provide a direct plot of the base-pair arrangements.

Force-extension experiments represent a unique approach to studying condensation, melting and polymorphism of double-helical nucleic acids in low humidity environments, because these transitions, other than electrostatic, are mechanochemical in character. Earlier experiments with this methodology were performed by linearly stabilizing many DNA fibres by a tethering weight, thus deriving information from ensemble measurements but with no control on the role of the constraints imposed by the interhelical interactions in the fibre-bundle samples. In these assays, the relative length of the oriented, aggregated bundle was observed as a function of temperature and ionic conditions, thus shedding light on the structural transitions promoted by the increase in alcohol concentrations.[21b, 81] With the advent of single-molecule techniques in the study of DNA,[8a] insight into intra and intermolecular interactions with sub-pN resolution and nanometre positioning could be investigated in an environment whereby hydration conditions are accurately controlled; by manipulating an individual DNA polymer, aggregation is avoided and condensation can be prevented by stretching the polymer above a critical force.[82] With this approach, not only the elastic properties of the polymer as a function of environmental conditions can be devised but transition forces, mechanical equilibrium conditions and dynamic information due to the intrinsically fluctuating nature of the system can also be derived.

The mechanical response of a polymer can be characterized by its force-extension curve, as explained in Fig. 4. The graph shows a complete stretch-relaxation cycle of dsDNA in aqueous buffer (black curve). Two main regimes of elasticity and the corresponding strands arrangement are shown: the *entropic elasticity* in which the molecule is stretched like an entropic spring until its characteristic contour-length (below some 5 pN), and the *enthalpic* or *intrinsic elasticity* in which it is stretched beyond its contour-length (below some 60 pN). In both regimes the molecule remains as a right-hand double-helix, although in the intrinsic regime it slightly changes its twist,[83] as discussed later. At about 65 pN, depending on ionic strength, pH, sequence and in the absence of torsional constraints (see below), the molecule experiences a structural transition to an almost totally unwound state. It is the so-called *overstretching transition*: the molecule lengthens to 1.7 times its contour-length over a narrow range of forces (~2 pN). The bases unstack and the strands may remain linked by partial base-pairing, like a parallel ladder (*S*-form),[14] or may separate into a totally molten state (*force-induced melting*).[84] Environmental conditions have been shown to dictate the stability of the two strands at high force in either one of these two states or in a heterogeneous combination of both (see recent, critical references [84b, 85] and [8c] for a review discussion). To illustrate this matter, we have just plotted in Fig. 4 a second stretch-relaxation cycle of the same molecule (red curve) in the same buffer at high ethanol concentration to show that the molecule relaxes as single-stranded DNA when the conditions (high force, low humidity and/or low salt concentration) do not favour strand-annealing. Likewise, Fig. 5 shows the dynamics of strand separation during the overstretching when there is a free strand end: the molecule unpeels like in an unzipping process leading to a sequence-dependent stick-slip pattern in physiological conditions (Fig. 5A) or to a process of coincident unwinding and unzipping at both high ethanol concentration and low salt conditions (Fig. 5B).

The stability of the A form and its transition to the B form in dsDNA and dsRNA must be testable by using mechanical force. Fig. 6 plots the hypothesis behind the single-molecule manipulation

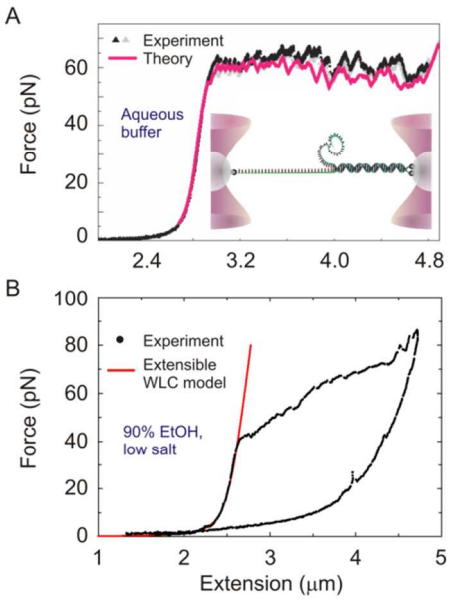

Fig. 5 Force-induced unpeeling of dsDNA. A duplex, held between two beads by only one strand in one of its ends, is melted from the free strand end as depicted in the inset of A. (A) Force-extension curve of an 8.4-kbp molecule (pKYB1 plasmid) taken at 10 nm/s in physiological conditions (50 mM NaCl, 10 mM Tris-HCl, pH 7.8, 20°C). The overstretching plateau reveals a sequence-dependent stick-slip dynamics. Reproduced from [111a] with permission, copyright (2011) Macmillan Publishers Ltd. (B) Force-extension curve of an 8.0-kbp molecule (pBACgus11) taken at 500 nm/s and 20°C in a mixture of 90% ethanol with 1 mM NaCl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). In this case, unwinding and unpeeling processes meet in an "ovestretching transition" that is no-longer a plateau. The molecule relaxes as ssDNA as a consequence of the low polarity solution. A fitting to the so-called *Worm-like chain* (WLC) model reveals a persistence length and stretch modulus dramatically lower than in physiological conditions (P = 13.7 nm, S = 878 pN) due to the presence of ethanol. Reproduced from [11b] with permission, copyright (2012) Wiley Periodicals, Inc.

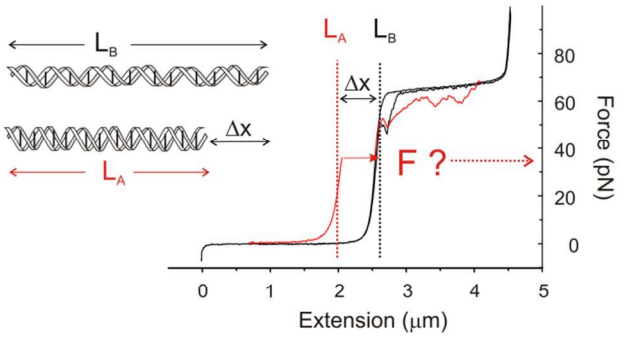

Fig. 6 Hypothesis of a force-induced A→B transition. A dsDNA molecule in high alcohol concentration and/or with a high G•C content switches from the A to the B form at a force *F* (red curve). As a reference of the B-form elastic behaviour, the previous stretch-relaxation cycle of the pBACgus11 DNA molecule (Fig. 4), stable in B-form in aqueous buffer solution, is plotted in black.

experiment: if the A↔B transition is cooperative and reversible, it should be possible to pull a fibre stable in the A-form to a force '*F*' at which the molecule changes its conformation to the longer B-form. This force cannot, in any case, be above the overstretching transition since the conformation of the molecule is certainly different from a double-helix (see above). If the A→B transition takes place in the intrinsic elasticity regime, a sudden jump between the A and B contour-lengths (L_A and L_B, respectively),[‡] should be observed. If the transition is coincident with the overstretching transition, near 65 pN, this transition should start at extensions near L_A and end at $1.7\times L_B$, thus giving rise to a total transition length of approximately $\Delta x + 1.7\times L_B$, being $\Delta x \approx L_B - L_A$.[‖] If the transition takes place at lower forces, namely, within the entropic elasticity regime, it may not be observed easily as the force-extension curve shows an almost horizontal slope in this regime. In such a case, a different experimental approach should be used to observe the change from the A to the B contour-length (see below).

