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Properties of DNA in concentrated aqueous solutions of LiCl suggest transition to C-DNA

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ABSTRACT

C-DNA is the canonical double helix form related to B-DNA. It is obtained in air-dried DNA fibers prepared from various aqueous salts, most commonly LiCl. The possibility of its existence in solution is unclear. We study conformational changes induced in supercoiled circular DNA by increasing LiCl concentrations. The physical properties of supercoiling allowed us to quantify changes in helical twist directly from circular dichroism (CD) spectra and to track conformations of DNA minicircles using atomic force microscopy (AFM). Both CD and AFM provide strong qualitative evidence that DNA helical twist increases with increasing LiCl concentration without any sign of saturation. The maximum increase estimated for the concentration range up to 8 M LiCl corresponds to the onset of the B-to-C DNA transition, suggesting stability of C-DNA at concentrations slightly above this level.

INTRODUCTION

C-DNA (together with A- and B-DNA) represents one of the three canonical right-handed double-helical forms discovered in the 1950s by molecular modeling based on X-ray fiber diffraction and other experimental data (1–3). Although C-DNA features distinct diffraction patterns and helical parameters, its valence and torsion angles (unlike those of A-DNA) do not deviate far from common B-DNA values; thus, C-DNA has been considered as a deformed B-DNA or its close relative (2, 4, 5). Originally, C-DNA fulfilled an important role in the validation of the Watson-Crick model, based on the fact that reversible transformations between some diffraction patterns were accounted for by the transitions between the A, B, and C forms of the same DNA molecule with unperturbed topology of hydrogen bonding (6). Yet, while free A- and B-DNA have been found *in vitro* and *in vivo*, C-DNA was only described convincingly in fibers under low air humidity (2, 7), and its existence in aqueous solutions, as well as its biological significance, remained subject to debate.

Recently, the ability of DNA to undergo the B-to-C transition was suggested to play a role in recombination-independent recognition of DNA homology (8, 9). The existence of a molecular mechanism that allows two DNA double helices with homologous sequences to recognize each other without strand separation and cross-hybridization follows from genetic studies of the genome protection processes called RIP (repeat-induced point mutation) and MSUD (meiotic silencing by unpaired DNA) common in filamentous fungi *Neurospora crassa* (9, 10). Based on analysis of homology requirements of RIP and MSUD, it was suggested that the detection of sequence similarity could rely on direct pairing of dsDNA, without involving single-stranded DNA or RNA intermediates (9, 10). These observations are accounted for by an all-atom model in which homologous dsDNAs are assumed to pair by a series of major-groove contacts, with each contact corresponding to a stack of 3-4 planar quartets, and each quartet formed by two identical Watson-Crick base-pairs (11). The contacts have to be spaced with periodicities compatible with DNA structures, and the pairing is improved if the participating dsDNAs are coiled into a right-handed plectoneme (11). The experimental optimal inter-contact distance for RIP and MSUD suggests that the two double helices should be indeed folded into a right-handed plectoneme, with the conformations of the intervening unpaired segments shifted from B- to C-DNA (9).

The C-form may be particularly suitable for homologous dsDNA-dsDNA pairing for at least two reasons. First, the very shallow major groove of C-DNA (while being as wide as that of B-DNA) permits almost perfect initial quartet contacts without atom-atom clashing (8). Second, the helical repeat of approximately 9 bp allows a pair of C-DNA duplexes coiled into a right-handed

plectoneme to have their major grooves facing each other every 22 bp, supporting the high density of homologous contacts necessary for the detection of relatively short repeats (8, 10). In the light of these considerations, the B-to-C transition emerged as an attractive strategy to lower the energy barrier for the formation of quartet stacks during homologous dsDNA-dsDNA pairing.

Until the 1990s, C-DNA was a subject of active investigations. The purported B-to-C transitions were typically monitored by measuring the circular dichroism (CD) of DNA in the near-UV (180–320 nm) range (12–15). The reference CD spectra of B- and C-DNA were obtained by analyzing unoriented DNA films under the conditions similar to those used in X-ray fiber diffraction experiments (16). At high air humidity, those spectra were indeed congruent with the results obtained in solution (16). Using this approach, partial B-to-C-DNA transitions were found in different biological media (12, 13), protein complexes (17, 18), chromatin (14) as well as concentrated aqueous solutions of common salts (19, 20) and in the presence of dehydrating co-solvents (19, 21). However, these results were inconsistent with those of other methods (22–25), and the controversy could not be properly resolved at that time. This situation led to the conclusion that the original assignment of the reference CD spectra was incorrect, and that the conformation of DNA giving rise to the “C-form” spectrum represented “a minor variant of the B-form” (22, 23, 26, 27). However, the following three questions remained unanswered: What is the structure of the B-form variant producing the CD spectrum that was originally attributed to C-DNA? What is the true CD spectrum of C-DNA? And, ultimately, can the B-to-C transition occur under the conditions other than the ones provided by dry DNA fibers?

In this study, we have revisited the above problem using CD of supercoiled circular DNA as a sensor of helical twist. The CD method is generally sensitive to conformational transitions between the canonical DNA forms (28, 29). At least at moderate salt concentrations, the CD signal clearly correlates with DNA twisting (30). Although supercoiled DNA was always indispensable in solution measurements of DNA twisting, the CD method was applied almost exclusively to linear molecules. There has only been one systematic study of supercoiled DNA using CD spectroscopy (31) which did not specifically address the salt effects. It was shown that the CD spectra of circular DNA vary smoothly with the degree of supercoiling, which offered a fundamental possibility to probe helical twist directly (32). We also use flat 178 bp DNA minicircles that are very rigid and resist changes of twist in low salt conditions (33). With increasing ion strength they should progressively become more flexible and this can be monitored using CD as well as AFM. Due to the simple fixed shape and small size of these minicircles, the onset of supercoiling or other structural changes associated with DNA twisting can be directly confirmed or refuted using AFM.

