nature portfolio

Peer Review File

Counterintuitive DNA destabilization by monovalent salt at high concentrations due to overcharging

Corresponding Author: Professor Xinghua Zhang

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Referee for “Counterintuitive DNA destabilization by monovalent salt at high concentrations” by Zhang et al.

The authors use a number of different techniques to probe the stability of double-stranded DNA at very high concentration of monovalent ions. They find evidence for a de-stabilization with increasing salt concentration above a critical salt concentration and interpret this in light of overcharging of the DNA helix.
These results are surprising, because overcharging was previously thought to only occur with higher valency ions (divalent and higher). This is, therefore, an interesting observation that the authors support with several different experiments and all-atom MD simulations. I enjoyed reading the manuscript and support its publication, but have some points of attention outlined below.

- In the Abstract, I would suggest to devote more than one sentence to the experiments? Reading only the abstract, one might get the impression that this is a simulation study.

- In the Introduction and Conclusion, the authors argue for the importance of different salt concentration for DNA (which I agree with) and then claim that it has an important role for “gene expression” (e.g. page 2 and again on page 11). In the cell, the salt concentration is tightly regulated and the conditions tested in the paper are almost certainly lethal to almost all cells! So I think this is simply in correct or at least an overstatement. Changing salt concentration by several molar within a living cell is simply not a way gene expression is regulated. This does not invalidate the relevance of the findings, though, since salt concentration plays a critical role for in vitro assays and many other contexts. For a topical review see e.g.
Understanding nucleic acid-ion interactions.
Lipfert J, Doniach S, Das R, Herschlag D. Annu Rev Biochem. 2014;83:813-41. doi: 10.1146/annurev-biochem-060409-092720. Epub 2014 Mar 5. PMID: 24606136

- The destabilization at high salt appears to be lower for RNA. Possibly due to the duplex having a larger radius? Or possibly due to the fact that RNA has a more negative potential (such that it takes more ions to overcharge?):
Quantitative Studies of an RNA Duplex Electrostatics by Ion Counting.
Gebala M, Herschlag D. Biophys J. 2019 Sep 17;117(6):1116-1124. doi: 10.1016/j.bpj.2019.08.007. Epub 2019 Aug 12. PMID: 31466697

- The authors raise an interesting point, namely the difference in the single-stranded state of a molecule stretched by force vs. free in solution. The authors note that “while in the random-coiled state, there are intra-strand base contacts and interactions which should depend on salt condition”. However, did the authors also consider the changes in the bending persistence length (and therefore polymer entropy) with salt? See e.g.

Ionic strength-dependent persistence lengths of single-stranded RNA and DNA.
Chen H, Meisburger SP, Pabit SA, Sutton JL, Webb WW, Pollack L. Proc Natl Acad Sci U S A. 2012 Jan 17;109(3):799-804. doi: 10.1073/pnas.1119057109. Epub 2011 Dec 27. PMID: 22203973

Salt dependence of the radius of gyration and flexibility of single-stranded DNA in solution probed by small-angle x-ray scattering.
Sim AY, Lipfert J, Herschlag D, Doniach S. Phys Rev E Stat Nonlin Soft Matter Phys. 2012 Aug;86(2 Pt 1):021901. doi: 10.1103/PhysRevE.86.021901. Epub 2012 Aug 1. PMID: 23005779

- In the section “Mechanism of DNA duplex destabilization by monovalent ions” it might be good to say earlier on that overcharging always refers to some specific volume. If integrated out to infinity, the total charge has to be zero.

- Can the authors give a clearer interpretation of the fitting parameters given after Equation 5?

- The authors claim an important role for excluded volume (just before “Discussion”). Is this supported by the simulations? Can one e.g. vary the ion size in the simulations?

Minor points:

- Abstract: Not so clear what is meant by “the uniform ion concentration versus valence phase diagram”. Rephrase?

- “cobalt hexamine” should not be capitalized (page 2).

- Results, section heading:
“Monovalent cations at high concentrations destabilize DNA duplex in single-molecule experiments.” -> “duplexes”?

- “thus we corrected ΔL accordingly the refractive index of the buffer (varies up to 4%) using the previous method.” -> Sounds strange. Rephrase. What us “the previous method”? “according to the change in refractive index”?

- Caption Figure 2: “error bars are” not “were”.

- “using the fluorescence quenching test” (page 4) – which one? This has not be introduced. “a fluorescence quenching test” or rephrase?

- I would describe briefly what “our CG Langevin dynamics simulations” do, when they are first introduced.

- Define “k_q” (Equation 1) at first use.

- Figure 4D: Lines overlap with the figure legend.

- Page 5: “fairly agrees” -> Rephrase? What does this mean?

Reviewer #2

(Remarks to the Author)
Report on NCOMMS-24-31616
Title: Counterintuitive DNA destabilization by monovalent salt at high concentrations
Authors: Zhang et al.

This study employs a multi-faceted approach, combining experimental and simulation techniques, including all-atom, oxDNA, and coarse-grained modeling, to explore the impact of high monovalent salt concentrations (>1M) on the stability of DNA, RNA, and RNA-DNA duplexes. While the results align with experimental observations, they fail to provide novel insights into the phenomenon. Instead, they reaffirm the established understanding that high monovalent salt concentrations destabilize nucleic acids due to overcharging, a mechanism well-documented for multivalent ions at high concentrations.

The authors’ assertion that their study challenges the long-held belief that overcharging is exclusive to multivalent ions is puzzling. At low concentrations, monovalent salts stabilize DNA through Debye screening, a phenomenon only applicable at low salt concentrations. It is unjustified to extrapolate this effect to high salt concentrations. In any case, the Tm vs. c curve of Fig 3B for NaCl matches that of Ref 11, and subsequent papers.

The logarithmic behaviour of Eq. 1 would have been a signature of strong correlations. However, the fit of Eq. 2 is not the linearized version of Eq. 1 (k_q/c* is not 0. 074). This raises doubts about any correlation effect from the “Wigner crystal” theory. The radial distribution plots in Fig 4F are liquid-like with no exotic features.

The concentration-valence phase diagram (Fig 5E) shows that $c*=1$ does not represent anything special, such as a phase transition or critical point. This implies that the explanation would essentially be similar to the multivalent case. Moreover, the minimum valence of 0.8 for reversal (from CG) should not be considered a "universal" value, as it is expected to have some scaling dependence on the length of DNA (for the large length limit).

A few other comments:

• The authors could have utilized the oxRNA model to explore RNA stability at low monovalent salt concentrations as well in figure 2D. (There is no fit for RNA stability as they did for DNA).

• Please check whether q_eff = 0 means that the absorbed counterions carry the same charges as DNA!

The proportionality of the potential and the electric charge (below Eq 3) is not surprising, but rather inbuilt in electrostatics/Gauss' law.

• The authors should explain why they chose a cylinder radius of 1-2 nm in their simulations to calculate q_cyl. It's particularly important to know the reasoning behind this choice since the best match with the data in Figure 2B occurs at r_cyl=2nm, and notably, q_max is always near ~1.5nm.

• It would be nice to investigate the layering of cations around DNA using all-atom simulations and to elucidate the layering structure. (radial distribution function at high salt concentration >1M)

• The hysteresis loop in Figure 1D requires further discussion, particularly regarding the determination of the unzipping force. The phenomenon of hysteresis in DNA unzipping has been a subject of study for several years.

The authors should establish a clear connection in the paper. In its current form, the paper is merely presenting the results, which, unfortunately, are not surprising (e.g., Ref 11 and other subsequent papers).

Publication in Nature Comm. cannot be recommended.

Reviewer #3

(Remarks to the Author)
The authors provide experimental evidence of DNA (and RNA) hairpin destabilization at high enough concentration of monovalent salt. They interpret their data in terms of overcharging (inversion of net charge) due to counterion correlations.

The experimental data provided by the authors are indeed quite interesting at their core, since there is in fact a general belief in the polyelectrolyte community that overcharging could only take place for multivalent cations. At the same time, given the molar concentration range needed to show this effect, much larger than the usual salt concentration in a typical biological context, I doubt this finding could be of practical relevance in DNA biophysics at large (with the exception of specialized organisms living in high salt concentration environments). Moreover, the authors study a very special case of DNA duplex, namely a small hairpin, again very far from typical DNA in a biophysical context.

Perhaps more importantly, in my opinion, the manuscript is not written clearly. It would definitely benefit from an extensive editing of english language, but its lack of clarity does not depend only on this. Several key points are not addressed properly as detailed below.

More generally, I would suggest the authors to emphasize the connection with the polyelectrolyte theory and tone down the claims about the biophysical relevance of their results. In that respect, they should definitely acknowledge previous work (https://doi.org/10.1021/jp010861+) where the possibility of overcharging for monovalent salt had been already suggested, based on analytical and numerical results.

More specifically, I would suggest the authors (1) to explain in clear terms on which grounds the "monovalent salt -> no overcharge" belief was established; (2) to highlight that their estimate of the threshold concentration c0 for DNA overcharging based on Wigner crystal theory, now obtained in supplementary section S6 does indeed recover the experimental result within a 2 factor. At the same time, they should explain more carefully how their estimate of c0 is obtained (in eq.s S4-S7 several numerical factors are present whose origin is not obvious).

Other major issues

- In fig. 1 and related discussion in the main text, it is not clear how the authors identify the transition force to then perform the exuilibrium experiment. Fig. 1D shows the hysteresis typical of the out-of-equililbrium setups used in single molecules stretching/unzipping experiments. How is the transition force then determined? I would suggest the authors to name it "transition" and not "equilibrium" force, since they describe it as the force for which the folded and the unfolded state have tha same probability.

- The estimation DeltaG = f DeltaL for the duplex stability at zero force, using DeltaL obtained at the transition force (see above for the naming), neglects the different extension of the unfolded and folded states at zero force. In other words, the unfolded minimum in Fig. 1F should changes its position on the horizontal axis. Can the authors show or at least argue that this is a reasonable approximation?

- The authors state that error bars are obtained from "at least" three independent experiments: they actually ought to detail how many of them were carried out for all data reported in the manuscript.

- The authors state at lines 130-131 that the differences observed in the tweezer vs thermal melting experiments are due to the different properties of the unfolded states in the two setups. This is in itself reasonable (plus see the above comment on DeltaG estimation in the tweezer experiments). However, the data in Fig. 2 and 3 show a difference in the salt ranking (look e.g. at NaCl for DNA). The authors should try and connect this observation with the above statement.

- Related to the above, at a more general level: the authors state repeatedly that they are discussing the stability of duplexes, whereas the typical experimental counterpart of overcharging is based on NA condensation and/or electrophoresis experiments. The point is again the non trivial properties of the unfolded state for single stranded NA. For example: could single stranded NA be as well overcharged. This is in my view a missing key point in the authors approach which could have been tackled with the help of numerical simulations (but see below)

- I do not get the point of emphasizing the fact that oxDNA simulations (at intermediate resolution) are able to reproduce the folding/unfolding transition, whereas the more coarse-grained LAMMPS simulations are used to investigate duplex-DNA modeled as a fixed linear charged rod. The really interesting model to simulate should be folding/unfolding with explicit ions (see also the above comment).

- Another crucial point: the authors present two different approximate equations (1 and 2) to model how the net charge depends on concentration. It is definitely unclear which of the two is used (together with Eq.s 4 and 5) to fit experimental data in Fig. 2B. A similar lack of clarity is present in the supplementary material for eq.s S10-S11. I actually guess eq. 2 is used; if that is the case: is eq. 1 not working? How would the fit appear in the non used case? What could be learned by the fact that only one of the two approximations is working (if that is the case).

- The charge patterns ni Fig. 5B-C are interesting. Oscillations from positive to negative net charge regions in Fig 5C can be seen (which are not present in Fig. 5B). Can the authors comment on this feature? Tehy should show the corresponding data (i.e for 1M and 3M concentrations) in Fig 5D. The "symbols are from previous experiments" phrase in Fig. 5C caption seems misplaced. Which symbols from which experiments?

- The authors present two different sets of experimental reults (hydrogen bonds destabilization and electrophoresis) in the discussion sections. This definitely does not improve the clarity of manuscript.

- The interpretation of electrophoresis experiments outlined in the supplementary material (and summarized in Fig. S19) is not convincing. In particular, the inset showing velocity of Cs+ atoms as a function of distance from DNA axis is not really showing, in my view, the existence of 3 different regimes, as instead claimed by the authors.

Minor issues

- Which salt is used for fig. 2B-D?

- The full geometry of the cylinder cylinder shell in Fig. 4A is not given. What is the value of the inner radius?

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my previous concerns in the revision and I support publication of the manuscript at this stage.

Reviewer #2

(Remarks to the Author)
We have not found the response and revisions to be significant enough to alter our evaluation. Specific issues regarding some of the points raised in the previous round are outlined below. Other points have been adequately addressed.

Points 1 and 2: The melting point versus NaCl concentration curve was previously reported in Khimji et al. (Fig 3B of current Ref 2, old Ref 11; see also Fig 3 of Tomac et al, JACS 118, 5544 (1996)), and the reported curve in the current paper is similar to that. The current paper find similar effects for other salts. The experimental part is a reconfirmation of known effects on melting.

Point 3: The entire discussion on Wigner crystal is insignificant as the authors themselves mention in the revised version that "In this theory, the interactions between monovalent ions are not strong enough to induce a Wigner crystal." The reported data do not satisfy Eq 1 (Fig 4c). Therefore, no remarkable phenomena would be expected.