Hormeño et al. first used force-extension experiments by OT to test the existence of the A-B transition in dsDNA.[11c] They prepared two types of molecules, one with a balanced, 48%, G•C content (pBACgus11, 8022 bp) and a second one with a 70% G•C content (piJ702, 5682 bp). Both molecules showed CD signals characteristic of the A form in a buffer with high ethanol concentration (above 70%). The second, 70% G•C content molecule also showed a CD signal compatible with an A-form in aqueous buffer (with no alcohol). Surprisingly, both polymers extended like B-form double helices, both in aqueous buffer and at high ethanol concentrations. Fig. 4 is an example of their results: the red curve illustrates that the dsDNA molecule at 80% ethanol concentration has the same contour-length as that in aqueous buffer since the intrinsic elasticity regime of the trace in 80% ethanol overlaps with that in physiological buffer. The overstretching plateau showed almost similar lengths for both traces, approximately equal to $1.7\times L_B$, hence also discarding a transition between A and B forms at high force.

In order to test whether an A→B transition takes place during the entropic elasticity regime, this team performed *constant-force* experiments with MT.[11c] In these assays, the molecule was held at a constant force in physiological buffer; then, this aqueous environment was substituted for a water ethanol mixture at high ethanol concentration while the molecule was held stretched at the same force. Their results, however, showed that both 48%-G•C and 70%-G•C molecules totally collapse, certainly reducing their contour length beyond the ~25% that should be expected for this transition (see Fig. 1), a result which is compatible with the condensation of the individual molecules induced by the presence of a low environmental polarity.

Finally, to examine whether individual DNA molecules with A-type base-stacking manifest a global arrangement compatible with corresponding crystallographic distances between base-pairs, this team used AFM. In these experiments, the polymers are deposited onto a freshly cleaved mica surface in air by using divalent cations (typically Mg^{2+}) and the contours of the single molecules can be directly traced from the images. Measurements over the above DNA

molecules again showed contour lengths compatible with the B-form.[11a] The 48%-G•C DNA was expected to be in this form but since the 70%-G•C DNA did exhibit A-form CD signal in all conditions, it should have been expected to show a reduced contour-length, compatible with the A-form, especially because the deposition conditions include low humidity and divalent cations that enhances the stabilization of this form. It is important to mention, however, that Fang et al.[86] measured contour-length changes compatible with an A-form DNA in their AFM experiments by using water-ethanol solutions. The authors observed that ethanol concentrations as low as 25% could stabilize the A-form molecules on a surface treated with Mg^{2+} although they found higher order condensed structures at >20% ethanol concentration, a result that will be discussed further in this review.

Intrinsic structure of RNA under tension

Single dsRNA molecules were for the first time stretched by Bonin et al.[87] In their study, in which they used AFM in scanning force mode, they compared the force-extension curves of several sequences of DNA and RNA duplexes. They found qualitatively distinct aspects, including the A-S transition in RNA. Abels et al. studied the force-extension curves of dsRNA at low forces.[25] This team used MT with which they were able to measure the contour and persistence lengths of the molecules in physiological conditions, respectively. Their experiments with AFM, used in imaging mode in air, yielded similar results.

Herrero-Galán et al.[18] used OT to obtain the complete force-extension curve of this polymer. To perform a rigorous comparison with dsDNA, this team prepared 4-kbp DNA molecules with balanced G•C content from the λ-phage genome and their sequence-equivalent dsRNA molecules. Fig. 7A shows the characteristic behaviour of each nucleic acid substrate in physiological buffer. Both molecular types overstretch at high force, although this transition in dsRNA takes place at lower forces and in a less cooperative fashion than in its DNA counterpart. The most remarkable features are that the intrinsic elasticity regime of dsRNA is shifted to shorter contour lengths, which clearly identifies the characteristic A-form of this nucleic acid substrate, and that the overstretching transition is also shorter than in dsDNA. To better observe these features, Fig. 7B plots the same dsRNA force-extension curve normalized in the extension axis to the A-form contour length together with that of dsDNA normalized to the B-form contour length. This representation indicates moreover that the intrinsic elasticity regime of dsRNA is tilted to lower slopes than that of dsDNA, hence demonstrating that the dsRNA stretch modulus is lower than that of dsDNA (see next section). Finally, the length of the overstretching transition is 0.7 times the contour length of its respective substrate, either in A or in B form. This result is related to the respective sugar-puckering of the ribonucleotides: in RNA, the $C_{3'}$-endo conformation constrains adjacent phosphates to shorter distances than in DNA, with $C_{2'}$-endo conformation for the deoxyribonucleotides. Fig. 8 addresses these facts. As shown, this model provides the sugar-puckering modes with a major role in

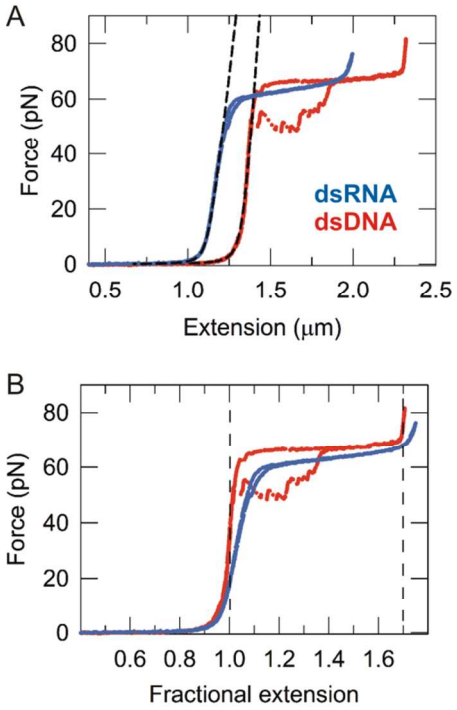

Fig. 7 Force−extension characterization of dsRNA and dsDNA substrates of equivalent sequence. (A) Force−extension curves in 10 mM Tris-HCl with 300 mM NaCl and 1 mM EDTA (pH 8.0). Stretch−relax cycles were performed at 500 nm/s. Blue and red dots correspond to data points of a dsRNA and a dsDNA molecule, respectively, with their corresponding fittings, black dashed curves, to the extensible WLC model. The fits for these examples yielded values of (contour length) L = 1.14 μm, (persistence length) P = 59.8 nm, and (stretch modulus) S = 632 pN for dsRNA and L = 1.36 μm, P = 48.5 nm, and S = 1280 pN for dsDNA. (B) Same as in (A) with extension axis of each substrate molecule normalized to its respective contour length. Dashed lines at fractional extensions 1.0 and 1.7 demarcate the elasticity regimes. Adapted from [18] with permission, copyright (2013) American Chemical Society.

demarking the possible base-stacking conformations and in limiting the elasticity of their respective double-stranded polymers.