We probe the properties of supercoiled DNA in concentrated aqueous solutions of LiCl, which is most interesting for three reasons. First, although Li^+ is not biologically relevant, it is the only simple cation that promotes the B-to-C transition while attenuating the B-to-A transition in both fibers and solution (25). Second, aqueous solutions of up to 16M LiCl remain stable, the property that allows the water activity to be reduced to 11% (34), which covers the entire range of air humidity used in X-ray fiber diffraction experiments. Third, concentrated LiCl solutions can still be analyzed by near-UV CD spectroscopy. Helical twist is the main measurable parameter distinguishing B-DNA from C-DNA, so information about its value at low water activity, i.e., very high concentrations of LiCl, is critical for drawing conclusions about the possibility of a B-to-C transition in solution. We are exploring the theoretical possibility of extracting such information directly from CD spectra of supercoiled DNA. Any structural interpretation of CD data must assume that similar CD spectra of the same polymer correspond to similar average secondary structures. Based on this principle alone, without more specific assumptions, we were able to evaluate the changes of the helical twist of circular DNA by measuring the salt-dependent difference between the CD spectra of two topoisomers with different superhelical densities. The twist value increases steadily in the concentration range from 1M to 8M, with the overall increase relative to normal salt conditions reaching $1.6 \pm 0.15^\circ$, which in DNA fibers would result in a change in the helical pitch from the canonical 10.0 to 9.6 pb/turn. According to previous X-ray fiber diffraction experiments, the latter value can be detected in both B and C conformations of DNA under conditions corresponding to the onset of the B-to-C transition (35).

RESULTS AND DISCUSSION

Supercoiling strain impedes the high-salt transition in DNA detected by CD

Figures 1A–D show the preparation of DNA constructs and explain the abbreviations used in the subsequent text and figures. Capital letters P and M refer to mother plasmid pEAG270A (2877 bp) and the 178 bp minicircle, respectively. Subscripts S, C, O and L, respectively, refer to the negatively supercoiled (native), closed circular, open circular (nicked), and linear forms of the corresponding DNA.

The near-UV (220–400 nm) CD spectra of DNA constructs sketched in Fig. 1B were measured in aqueous LiCl solution for concentrations ranging from 0.1M to 8M. Figure 1E shows the results for the open circular (P_O) and linear (P_L) forms of the plasmid DNA. The collapse and inversion of the

intensity of the broad band at 275 nm is a characteristic pattern originally attributed to the B-to-C transition (16, 19, 36) . The accompanying spectral changes at shorter wavelengths are not monotonous and probably reflect other factors. At every LiCl concentration of this range, the CD spectra of the open circular plasmid and its linear form are superimposed within the errors (37) .

Figure 1F compares the same CD spectra of P_O with those of closed circular forms P_C and P_S . In 100 mM LiCl all of them have typical B-DNA shapes with different positive intensity of the 275 nm band which changes as $P_O < P_C < P_S$. This ranking reflects conformational changes in DNA caused by supercoiling stress because its magnitude is proportional to the superhelical density (31) while all three constructs are topological isomers of the same circular DNA. This effect has long been known (37) , but poorly understood, including environmental influences that have never been tested. Increased LiCl concentrations affect these three spectra similarly to Fig. 1E, with smaller changes in amplitude of the 275 nm CD band for covalently closed supercoiled topoisomers. As a result, in 8M LiCl the spectra are arranged in the same order as in 0.1M LiCl, with 275 nm bands inverted and more separated than at the beginning. Interestingly, the lower gap between the spectra of P_O and P_C has increased stronger than the upper gap between P_S and P_C which mainly expanded horizontally.

The CD spectra of 178 bp minicircles M_O and M_C measured in similar conditions are shown in Fig. 2A. In this series, the linearized plasmid P_L was used as a reference since we could not afford systematic linearization of minicircle DNA for comparisons. The minicircle and plasmid sequences are quasi-random and statistically similar. In additional experiments we found that their CD spectra are almost identical at 0.1M LiCl and sufficiently close at all higher concentrations. Fig. 2A indicates that in 0.1M LiCl the M_O and M_C spectra are similar to each other as well as the P_L spectrum. With the helical repeat of 10.5 bp/turn, the DNA length of 178 bp corresponds to exactly 17 turns. Therefore, closed minicircles M_C are not torsionally strained and the difference from M_O should be negligible. DNA in minicircles is constantly bent along its entire length, which apparently does not affect at least this segment of CD spectra despite the obvious difference of 3D structures from linear DNA. These considerations explained the results for 0.1M LiCl, but the changes observed with increasing LiCl concentration were surprising.

Under low salt conditions, closed 178 bp minicircles are flat rigid rings restrained by strong interhelical electrostatic repulsion (33) . They can neither twist nor bend and the CD spectrum of M_C should be almost constant until the LiCl concentration increases. In fact, the M_C spectrum remains constant only at concentrations below 0.1M (see Fig. S1), but it noticeably changes already

between 0.1M and 0.5M. In contrast, DNA in open minicircles can twist even in rigid flat rings and the spectrum of M_O was expected to behave similarly to the P_O spectrum in Fig. 1E, i.e., remain close to that of P_L . However, Fig. 2A shows the opposite, namely, the CD spectrum of M_O diverges from the spectrum of linear DNA and always remains close to the spectrum of M_C . Up to LiCl concentration of 8 M there are hardly any significant difference in the CD spectra of M_O and M_C and only in 8M LiCl the M_O spectrum deviates towards that of linear DNA. According to the common knowledge, a single-strand break in a covalently closed circular DNA should remove all supercoiling constraints and transform its CD spectrum to that of linear DNA (37). This is confirmed for P_L and P_O in Fig. 1E, but Fig. 2A suggests that the strand break in M_O did not remove the ring closure constraint. Note also that although the effect of increasing LiCl concentration on the CD spectra of M_C is qualitatively similar to that for P_C , it is much smaller. The positive intensity of the 275 nm band in the minicircle spectra is not inverted even in 8M LiCl.

Minicircles are particularly useful for this study because they are suitable for AFM imaging. Unlike larger DNA circles, they are not a mixture of topoisomers but are conformationally homogeneous. Their original ideal shape should deteriorate with increased LiCl concentrations, which can be visualized using AFM. It was previously shown that nickel treated mica can effectively adsorb DNA from monovalent salt solutions directly, without the addition of other ions (38, 39). We found that this approach can be adapted for DNA in LiCl solutions with concentrations above 2M. With available resolution we could distinguish the main types of structures present and qualitatively estimate the evolution of the ensemble of minicircle conformations at high LiCl concentrations.

According to Fig. 2B, in 4 M LiCl, 75% of minicircles are still ring-shaped and seen on AFM images as roughly circular spots with a central hole. Another characteristic highly populated structure is an elongated straight conformation with the length and thickness corresponding to a single minicircle collapsed into a straight rod along a diameter. In 6M LiCl, the proportion of rings is reduced to 0.49 and collapsed structures already begin to predominate. In 8M LiCl, the percentage of rods appears to have increased further, although simple population count is no longer possible due to the presence aggregates of different sizes. Each of the scans of Fig. 2B also contains amorphous structures without holes which may correspond to tilted deformed rings. Since the LiCl concentration in the deposited solution was the only varied parameter we can reasonably assume that these scans reflect differences between solution ensembles of DNA conformations. Repeated scans of the same samples a few days later showed similar mixtures of structures with gradually growing aggregates in 8 M LiCl. Consequently, transitions between ring and rod-shaped structures

are likely reversible and their mixture is in equilibrium. Rod-like structures were also visible in 2 M LiCl in closed and open minicircle samples, but the AFM images were much less clear.