Point 6: q_eff=0: Shouldn't it be "equal but opposite charge"?

Reviewer #3

(Remarks to the Author)
The authors did overall a great job in addressing my comments and concerns. The quality of the manuscript greatly improved.

I have few requests left plus (again) the general requirement that the quality of the English language should be improved.

- introduction, 2nd linw: DNA and DNA-> DNA and RNA (I guess)

- to further clarify the discussion about the 3 states (D,C,S as in fig. 3c), the labels could be used for the corresponding minima in fig. 1g

- if I understand correctly: DeltaG_SD is measured from MT experiments (see fig. 2 data); DeltaG_CD is computed in Section S3; DeltaG_SC is computed as described at page 6. Could not then the authors test quantitative the relationship DeltaG_SD = DeltaG_SC + DeltaG_CD, at least for one salt and for one value of salt concentration? This would strengthen even more the analysis carried out by the authors

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The document includes

Reply to Reviewer #1

The authors use a number of different techniques to probe the stability of double-stranded DNA at very high concentration of monovalent ions. They find evidence for a de-stabilization with increasing salt concentration above a critical salt concentration and interpret this in light of overcharging of the DNA helix. These results are surprising, because overcharging was previously thought to only occur with higher valency ions (divalent and higher). This is, therefore, an interesting observation that the authors support with several different experiments and all-atom MD simulations. I enjoyed reading the manuscript and support its publication, but have some points of attention outlined below.

—Reply: Thank you very much for recognizing the significance of our work and supporting its publication.

1.- In the Abstract, I would suggest to devote more than one sentence to the experiments? Reading only the abstract, one might get the impression that this is a simulation study.

—Reply: Thank you for your good suggestions. In the abstract, we have added: "*Unexpectedly, our force-induced hairpin unzipping experiments and thermal melting experiments show that LiCl, NaCl, KCl, RbCl, and CsCl with concentrations beyond ~1 M destabilize DNA, RNA, and RNA-DNA duplexes. The two types of experiments yield different changes in free energy during melting, while the results that high concentration monovalent salts destabilize duplexes are common. The effects of these monovalent ions are similar but also have noticeable differences. From 1 M to 4 M, DNA duplex is destabilized by about 0.3 k_BT/bp and the melting temperature decreases by about 10 ^oC.*"

—Revision: Abstract. We have added four sentences about experiments as shown above.

2. - In the Introduction and Conclusion, the authors argue for the importance of different salt concentration for DNA (which I agree with) and then claim that it has an important role for

"gene expression" (e.g. page 2 and again on page 11). In the cell, the salt concentration is tightly regulated and the conditions tested in the paper are almost certainly lethal to almost all cells! So I think this is simply incorrect or at least an overstatement. Changing salt concentration by several molar within a living cell is simply not a way gene expression is regulated. This does not invalidate the relevance of the findings, though, since salt concentration plays a critical role for in vitro assays and many other contexts. For a topical review see e.g. Understanding nucleic acid-ion interactions. Lipfert J, Doniach S, Das R, Herschlag D. Annu Rev Biochem. 2014;83:813-41. doi: 10.1146/annurev-biochem-060409-092720. Epub 2014 Mar 5. PMID: 24606136

—Reply: Thank you for pointing out this issue. We totally agree with your comment. We have completely re-written the first paragraph: *"Ion-nucleic acid interactions play essential roles in biological processes, because nucleic acids, including DNA and DNA, carry high density of negative charges (see a review in[1]). One of the important roles of ions, such as Na^+ and K^+, is screening the repulsions among negative charges in nucleic acids[2, 3, 4, 5 6]. Without such screening, DNA duplex would be unstable, and RNA folding would not occur due to the strong repulsions in nucleic acids[1]. "*

—Revision:
[i] Abstract. We have switched the order of "DNA biophysics" and "polyelectrolyte theory" to highlight the primary implication of our work in polyelectrolyte theory.
[ii] Page 2. We have re-written the 1st paragraph.
[iii] Page 14. When discussing the implications of our work, we first describe the implication in polyelectrolyte theory. We place the implication in biophysics at last.

3. - The destabilization at high salt appears to be lower for RNA. Possibly due to the duplex having a larger radius? Or possibly due to the fact that RNA has a more negative potential (such that it takes more ions to overcharge?):Quantitative Studies of an RNA Duplex Electrostatics by Ion Counting. Gebala M, Herschlag D. Biophys J. 2019 Sep 17;117(6):1116-1124. doi: 10.1016/j.bpj.2019.08.007. Epub 2019 Aug 12. PMID: 31466697

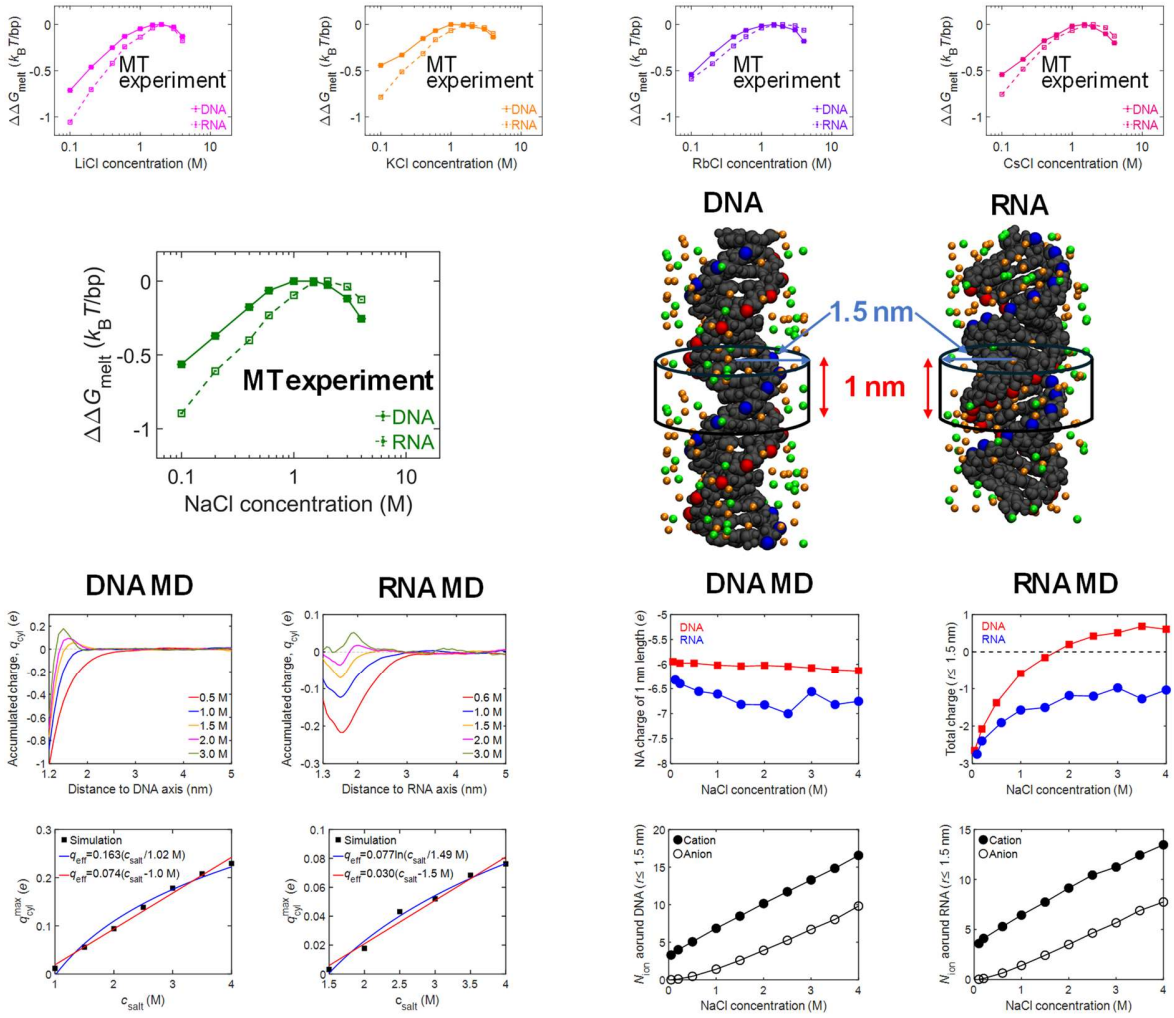
—Reply: Thank you for pointing out the difference between RNA and DNA. Yes, our experiments show that RNA overcharging requires high salt concentrations and RNA destabilization has smaller magnitudes at given ion concentrations, compared to DNA. Our MD simulations can capture this difference: DNA overcharges with 1 M NaCl, and RNA overcharges with 1.5 M NaCl. The difference is probably caused by the geometrical differences: helical pitch (RNA 2.7nm vs DNA 3.4 nm), helical radius and groove structures. We analyzed the influence of helical pitch. As shown by the figure below, over an axis of 1 nm, DNA carries -6*e* and RNA carries ~-7*e* (depending on c_{salt} due to NA deformation). When counting the total charge of ions in the cylinder with 1.5 nm radius and 1 nm height, we obtained +6.5*e* for DNA and +6*e* for RNA. Then, DNA overcharges (-6*e*+6.5*e*>0) but RNA

does not (-7*e*+6*e*<0). So, the larger c_{salt}^{*} for RNA are caused by multiple factors, including the higher linear charge density and the ion distribution pattern of RNA.

—Revision:

[i] Page 5. We have added: "*The values of c_{salt}^{*} for RNA are typically slightly less than the ones for DNA. Under a given high c_{salt}, the magnitude of destabilization for RNA is smaller than the case of DNA. The differences between RNA and DNA are possibly caused by different charge densities and ion distribution patterns[25] (see more discussions in Sec. S6 of SI). Previous ion counting experiments have also observed that ion distributions around RNA and DNA are different[1, 26].*"

[ii] SI. Sec S6. We have added the results mentioned above.



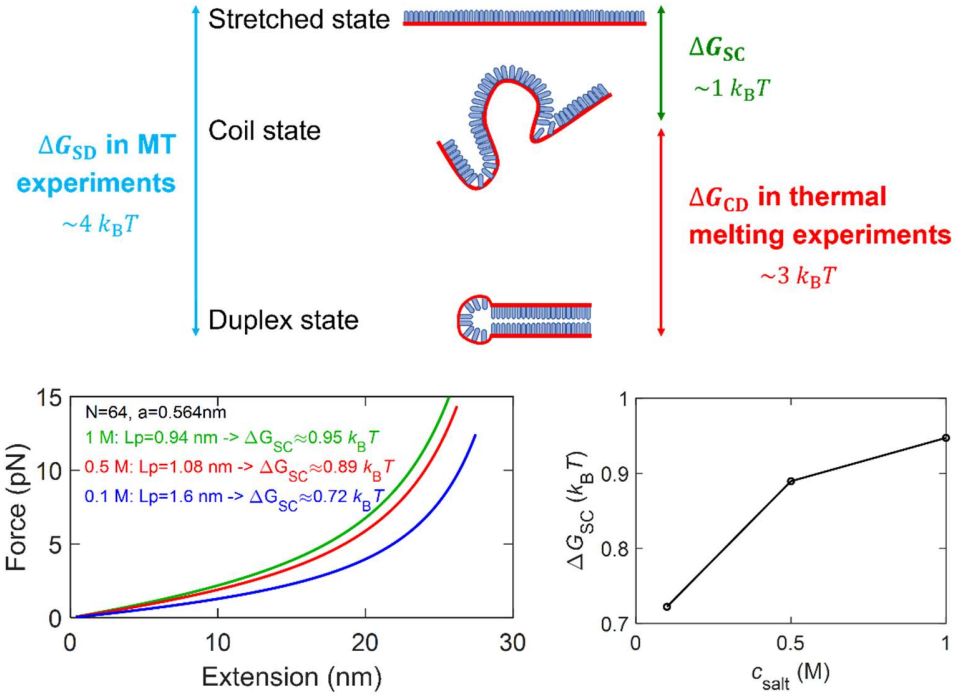
4. - The authors raise an interesting point, namely the difference in the single-stranded state of a molecule stretched by force vs. free in solution. The authors note that "while in the random-coiled state, there are intra-strand base contacts and interactions which should depend on salt condition". However, did the authors also consider the changes in the bending

persistence length (and therefore polymer entropy) with salt? See e.g. Ionic strength-dependent persistence lengths of single-stranded RNA and DNA. Chen H, Meisburger SP, Pabit SA, Sutton JL, Webb WW, Pollack L. Proc Natl Acad Sci U S A. 2012 Jan 17;109(3):799-804. doi: 10.1073/pnas.1119057109. Epub 2011 Dec 27. PMID: 22203973 Salt dependence of the radius of gyration and flexibility of single-stranded DNA in solution probed by small-angle x-ray scattering. Sim AY, Lipfert J, Herschlag D, Doniach S. Phys Rev E Stat Nonlin Soft Matter Phys. 2012 Aug;86(2 Pt 1):021901. doi: 10.1103/PhysRevE.86.021901. Epub 2012 Aug 1. PMID: 23005779

—Reply: Thank you very much for telling us these two papers. Inspired by your comment, we did more analysis on the free energy changes in MT and thermal melting experiments. As shown by the figure below, there are three DNA states: stretched state (S), coil state (C), and duplex state (D). The two experiments correspond to ΔG_{SD} and ΔG_{CD}, respectively. They differ by $\Delta G_{\mathrm{SD}} - \Delta G_{\mathrm{CD}} = \Delta G_{SC}$. The value of ΔG_{SC} can be estimated through the integration of the force-extension curve $\Delta G_{\mathrm{SC}}^{\mathrm{chain}} = \int_{z_c}^{z_s} f \mathrm{dz}$ from the coil state to the stretched state. Here, z_s and z_c are the extensions of the stretched and coil states, respectively, and *f* is the stretching force at a given extension, *z*. After approximating ssDNA as a wormlike chain [PRL 102:068301 (2009)], the dependence of the force on the extension is described by the Marko-Siggia equation: $f = \frac{k_B T}{L_p}\left[\frac{1}{4(1-z/L)} - \frac{1}{4} + \frac{z}{L}\right]$. Here, L_p is the ssDNA persistence length, $L = Na$ is the ssDNA contour length, $N = 64$ is the number of nucleotides for DNA hairpin in MT experiments, and $a \approx 0.564\, nm$ is the contour length per nucleotide [PNAS 109:799 (2012); PRE 86:021901 (2012)]. Previous experiments have obtained L_p ≈0.94 nm, 1.08 nm, and 1.6 nm at 1 M, 0.5 M, and 0.1 M NaCl, respectively. Based on the Marko-Siggia equation and f^*=12.5, 14.4, and 15 pN at 0.1, 0.5, and 1 M NaCl, we can obtain the values of z_c. Eventually, we obtained $\Delta G_{\mathrm{SC}} = \Delta G_{\mathrm{SC}}^{\mathrm{chain}}/28 \approx$ 0.72, 0.89 and 0.95 k_BT/bp at 0.1, 0.5 and 1 M NaCl, correspondingly. Here, the 28 is the number of base pairs of the DNA stem region in MT experiments.