The force-induced melting behaviour of dsRNA is also different from that of dsDNA. Fig. 7 shows that the former molecular substrate displays very little mechanical hysteresis in the stretch-relaxation cycle as compared to dsDNA. This fact was found to correlate with the different melting behaviour of dsRNA, whose melting temperature is always higher than that of dsDNA of equivalent sequence.[18]

Importantly, dsRNA does not exhibit a mechanical transition A→B, a result that is compatible with force-free structural observations in different environmental conditions to date.[41a] This result concurs with the above explained behaviour of dsDNA, which does not exhibit either A-form elastic response or A-form contour-length at the single-molecule level, even for G•C-rich sequences and/or in low-humidity conditions.

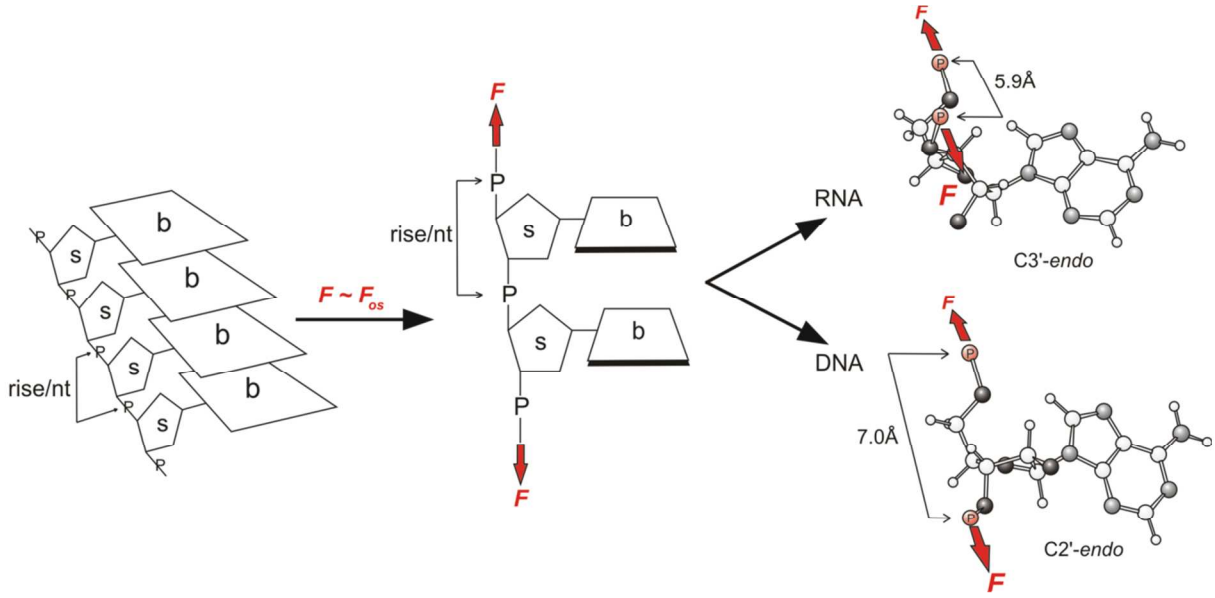

Fig. 8 Schematic depiction of the elastic response of nucleic acids under force. Rise per nucleotide (rise/nt) depends on the stacking of bases (*b*) in the absence of stress. When force is applied at overstretching values ($F \approx F_{os}$), bases unstack and rise per nucleotide is restricted by the distance between phosphates linking adjacent sugar rings (*s*), which is of 5.9 Å in dsRNA, where sugar rings are in $C_{3'}$-endo conformation, and of 7.0 Å in dsDNA, where they adopt the $C_{2'}$-endo conformation. Reproduced from [18] with permission, copyright (2013) American Chemical Society.

Condensation coexists with A-type base-stacking in DNA

DsDNA is known to condense. When many DNA molecules are present, it also aggregates and precipitates at high alcohol concentration. The presence of multivalent cations favours this intramolecular transition to a compact structure in which lateral chain interactions take place. In contrast, dsRNA has been so far reported to resist condensation.[88] The fact that dsDNA can condense in low humidity environments and with the help of polycations has been used to study DNA organization in free form and to mimic environmental conditions similar to those inside viral capsids.[80]

The condensation of DNA molecules at the single-molecule level has been studied by a number of authors.[86, 89] Fig. 9 reveals the abrupt condensing effects that high ethanol concentration produce in the force-extension curves of 70% G•C-rich piJ702 DNA. In these experiments, each molecule was first stretched and relaxed in physiological conditions (aqueous buffer with 80 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and subsequently stretched and relaxed twice in a buffer-90 % ethanol mixture (Fig. 9A) or in the same buffer with 1 mM NaCl and 17.5 µM spermine (Fig. 9B). Force-extension curves of DNA in the ethanol or spermine mixtures show either a plateau at entropic forces of ~3 pN or a stick-release pattern. These effects indicate the unravelling of collapsed structures, such as coiled DNA or toroidal supercoiling, in the form of a smooth decondensation process or a violent unfolding of the polymer (see [11c] and references therein), respectively. These experiments together with those at constant force with MT and those of Fang et al. with AFM[86] confirm that the conditions that induce A-type base-stacking in DNA do globally produce condensation of the single molecule.

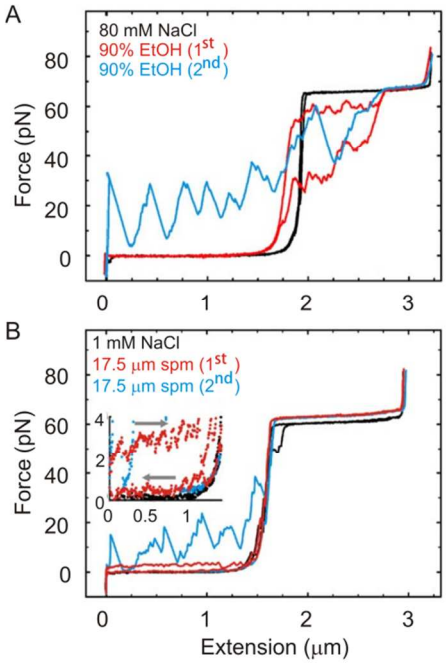

Fig. 9 Condensation of a single DNA molecule under force. In each graph, a single molecule of dsDNA (piJ702 plasmid, 5682 bp, 70% G•C content) is first stretched and relaxed in physiological buffer (black curve), followed by two successive stretch/relax cycles (red and blue curves) in a buffer-90 % ethanol mixture (A) or in buffer with a low concentration of spermine (B). Reproduced from [11c] with permission from Elsevier, copyright (2011).