Overall, the results shown in Figs. 1E-2A indicate that in closed DNA loops the conformational changes caused by increasing LiCl concentration are qualitatively similar to that in linear DNA. These changes are markedly impeded by the ring closure constraints and negative supercoiling, consistent with increased DNA helix twisting as a major effect of increasing LiCl concentration. This increase is continuous up to a maximum at 8 M LiCl and probably beyond.

Structural interpretation of CD spectra

The characteristic CD spectrum of DNA in the near-UV range is produced by electrons of non-chiral nucleobases under the influence of two external conditions: (1) the chemical chirality of the proximal deoxyribose rings and (2) the overall helicity of the structure (40). The first factor is considered negligible due to the relatively large distances to the chiral sugar atoms, and a very large number of closer atom-atom contacts in the base-pair stacks. Indeed, reasonably good qualitative and sometimes quantitative agreement with experiment is achieved by theoretical predictions of CD-spectra that take into account only base-base interactions (41). The helical chirality emerges due to base-pair twisting which is the key element of this theory (42). It is natural, therefore, that the influence of supercoiling and solution conditions on CD spectra was initially attributed to altered twist angles (19, 37). This simple interpretation later became increasingly questionable as new data accumulated. It was argued that due to a comparatively low intensity of the 275 nm CD band it may involve a non-negligible contribution from interactions with proximal sugars, consequently, it cannot be used as a quantitative reporter of the base-pair twisting and/or an evidence of the C-DNA structure (22, 43). We agree with this physical argument, but not with the conclusion drawn.

We assume that the intensity of the 275 nm band in the CD spectrum of DNA is directly or indirectly affected by many factors, in particular, transitions between the backbone conformers BI and BII which invert the orientation of the phosphate groups and modulate the helical electrostatic field at the stacked bases (5). The low relative intensity of the 275 nm CD band does indicate that such factors are significant, and they still cannot be taken into account in the calculations of CD spectra (44). However, in many cases there are strong reasons to believe that, in the first approximation, all of these interactions, and with them the CD signal, change in concert and in proportion to the twist angle. For supercoiling this follows from the earlier established experimental

dependence of CD spectra of circular DNA on the magnitude and sign of the superhelical density. Indeed, the effects of negative and positive supercoiling on the intensity of the 275 nm CD band have opposite signs and their magnitude varies linearly over a wide range around the zero superhelical density (31). Below we show that our present data suggest that this is also true for changes in CD spectra caused by variations in LiCl concentration.

Closed loops of long double stranded DNA are well described by a simple topological model using three parameters, namely, the linking number (Lk), twist (Tw) and writhe (Wr) related by equation (45, 46)

$$Lk = Wr + Tw. \quad (1)$$

Twist and writhe are real values and Lk is an integer defined as the number of times the two strands are intertwined. It is a topological invariant changed by integer increments when one of the strands is broken and rejoined after rotation around the opposite strand. In contrast, parameters Tw and Wr are real numbers that change with the shape of the loop; they are mutually balanced and are in thermodynamic equilibrium. Mixtures of plasmid topoisomers, such as samples P_C and P_S produced by topoisomerases or isolated from cells, are characterized by averages $\langle Lk \rangle$, $\langle Wr \rangle$, and $\langle Tw \rangle$, all of which are real numbers also related by Eq. (1). Since we are dealing only with such averages the angular brackets will be omitted.

A closed circular DNA relaxed by a topoisomerase has $Wr = 0$ and $Lk = Tw_o = Lk_o$, where Tw_o is the twist in linear DNA and in open DNA circles. Note that according to this definition Lk_o is a real number that changes continuously. In the harmonic approximation, the minimum energy conformation of open circles is a planar ring ($Wr = 0$) free from twisting strain ($Tw = Tw_o$) and with the minimum bending strain required for loop closure. According to Eq. (1), with $Lk < Lk_o$, DNA in flat circles is unwound and torsionally stressed ($Wr = 0, Tw < Tw_o$), while without twisting ($Tw = Tw_o, Wr < 0$) it is supercoiled, i.e., additionally bent. The supercoiling stress can be relieved by balancing the bending and twisting strains to obtain a global energy minimum with $Wr < 0$ and $Tw < Tw_o$. In the general case the excess or deficit in the linking number is partitioned between a non-zero writhe and a shift of the twist from that in linear DNA:

$$\Delta Lk = Lk - Lk_o = Wr + \Delta Tw. \quad (2)$$

This partitioning was previously studied by analysis of electron microscopy (EM) images of supercoiled plasmids and also by Monte-Carlo (MC) simulations. According to EM data, the proportions of the linking number deficit compensated by writhing and twisting are nearly constant: $qWr = Wr / \Delta Lk \approx 0.72$ and $qTw = 1 - qWr = 0.28$, respectively (47), which was checked for the

natural range of superhelical density $\sigma = \Delta Lk / Lk_0$. The MC simulations predicted an increase of qWr from 0.72 at $\sigma \approx 0$ to 0.8 at $\sigma \approx 0.06$ (48). Accordingly, the twist of supercoiled topoisomers is

$$Tw_S = Lk_0 + qTw(Lk_S - Lk_0) = qWr \cdot Lk_0 + qTw \cdot Lk_S, \quad (3)$$

and the twist difference between topoisomers P_C and P_S is:

$$\Delta Tw_{CS} = Tw_C - Tw_S = qTw(Lk_C - Lk_S). \quad (4)$$

In the above equations, all variables are measured in helical turns. Twisting is also measured by the average angle between adjacent base pairs, that is, $\delta Tw = \Delta Tw \cdot 360^\circ / N$ where N is the length of the DNA double helix in base pairs.

Eq. (3) indicates that when the twist of linear DNA is increased due to temperature or salt effects, it is also increased in supercoiled DNA, but to a lesser extent because of the concomitant increase of the superhelical density. In the first approximation, these effects are additive and Eq. (4) states that in our experiments the twist difference between topoisomers P_S and P_C should not vary with LiCl concentration. It follows that the initial decrease of twist in P_S caused by torsional stress in 100 mM LiCl may be compensated, and at some higher LiCl concentration it will become similar that of relaxed topoisomer P_C in 100 mM LiCl (see Fig. 1F). Note that the corresponding 3D structure of P_S will not at all resemble a flat circle of relaxed plasmid P_C . This supercoiled conformation would be adopted by P_S in 100 mM LiCl if the torsional rigidity of DNA were much larger ($qTw \approx 0$). However, if the CD spectrum of DNA is determined by the helical twist, the corresponding spectrum of P_S should be similar to the spectrum of P_C in 100 mM LiCl. This reasoning is valid also for other LiCl concentrations and other topoisomer pairs like P_S-P_O , P_C-P_O , etc. In other words, the theory predicts that, with increased LiCl concentration, the CD spectra of all supercoiled topoisomers of a plasmid DNA follow along the same pathway behind the spectra of P_O and P_L , and pass through similar configurations at different salt concentrations.