The increase of ΔG_{SC} with c_{salt} should be the reason why the peak locations in Fig. 3b, i.e. c_{salt}^*, are typically smaller than the peak locations in Fig. 1g, considering that MT and thermal melting experiments correspond to ΔG_{SD}, and ΔG_{CD}, respectively, which differ by ΔG_{SC}.

The value of ΔG_{SC} depends not on only c_{salt}, but may also depend on the ion species, because ion distribution around nucleic acids vary among ion species. Furthermore, ions may mediate intra-strand interaction in the random-coiled state and affect the effective ssDNA persistence length. For example, the smaller ΔG_{CD} for Na^+ and Cs^+ at high concentrations in Fig. 3b may be caused by greater intra-strand attraction mediated by Na^+ or Cs^+, which enlarges the dependence of ΔG_{SC} on c_{salt}.



—Revision:

[i] Figure 1. We have revised Fig 1f, which shows the free energy difference between the folded and unfolded hairpin.

[ii] Figure 3. We have added Fig 3c.

[iii] Page 6. We have added the discussion about ΔG_{SD} and ΔG_{CD} in MT and thermal melting experiments.

[iv] SI. We have added a new section Sec S3 about the above calculation:

"*In MT experiments, NA hairpins transit between the folded duplex state and unfolded stretched state under tension, and the free energy difference is denoted as ΔG_{SD}. In thermal melting experiments, NA duplexes transit between the duplex state and a single-stranded random-coiled state, and the free energy difference is denoted as ΔG_{CD}. As shown in Fig. 3c, we have $\Delta G_{SD} = \Delta G_{CD} + \Delta G_{SC}$, where ΔG_{SC} is the free energy between the stretched state and coil state.*

The value of ΔG_{CD} at 1 M NaCl can be estimated for a given sequence using an empirical equation[29]. For our DNA hairpin sequence, ΔG_{CD} at 22 °C is 2.77 k_BT/bp for 1 M NaCl. See the calculation details in Sec. S3 of SI.

The value of ΔG_{SC} can be estimated through the integration of the force-extension curve $\Delta G_{SC}^{chain} = \int_{z_c}^{z_s} f dz$ from the coil state to the stretched state. Here, z_s and z_c are the extensions of the stretched and coil states, respectively, and f is the stretching force at a given extension, z. After approximating ssDNA as a worm-like chain[30], the dependence of

the force on the extension is described by the Marko-Siggia equation: $f = \frac{k_B T}{L_p}\left[\frac{1}{4(1-z/L)} - \frac{1}{4} + \frac{z}{L}\right]$. Here, L_p is the ssDNA persistence length, $L = Na$ is the ssDNA contour length, $N = 64$ is the number of nucleotides for DNA hairpin in MT experiments, and $a \approx 0.564\ nm$ is the contour length per nucleotide[31, 32]. Previous experiments have obtained L_p ≈0.94 nm, 1.08 nm, and 1.6 nm at 1 M, 0.5 M, and 0.1 M NaCl, respectively. Based on the Marko-Siggia equation and f^*=12.5, 14.4, and 15 pN at 0.1, 0.5, and 1 M NaCl, we can obtain the values of z_c. Eventually, we obtained $\Delta G_{SC} = \Delta G_{SC}^{chain}/28$ ≈0.72, 0.89, and 0.95 k_BT/bp at 0.1, 0.5 and 1 M NaCl, correspondingly. Here, the 28 is the number of base pairs of the DNA stem region in MT experiments.

The increase of ΔG_{SC} with c_{salt} should be the reason why the peak locations in Fig. 3b, i.e. c_{salt}^*, are typically smaller than the peak locations in Fig. 1g, considering that MT and thermal melting experiments correspond to ΔG_{SD}, and ΔG_{CD}, respectively, which differ by ΔG_{SC}.

The value of ΔG_{SC} depends not only on c_{salt}, but may also depend on the ion species. Ions may mediate intra-strand interaction in the random-coiled state and thus affect the effective ssDNA persistence length or make the force-extension curve deviating from the worm-like chain behavior[30]. For example, the smaller ΔG_{CD} for Na^+ and Cs^+ at high concentrations in Fig. 3b may be caused by greater intra-strand attraction mediated by Na^+ or Cs^+, which enlarges the dependence of ΔG_{SC} on c_{salt}."

5. - In the section "Mechanism of DNA duplex destabilization by monovalent ions" it might be good to say earlier on that overcharging always refers to some specific volume. If integrated out to infinity, the total charge has to be zero.

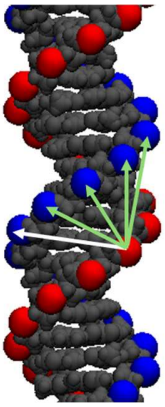
—Reply: Yes. Thank you for raising this important point. Yes, the overcharging corresponds to the total charges of DNA and ions within a certain short distance around DNA.

—Revision: Page 8. We have added: "*It is worth noting that q_{eff} correspond to the total charges of DNA and ions within a certain short distance around DNA, because the total charges of DNA and all ions, i.e., the entire system, are always zero.*".

6. - Can the authors give a clearer interpretation of the fitting parameters given after Equation 5?

—Reply: Thank you for raising this question. We made many approximations in calculating the inter-strand electrostatic interactions. One approximation is that we only consider the charge-charge interaction within a base pair, as shown by the white arrow in the figure below.

Actually, the charge on a DNA strand can interact with all charges on the other DNA strand. This approximation causes the underestimation of the interaction. Accordingly, we need a coefficient, which is larger than 1, to compensate this underestimation.

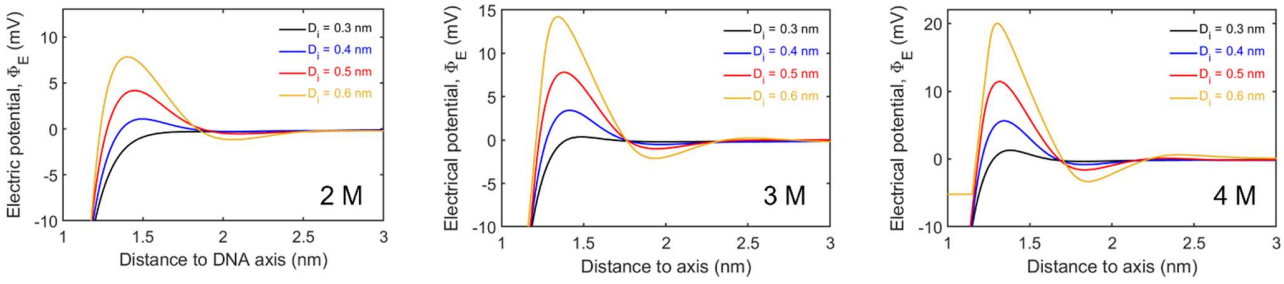


—Revision:

[i] Page 10. We have added: "*One reason that causes $k_{fit} > 1$ is that the calculation of E_0 only considers the charge-charge interaction within a base pair, and actually the charge on a DNA strand can interact with all charges on the other DNA strand.*"

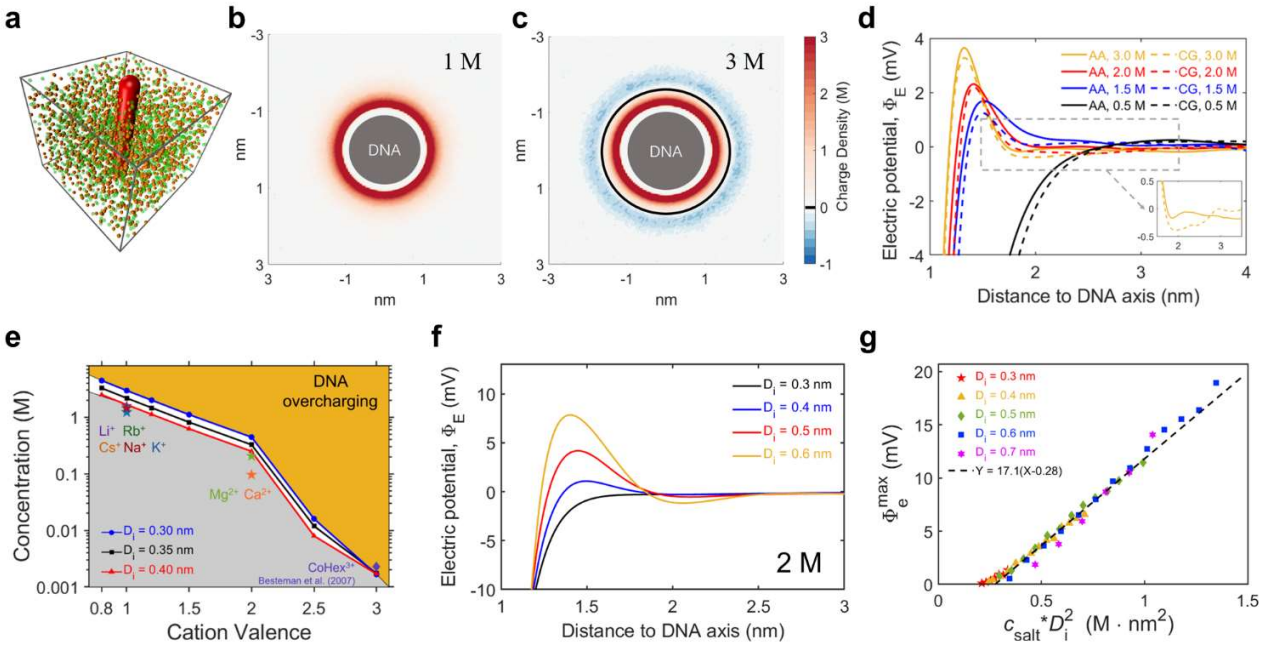
7. - The authors claim an important role for excluded volume (just before "Discussion"). Is this supported by the simulations? Can one e.g. vary the ion size in the simulations?

—Reply: Please see the figures below. When reducing the ion diameters, the overcharging electrical potential becomes smaller. When the ion diameter is less than a critical value, the overcharging phenomenon disappears.



—Revision:

[i] Figure 5. We have added Fig 5f and 5g to show the effect of excluded volume interactions on DNA overcharging potential.



Minor points:

8. - Abstract: Not so clear what is meant by “the uniform ion concentration versus valence phase diagram”. Rephrase?

—Reply: We apologize for this unclear writing. We have revised it to: "*our coarse-grained simulations obtained a phase diagram that indicates whether DNA overcharging occurs at a given ion valence and concentration.*"

—Revision: Abstract. We have added the above sentence.

9. - “cobalt hexamine” should not be capitalized (page 2).

—Reply: Thank you for pointing out this issue.

—Revision: Page 2, paragraph 3. We have changed “Cobalt Hexamine” to “cobalt hexamine ($CoHex^{3+}$)” and subsequently refer to it as $CoHex^{3+}$ for short.

10. - Results, section heading: “Monovalent cations at high concentrations destabilize DNA duplex in single-molecule experiments.” -> “duplexes”?

—Reply: Thank you. We have changed it.

—Revision: Page 3, paragraph 3. We have changed “duplex” to “duplexes”.

11. - “thus we corrected ΔL accordingly the refractive index of the buffer (varies up to 4%) using the previous method.” -> Sounds strange. Rephrase. What us “the previous method”? “according to the change in refractive index”?

—Reply: We apologize for this unclear writing. We have changed the sentence. Followings are the related content in Ref.24.

Figure Redacted

—Revision: Page 3. We have added: "*Note that varying salt concentration affects the refractive index of the buffer (up to 4%). We have considered this factor in the measurement of ΔL using a previous method[24]. Eventually, we found that ΔL very weakly depends on the salt condition (See Supplementary Fig. 1b).*"

12. - Caption Figure 2: "error bars are" not "were".

— Reply: Thank you. We have changed it.

13. - "using the fluorescence quenching test" (page 4) – which one? This has not be introduced. "a fluorescence quenching test" or rephrase?