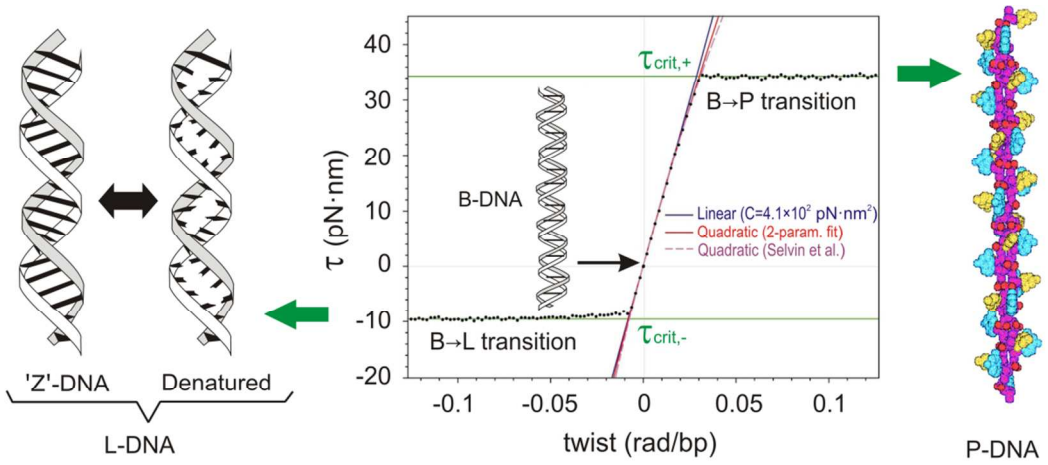

Fig. 10 Torque-twist characterization of dsDNA. The central diagram shows a torque-angle curve at constant tension; different forces generate similar curves according to the force-torque diagram of Fig. 11. Reproduced from [71] with permission, copyright (2003) Macmillan Publishers Ltd. The left schematic depictions address the two mixed phases that can take place at underwinding torques, the Z-like ('Z') DNA and the denatured phase, which, apart from environmental conditions, depend on sequence. The right figure is a molecular model of P-DNA, which takes place at overwinding torques. Reproduced from [13a] with permission, copyright (1998) National Academy of Sciences, U.S.A.

From the numerical simulations by Noy et al.,[90] it was pointed out that the A-type base-stacking is loosely stable in ethanolic conditions under the effect of thermal fluctuations in comparison to B-type base-stacking.[11c] In particular, the force deduced from these simulations in which the B-A transition should take place is ~5 pN,[11c] clearly within the condensation regime shown in Fig. 9. This result supports the fact that interhelical interactions are required to strengthen the global stability of A-DNA, a scenario that takes place in measurements by X-ray crystallography and in ensemble-average techniques in which the molecules strongly pack or are left free to condense and aggregate.

The fact that torsion has a role in DNA condensation in the presence of "collaborative" salts has been verified recently.[15, 91] The formation of compact (tertiary) morphologies which involve rotation and the (secondary) conformations that can be induced by an imposed twist are analysed next.

Chirality and mechanical stress yield new double-stranded structures

DsDNA and dsRNA are helices; hence, their deformations under stress cannot only be explained by stretching and bending deformations, which suffices for the mechanical description of an isotropic rod. Chirality unavoidably transduces part of the stretching energy, which majorly extends the molecule, into twist and vice-versa. Such simple reasoning has been used to find and explain new conformations of double-stranded nucleic acids by separately using either force or torque, or a combination of both.

The first twisting experiments were conducted by Strick et al.[92] with magnetic tweezers, soon after the dsDNA had been stretched with the same technique.[93] Magnetic tweezers offer a simple method to coil a polymer by gyrating the magnets around the stretching axis (see Fig. 3C), although rotating pipettes (Fig. 3B)[71, 94] and other all-optical strategies can also be incorporated into an optical tweezers apparatus[72b] (see previous section), leading to the same physics over the sample molecule. To accumulate turns in the dsDNA, its two strands, besides being nick-free, must be attached by both ends to steady surfaces. The molecule is then torsionally-constrained. In these conditions, the molecule self-induces a torque as it is stretched due to its chirality. The overstretching plateau then takes place at around 110 pN. In torsionally-unconstrained conditions, the accumulated torque is released as the molecule lengthens because the discontinuous strand is free to rotate around the continuous one, thus producing the previously discussed ~65-pN overstretching plateau (Figs. 4 and 5).

By keeping a constant tension to prevent writhing, which leads to the formation of plectonemes, the molecule can be under- and overwound, giving rise to the so-called L-DNA (left DNA) and the P- (Pauling-like) DNA,[13b] respectively (see Fig. 10). Like for the S-DNA, the structure of these conformations can be modelled by indirect methods. L-DNA is a denatured form of B-DNA[13a, 95] with a net left-handed twist upon completion of the torsional B-L transition;[8a, 71] L-DNA is not stable at zero torque in physiological conditions and its structure is related to either a state of separated strands or a Z-like DNA depending on sequence (see Fig. 10, left).[12, 96] In any case, the effective rise per residue is similar to that of Z-DNA, thus making it slightly longer than B-DNA.[12, 71, 94, 96b] P-DNA involves an inter-winding of the two phosphate backbones at the centre of the conformation and exposed (unpaired) bases on the outside (see Fig. 10, right). The predicted number of bases per turn for P-DNA is between 2.4-2.62 and the apparent rise per residue is 0.535-0.585 nm.[13a, 94, 97] Bryant et al. observed these transitions and quantified the force-torque pairs, Fig. 11A, that trigger the structural changes so mechanically induced, including triple points,[71] which had been predicted by Sarkar et al.[97] partly on the basis of the experimental data gathered by the same team.[94] This diagram has been recently modified at low forces, Fig 11B, to include the