Figure 3A shows that such behavior is indeed observed. In Fig. 1F, the intensity of the 275 nm band decreases with increasing LiCl concentration and since the P_C and P_S plots stay a little apart, every P_C plot is bounded from above and below by two P_S plots for consecutive LiCl concentrations. This is shown in the left panel of Fig. 3A where the P_C spectrum in 0.1 M LiCl is bounded by the P_S spectra in 0.1 and 0.5 M. Between these boundaries the P_S spectra change smoothly and we can find the LiCl concentration at which the P_S spectrum best matches the bounded P_C spectrum.

In the middle panel of Fig. 3A, the result of prediction obtained by linear interpolation between the bounding P_S spectra is compared with the target P_C spectrum as well as the verification P_S spectrum measured at the predicted LiCl concentration (0.375 M). The three spectra match within the errors, which means that in closed circular DNA, the change of the CD spectrum due to reduced linking number can be compensated by an increase in LiCl concentration, and *vice versa*. Indeed, the changes obtained in 0.1 M LiCl by reducing the linking number of topoisomer P_C to that of P_S are compensated by raising the LiCl concentration to 0.375 M. The linking number, the twist, and the CD spectrum are associated and change concertedly. The CD signal can be used as a reporter of these changes even if its immediate sources are not clear.

We can conclude that the increase in twist upon the transfer of P_S from 0.1 M to 0.375 M LiCl is equal to the twist difference between P_S and P_C in 0.1 M LiCl. This conclusion requires only one assumption, namely, that similar CD spectra of this circular DNA correspond to similar conformations around the π -electrons of stacked bases. The right panel of Fig. 3A shows that the same properties are also inherent in the pair of topoisomers P_S and P_O . Here the CD spectrum of P_S in 0.5M LiCl and that of P_O in 0.1M LiCl are almost identical even without refinement of concentrations. This lucky coincidence proves that the CD spectra of P_S and P_O behave similarly to the P_S and P_C spectra in Fig. 1F, and for each P_S spectrum one can find an equivalent P_O spectrum at a smaller LiCl concentration. The twist of DNA in P_O is similar to that of P_L (See Fig. 1E), therefore, one can use the literature twist value for linear DNA in 0.1M LiCl as an estimate for topoisomer P_S in 0.5M LiCl and further refine this concentration if a better estimate is necessary. This approach is generally applicable to topoisomers of the same DNA and we use it below to roughly estimate the effect of LiCl on DNA twisting over the entire range of concentrations.

Figure 3B compares the effect of LiCl on the maximum intensity of the 275 nm CD band for DNA constructs P_S , P_C and P_O . It was averaged over the minimum required interval covering the positions of the maximum in all spectra. The P_S and P_C traces are vertically shifted already in low salt. This shift decreases at LiCl concentrations up to 1M, passes through a broad minimum and then increases again. The trace of P_O in low salt is close to that of P_C and gradually separates downwards with increasing LiCl concentration. This behavior is qualitatively consistent with the above prediction of Eqs. (3) and (4), as well as the supposed relationship between changes in the CD signal and the superhelical density. At the same time, the shift between the P_S and P_C traces varies slowly with increasing LiCl concentration suggesting that these topoisomers are involved in dissimilar structural changes that influence the twist/writhe balance.

Fig. 3B provides a tempting way to roughly estimate the magnitude of the increase in DNA twist due to increasing LiCl concentration across the entire range. Based on Eq. (4) and using the approach shown in Fig. 3A we can continue to move towards higher LiCl concentrations by alternating vertical and horizontal steps between the P_S and P_C plots in Fig. 3B. Horizontal steps correspond to finding the next LiCl concentration at which the P_S spectrum best matches the P_C spectrum at the previous LiCl concentration. Vertical steps correspond to measuring the corresponding next P_C spectrum. This algorithm will produce a descending stepwise line plot, with the vertices approximately on P_S and P_C plots and every step corresponding to a twist increment of ΔTw_{CS} . The total twist increase depends only on the total number of steps, and a first-approximation estimate can be obtained rapidly from a similar stepwise line plot with vertices on the P_S and P_C traces without an exact match of the corresponding CD spectra in the vertices.

In principle, parameter ΔTw_{CS} can be obtained directly from Eq. (4) using the known value of qTw and the linking number difference $Lk_C - Lk_S$ measured independently by the gel band counting method (49). The linking number of open circles is $Lk_O = N/10.5 = 274$ where N is the plasmid length (2877 bp). In the gel buffer (40 mM Tris-acetate), the corresponding averages for topoisomer distributions of P_S and P_C were shifted from Lk_O by $\Delta Lk_C = -0.5 \pm 0.2$ and $\Delta Lk_S = -16.8 \pm 0.3$, respectively. Since the LiCl concentration does not affect Lk_C and Lk_S , Eq. (4) gives $\Delta Tw_{CS} \approx 4.6$ and the corresponding angular increment per base-pair $\delta Tw_{CS} = \Delta Tw_{CS} \cdot 360/n = 0.57^\circ$. As shown in the previous section, ΔTw_{CS} is equal to ΔTw_S due to the increase of the LiCl concentration from 0.1 M to 0.375 M. The corresponding increase for linear DNA is found using Eq. (3) as

$$\Delta Tw_O = \Delta Lk_O = \Delta Tw_{CS} / qWr, \quad (5)$$

which gives approximately 6.4 of helical turns and $\delta Tw_O = 0.80^\circ$ in terms of base-pair twisting. The last value cannot be correct as it is at least five times higher than previous consistent estimates obtained by different methods (50, 51). This large discrepancy has two plausible, non-mutually exclusive explanations. The qTw value in our experiments may be somewhat lower than the previously estimated range of 0.20–0.28 due to specific salt conditions and because the coarse-grained models do not account for the distinction between twisting and writhing for short DNA segments. We discuss this issue further in the Supplementary Data. Also, in our salt conditions the topoisomer P_S may collapse into a tightly interwound plectonemic form (33, 52–54). For native superhelical density such transitions occur at ionic strengths below 1M (54) and they should be accompanied by a strong increase of the negative writhe with a corresponding positive increase of twist (33). Indeed, the vertical shift between the P_S and P_C plots in Fig. 3B is significantly reduced

at LiCl concentrations of 0.1M to 1M because the CD intensity decreases faster for P_S , which corresponds to faster growth of twist.