— Reply: We apologize for this unclear writing. We have changed the sentence.

—Revision: Page 5. We have added: "*We measured the melting temperature (T_m) of NA duplexes at various c_{salt} using a fluorescence quenching test, as detailed in the methods section (Fig. 3). In the duplex state, the BHQ1 quencher on one strand effectively suppressed the proximate FAM fluorescence on the complementary strand. We raised the temperature incrementally at a rate of 0.1℃/second. As the NA duplex gradually melted and the FAM strand deviated from the BHQ1 strand, we observed a corresponding increase in fluorescence intensity. The peak of the derivative of the fluorescence intensity determined the T_m under each salt condition*[28] (*Figs. 3a and S2)*. "

14. - I would describe briefly what "our CG Langevin dynamics simulations" do, when they are first introduced.

—Reply: Thank you for pointing out this issue. The style of *Nature Communications* typically places the Methods section after the Discussion section. We have added "which are described in the method section" to direct readers to the method section for the simulation details.

—Revision: Page 8. We have added: "…, *which are described in the method section,* …".

15. - Define "k_q" (Equation 1) at first use.

—Reply: Thank you for pointing out this issue. k_q is a fitting parameter.

—Revision: Page 9. We have added: "*Here, k_q is a fitting parameter*".

16. - Figure 4D: Lines overlap with the figure legend.

—Reply: Sorry for the issue, we have changed it.

—Revision: We have revised Fig 4d to remove the overlap.

17. - Page 5: “fairly agrees” -> Rephrase? What does this mean?

—Reply: Thank you for pointing out this issue. We have added a few sentences for this comparison,

—Revision: Page 11. We have added: *“Our theoretical phase boundary for D_i = 0.4 nm (red line in Fig. 5e) agrees with our experimental results for monovalent ions: Li^+, Na^+, K^+, Rb^+, and Cs^+. Our theoretical phase boundaries are above the experimental results for divalent ions, Mg^{2+} and Ca^{2+}, possibly because the hydrated ion diameters of Mg^{2+} and Ca^{2+} are larger than 0.4 nm. Our theoretical phase boundaries are close to a previous experimental result[12] for $CoHex^{3+}$.”*

Reply to Reviewer #2

1. This study employs a multi-faceted approach, combining experimental and simulation techniques, including all-atom, oxDNA, and coarse-grained modeling, to explore the impact of high monovalent salt concentrations (>1M) on the stability of DNA, RNA, and RNA-DNA duplexes. While the results align with experimental observations, they fail to provide novel insights into the phenomenon. Instead, they reaffirm the established understanding that high monovalent salt concentrations destabilize nucleic acids due to overcharging, a mechanism well-documented for multivalent ions at high concentrations.

—Reply: Thank you for reviewing our manuscript and giving us useful suggestions. However, we do not agree with you that our results are not new. You commented that: (i) our experimental results are not new, because Khimji et al ChemComm 49:1306 (2013) and subsequent papers presented similar results; (ii) our theoretical explanation is not new, because our theory is based on DNA overcharging, just extending multivalent-ion-induced DNA overcharging to monovalent-ion-induced DNA overcharging. However, the literature has never suggested that (i) monovalent-ion-induced DNA destabilization is caused by (ii) DNA overcharging.

ChemComm (2013) does not spend any words on DNA destabilization by overcharging, because they focused on the effect of anions on DNA stability. The subsequent papers, such as Eur Biophys J 46:33 (2017) by Maity, Singh, and Singh used a different mechanism to explain DNA destabilization, without any word of DNA overcharging or charge inversion. So, the scientific community of DNA structures and interactions does not know monovalent-ion-induced DNA destabilization is caused by DNA overcharging.

Our work is the first to reveal the mechanism of this counterintuitive phenomenon, and revealing mechanism is the core value of scientific research.

We would like to reinforce the primary contributions of our work:

(i) precisely measuring DNA, RNA, and RDH destabilization by Li^+, Na^+, K^+, Rb^+, Cs^+, Mg^{2+}, Ca^{2+} by force-induced hairpin unzipping experiments and thermal melting experiments;

(ii) showing DNA destabilization by high concentrations of monovalent ions including Li^+, Na^+, K^+, Rb^+, Cs^+ is a common phenomenon not ion-specific;

(iii) proving that such DNA destabilization is caused by DNA overcharging using extensive all-atom and CG simulations, Wigner crystal theory, and electrophoresis experiments;

(iv) Discovering that overcharging can also occur for ssDNA through new experiments and simulations, a finding inspired by Reviewer #3's insightful comments.

Our results have both fundamental and practical values:

(i) Fundamentally, we advance the polyelectrolyte theory of overcharging using DNA as a model polyelectrolyte. We highlight that single-DNA technique can achieve a high

precision (~0.01 pN & 1 nm) that is unachievable in traditional experiments for other polyelectrolytes.

(ii) Practically, our systematical experimental data of DNA, RNA, RHD duplex stabilization under various salt conditions act as the table of physical chemical thermochemical and other physical property data for nucleic acids, which can be used in many bionanotechnological applications, such as DNA origami, and DNA nanopore sequencing.

—Revision: Page 15. We have added: "*In conclusion, we summarize overall results of this work: (i) precisely measuring DNA, RNA, and RDH destabilization by Li^+, Na^+, K^+, Rb^+, Cs^+; (ii) showing DNA destabilization by high concentrations of monovalent ions is a common phenomenon, not ion-specific; (iii) proving that such DNA destabilization is caused by DNA overcharging using extensive all-atom and CG simulations, Wigner crystal theory, and electrophoresis experiments. Our results have both fundamental and practical value: (i) Fundamentally, we refresh the polyelectrolyte theory of overcharging using DNA as a model polyelectrolyte. We highlight that single-DNA technique can achieve a high precision (~0.01 pN & 1 nm) that is unachievable in traditional experiments for other polyelectrolytes. (ii) Practically, our systematical experimental data of DNA, RNA, RHD duplex stabilization under various salt concentrations act as the table of physical chemical data for nucleic acids, which can be used in many bionanotechnological applications, such as DNA origami, and DNA nanopore sequencing.*"

2. -The authors assertion that their study challenges the long-held belief that overcharging is exclusive to multivalent ions is puzzling. At low concentrations, monovalent salts stabilize DNA through Debye screening, a phenomenon only applicable at low salt concentrations. It is unjustified to extrapolate this effect to high salt concentrations. In any case, the Tm vs. c curve of Fig 3B for NaCl matches that of Ref 11, and subsequent papers.

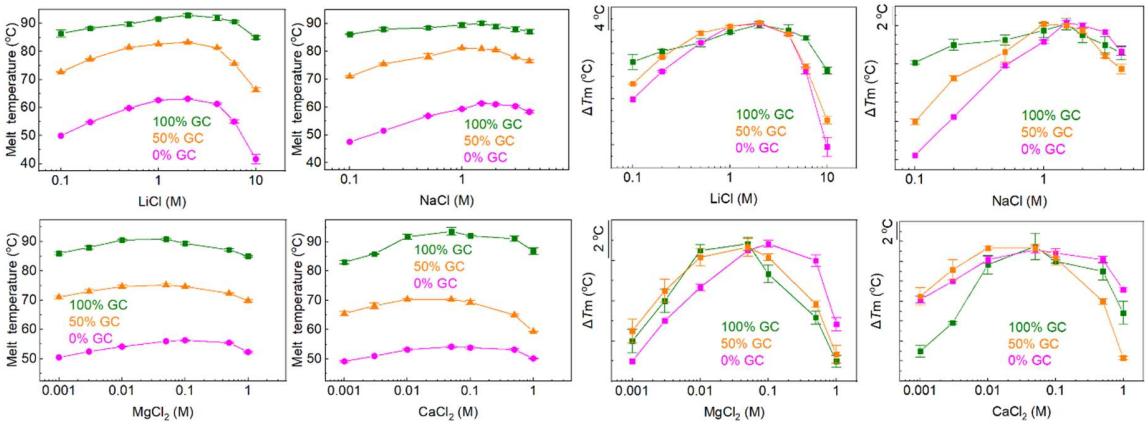
—Reply: Thank you for pointing out this issue. Our results are far beyond Khimji et al. ChemComm 49:1306 (2013) in many terms:

(i) Khimji et al ChemComm 49:1306 (2013) did not spend any words on DNA destabilization by overcharging. The focus of Khimji et al. is investigating the effects of anions on DNA stability, not DNA destabilization by ions.

(ii) ChemComm 49:1306 (2013) performed experiments only for one monovalent cation: Na^+. From this data, we are not sure whether the DNA destabilization is caused by specific binding of Na^+. In biophysics, it is quite common that some ions have specific effects due to specific binding patterns. Here, we performed experiments for DNA, RNA, and RDH duplexes with Li^+, Na^+, K^+, Rb^+, Cs^+, Mg^{2+}, Ca^{2+}. With this systematical data, we can make sure duplex destabilization by high concentrations of ions is a common physical mechanism.

(iii) Our MT experiments can directly give the energetic changes, as shown in Fig 2, which cannot be directly obtained by the thermal melting experiments in ChemComm 49:1306 (2013).

(iv) We assume the "subsequent papers" refer to Eur Biophys J 46:33 (2017) by Maity, Singh, and Singh, because among the papers citing ChemComm 49:1306 (2013), only this EBJ paper analyzed DNA destabilization. However, this EBJ has no word about DNA overcharging. This EBJ paper considered that DNA destabilization is caused by the weakening of base-pairing hydrogen bond interactions, i.e. the decrease of Di when $c_{\mathrm{salt}} > 1$ M. If base-pairing hydrogen bonds is the reason for DNA destabilization, the critical c^*_{salt} should show clear dependence on GC content in DNA sequences. However, our experiments do not observe such dependence. As suggested by Reviewer #3, we have removed the dependence on GC content to enhance the clarity of the manuscript.



(v) Most importantly, we revealed the mechanism for duplex destabilization: overcharging. The scientific community of DNA structures and interactions does not know monovalent-ion-induced DNA destabilization is not ion-specific and it is caused by DNA overcharging. We provide extensive experimental, simulation, and theoretical results to show that the destabilization is caused by overcharging, which is the core value of this work.

—Revision: End of introduction section. We have added: "*DNA thermal melting experiments by Khimji et al. observed that DNA duplex is destabilized at high concentrations of Na^{+2}. The authors did not discuss the mechanism of DNA destabilization, because their focus was on the effect of polyanion on DNA stability. Their experiments used only one cation: Na+. It is unclear whether any ion-specific effect causes DNA destabilization under high salt concentrations. Later, a theoretical work by Maity, Singh, and Singh[5] reproduced the DNA destabilization at high salt concentrations after assuming the effective base-pairing hydrogen bonds weaken at high salt concentrations. This assumption has not yet been validated.*
In this work, we performed precise and systematical experimental measurement for DNA, RNA, and RDH duplex stability with wide ranges of salt concentrations of LiCl, NaCl, KCl,

RbCl, and CsCl. The experimental results constantly showed duplex destabilization by high concentrations of monovalent ions. Furthermore, we prove that such duplex destabilization is caused by overcharging using extensive all-atom molecular dynamics (MD) and CG Langevin dynamics simulations, as well as Wigner crystal theory."

3. -The logarithmic behavior of Eq. 1 would have been a signature of strong correlations. However, the fit of Eq. 2 is not the linearized version of Eq. 1 (k_q/c* is not 0. 074). This raises doubts about any correlation effect from the "Wigner crystal" theory. The radial distribution plots in Fig 4F are liquid-like with no exotic features.

—Reply: We apologize for the confusion. Please note that Eq 2, a linear equation, is just for convenient usage, not based on rigorous mathematical simplification of Eq 1.

It is worth pointing out that Wigner crystal theory was developed for multivalent ions, because the strong repulsions among multivalent ions favor the formations of crystal-like structure. Here, we applied the theory to monovalent ions, and found the functional form, the logarithmic function in Eq. 1, seems to work, but the quantitative values, such as c^*_{salt}, deviate greatly from the prediction of the theory. Such deviation is not surprising, because monovalent ions do not form a Wigner crystal, as shown by the liquid-like structure in Fig. 4f.

—Revision:

[i] Page 9. We have added: "*Note that the above linear equation is just for convenient usage, not based on rigorous mathematical simplification of Eq. 1.*"

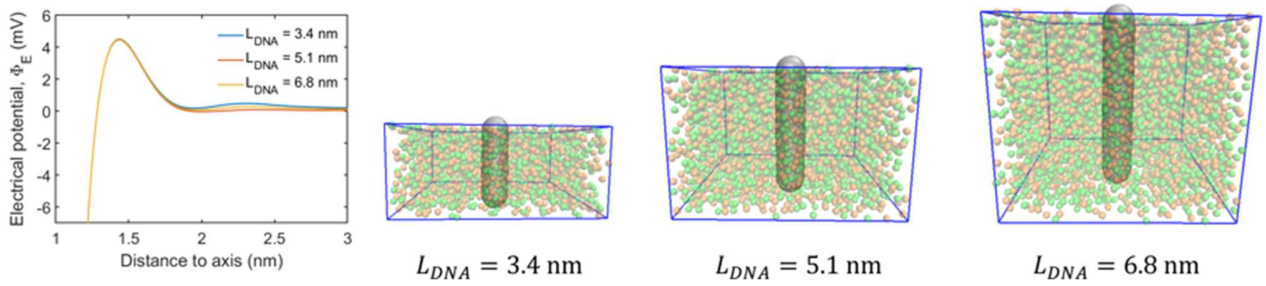
[ii] Page 13. We have added: "*It is worth pointing out that Wigner crystal theory was developed for multivalent ions, because the strong repulsions among multivalent ions favor the formations of crystal-like structure. Here, we applied the theory to monovalent ions, and found the functional form, the logarithmic function in Eq. 1, seems to work, but the quantitative values, such as c^*_{salt}, deviate greatly from the prediction of the theory. Such deviation is not surprising, because monovalent ions do not form a Wigner crystal, as shown by the liquid-like structure in Fig. 4f.*"

4. The concentration-valence phase diagram (Fig 5E) shows that $c*=1$ does not represent anything special, such as a phase transition or critical point. This implies that the explanation would essentially be similar to the multivalent case. Moreover, the minimum valence of 0.8 for reversal (from CG) should not be considered a "universal" value, as it is expected to have some scaling dependence on the length of DNA (for the large length limit).