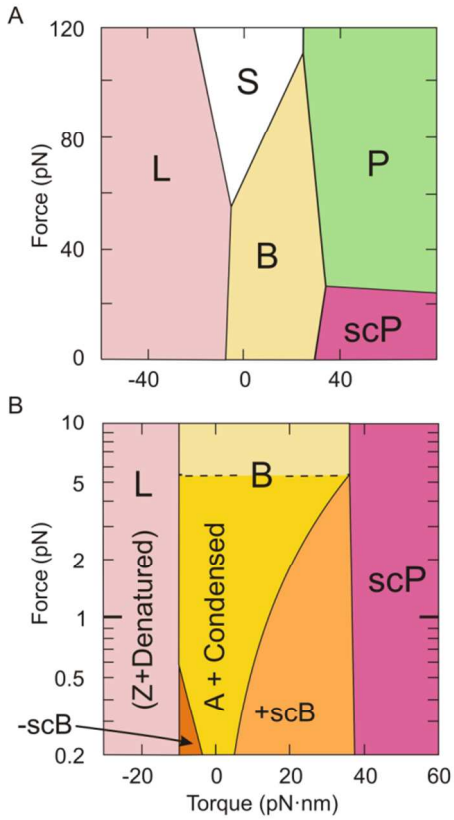

Fig. 11 DNA force-torque phase diagram near physiological conditions. Colour regions represent conditions for the stability of pure structures; lines indicate conditions for structural coexistence of two conformations in a single dsDNA molecule. (A) Early diagram adapted from [97], copyright (2001) American Physical Society. (B) Phase diagram at low forces. Conditions for Z-DNA and A-DNA plus condensation often require high ionic strengths and/or polycations and low humidity. Revised from [119] and [96b] with permission, copyright (2007 and 2011) American Physical Society, following these sources and the present review report. scP, supercoiled P-DNA; +scB and -scB, supercoiled B-DNA at overwinding and underwinding torques, respectively. Changes in ambient temperature, pH and salt type and concentration, which may modify coexistence lines and triple points, have not been considered in depth.

competing structures that take place at underwinding torques[8b, 73a] and is here revised to take account of condensation and its coexistence with the A-form.[11c]

Under low enough tensile strength, supercoiling leads to molecular buckling and formation of plectonemes, the understanding of which are important for DNA condensation and packing, besides being related to *in vivo* DNA processing by motor proteins. It has been observed that the buckling transition in overwound DNA follows an abrupt change in extension that nucleates the formation of a plectoneme.[8b, 12, 74b, 98] In underwound DNA, a similar mechanism has been observed for the B-Z transition in GC-repeat sequences, henceforth showing the presence of an energetic barrier for buckling.[12]

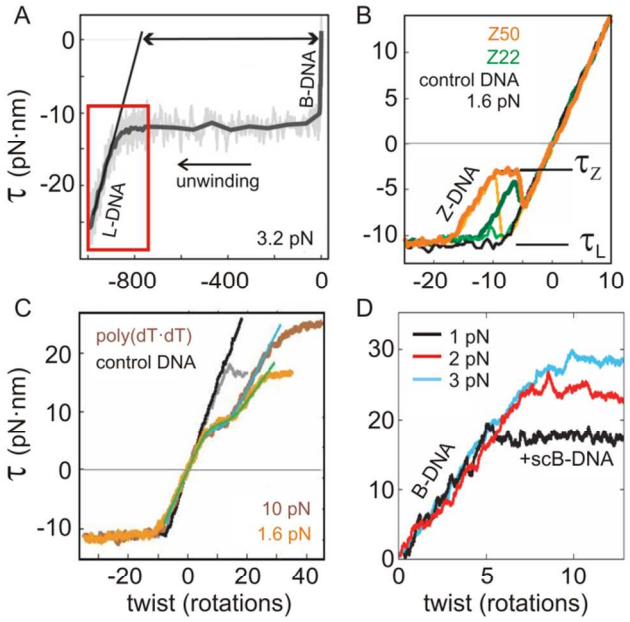

Fig. 12 Torque-twist curves of dsDNA at low force. (A) B-L transition of a 4.6-kbp control dsDNA molecule. (B) B-Z transition of either a 50-bp or a 22-bp long GC-insert molecule in a PBS (phosphate buffered saline) solution (137 mM NaCl, 2.7 mM KCl, 0.5 mg/mL BSA, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 5 mM EDTA, pH 7.4). (C) B-to-denatured DNA transition for a 100-bp insert with mismatched dT bases in the same PBS solution. (D) B-scB (positively supercoiled B-DNA) transition of a 2.2-kbp dsDNA molecule in 150 mM NaCl PBS solution, 23.5°C. (A-C, reproduced from [12] with permission; D, reproduced from [101] with permission, copyright (2008) American Physical Society).

B-L and B-P transitions occur at critical torques, as shown in the force-torque phase diagrams of Fig. 11, which are nearly force-independent.[13a, 71, 94] Force-extension curves at constant torque also reveal the dsDNA chirality. Specifically, the overstretching force for a torsionally-constrained molecule depends on the applied torque, thus leading to a ~50-pN plateau in the presence of slightly negative (unwinding) torque due to the B+L→S transition, and to a ~25-pN plateau in the presence of positive (overwinding) torque due to the transition from plectonemically supercoiled P-DNA (scP) to P-DNA.[8a, 94, 97] The length of these plateaus depends on the number of accumulated turns, i.e., their widening are at the expense of the narrowing of the 110 pN plateau.[97] These experiments additionally suggested a quantification for the S-DNA helicity, namely, 33-37.5 base pairs per turn and a rise per residue of 0.58 nm,[71, 94, 97] these estimations being based on the observation that S-DNA is not a totally unwound configuration.

Both L- and P-DNA involve a lengthening of the molecule, underlying the fact that twist couples to extension. Conversely, stretching a dsDNA molecule slightly induces twist even before the overstretching transition.[99] Experiments performed by Lionnet et al.[100] and Gore et al.[83] demonstrated such twist within the enthalpic elasticity regime, which would intuitively underwind the molecule.

However, dsDNA turned out to slightly overwind for forces below <30 pN, the reason possibly related to a radial contraction of the double-helix.[83, 100] Above 30 pN, the molecule loses helicity, the major underwinding contribution taking place abruptly at the overstretching transition, as explained above. When the liquid environment is far from physiological, however, slight underwinding just above 35 pN can trigger the unpeeling of the double helix. Low humidity conditions can generate these dynamics, see Fig. 5B: the force-extension curve fits to the extensible *Worm-like chain* (WLC) model (no twist incorporated) until almost the start of the unwinding, which subsequently triggers the unpeeling. Overall, unwinding and upeeling leads to a dramatically tilted overstretching transition in these low-polarity conditions.

The most recent reports on the unwinding and overwinding transitions have focused in the low tension regime, diagram 11B. These studies have discerned the B-Z transitions for appropriate sequences (G•C steps) and the strand separation for mismatched bases at unwinding, see Fig. 12A-C.[12, 73a, 96a] A buckling transition for B-DNA has also been found at both unwinding (-scB) and overwinding torques (+scB),[8b, 101] see Fig. 12D, flanking the condensation of the A-like DNA[11c] at near zero torque (diagram 11B).

The analysis of twist, including the coupling between stretch-twist elasticity and the recently found torque-induced structures, is very detailed to date for dsDNA in physiological conditions and in the presence of small, binding molecules that act as intercalators.[74b, 102] Although a theoretical treatment has advanced the twist transitions and stabilities for dsRNA,[103] no experiments of this kind have been published for the RNA duplex as yet.