For our present purposes the above uncertainty is not critical. At concentrations above 1M, the P_S and P_C plots become more parallel, and further changes in twist can be approximated by continuing to treat δTw_{CS} as a constant and using a different, more reasonable estimate for it, which can be obtained from the literature data for linear DNA. To this end, we predicted for all P_C and P_S spectra the LiCl concentrations of good matches with P_O and then estimated the corresponding values of δTw_{CS} with a literature three-parameter power-law expression fit to direct experimental measurements (51). The resulting plot shown in Fig. 3C reveals a significant decrease of δTw_{CS} between 0.5M and 1M LiCl with a qualitative correlation with fluctuations of the vertical shift between the P_C and P_S traces in Fig. 3B. Although the points in Fig. 3C at 2M and 3M represent extrapolation beyond the range of concentrations studied experimentally (51) we assumed that from 1M to 8M LiCl the δTw_{CS} value stays between 0.07° and 0.09° which corresponds to $qTw \approx 0.04$.

Fig. 3D shows predictions of the DNA helical twist at high LiCl concentrations based on CD data. A stepwise line plot was constructed as explained above between traces P_C and P_S in Fig. 3B and scaled with δTw_{CS} values of 0.07° , 0.08° and 0.09° , respectively. This gave three plots of increasing twist with LiCl concentration that were then shifted vertically to match the experimental value at 1M (51). In Fig. 3D these dependences are compared with the power law (51) extrapolated beyond 1M. All plots smoothly continue the experimental data for lower concentrations, and with $\delta Tw_{CS} = 0.8^\circ$ the CD-based graph is surprisingly close to the power-law extrapolation. This agreement indicates that the intensity of the 275 nm band in CD spectra of DNA changes with LiCl concentration in qualitative agreement with the coarse-grained model of supercoiled DNA and the assumed relation with the helical twist. The predicted increase of twist is around 1.1° for the concentration range from 1 M to 7.5 M and $1.6 \pm 0.15^\circ$ for the total increase relative to the reference conditions in 50 mM KCl (51) Although this analysis gives only rough estimates, the concerted behavior of CD spectra of supercoiled topoisomers suggests that the helical twist of DNA steadily increases with LiCl concentrations between 100 mM and 8 M without reaching a plateau.

Twist changes in minicircles

Taking into account the conclusions of the previous section, a consistent interpretation of the spectra shown in Fig. 2A is that the DNA twisting in minicircles also increases, but less than in large circles and linear DNA. With some caveats, the approach of the previous section can also be used to roughly quantify this increase. Using linear interpolation between P_O spectra for consecutive LiCl concentrations, we predicted for all M_C spectra the closest P_O spectra and the LiCl concentrations at which they would be obtained. From these, we then estimated the corresponding twist values using the empirical power law formula for linear DNA (51). The quality of the fit can be assessed from Fig. S2, which compares the best-fit computed P_O spectra with the corresponding M_C spectra. In 0.5M and 1M LiCl, the agreement between them is as good as for 0.1M in Fig. 2A, suggesting that between 0.1M and 1M the spectra of minicircles follow the same path as the P_O spectra. A slight discrepancy in spectrum shapes appears in 2M LiCl (Fig. S2) and then gradually increases with salt concentration. This pattern indicates that some qualitative changes begin to develop in minicircle structures at LiCl concentrations above 1M, which can be interpreted using the AFM data in Fig. 2B. At LiCl concentrations below 1M the minicircle DNA at short length scales is a regular double helix indistinguishable by CD from linear DNA at some other LiCl concentration. In collapsed structures, DNA may be deformed near the terminal loops, which in minicircles have significant statistical weight, and also at sites of close DNA-DNA contacts. The discrepancy in spectrum shapes in Fig. S2 at LiCl concentration above 1M probably reflects the increasing proportion of collapsed structures in Fig. 2B.

To measure the writhe we also need the point of zero linking number deficit (ΔLk), that is, the LiCl concentration at which the helical twist is the same in minicircles and linear DNA. In our case, the best approximation is 0.1M LiCl where the corresponding CD spectra are almost identical (Fig. 2A). In 10mM LiCl (Fig. S1) they diverge in the direction opposite to Fig. 2A, indicating that the twist of linear DNA is smaller than in minicircles, so the ΔLk value has changed the sign and become positive. The CD spectra of minicircles do not differ in 10mM and 0.1M LiCl (Fig. S1), that is, the twist did not change, the writhe is close to zero at both concentrations, and the newly emerged positive ΔLk was completely compensated by the positive twist difference from linear DNA, which is consistent with previous findings (33).

The estimated twist of minicircles depends on LiCl concentration as shown in Fig. 3E. Between 0.1M and 0.5M it increases similarly in M_C and the larger circular plasmid P_C (2877 bp). The increase almost stops in M_C between 0.5 and 1M, causing the two plots to diverge, but resumes after

1M simultaneously with the appearance of the discrepancy in the shapes of CD spectra in Fig. S2. This renewed growth occurs at an increasing rate, indicating that it is driven by a mechanism different from that in P_C and linear DNA. According to Eq. (2), the corresponding negative writhe equals the twist increase relative to the zero ΔLk state. Measured in helix turns, its value is about 0.07 in 2M LiCl and the maximum at 8M is about 0.6. These observations are also consistent with the AFM data. As already mentioned, the collapse of minicircles is likely reversible. It probably begins at high salt concentrations when the electrostatic repulsion decreases and allows juxtaposed DNA segments to approach each other. At small distances ion bridges begin to form, which inverts the repulsion and prompts a transition to a tightly interwound plectonemic conformation. At salt concentration above 1M, such strong fluctuations can apparently occur with small negative writhe, but the transition should result in a stepwise increase accompanied by a compensating increase in twist (33). The twist plot in Fig. 3E reflects the change in the proportions of rings and rods as well as the direct salt effect.

Quasi-identity of the CD spectra of M_C and M_O in Fig. 2A indicates that the stacking energy is high and conformations with small twist changes due to unstacked nicks are rare in M_O . In large circles with $Wr \approx -0.5$, transitions to conformations with $\Delta Lk = +1$, increased twist and positive writhe would be possible, but here in tight plectonemes they are prevented by ionic bridges that must be broken on the way through conformations with zero writhe. The described behavior of supercoiled 178 bp minicircles significantly differs from the previous detailed CryoEM study (33). This is certainly due to much higher ionic strengths and transitions to the tightly interwound plectonemes.