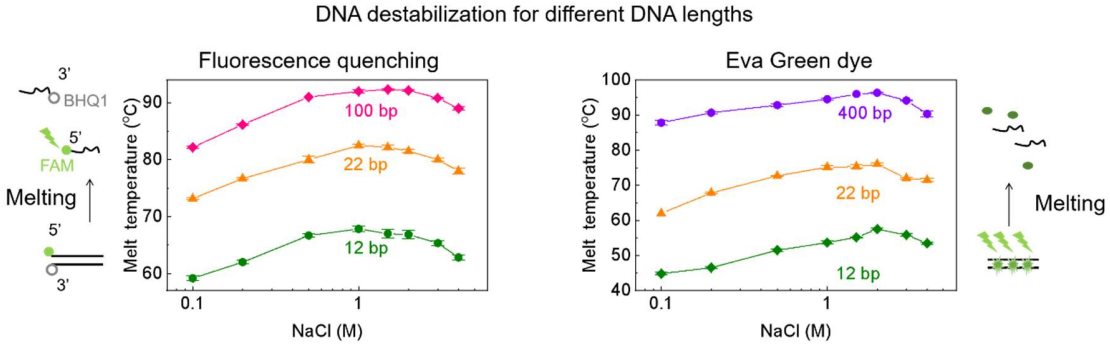
—Reply: As pointed by you in the above comment, it is completely unknown whether the Wigner crystal theory can be applied to monovalent ions (Z=1), considering that the ion distributions exhibit liquid-structure rather than crystal structures. Here is a quote from Rev. Mod. Phys 74:329 (2002): "*A system of monovalent ions, Z=1, is weakly coupled, Γ∼1, and*

this is why classical mean-field theory applies. By contrast, a system in which Z-ions have large Z is strongly coupled, and we see that R becomes larger than λ. For example, at Z=3 and σ=1.0 e/nm2, we get Γ=6.4, λ≈0.1 nm, and R ≈ 1nm." Accordingly, the authors of Rev. Mod. Phys paper did not proceed with the calculation for Z=1. We push the Wigner crystal theory to Z=1 and found that the functional forms appear to marginally work, which is something straightforward and easily anticipated. We found that the predicted values of c_{salt}^{*} by Wigner crystal theory deviate greatly from simulation results (see Sec S8 in SI). In this sense, our result and analysis are not something "not special".

We would like to point out that DNA overcharging and DNA melting temperature are different. DNA melting depends on DNA length, because DNA conformational entropy plays a crucial role in DNA melting and such entropy depends on DNA length. However, DNA overcharging does not exhibit a strong dependence on DNA length, at least for short DNA as straight segments. Our AA and CG simulation results of DNA overcharging do not depend on DNA length. Please see the figure below.



To address your concern about DNA length, we have performed new thermal melting experiments for different DNA lengths using the fluorescence quenching test or Eva Green dye. DNA destabilization at high c_{salt} constantly occurs for different DNA lengths.



—Revision:

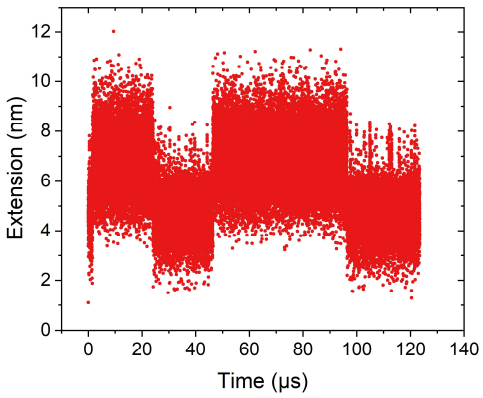
[i] Page 1. Abstract. We have deleted the sentence "The minimum ion valence for overcharging is ~0.8".

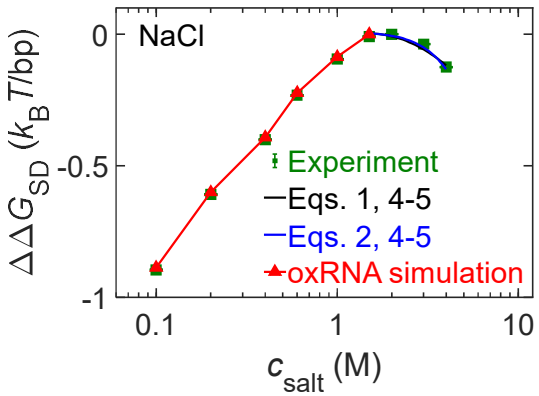
[ii] Page 5. We have added: "*DNA destabilization at high c_{salt} constantly occurs for different DNA lengths from 12 to 400 bp in thermal melting experiments, as shown in Supplementary Fig. 6.*"
[iii] Page 11. We have revised one sentence to "*When we set the maximum c_{salt} as 3 M, the minimum ion valence for DNA overcharging is about 0.8 for short DNA segments with straight rod conformations*."
[iv] SI. We have added the above experimental results for different DNA lengths as Fig. S6.

A few other comments:

5. -The authors could have utilized the oxRNA model to explore RNA stability at low monovalent salt concentrations as well in figure 2D. (There is no fit for RNA stability as they did for DNA).

—Reply: Thank you for your suggestion. We have utilized oxRNA simulation to explore RNA unzipping and obtain the unzipping-rezipping kinetics for 120 μs in simulations. The simulation results of oxRNA model also agree with our experimental results. Please see the figures below.





—Revision:
[i] Figure 2. We have updated Fig 2d.
[ii] Page 8, paragraph 3. We have added: "*Similar oxRNA simulations were performed and the results agree with experiments for $c_{salt} < c^*_{salt}$ (red curve in Fig. 2d).*"
[iii] Page 16, paragraph 4. We have added: "*We also use oxRNA model[59] to observe the zipping/unzipping of RNA hairpin. In oxRNA simulations, the sequence of the RNA is similar as that of DNA except T is replaced by rU.*"
[iv] SI. We have updated Fig. S8.

6. -Please check whether q_eff = 0 means that the absorbed counterions carry the same charges as DNA!

—Reply: Yes. q_eff = 0 means that the absorbed counterions carry the same charges as DNA

—Revision: Page 8. We have added: "*It is worth noting that* q_{eff} *correspond to the total charges of DNA and ions within a certain short distance around DNA, because the total charges of DNA and all ions, i.e., the entire system, are always zero.*"

7. -The proportionality of the potential and the electric charge (below Eq 3) is not surprising, but rather inbuilt in electrostatics/Gauss law.

   —Reply: Yes. We have added a sentence to mention it after Eq 3.

   —Revision: Page 9. We have added: "*Note that because of the Gauss's law, the linear behavior of the accumulated charge in Eq 2 can lead to a linear behavior of the electrical potential.*"

8. -The authors should explain why they chose a cylinder radius of 1-2 nm in their simulations to calculate q_cyl. It's particularly important to know the reasoning behind this choice since the best match with the data in Figure 2B occurs at r_cyl=2nm, and notably, q_max is always near ~1.5nm.

   —Reply: Sorry for the confusion. The 2 nm in Eq 4 and Fig 2B corresponds to the inter-strand P-P distance (or DNA diameter), not r_cyl used for overcharging calculation.

   —Revision: Page 9. We have added: "(inter-strand P-P distance or DNA diameter)."

9. -It would be nice to investigate the layering of cations around DNA using all-atom simulations and to elucidate the layering structure. (radial distribution function at high salt concentration >1M)

   —Reply: As shown in Fig 4f, there is no clear layer structure at 4 M NaCl. Please see more details in our replies to points #3 and #4 above.

10. - The hysteresis loop in Figure 1D requires further discussion, particularly regarding the determination of the unzipping force. The phenomenon of hysteresis in DNA unzipping has been a subject of study for several years.

    —Reply: The unfolding and refolding processes in our experiments are just used to estimate the range of the transition force, not to determine the transition force. We precisely determine the transition force by finely tuning the force until the equal probability of folded and unfolded

states. The focus of our work is the transition force, not the kinetics of unfolding and refolding (hysteresis).

—Revision: Page 3. We have added: "*Within the range from the refolding force to the unfolding force, we searched a transition force, f^*, where the folded and stretched states of the NA hairpin had the same probability (Fig. 1e)*".

11. -The authors should establish a clear connection in the paper. In its current form, the paper is merely presenting the results, which, unfortunately, are not surprising (e.g., Ref 11 and other subsequent papers).

—Reply: Thank you for your comment. As mentioned in our reply to point #1, ChemComm (2013) does not spend any words on DNA destabilization by overcharging, because they focused on the effect of anions on DNA stability. The subsequent papers, such as Eur Biophys J 46:33 (2017) by Maity, Singh, and Singh used a different mechanism to explain DNA destabilization, without any word of DNA overcharging or charge inversion. So, the scientific community of DNA structures and interactions does not know monovalent-ion-induced DNA destabilization is caused by DNA overcharging.

We carefully read Eur Biophys J 46:33 (2017). We found that the last two terms in Eq 2 of that paper can capture DNA destabilization at high salt concentrations. The authors considered Di in Eq 2 as hydrogen bonding between the bases in a pair. If we treated D_i as the effective inter-strand interaction, including electrostatic interactions, then we may consider the nonmonotonic behavior of D_i is caused by overcharging. The calculation is as follows.

Maity, Singh, and Singh used a modified Peyrard Bishop Dauxois (PBD) model to capture DNA destabilization by high salt concentrations [Maity, Singh and Singh, Eur Biophys J, 46, 33 (2017)]. Here, we re-calculated the DNA energy change at high salt concentrations in their model. The dissociation energy of one base pair follows:

$$V = V_m(y_i) + V_{sol}(y_i)$$

$$V_m(y_i) = D_i(e^{-a_i y_i} - 1)^2$$

$$V_{sol} = -\frac{1}{4} D_i[\tanh(\gamma y_i) - 1]$$

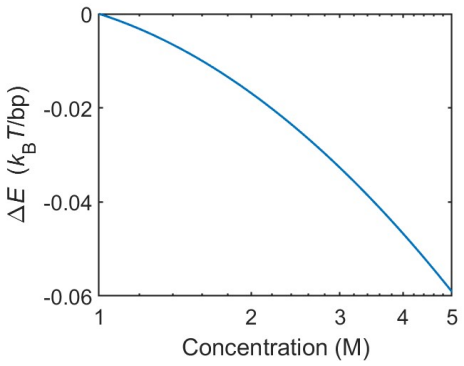
$$D_i = D_0\left(1 + \lambda_1 \ln\frac{C}{C_0} - \lambda_2 \ln^2\left(\frac{C}{C_0}\right) + \chi\left(\frac{C_0}{C^t}\right)\right)$$

where y_i is the base-base distance with $y_i = 0$ corresponding to the minimum energy, D_i is the interaction strength, C is the monovalent salt concentration and C_0 is the critical salt

concentration. Other parameters are $D_0 = 0.043\,eV$, $\lambda_1 = 0.01$, $\lambda_2 = 0.011$, $C_0 = 1\,\mathrm{M}$, $\chi = 1.2$ and $t = 0.01$. DNA melting corresponds to the change of y_i is from 0 to ∞. Accordingly, we have $\Delta V \approx \frac{3}{4} D_i$. DNA destabilization is related to the terms $\lambda_2 \ln^2\left(\frac{C}{C_0}\right) + \chi\left(\frac{C_0}{C^t}\right)$ in the above equations. Hence, we calculate the magnitude of destabilization as

$$\Delta E = -\frac{3}{4} D_0 \left(\lambda_2 \ln^2\left(\frac{C}{C_0}\right) - \chi\left(\frac{C_0}{C^t}\right)\right)$$

The figure below shows the dependence of ΔE on the monovalent salt concentration.



—Revision:

[i] Page 13. We have added: "*In addition to the Wigner crystal theory, a previous study has added two salt-dependent energetic terms for inter-strand interaction to capture DNA destabilization under high salt concentrations[5]. See Sec. S11 of the SI.*"
[ii] SI. We have added a new section Sec S11.