The mechanical response of DNA and RNA

Statistical interpretation of the mechanical parameters

Polymers in solution are dynamic structures which are subjected to *thermal fluctuations*. This noise represents a natural randomness (the system is said to be stochastic or non-deterministic) with which molecular motors have to deal when they operate over their substrates. Therefore, the role of thermal fluctuations must be assessed in order to fully understand the information processing machinery. This means that, apart from the purely mechanical and electrostatic nature of the elastic parameters, there is a statistical contribution which is inherent to the system and that carries biological meaning. Mechanical parameters are primarily represented by the *mean*, which characterizes the ensemble average and should be obtained in the limit of many assays (i.e., in a bulk experimental framework), and by the *distribution width*, which is typically provided by the standard deviation in Gaussian distributions.

Distribution widths may be confounded with experimental errors and this represents a common misunderstanding in single-molecule experiments. In particular, in a system made up of one or a few different but related macromolecules (as, for example, during replication) in an aqueous solution at temperatures near or above room temperature, distribution widths of measured observables cannot be made indefinitely narrow in the limit of an infinite number of assays, according to the definition of error in a deterministic system, but they converge to a defined value because of the presence of thermal fluctuations. In contrast, experimental errors, which cannot be separated from this Brownian noise in many situations, can be made indefinitely narrow because they are not inherent to the system dynamics.[⊥]

In a real situation, the mean value of the DNA persistence length (see next subsection) can help us understand, for example, the mean size of a condensate and its standard deviation can address the probability of spontaneous high bending. The latter could be useful in interpreting the energetic barrier that a molecular motor has to break in order to curve the DNA. The mean value of the stretch modulus and the torsional rigidity are useful in interpreting the energies, forces and torques that, for example, a helicase needs to exert in order to unstack and unwind individual bases, and whether a passive mechanism in which they just wait for spontaneous breathing of the bases to step forward would work.

Direct reference to thermal noise mechanisms has been made in the literature, for example, to explain the binding of RecA to DNA[94] by spontanous stretching fluctuations. More general thermal ratchet mechanisms are present in DNA-binding molecular motors, which comprise both the Brownian action of the protein and the structural fluctuations of the DNA substrate.

Flexural and bending elasticity of dsDNA and dsRNA

There are three main mechanical parameters to characterize the tensile elastic response of a double-helix polymer in solution: the persistence length, P, which quantifies the tendency of a polymer to remain straight,[104] the stretch modulus, S, which accounts for the flexural rigidity (*Young modulus* of the polymer divided by its cross section),[105] and the overstretching transition force, which represents the capacity of a polymer to remain in double-helix form against a stretching force, F_{os}.[8a, 14] The last parameter can be directly quantified from the force-extension curves and the first two parameters can be obtained by fitting these experimental data to the WLC model.[106] The persistence length can also be obtained from AFM images; this technique is moreover an excellent tool to measure the contour-length and to directly compare the diameters of individual molecules co-adsorbed on the same surface.

The mechanical properties of dsDNA have been extensively analysed previously. Baumann et al. found that the persistence length of this polymer diminishes with physiological, monovalent salt concentration (80-500 mM NaCl), ranging typically from 45−53 nm.[82] Multivalent salt concentration produces a similar decreasing trend but lower concentrations are required. This behaviour is mainly explained due to the partial shielding of the negative DNA surface charge by free cations, which bind to the DNA phosphate groups. Under these conditions, high electrostatic repulsion between different chain segments is prevented when the molecule bends.

The stretch modulus increases with salt concentration and the typical values for the mentioned salt concentrations are 1000−1400 pN. The overstretching force also increases with salt concentration, approximately from 63 to 67 pN.[84a] The increase in stretch modulus and overstretching force with increasing salt concentration is mainly

explained by an enhancement of the hydrophobic, base-stacking interactions in the presence of bound cations to the DNA surface. Overall, the behaviour of these three parameters indicates that the mechanical stability of the double-helix increases with salt concentration.

Hormeño et al.[11a] analysed the mechanical properties of a dsDNA molecule with A-type base-stacking (70% G•C content, previous piJ702) at similar physiological conditions (80 mM [NaCl]) by OT and MT. They found that the persistence length is similar to that of canonical B-DNA molecules (48% G•C-rich pBACgus11 control molecules; see also the values given for the λ-phage DNA).[82] The stretch modulus and the overstretching transition force are, however, higher, S=1325 ± 93 pN and F_{os}=65.4 ± 0.2 pN, respectively (compare with 1114 ± 61 pN and 63.8 ± 0.1 pN for the balanced G•C content control molecules). The cooperativity of the overstretching transition is also higher for 70% G•C-rich DNA (see Fig. 9) and the molecules hardly show hysteresis between the stretch and relax paths (high melting stability). These results indicate that the higher the G•C content, the higher the base-stacking and base-pairing stability in DNA. However, no apparent sign of the A-type base-stacking was present in these measurements. The authors also measured the persistence length of the molecules in the absence of force by using AFM. In this case, they found an abrupt change in the persistence length, P=74.5 ± 0.5 nm, that, although reminiscent of the A-type base-stacking, did not give rise to a shorter contour-length characteristic of the A-DNA, as explained in the previous section. The difference of this persistence length value with respect to that in the presence of force indicates that, as measured by CD, there exists local distortions from the B-type base-stacking in DNA molecules with a high-G•C content, but that these distortions are compromised under stress.[11a]

In a subsequent study, this team investigated the effect of low humidity and polycations in the mechanical properties of dsDNA.[11b] The authors used ethanol at increasing concentrations and spermine in micromolar concentration. They found that the persistence length of balanced G•C content molecules (pBACgus11) decreases almost monotonically with ethanol concentration, reaching values as low as 20 nm at 100% ethanol, and that the stretch modulus shows an abrupt reduction, down to ~700−800 pN, at low ethanol concentration (10%), which was conserved for almost the whole range of ethanol concentrations. The cooperativity of the overstretching transition force is considerably reduced in ethanolic solutions and the mechanical hysteresis between the stretch and relax paths also shows an abrupt increase at low ethanol concentrations (see Figs. 4 and 5B and other force-extension curves in[11b, c]). These strong denaturing effects are partially smoothed by increasing the monovalent salt concentrations (in millimolar concentrations), but more remarkably by preparing the buffer-ethanol solutions with spermine just in micromolar concentration.[11b] The overstretching transition force and the hysteresis energy, though lower than in aqueous buffer, show a maximum near 60% ethanol concentration. This re-entrant behaviour was explained as due to the critical balance between the weakening of base-stacking interactions –mainly hydrophobic− and the enhancement of both cation binding to the DNA backbone and base-pairing interactions –mainly electrostatic− in low polarity environments.[11b]