Contrasting views on the structure of DNA in concentrated ionic solutions

Previous conclusions rejecting CD data on significant changes in DNA structure in concentrated simple salts, including the formation of the C-form, followed from results of several studies (22, 23, 35, 50). The most important objection came from X-ray diffraction experiments with swollen fibers (35). In this method, a DNA fiber is immersed in a solvent and allowed to swell in a narrow capillary with sealed ends. Comparisons of these conditions with solution involves important assumptions, of which we will discuss one that had previously escaped attention. For some reason, ions of concentrated salts in these studies were considered to affect DNA only directly and not via water activity (a_w). Water spontaneously diffuses into areas where a_w is lower and swelling indicates that it is increased in the fiber. Therefore, transitions from B-DNA conformation to other forms cannot occur during or after swelling. The swelling can only preserve the original structure of B-DNA or lead to transitions to this conformation, which has been observed in all such experiments

without exception (35, 55, 56) . The B-DNA X-ray diffraction patterns were obtained even for swelling in 6.2M and 8M LiCl corresponding to a_w of 0.60 and 0.43, respectively (34) . The latter result differs from the X-ray data for dry Li-DNA fibers and cast doubts on the reality of salt effects on DNA twist in solution, however, it has a simple alternative explanation.

Fiber wetting in multi-component solvents like electrolytes is not as simple as in pure water. It may be accompanied by significant diffusion flows of ions and water and result in noticeable changes of the original solvent composition. With fibers prepared from 1 mM salt solutions (56) , the maximum salt concentration obtained after swelling in pure water was only 0.1M (55) . In experiments with concentrated LiCl such fibers certainly absorbed ions and could noticeably reduce the LiCl concentration in the surrounding solution. The magnitude of this effect can be partially estimated from published data. The initial volume of solvent was at least 20 times larger than that of the fiber, but the fiber volume significantly increases during swelling (56) and the final difference should be several times smaller. Taking into account the unknown ion capacity of fibers and possible boundary effects of the capillary on all chemical potentials, such experiments can be compared with solution only qualitatively. To control the water activity near DNA, the sample must be equilibrated with bulk electrolyte, which is impossible in sealed capillaries.

The same X-ray approach was used to test another example of a CD-detected C-DNA structure (23) . Previously it was found that formaldehyde cross-linking of exocyclic amino groups of bases to various of simple amines changed CD spectra above 260 nm similarly to concentrated electrolyte solutions (57) . The similarity was not exact since below 260 nm the spectra of modified DNA gradually diverged from the results in electrolytes. The X-ray verification showed that regardless of the CD spectrum, this DNA remained in a B-DNA conformation with nearly canonical helical twist (23) . These results do not contradict our data; on the contrary, they support the proposed direct relationship between the amplitude of the 275 nm CD band and the proportion of BII conformers in DNA. Although the attached positively charged amines could replace salt cations, some unexplained observations suggest that these modifications also affected the BI/BII balance. For instance, in otherwise similar conditions, the effect of crosslinking on CD spectra was smaller in sonicated DNA samples (57) , likely because double helical products of sonification contained many single-strand breaks that do not participate in BI-BII dynamics. This dynamics should influence electrons in stacked bases stronger than attached amines since the phosphate groups are closer and are not screened from bases by intermediate water layers.

The BI-BII transitions can occur without changes of the global 3D structure, since the induced increase of base-pair twisting can be compensated by a similar decrease at an adjacent base-pair step. This gives rotation of a single base pair in a stack and takes place even in DNA crystals (58) . The strange increase of positive CD intensity observed below 260 nm (57) can be due to this compensation effect in modified DNA. In air-dried DNA fibers, persistent BII conformers should block B-to-A transitions, which explains a striking observation of C-DNA diffraction pattern for NaDNA fibers of this DNA in conditions where only A-form is generally observed (23) . When the average helical twist is increased by hydration forces or external torques the increased proportion of BII conformers is an indicator of global structural changes, but this does not mean that the same global changes can be induced by chemical modifications that stabilize BII conformers. Other relevant previous results are discussed in the Supplementary Data.

The true C-DNA should be observed at LiCl concentrated above 8M

In the Introduction we asked what is the structure of “a minor variant of the B-form” responsible for the characteristic CD spectrum originally attributed to C-DNA? This CD spectrum was measured for an unoriented DNA film at 75% air humidity (16) , and it is very similar to those in Fig. 1E for water activity $a_w \approx 0.75$. With a simple visual inspection it is obvious that if this spectrum were shown in Fig. 1E, it would fall between LiCl concentrations of 4M and 6M at which a_w is about 0.79 and 0.61, respectively (34) . The value $a_w \approx 0.75$ is reached in 4.5M LiCl and the corresponding spectrum predicted by linear interpolation similarly to Fig. 3A gives a very reasonable match with the previous CD standard for the C-form (16) (Fig. S3) . This result supports the early view that at high salt concentrations, DNA secondary structure and CD spectra strongly depend on water activity as well as on direct DNA-ion interactions (20, 59, 60) .

The steady decrease of the intensity of the 275 nm CD band is probably caused by backbone transitions from BI to BII conformation (61) rather than directly by base-pair orientations. The BII conformations are associated with DNA flexibility and high helical twist (62) . The BI/BII transitions involve rotations of charged groups placed along a 3D spiral trace around stacked bases, which modulates long-range chiral electrostatic field around electrons and should concertedly affect a number of electron transitions involved in the integral CD signal. This effect was not previously considered because originally only BI conformers were used for building models of the DNA double helix and only a handful of instances BII conformations were found in refined X-ray structures. For natural base-pair sequences, the BII conformers are absent in A-DNA, reach nearly 50% in C-DNA, and vary between these two limits in B-DNA (5) . The BI/BII transition is one of

the possible responses to external forces that can change the shape of long DNA. Due to the relatively high frequency of these transitions in natural DNA and the spiral chirality of the backbone conformation, the BI/BII dynamics serendipitously emerged as a suitable reporter for CD spectroscopy. This interpretation explains previous controversies, notably, the contrasting behavior of the 275 nm CD band and neighboring large peaks at shorter wavelengths (27) , perplexing sequence effects (63) , and other data.