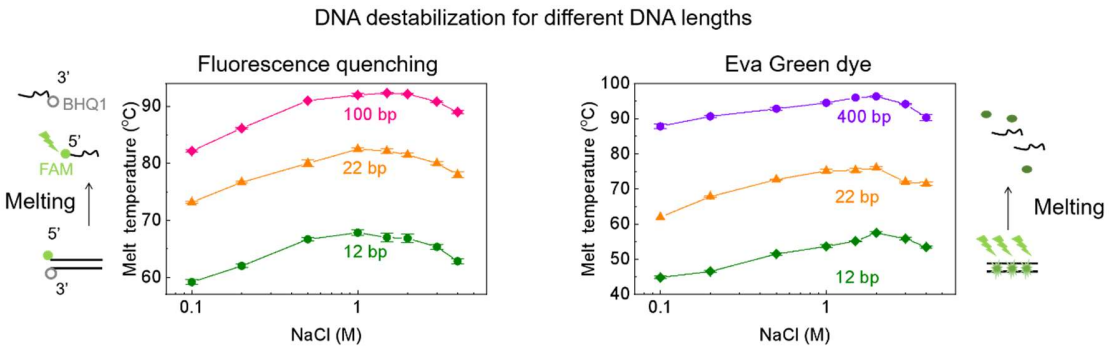
Reply to Reviewer #3

The authors provide experimental evidence of DNA (and RNA) hairpin destabilization at high enough concentration of monovalent salt. They interpret their data in terms of overcharging (inversion of net charge) due to counterion correlations.

1. The experimental data provided by the authors are indeed quite interesting at their core, since there is in fact a general belief in the polyelectrolyte community that overcharging could only take place for multivalent cations. At the same time, given the molar concentration range needed to show this effect, much larger than the usual salt concentration in a typical biological context, I doubt this finding could be of practical relevance in DNA biophysics at large (with the exception of specialized organisms living in high salt concentration environments). Moreover, the authors study a very special case of DNA duplex, namely a small hairpin, again very far from typical DNA in a biophysical context.

   —Reply: Thank you for pointing out this issue. We totally agree with your comment about the biological relevance. We have completely re-written the first paragraph: *"Ion-nucleic acid interactions play essential roles in biological processes, because nucleic acids, including DNA and DNA, carry high density of negative charges (see a review in[1]). One of the important roles of ions, such as Na^+ and K^+, is screening the repulsions among negative charges in nucleic acids[2, 3, 4, 5 6]. Without such screening, DNA duplex would be unstable, and RNA folding would not occur due to the strong repulsions in nucleic acids[1]"*

   To address your concern about DNA length, we have performed new thermal melting experiments for different DNA lengths using the fluorescence quenching test or dsDNA dye (Eva Green). DNA destabilization at high c_{salt} constantly occurs for different DNA lengths.



   —Revision:

   [i] Abstract. We have switched the order of "DNA biophysics" and "polyelectrolyte theory" to highlight the primary implication of our work in polyelectrolyte theory.

[ii] Page 2. We have re-written the 1st paragraph.
[iii] Page 5. We have added: "*DNA destabilization at high c_{salt} constantly occurs for different DNA lengths from 12 to 400 bp in thermal melting experiments, as shown in Supplementary Fig. 6.*"
[iv] Page 14. When discussing the implications of our work, we first describe the implication in polyelectrolyte theory. We place the implication in biophysics at last.
[v] SI. We have added the above experimental results for different DNA lengths as Fig S6.

2. Perhaps more importantly, in my opinion, the manuscript is not written clearly. It would definitely benefit from an extensive editing of english language, but its lack of clarity does not depend only on this. Several key points are not addressed properly as detailed below.

—Reply: We apologize for the unclear writing. We have carefully revised the language of the entire manuscript.

3. More generally, I would suggest the authors to emphasize the connection with the polyelectrolyte theory and tone down the claims about the biophysical relevance of their results. In that respect, they should definitely acknowledge previous work (https://doi.org/10.1021/jp010861+) where the possibility of overcharging for monovalent salt had been already suggested, based on analytical and numerical results.

—Reply: We agree with you. We have completely changed the first paragraph of the introduction, not mentioning the gene regulation. In the discussion, we have removed most sentences about gene regulation, but focus on the implication on polyelectrolyte theory.

Thank you for telling us this reference. Yes, DNA overcharging with monovalent ions has been observed in simulations and theoretical calculations. In these simulations and calculations, DNA was modelled as a cylinder and water molecules were not considered. It is not sure that the phenomenon in such a simple model can be applied to realistic DNA molecules. After two decades of this work, no experimental results have confirmed that DNA overcharging can occur with monovalent ions.

—Revision:

[i] Abstract. We have switched the order of "DNA biophysics" and "polyelectrolyte theory" to highlight the primary implication of our work in polyelectrolyte theory.
[ii] Page 2. We have re-written the 1st paragraph.
[iii] Page 2. We have added: *"In addition, DNA overcharging induced by monovalent ions has been observed in coarse-grained (CG) simulations and theoretical calculation[19]. In these simulations and calculations, DNA was modelled as a cylinder and water molecules were not considered. It is not sure that the phenomenon in such a simple model can be applied to*

realistic DNA molecules. After two decades of this work, no experimental results have confirmed that DNA overcharging can occur with monovalent ions. Whether DNA overcharging by monovalent ions can occur remains not completely clear."

[iv] Page 14. When discussing the implications of our work, we first describe the implication in polyelectrolyte theory. We place the implication in biophysics at last. We have shortened the discussion about the implications on biophysics.

4. More specifically, I would suggest the authors (1) to explain in clear terms on which grounds the "monovalent salt -> no overcharge" belief was established; (2) to highlight that their estimate of the threshold concentration c0 for DNA overcharging based on Wigner crystal theory, now obtained in supplementary section S6 does indeed recover the experimental result within a 2 factor. At the same time, they should explain more carefully how their estimate of c0 is obtained (in eq.s S4-S7 several numerical factors are present whose origin is not obvious).

—Reply: Thank you for pointing out this important issue.

Regarding DNA overcharging by monovalent ions, we think the status is as follows:

(1) Deserno et al. [JPCB 105:10983 (2001)] have observed it in coarse-grained simulations and theoretical calculations.

(2) Shklovskii, Grosberg, and coworkers have investigated DNA overcharging using the Wigner crystal theory. But they think DNA overcharging can only occur with multivalent ions and carried out the quantitative calculations only for multivalent ions, not for monovalent ions. Basically, they made many approximations which are supposed to work for multivalent ions. Here is a quote from Rev. Mod. Phys 74:329 (2002): "*A system of monovalent ions, Z=1, is weakly coupled, $\Gamma \sim 1$, and this is why classical mean-field theory applies. By contrast, a system in which Z-ions have large Z is strongly coupled, and we see that R becomes larger than λ. For example, at Z=3 and $\sigma = 1.0$ e/nm^2, we get $\Gamma = 6.4$, $\lambda \approx 0.1$ nm, and $R \approx 1$nm." Accordingly, the authors did not proceed with the calculation for Z=1.*

(3) No experiments have observed DNA overcharging with monovalent ions.

We have modified and added a few sentences in the introduction and result sections to describe the current understanding of this area. In the introduction, we have added:

"*In the theory based on the Wigner crystal, the interactions between multivalent ions are so strong that they form a strongly correlated liquid or Wigner crystal, which causes DNA-DNA attraction and DNA charge inversion[13, 14]. In this theory, the interactions between monovalent ions are not strong enough to induce a Wigner crystal.*"

"*In addition, DNA overcharging induced by monovalent ions has been observed in coarse-grained (CG) simulations and theoretical calculation[19]. In these simulations and calculations, DNA was modelled as a cylinder and water molecules were not considered.*

It is not sure that the phenomenon in such a simple model can be applied to realistic DNA molecules. After two decades of this work, no experimental results have confirmed that DNA overcharging can occur with monovalent ions. Whether DNA overcharging by monovalent ions can occur remains not completely clear."

In the result section, we have added: "*While DNA overcharging with high-valent ions has been observed in experiments[12] and explained by theory[13, 14], DNA overcharging by monovalent ions is much less explored[19] and whether it can occur remains not completely clear.*"

Regarding the coefficients in our equations, here are the explanations:

(1) In Eq. S7, the parameter 1 nm is the DNA radius and the parameter 0.17 is the distance between two charges along the DNA axis.

(2) Eq. S6 and Eq. S8 are adapted from Eq. 10 and Eq. 7 in [Physica A, 274:446 (1999)], respectively.

(3) Eq. S9 is adapted from Eq. 3 and Eq. 4 in [Physica A, 274:446 (1999)], and the coefficient 1.76 is the multiplication of the two coefficients of 1.96 and 0.9.

—Revision:

[i] page 2, we have added: "*In the theory based on the Wigner crystal, the interactions …*"

[ii] page 2, we have added: "*In addition, DNA overcharging induced by monovalent …*"

[iii] page 8, we have added: "*While DNA overcharging with high-valent ions has been observed in experiments[12] and explained by theory[13, 14], DNA overcharging by monovalent ions is much less explored[19] and whether it can occur remains not completely clear.*"

[iv] SI, Sec S8. We have explained the numerical factors in equations.

Other major issues

5. - In fig. 1 and related discussion in the main text, it is not clear how the authors identify the transition force to then perform the equilibrium experiment. Fig. 1D shows the hysteresis typical of the out-of-equilibrium setups used in single molecules stretching/unzipping experiments. How is the transition force then determined? I would suggest the authors to name it "transition" and not "equilibrium" force, since they describe it as the force for which the folded and the unfolded state have the same probability.

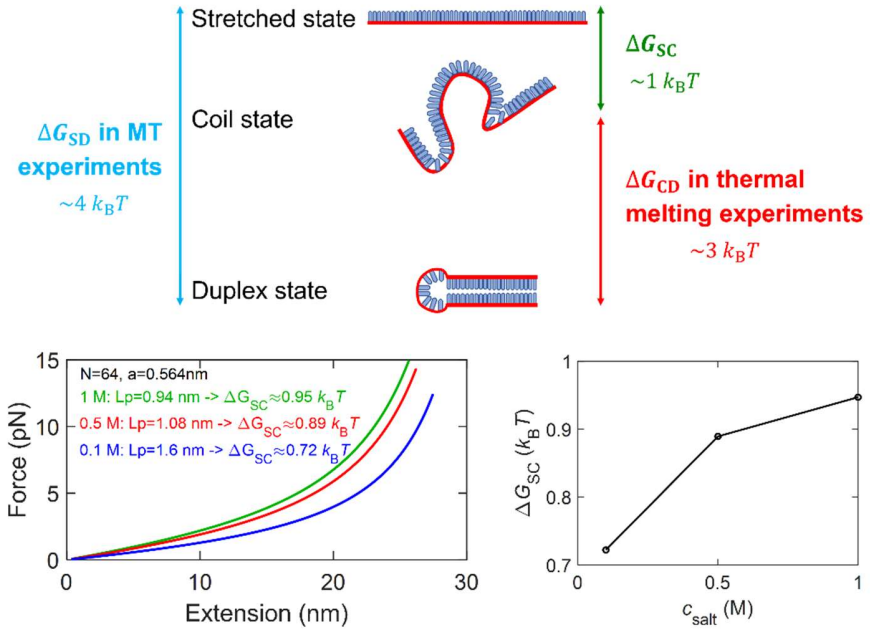
—Reply: We apologize for the confusion. In Fig 1d, the folding and refolding act as a fast scan to quickly determine the range where the transition force is located in. Then, we finely tuned the force to precisely determine the transition force where the two DNA states have the same probability. Following your suggestion, we have changed the equilibrium force to the transition force.

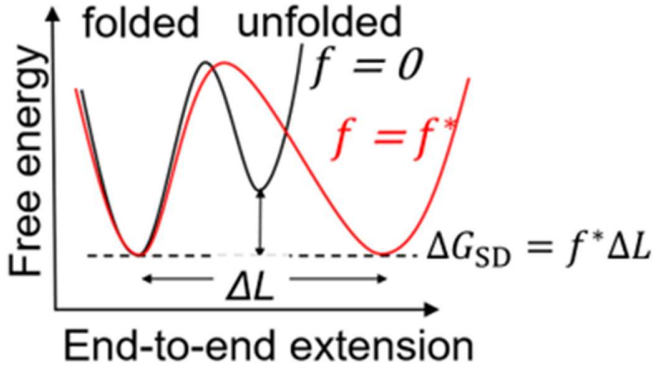
—Revision:
[i] Page 3. We have added: "*Within the range from the refolding force to the unfolding force, we searched a transition force, f^*, where the folded and stretched states of the NA hairpin had the same probability (Fig. 1e).*"
[ii] Fig 1 caption. We have replaced the "equilibrium force" by the "transition force".

6. - The estimation DeltaG = f DeltaL for the duplex stability at zero force, using DeltaL obtained at the transition force (see above for the naming), neglects the different extension of the unfolded and folded states at zero force. In other words, the unfolded minimum in Fig. 1F should changes its position on the horizontal axis. Can the authors show or at least argue that this is a reasonable approximation?