With regards to dsRNA, its mechanical properties were recently studied by using the same techniques in physiological conditions and compared to those of dsDNA molecules of the same sequence, as in the previous section.[18] The elasticity of dsRNA keeps some similarities with that of dsDNA in ethanolic conditions. Specifically, the overstretching transition is less cooperative and takes place at a lower force than that of equivalent dsDNA molecules (approximately by 2 pN; F_{os} compared at half-plateau length). The stretch modulus is lower (approximately by one half, compare S=500 ± 29 pN, RNA, to 935 ± 121 pN, DNA, at 150 mM [NaCl]) and the persistence length is higher (approximately by 10 nm, compare P=61 ± 3 pN vs. 48 ± 2 nm at 150 mM [NaCl]), in agreement with Abels et al.[25] In particular, both the instrinsic and electrostatic components of the persistence length are lower for dsRNA than for dsDNA. The electrostatic component was moreover shown to decrease with the ionic strength for dsRNA, a trend that was expected from the similar behaviour of its DNA counterpart under equivalent conditions.[18, 82]

The distinctive mechanical properties of dsRNA relative to those of dsDNA are explained by their different global structures, either A or B forms, respectively. In particular, the fact that the base-stacking geometry is less planar and dislocated with respect to the helix axis in the A form than in the B form makes dsRNA a more stretchable molecule than its DNA counterpart, thus giving rise to a lower stretch modulus and overstretching transition force. The fact that the charge density of dsRNA is higher than in dsDNA, due to the reduced distance between phosphates in the A form (see Fig. 1, 2 and 7), makes dsRNA electrostatic persistence length greater; and the fact that the rod diameter of dsRNA is larger than that of dsDNA, makes dsRNA an intrinsically less bendable polymer with respect to dsDNA, a fact that is consequently reflected in a greater intrinsic persistence length.

Torsional elasticity of dsDNA

To fully characterize nucleic acids from the mechanical point of view, two other parameters are required: the torsional rigidity, C, which measures the strength of the polymer under torque, and the twist-stretch coupling, g, which quantifies how changes in extension influence those in twist and vice-versa. These parameters have been analysed for moderate forces, within the intrinsic elasticity regime, where the extension of the molecule, free from entropic, bending fluctuations and condensation, is proportional to the applied force. The analysis is also done within linear elasticity theory for torsion, i.e. within the region in which torque is proportional to twist angle; in this regime, torque must be low enough to induce neither B-P nor B-L transitions, nor a writhing dynamics. A coupling between the bending and twisting fluctuations can also be formulated but no experimental evidence has been observed to date.[99b]

Twist experiments have only been performed on dsDNA molecules, henceforth limiting the present analysis to its double-helix. The study of the torsional rigidity for the B-DNA duplex shows very little dependence on the winding direction, see Fig. 10, i.e. this parameter does barely reflect the chirality of the double-helix

for low force and low torque.[71, 107] Measurements on the torsional rigidity agreed in values of C= 410-460 ± 40 pN·m^2 (ref. [71, 83, 108]), larger than initial analyses and measurements (refs. [104a, 105a, 107, 109]). More recent studies showed that this parameter is actually force-dependent, increasing with force up to a saturation point.[8b, 76, 110] This anomalously large torsional rigidity was suggested to stem from the radial contraction of the dsDNA rod, as mentioned in a previous section.[83]

With regards to tension, counterintuitive overwinding as dsDNA is stretched was observed below some 30 pN, while underwinding took place above that tension, as explained in a previous section. This fact affects parameter g, which was observed to remain constant and negative below 30 pN (g=−(90-100) pN·m) and to depend on tension above that critical force, changing sign (i.e. switching from overwinding to underwinding) above ~35 pN.[8b, 83, 100, 111] A simple theoretical DNA elasticity model that includes extension, torsion and melting changes has been recently proposed, proving to describe the complete-force-extension curves of dsDNA.[111a]

Discussion

Single-molecule experiments have provided the way to investigate the dynamic behaviour of nucleic acids in solution: the stability of their structural states, their transitions and their mechanical properties.

To date, tensile experiments have demonstrated that, (i), dsRNA is an intrinsic A-form molecule, (ii), dsDNA is an intrinsic B-form molecule, (iii), both dsRNA and dsDNA do not transition between A-B conformations in the presence of force, and (iv), condensation and aggregation coexists with the A-type base-stacking in DNA. This coexistence suggests that low-humidity conditions locally change the base-stacking arrangement and globally induce DNA condensation/aggregation. This effect may eventually stabilize a molecular contour-length reduction in this polymer thus explaining the rise per residue obtained by bulk (ensemble-averaged) microscopy techniques.

Torque and its combination with longitudinal force have so far only been applied to DNA. These experiments have shown that, (v), Z-DNA and P-DNA can be induced by both torque and moderate tension, (vi), torque and low force leads to supercoiled condensation, (vii), S-DNA is induced at high force and favoured by low unwinding torque, and (viii), melted DNA can be induced by high force or moderate unwinding torque in the presence of other denaturing conditions like low polarity environments or A•T-rich sequences.

The intrinsic structures of dsRNA and dsDNA are determined by the sugar-puckering modes. The stability of the A and B forms in dsRNA and dsDNA, respectively, is thus compatible with former backbone- and base-centred interpretations but according to single-molecule experiments, the sole bistability of the base-stacking modes in DNA is not sufficient to promote a global A-DNA conformation stable in low humidity conditions. The mechanical properties of this polymer under these conditions, however, resemble those of dsRNA. Specifically, dsDNA in buffer-ethanol mixtures has lower stretch modulus, lower overstretching force and less cooperative overstretching transition than in aqueous buffer. The persistence length decreases with ethanol concentrations below that of the physiological range and the hysteresis energy between stretch and relax paths in the force-extension curves, which is related to the melting stability of the two strands, is higher than in aqueous buffer. DsRNA in physiological conditions exhibits reduced values of the stretch modulus and overstretching force, as well as a reduced cooperativity in the overstretching transition, with respect to equivalent dsDNA molecules in the same aqueous conditions. The persistence length is, however, higher and the hysteresis energy lower than in dsDNA; hence, dsRNA shows better melting stability in the presence of force than dsDNA.

These properties might have been of importance to favour the more extended use of dsDNA as an information carrier: the shorter persistence length for dsDNA with respect to dsRNA could be of selective advantage when dealing with ordered packaging, as required for chromosome condensation. Also, the handling of nucleic acid polymers by the enzymes responsible for their replication, repair and transcription would be less constrained by a polymer with a lower bending flexibility. The higher contrast between the elastic regimes exhibited by dsDNA might also be related to a more controllable and efficient response of this polymer when dealing with its processing mechanisms. The larger stretch modulus and overstretching force of dsDNA and the fact that this transition is sharper for this substrate indicate a higher resilience of its double-helix geometry relative to that of dsRNA. These features may have made the ancestral development of recognition mechanisms of nucleic-acid-binding proteins more advantageous for the double-helix geometry of DNA than for that of RNA in situations of mechanical stress.