In air-dried fibers, C-DNA conformations begin to appear at humidity of 75-79% and they have the helical twist of 37.1-37.5°, that is only 1.1-1.5° higher than in canonical B-DNA (2, 35) . As shown here, the transition pathway from typical B-DNA structure to that at $a_w \approx 0.75$ can be divided into a series of steps with structural changes similar to those in supercoiled circular DNA due to variation of superhelical density. This pathway is accompanied by a 1.2° integral increase of helical twist (Fig. 3D), therefore, the reference CD spectrum of C-DNA (16) corresponds to an intermediate on the B-to-C pathway rather than the “true C-form”. This intermediate is close to the upper boundary of water activity at which the C-DNA may exist. The lower limit of water activity at which B-DNA diffraction patterns of LiDNA cease to appear occurs at air humidity of 66% (64) , which means that there is a transition range of water activity between $a_w \approx 0.75$ and 0.66 and the “true C-form” should be found at lower a_w values.

The environment of DNA in solution is physically very different because the fiber packing prevents DNA bending and essentially selects extended approximately straight DNA conformations, which in solution have negligible entropy. These geometrical restrictions apparently favor a higher twist of the B-form and facilitate the B-to-C transition in fibers (65, 66) . If so, the onset of the B-to-C transitions in solution should be shifted from the transition zone in fibers to lower water activity, i.e., above the range of LiCl concentrations from 4.5 to 5.5 M corresponding to a_w from 0.75 to 0.66 (34) . In saturated LiCl, $a_w \approx 0.11$ (34) which is significantly lower than the water activity in true C-form fibers, and a true B-to-C transition is likely achievable in aqueous LiCl at concentrations above 5.5 M. The corresponding CD spectrum should continue to evolve towards stronger negative intensities of the 275 nm CD band below the minimum in Fig. 1E.

DECLARATION OF INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

A.K.M. & E.G. conceived and designed the study; A.K.M developed the theoretical framework;

M.M. & E.G. performed experiments; A.K.M. & E.G. analysed the results and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. CD spectra of supercoiled circular DNA constructs in aqueous LiCl solutions.

(A) Obtaining minicircles from the mother supercoiled plasmid. The minicircles were excised with Cre recombinase and purified on Superose 6. The DNA contours are not shown to scale.

(B) Designations of DNA constructs used in the text with sketches of the topology. The Contours are not shown to scale.

(C) A chloroquine agarose gel of plasmid constructs. Pl, Pc, and Po were prepared from Ps by enzymatic treatment with ScaI, Nt.BspQI, and vaccinia Top1, respectively, and purified by agarose gel electrophoresis followed by electroelution.

(D) A chloroquine agarose gel of minicircle constructs. Mo was prepared from Mc by enzymatic treatment with Nt.BsmAI and purified by phenol-chloroform extraction.

(E) CD spectra of linear plasmid DNA and its open circles in LiCl solutions. The LiCl concentrations are indicated above the plots.

(F) CD spectra of three circular constructs of plasmid DNA in LiCl solutions. The LiCl concentrations are indicated in the boxes. The relative average linking numbers measured in low salt conditions by the gel band counting method (49) were approximately 0, -0.5 and -16.8 for P_O, P_C and P_S, respectively.

Figure 2:

(A) CD spectra of closed and nicked minicircles in LiCl solutions as compared with the corresponding spectra of linear DNA. The LiCl concentrations are indicated in the boxes.

(C) AFM scans of ensembles of minicircle DNA structures in concentrated LiCl solutions. The corresponding LiCl concentrations are indicated above the images.

Figure 3:

(A) The CD spectra of topoisomers P_C and P_O in 0.1M LiCl are similar to the spectra of P_S in 0.375M and 0.5M LiCl, respectively. (Left) CD spectra of P_S and P_C at three different LiCl concentrations. The two bounding P_S spectra were used for predicting the LiCl concentration (0.375M) at which P_S should have a CD spectrum similar to that of the bounded P_C spectrum. (Middle) The CD spectrum of P_S in 0.375M compared with that of P_C in 0.1M LiCl. The predicted spectrum is also shown for comparison. (Right) The CD spectrum of P_S in 0.5M LiCl and that of P_O in 0.1M LiCl.

(B) Dependence of average CD intensity at 275-278 nm on LiCl concentration for three circular topoisomers of plasmid DNA.

(C) Dependence of the twist difference between topoisomers P_S and P_C on LiCl concentration estimated from comparison of CD spectra. Also shown is the corresponding proportion of the linking number difference compensated by twisting (qTw).

(D) Changes in DNA double helix twisting estimated from CD data at high LiCl concentrations assuming three different values of the twist difference between topoisomers P_C and P_S . The red curve shows the extrapolation of a power law fit to experimental values over the concentration range from 50mM to 1M (Cruz-León et al., 2022)

(E) Changes of twist in minicircles estimated from CD data at high LiCl concentrations as compared with the similarly measured dependence for plasmid topoisomer P_C .

MATERIALS AND METHODS

DNA constructs and Circular Dichroism

The DNA constructs were obtained in this laboratory using genetic methods and enzymatic synthesis *in vitro*. The CD experiments were carried out at the Molecular Biophysics facility of Pasteur Institute. The corresponding detailed experimental protocols will be published later.

Atomic Force Microscopy: Sample preparation and scanning

DNA was diluted to a concentration of 2 ug/ml in a buffer solution containing different concentrations of LiCl. Samples were prepared on freshly cleaved mica discs. Following cleavage, the mica was treated with $NiCl_2$ solutions for 10 seconds, rinsed with ultra-pure water, and then dried with a stream of dry nitrogen. A 5- μ l droplet of DNA solution was deposited onto nickel treated mica surface for 1 min. Then, the surface was rinsed with 0.01% diluted Uranyl Acetate solution in order to stabilize the DNA molecules for AFM imaging in air. The sample is then rapidly rinsed with pure water (Millipore) to obtain a clean surface after drying with filter paper.

AFM images of the grids prepared for TEM were obtained by scanning the samples using a Dimension-Icon AFM microscope from Bruker operated in peak-force tapping mode. Ultra-sharp silicon cantilevers (ScanAsyst 0.4 N.m⁻¹) with a nominal tip radius < 5 nm were used. AFM images were acquired under an air atmosphere at room temperature. The force was reduced in order to avoid dragging of molecules by the tip. Integral gain was adjusted to give sharp images. Images were taken without on-line filtering and were subsequently processed only by flattening to remove the background slope.