—Reply: Thank you for pointing out this important issue. Inspired by your comment, we did careful analysis for DNA free energy change in MT experiments. As shown by the figure below, there are three DNA states: stretched state (S), coil state (C), and duplex state (D). The two experiments correspond to ΔG_{SD} and ΔG_{CD}, respectively. They differ by $\Delta G_{\mathrm{SD}} - \Delta G_{\mathrm{CD}} = \Delta G_{\mathrm{SC}}$. The value of ΔG_{SC} can be estimated through the integration of the force-extension curve $\Delta G_{\mathrm{SC}}^{\mathrm{chain}} = \int_{z_c}^{z_s} f \mathrm{dz}$ from the coil state to the stretched state. Here, z_s and z_c are the extensions of the stretched and coil states, respectively, and f is the stretching force at a given extension, z. After approximating ssDNA as a wormlike chain [PRL 102:068301 (2009)], the dependence of the force on the extension is described by the Marko-Siggia equation: $f = \frac{k_B T}{L_p}\left[\frac{1}{4(1-z/L)} - \frac{1}{4} + \frac{z}{L}\right]$. Here, L_p is the ssDNA persistence length, $L = Na$ is the ssDNA contour length, $N = 64$ is the number of nucleotides for DNA hairpin construct in MT experiments, and $a \approx 0.564\ nm$ is the contour length per nucleotide [PNAS 109:799 (2012); PRE 86:021901 (2012)]. Previous experiments have obtained L_p ≈0.94 nm, 1.08 nm, and 1.6 nm at 1 M, 0.5 M, and 0.1 M NaCl, respectively. Based on the Marko-Siggia equation and f^*=12.5, 14.4, and 15 pN at 0.1, 0.5, and 1 M NaCl, we can obtain the values of z_c. Eventually, we obtained $\Delta G_{\mathrm{SC}} = \Delta G_{\mathrm{SC}}^{\mathrm{chain}}/28$ = 0.72, 0.89 and 0.95 k_BT/bp at 0.1, 0.5 and 1 M NaCl, correspondingly. Here, the 28 is the number of base pairs for DNA duplex in MT experiments.

In terms of investigating DNA overcharging, we think the stretch-duplex transition is more suitable than the coil-duplex transition, because we focus on the overcharging of the duplex state, the stretched state is a simple state, and the intra-strand interaction in the coil state may complicate the situation.





—Revision:

[i] Figure 1. We have revised Fig 1f, which shows the free energy difference between the folded duplex and unfolded stretch states.
[ii] Figure 3. We have added Fig 3c.
[iii] Page 6. We have added the discussion about ΔG_{SD} and ΔG_{CD} in MT and thermal melting experiments.
[iv] SI. We have added a new section Sec S3 about the above calculation.

7. - The authors state that error bars are obtained from "at least" three independent experiments: they actually ought to detail how many of them were carried out for all data reported in the manuscript.

—Reply: Thank you very much for pointing out this issue. We have shown the specific number of measurements

The number of experimental measurements for different ions

	LiCl	NaCl	KCl	RbCl	CsCl
MT experiments	3	3	3	3	4
thermal melting	3	6	3	3	3

—Revision:

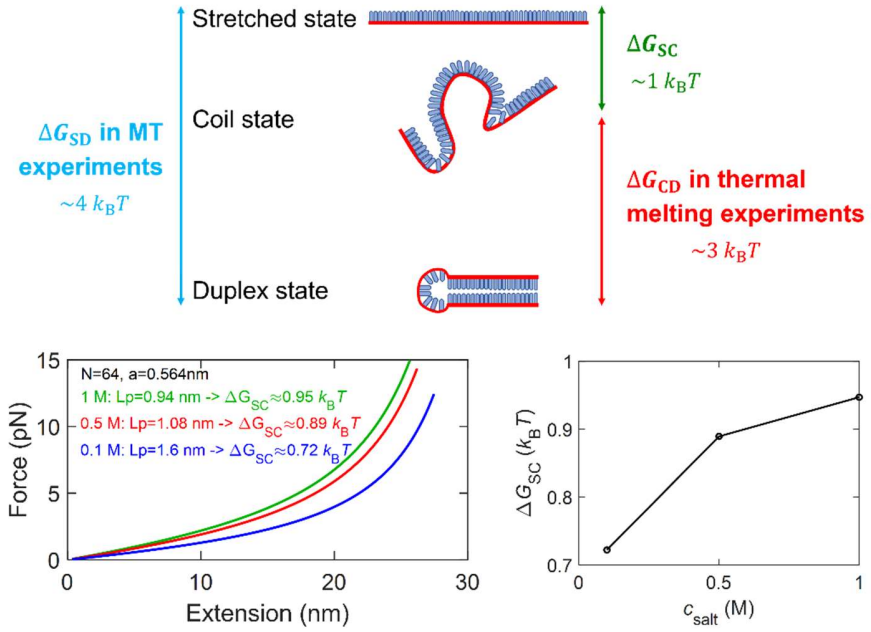
[i] Figure 1 caption. For hairpin unfolding experiments, we have added: "*The error bars are the standard errors among several independent experiments (four experiments for CsCl and three for other ions).*"

[ii] Figure 3 caption. For thermal melting experiments, we have added: "*the error bars are the standard errors among several independent experiments (six experiments for NaCl and three for other ions).*"

8. - The authors state at lines 130-131 that the differences observed in the tweezer vs thermal melting experiments are due to the different properties of the unfolded states in the two setups. This is in itself reasonable (plus see the above comment on DeltaG estimation in the tweezer experiments). However, the data in Fig. 2 and 3 show a difference in the salt ranking (look e.g. at NaCl for DNA). The authors should try and connect this observation with the above statement.

—Reply: Thank you for pointing out this important issue. As described in the reply to point #6, we did careful analysis for DNA free energy changes in MT and thermal melting experiments. The increase of ΔG_{SC} with c_{salt} should be the reason why the peak locations in Fig. 3b, i.e. c^*_{salt}, are typically smaller than the peak locations in Fig. 2b, considering that MT and thermal melting experiments correspond to ΔG_{SD}, and ΔG_{CD}, respectively, which differ by ΔG_{SC}.

The value of ΔG_{SC} depends not only on c_{salt}, but may also depend on the ion species, because ion distribution around nucleic acids varies among ion species. Furthermore, ions may mediate intra-strand interaction in the random-coiled state and affect the effective ssDNA persistence length. For example, the smaller ΔG_{CD} for Na^+ and Cs^+ at high concentrations in Fig. 3b may be caused by greater intra-strand attraction mediated by Na^+ and Cs^+, which enlarges the dependence of ΔG_{SC} on c_{salt}.



In MT experiments, NA hairpins transit between the folded duplex state and unfolded stretched state under tension, and the free energy difference is denoted as ΔG_{SD}. In thermal melting experiments, NA duplexes transit between the duplex state and a single-stranded random-coiled state, and the free energy difference is denoted as ΔG_{CD}. As shown in Fig. 3c, we have $\Delta G_{\mathrm{SD}} = \Delta G_{\mathrm{CD}} + \Delta G_{\mathrm{SC}}$, where ΔG_{SC} is the free energy between the stretched state and coil state.

The value of ΔG_{CD} at 1 M NaCl can be estimated for a given sequence using an empirical equation[29]. For our DNA hairpin sequence, ΔG_{CD} at 22 °C is 2.77 $k_{\mathrm{B}}T$/bp for 1 M NaCl. See the calculation details in Sec. S3 of SI.

The value of ΔG_{SC} can be estimated through the integration of the force-extension curve $\Delta G_{\mathrm{SC}}^{\mathrm{chain}} = \int_{z_c}^{z_s} f \mathrm{dz}$ from the coil state to the stretched state. Here, z_s and z_c are the extensions of the stretched and coil states, respectively, and f is the stretching force at a given extension, z. After approximating ssDNA as a worm-like chain[30], the dependence of the force on the extension is described by the Marko-Siggia equation: $f = \frac{k_B T}{L_p}\left[\frac{1}{4(1-z/L)} - \frac{1}{4} + \frac{z}{L}\right]$. Here, L_p is the ssDNA persistence length, $L = Na$ is the ssDNA contour length, $N = 64$ is the number of nucleotides for DNA hairpin in MT experiments, and $a \approx 0.564$ nm is the contour length per nucleotide[31, 32]. Previous experiments have obtained L_p ≈0.94 nm, 1.08 nm, and 1.6 nm at 1 M, 0.5 M, and 0.1 M NaCl, respectively. Based on the Marko-Siggia equation and f^*=12.5, 14.4, and 15 pN at 0.1, 0.5, and 1 M NaCl, we can obtain the values of z_c. Eventually, we obtained $\Delta G_{SC} = \Delta G_{\mathrm{SC}}^{\mathrm{chain}}/28$ ≈0.72, 0.89, and 0.95 $k_{\mathrm{B}}T$/bp at 0.1, 0.5

and 1 M NaCl, correspondingly. Here, the 28 is the number of base pairs of the DNA stem region in MT experiments.

The increase of ΔG_{SC} with c_{salt} should be the reason why the peak locations in Fig. 3b, i.e. c^*_{salt}, are typically smaller than the peak locations in Fig. 2b, considering that MT and thermal melting experiments correspond to ΔG_{SD}, and ΔG_{CD}, respectively, which differ by ΔG_{SC}.

The value of ΔG_{SC} depends on not only c_{salt}, but may also depend on the ion species. Ions may mediate intra-strand interaction in the random-coiled state and thus affect the effective ssDNA persistence length or make the force-extension curve deviating from the worm-like chain behavior[30]. For example, the smaller ΔG_{CD} for Na^+ and Cs^+ at high concentrations in Fig. 3b may be caused by greater intra-strand attraction mediated by Na^+ or Cs^+, which enlarges the dependence of ΔG_{SC} on c_{salt}.

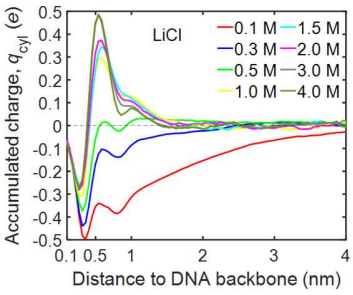
—Revision:
[i] Figure 1. We have revised Fig. 1f, which shows the free energy difference between the folded and unfolded hairpin.
[ii] Figure 3. We have added Fig 3c.
[iii] Page 6. We have added the above text.
[iv] SI. We have added a new section Sec S3 about the above calculation.

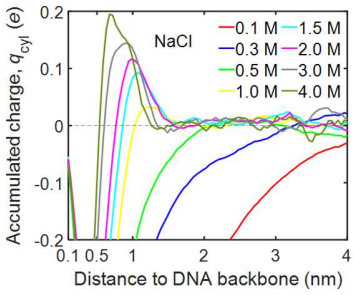
9. - Related to the above, at a more general level: the authors state repeatedly that they are discussing the stability of duplexes, whereas the typical experimental counterpart of overcharging is based on NA condensation and/or electrophoresis experiments. The point is again the non trivial properties of the unfolded state for single stranded NA. For example: could single stranded NA be as well overcharged. This is in my view a missing key point in the authors approach which could have been tackled with the help of numerical simulations (but see below)

—Reply: Thank you for this great suggestion. Following your suggestion, we have performed all-atom simulations of ssDNA and observed overcharging as expected. We have performed electrophoresis experiments for ssDNA and observed overcharging (inversion of electrophoresis).

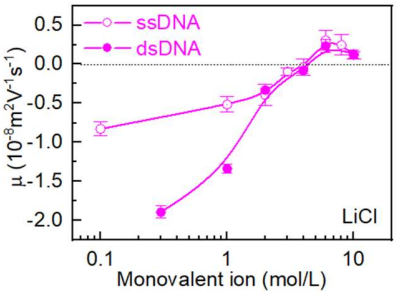
In addition, through magnetic tweezers experiments using 13751-nt ssDNA, we observed that the extension of ssDNA reversed at high concentrations of monovalent ions under constant forces, possibly due to charge reversion of the ssDNA. Under a constant force, the extension of ssDNA decreased with increasing salt concentration, likely due to the neutralization of its negative charge. However, at concentrations exceeding a threshold, the extension of ssDNA increased with increasing salt concentration, which may be attributed to charge reversion and the resulting increased repulsion between the net positive charges of the ssDNA.

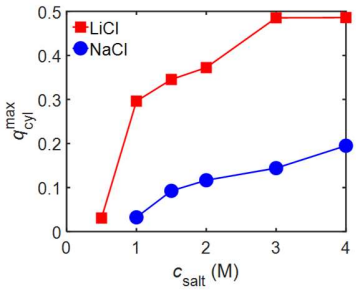
Simulation results:
to show ssDNA overcharging

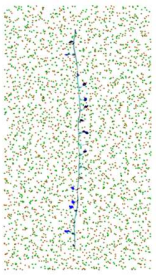




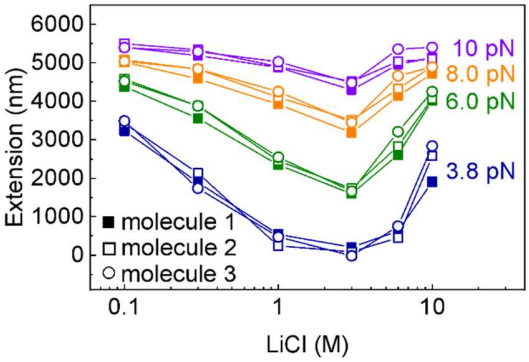
Experimental results:
to show ssDNA overcharging

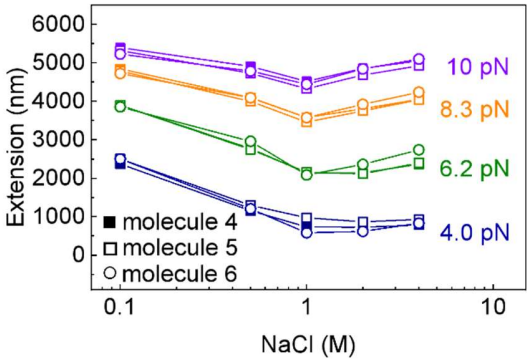


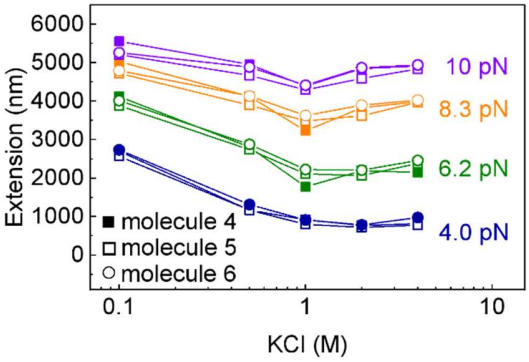


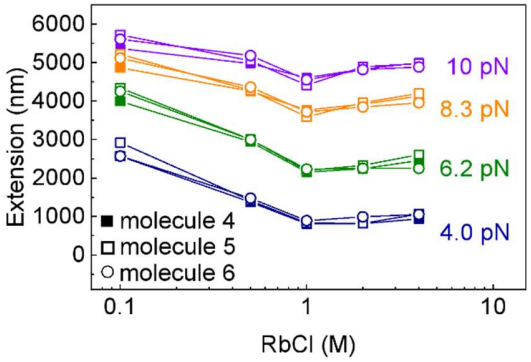


Extension of ssDNA indicates overcharging









—Reply:

[i] Page 14. We have added: "*Our all-atom simulations and electrophoresis experiments also observed single-stranded DNA overcharging at large* c_{salt}. *See Sec. S10 of SI.*"
[ii] SI. We have added a new section Sec S10 to include the results of ssDNA overcharging.