In vivo regulation of DNA hydration may influence processes such as transcription, recognition and encapsulation. Most of these processes take place locally, at the level of a few base-pairs, due to the binding of the proteins; they reduce the exposure of the DNA surface to the available water molecules and, as a result, change the stability of the double-helix. Single-molecule experiments show that the mechanical stability of DNA decreases in the presence of water-ethanol mixtures, used in *in vitro* experiments as an analogue of more complex *in vivo* situations, thus giving rise to a higher DNA malleability. These experiments then support early conjectures on DNA stability changes in transcription and other DNA-binding processes,[3] although these changes may not strictly be tied to an A-DNA conformational role.

In contrast, it is established that many biological processes require the mechanical characterization of double-stranded nucleic acids as chiral structures.[8d, 112] Plasmid and genomic DNA in prokaryotes are often found slightly underwound. In eukaryotes, DNA packaging into chromosomes also proceeds via negative supercoiling.[19] DNA-binding molecular motors can be aided or have to deal with torsional constraints which involve energetic barriers for their activity. For example, RNA polymerase generates positive supercoiling ahead of the transcription complex and negative supercoiled domains behind, hence involving the additional action of another class of

enzymatic machines, the topoisomerases, which are involved in the relaxation of DNA entanglement. In this regard, single-molecule techniques have not only provided additional sources of structural information: dynamic information, elasticity and torque/force-induced transitions of nucleic acids constitute unparalleled information not accessible by ensemble methods or imaging techniques.

Perspectives

The study of nucleic acids from a single-molecule point of view integrates mechanical, chemical and structural aspects into the same body of knowledge, which is essential to understanding information storage and processing in biological systems.

To date, understanding of force-induced structural transitions and of both flexural and bending properties of RNA and DNA duplex has made possible the comparative discussion reported in this review. Moving forward, the investigation of torque-induced transitions and torsional properties, including twist-stretch coupling, for dsRNA will lead to a complete comparison and to an integrated knowledge of the physico-chemical principles underlying their different structures and roles as the information blocks of life.

Likewise, although the single-molecule approach was applied to DNA-binding proteins almost twenty years ago,[8c, 113] no RNA-binding molecular motor has been investigated from a mechanochemical perspective to date. The mechanical behaviour of dsRNA sets the basis for a deeper understanding of a number of processes performed by dsRNA-interacting proteins (genome processing enzymes in dsRNA viruses such as RNA-based RNA polymerases, dsRNA helicases and dsRNA topoisomerases), in particular in understanding the physical constraints they must face during their function. Likewise, they are important for the design of nanotechnological applications like those derived from short interfering RNAs (siRNAs), ribozymes, antisense RNAs, aptamers and riboswitches, among others.[17] A comprehensive knowledge of the hybridization and stacking thermodynamic stability of dsRNA under tension shows promise in better designing siRNAs with controlled specificity and potency for therapeutic applications.[114]

Information processing by molecular motors is intimately linked to the mechanical properties of their substrate.[8a, 115] In this regard, it is intriguing to pose to which extent the differences between dsRNA and dsDNA affect their respective molecular machinery. The structural identities under force and subsequent mechanochemical properties will be essential to interpret differences in stepping motion and in efficiency for their respective protein motors.

Other double-stranded structures are also awaiting to be studied at the single-molecule level, especially the DNA:RNA hybrid, which is known to adopt the A-form according to structural, bulk analyses.[6] DNA:RNA hybrids are generated during transcription and commonly used as mechanical handles to study ssRNA structures, like hairpins[116] and RNA G-quadruplexes.[117] It is expected that the RNA backbone constrains the hybrid structure to the A-form since it possesses a lower distance between phosphates ($C_{3'}$-endo sugar conformation) than the DNA backbone (Fig. 8). Likewise, it is expected that the hybrid structure does not switch to the B-form in the presence of force due to similar reasons as those explained for the dsRNA polymer (Fig. 7).

The rare Z form was long ago shown to exist in the absence of stress. Z-DNA and Z-RNA stabilities are, however, more strongly dependent on sequence, besides humidity conditions and polycation binding, than are other conformations. Although the B-Z transition for DNA has recently been tested in single-molecule experiments, it remains to elucidate whether this structure could exist at the single-molecule level in the absence of torque or whether it is a transient form, strengthened by interhelical interactions as in the case of A-DNA. The Z form is the left-handed version of the A form in RNA and of the B form in DNA. Single-molecule experiments could further distinguish whether structural differences between Z-RNA and Z-DNA are of the same quantitative extent as the differences between A-RNA and B-DNA to rationalize different nomenclatures for the right-handed forms of RNA and DNA.

Finally, there exist non-canonical nucleic acid topologies that correspond to strict, short sequences and that involve more than two strands, the best known being the (guanine-rich) G-quadruplex and (cytosine-rich) i-motif conformations. These are rapidly gaining attention in the Biophysics and Nanomedicine communities due to their therapeutic and nanotechnological interest, as reviewed in Yu and Mao.[118] Both bulk and single molecule approaches are recently joining efforts to study these conformations. Single-molecule studies have provided key information about their stability in force-unfolding experiments, showing an alternative, mechanical path to chemical affinity to probe their interaction with drugs and other modulation factors. The use of torsional single-molecule strategies to understand the control that these cellular components exert in biomolecular processes is promising. In particular, structural reports suggest that torque may also have a role in the chiral selectivity, recognition and processing by (passive) ligands and (active) motor proteins.

Acknowledgements

We are sincerely indebted to S. Hormeño, F. Moreno-Herrero, B. Ibarra, J.L. Carrascosa, J.M. Valpuesta, M. Fuentes-Pérez and C. Carrasco for work through the years. C. Flors and A. Villasante are acknowledged for critical revision. Work supported by Fundación IMDEA Nanociencia.

Notes and references

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† For long dsDNA molecules, i.e., those with a contour length much greater than their so-called *persistence length* (see next sections), condensation can be regarded as aggregation of one molecule around itself due to the polymer's bending elasticity.

‡ The contour length of a linear polymer, L, is the total chain length of the molecule, considering its curvature. For nucleic acids, it can be estimated from L = [number of base pairs] × [mean rise per base-pair].

Crystallographic distances between base-pairs, see Fig. 1, can be used for A and B forms to get their respective contour lengths, L_A and L_B.

|| This estimation should be taken with care since the so called *stretch modulus* (see next section) of the molecule in A and B forms could be different thus leading to different slopes in the intrinsic elasticity regime of the force-extension curves.

⊥ In a macroscopic system, devoid of thermal fluctuations or other natural noise, the standard deviation should converge to zero in the limit of many equivalent experiments because the distribution width only stems from experimental errors. The system is said to be deterministic.

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