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FIGURES

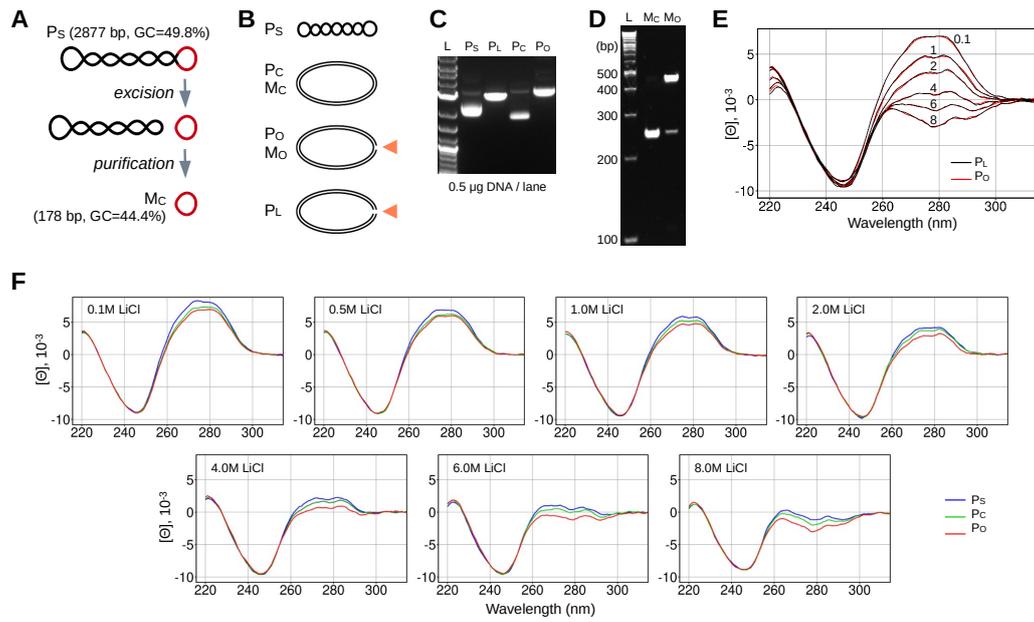


Figure 1

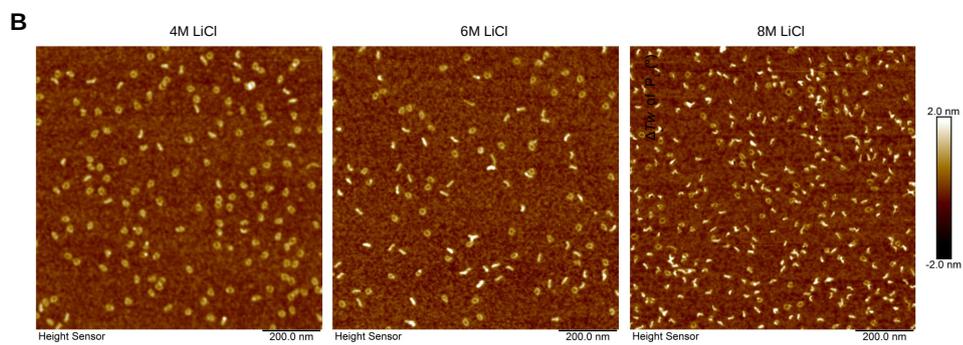
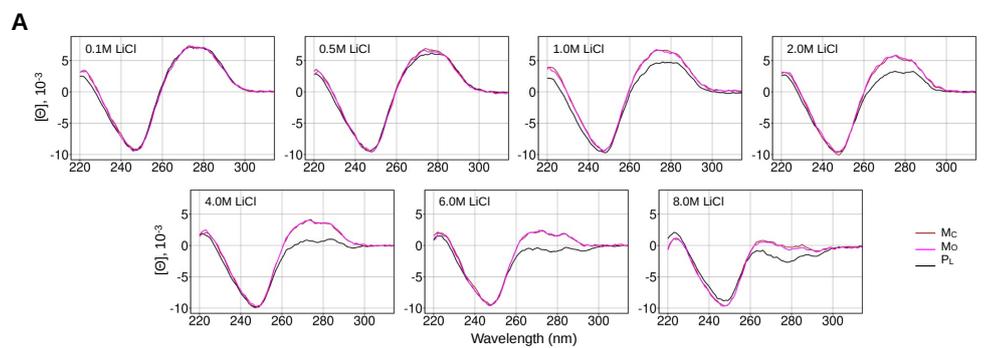


Figure 2

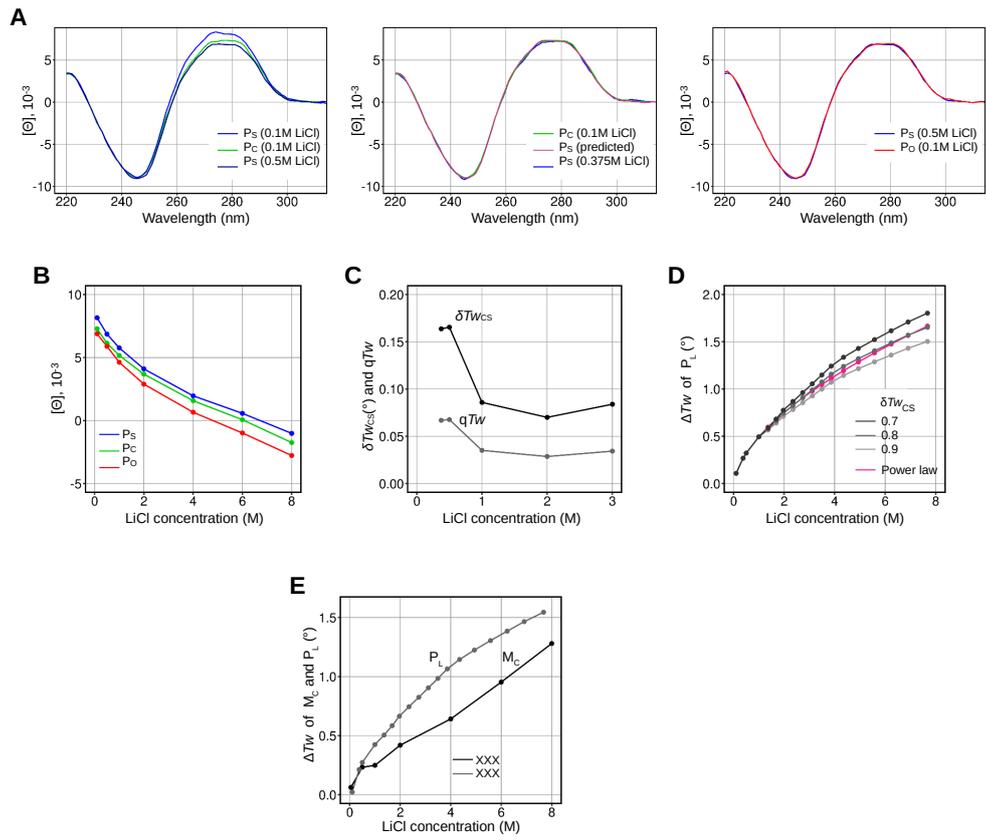


Figure 3