10. - I do not get the point of emphasizing the fact that oxDNA simulations (at intermediate resolution) are able to reproduce the folding/unfolding transition, whereas the more coarse-grained LAMMPS simulations are used to investigate duplex-DNA modeled as a fixed linear charged rod. The really interesting model to simulate should be folding/unfolding with explicit ions (see also the above comment).

—Reply: Thank you for your suggestion. Yes, investigating DNA overcharging using oxDNA would be a good option. However, the entire oxDNA parameterization is based on implicit ions with the Debye screening to obtain a double-helical DNA structure with properties close to experimental results. Adding explicit ions to oxDNA is very difficult, because it requires adjusting almost all oxDNA parameters.

—Revision: Main text, Page 8. We have added: "*Note that the entire oxDNA parameterization is based on inexplicit ions with the Debye screening to obtain a double-helix DNA structure with properties close to experimental results. Due to the absence of explicit ions, oxDNA cannot capture DNA overcharging with ions*."

11. - Another crucial point: the authors present two different approximate equations (1 and 2) to model how the net charge depends on concentration. It is definitely unclear which of the two is used (together with Eq.s 4 and 5) to fit experimental data in Fig. 2B. A similar lack of clarity is present in the supplementary material for eq.s S10-S11. I actually guess eq. 2 is used; if that is the case: is eq. 1 not working? How would the fit appear in the non used case? What could be learned by the fact that only one of the two approximations is working (if that is the case).

—Reply: We apologize for the confusion. We showed Eq. 1 because it is close to the theoretical equation, and we showed Eq. 2 because it has a simple linear form and is numerically close to Eq. 1 over the range of $1\ \mathrm{M} \leq c_{\mathrm{salt}} \leq 4\ \mathrm{M}$. We used Eq. 2 when deriving the overcharging electrical potential $\Phi_{\mathrm{E}}^{\mathrm{max}}$ in Eq. S15. We have added a few sentences to describe which equation is used on each occasion.

—Revision:

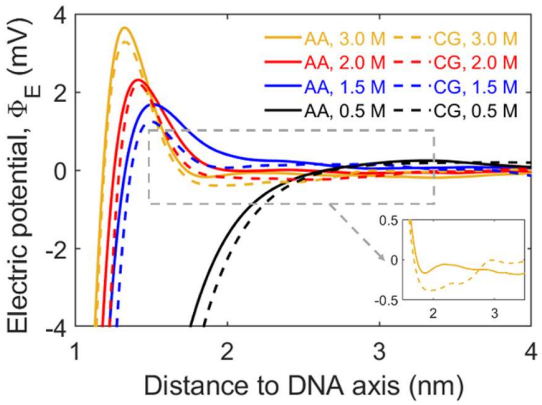
[i] Figure 2b: We have included two curves corresponding to Eq. 1 and Eq. 2, respectively.
[ii] Page 9. We have added: "*Alternatively, we can obtain an approximation of* Φ_E^{max} *from* q_{cyl}^{max} *in Eq (2). After approximating the thickness of the overcharging layer as 0.71 nm, we can obtain* $\Phi_E^{max} \approx 2.13(c_{salt} - 1\ \mathrm{M})$ *from Eq. 2, which is close to Eq. 3.*"

[iii] SI, Sec S8. We have added: "*As shown in Fig. 4c, the above equation is numerically close to a linear relationship over the range of* $1\ \mathrm{M} \le c_{salt} \le 4\ \mathrm{M}$*:* $q_{cyl}^{max} \approx 0.074(c_{salt} - 1\ \mathrm{M})$ *. (S13) For convenience, we will use the linear equation to derive the overcharging electrical potential.*"

12. - The charge patterns in Fig. 5B-C are interesting. Oscillations from positive to negative net charge regions in Fig 5C can be seen (which are not present in Fig. 5B). Can the authors comment on this feature? They should show the corresponding data (i.e for 1M and 3M concentrations) in Fig 5D. The "symbols are from previous experiments" phrase in Fig. 5C caption seems misplaced. Which symbols from which experiments?

—Reply: Yes, the curves in Fig 5d also have oscillations. We have added an inset in Fig 5d to show the oscillations. Please note that the oscillation is clear when displayed in the color map of Fig 5d, and not so clear when displayed in the linear scale in Fig 5d.
Yes, Fig 5 caption has misplacement of text, because we re-organized the sub figures a few times and forgot to adjust the figure caption accordingly. Now we have corrected it.

—Revision:
[i] Fig 5 caption. We have added: "***e*** *Phase diagram of DNA overcharging. The symbols are from experiments in our work and a previous study*[12]*.*"



13. - The authors present two different sets of experimental results (hydrogen bonds destabilization and electrophoresis) in the discussion sections. This definitely does not improve the clarity of manuscript.

—Reply: Thank you for your suggestion. We agree with you. We have removed the results and discussion about hydrogen bonds destabilization and duplex electrophoresis.

—Revision:

[i] Main text, page 11. We have removed the paragraph: “The strength of hydrogen bond is influenced by the type of monovalent salt and its…”
[ii] SI, Sec S8. We have removed this section.

14. - The interpretation of electrophoresis experiments outlined in the supplementary material (and summarized in Fig. S19) is not convincing. In particular, the inset showing velocity of Cs+ atoms as a function of distance from DNA axis is not really showing, in my view, the existence of 3 different regimes, as instead claimed by the authors.

—Reply: Following your above suggestion, we removed the results and discussion about duplex electrophoresis. Regarding the three regimes, there is no clear boundary between these regimes. These regimes were defined based on the theoretical expectation: (i) the strongest bound counterions on DNA move with the same speed as DNA; (ii) the counterions sufficiently far away from DNA cannot feel the influence of DNA and move like bulk ions; (iii) in the intermediate region, there is a gradual transition. Anyway, this part is not the essential part of this work, and we removed it following your suggestion.

—Revision:
[i] SI, Sec S8. We have removed this section about the interpretation of electrophoresis experiments.

Minor issues

15. - Which salt is used for fig. 2B-D?

—Reply: In Fig 2B and 2D, the salt for experimental data is NaCl.

—Revision: Figure 2 caption. We have added: “for the salt type of NaCl”. Also, we add the text “NaCl” in the Figures.

16. - The full geometry of the cylinder cylinder shell in Fig. 4A is not given. What is the value of the inner radius?

—Reply: We apologize for missing this information. The smallest cylinder has a radius of 1 nm, corresponding to DNA radius. The middle cylinder has a radius of 1.5 nm, corresponding to the position of the maximum overcharging. The largest cylinder has a radius of 2 nm, where the ion concentrations approach the bulk values.

—Revision: Figure 4 caption. We have added: *“The smallest cylinder has a radius of 1 nm, corresponding to DNA radius. The middle cylinder has a radius of 1.5 nm, corresponding to*

the position of the maximum overcharging. The largest cylinder has a radius of 2 nm, where the ion concentrations approach the bulk values."

Reply to Reviewer #4

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

—Reply: Thank you for taking the time to review our manuscript.

The document includes

Reply to Reviewer #1

Reviewer #1 (Remarks to the Author):

1. - The authors have addressed my previous concerns in the revision and I support publication of the manuscript at this stage.

   —**Reply:** Thank you very much for reviewing our manuscript, helping us improve the manuscript, and recognizing the significance of our work.

Reply to Reviewer #2

Reviewer #2 (Remarks to the Author):

We have not found the response and revisions to be significant enough to alter our evaluation. Specific issues regarding some of the points raised in the previous round are outlined below. Other points have been adequately addressed.

1. Points 1 and 2: The melting point versus NaCl concentration curve was previously reported in Khimji et al. (Fig 3B of current Ref 2, old Ref 11; see also Fig 3 of Tomac et al, JACS 118, 5544 (1996)), and the reported curve in the current paper is similar to that. The current paper find

similar effects for other salts. The experimental part is a reconfirmation of known effects on melting.

—Reply: We would like to highlight our unique contribution: explaining a counterintuitive and important phenomenon, destabilization of DNA duplex at high concentrations of monovalent salts through DNA overcharging. We do believe that revealing a mechanism for an important phenomenon is a significant contribution of scientific discovery.

[i] Previous experimental work reported the phenomena: destabilization of DNA duplex at high concentrations of monovalent salts.

[ii] some previous theoretical work investigated DNA charge inversion with multivalent ions.

However, the above two groups of studies are completely isolated. Nobody said [i] is caused by [ii]. Even the authors of [ii] do not expect DNA charge inversion can occur for monovalent ions based on rough estimation. We have added "due to overcharging" to emphasize the mechanism we reported in this work.

Also, in this work, we reported that RNA and RNA-DNA hybrid duplexes are also destabilized by high concentrations of monovalent salts. We also found that overcharging can occur for single-stranded DNA. These results are new and have not been reported previously.

2. Point 3: The entire discussion on Wigner crystal is insignificant as the authors themselves mention in the revised version that "In this theory, the interactions between monovalent ions are not strong enough to induce a Wigner crystal." The reported data do not satisfy Eq 1 (Fig 4c). Therefore, no remarkable phenomena would be expected.

—Reply: The Wigner crystal theory relies on a series of crude approximations, while our all-atom and coarse-grained simulations are much more realistic, and our experimental observations are facts. With the realistic data, we can evaluate which approximations in the theory are precise, qualitatively correct, or completely wrong. The authors of theory did not expect the theory works for the monovalent ions, while our simulations show the predicted charge inversion also occurs for monovalent ions.

3. Point 6: q_eff=0: Shouldn't it be "equal but opposite charge"?

—Reply: Yes. q_eff = 0 means that the absorbed counterions carry the same amount but opposite charges as DNA.

—Revision: Page 8. We have added "amount but opposite".

Reply to Reviewer #3

Reviewer #3 (Remarks to the Author):

The authors did overall a great job in addressing my comments and concerns. The quality of the manuscript greatly improved.

1. I have few requests left plus (again) the general requirement that the quality of the English language should be improved.

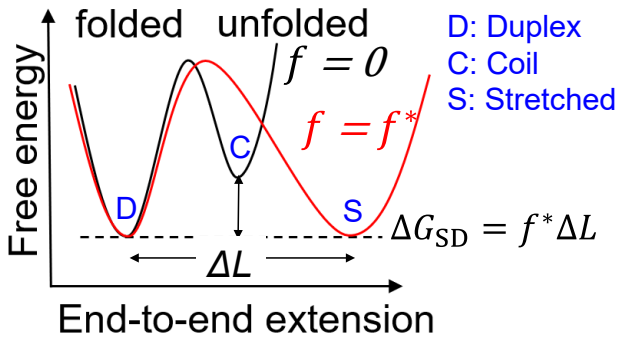
   —Reply: Thank you. We have carefully examined the writing of the entire manuscript and corrected a few language errors, marked in red.

2. - introduction, 2nd linw: DNA and DNA-> DNA and RNA (I guess)

   —Reply: Thank you. We have corrected it.

3. - to further clarify the discussion about the 3 states (D,C,S as in fig. 3c), the labels could be used for the corresponding minima in fig. 1g

   —Reply: Thank you for your suggestion. We have labelled them.



   —Revision: We have revised Fig. 1f.

4. - if I understand correctly: DeltaG_SD is measured from MT experiments (see fig. 2 data); DeltaG_CD is computed in Section S3; DeltaG_SC is computed as described at page 6. Could not then the authors test quantitative the relationship DeltaG_SD = DeltaG_SC + DeltaG_CD, at least for one salt and for one value of salt concentration? This would strengthen even more the analysis carried out by the authors

—Reply: Yes, your understanding is fully correct. Thank you for suggesting this useful calculation. At 1 M NaCl, $\Delta G_{CD} = 2.77\ k_BT/\text{bp}$ (from the nearest-neighbor model), $\Delta G_{SC} = 0.95\ k_BT/\text{bp}$ (from Marko-Siggia equation) and $\Delta G_{SD} = 3.53\ k_BT/\text{bp}$ (from MT experiments). So, $\Delta G_{CD} + \Delta G_{SC} = 2.77 + 0.95 = 3.72\ k_BT/\text{bp}$, which is close to $G_{SD} = 3.5\ k_BT/\text{bp}$, in agreement with expectation.

—Revision: Page 6, paragraph 4, we have added above description.

Reply to Reviewer #4

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

—Reply: Thank you for taking the time to review our manuscript and coaching junior reviewers.