

UNVEILING THE SOLUTION STRUCTURE OF A DNA DUPLEX WITH CONTINUOUS SILVER-MODIFIED WATSON-CRICK BASE PAIRS



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors report the first experimental structure determination of a fully metalated B-type DNA duplex. Towards this end, they prepared and investigated a palindromic dodecamer oligonucleotide, in which all purine residues are replaced by 7-deazapurines. While previously the authors had been able to confirm indirectly (based on titration studies) that such oligonucleotides form Watson-Crick-type silver(I)-mediated base pairs, the actual three-dimensional structure of such a fully metalated duplex had remained elusive. The present manuscript now reports this structure, determined by NMR spectroscopy and corroborated by further extensive characterization (SAXS, CD, MS, UV). The structure is highly significant for such fields as DNA nanotechnology, bioinorganic chemistry, and coordination chemistry. It shows that B-type DNA is fully compatible with metal-mediated base pairing. Importantly, the arrangement of the silver ions is unprecedented and contrasts that of previous reports in (different) duplexes in that the silver ions are arranged helically rather than linearly along the helix axis.

I recommend accepting this manuscript for publication, provided that the following minor issues are addressed:

UV melting: Why are the melting curves reported up to 0.75 equiv. of Ag(I) only? For all other titrations, data are also reported for stoichiometric conditions and for excess Ag(I). Moreover, why are different experimental conditions (salt concentration) used for the melting studies compared to the CD-spectroscopic studies?

ESI-MS: Why are no ESI-MS studies reported for the reference duplex?

SAXS: The authors report that with increasing Ag(I) concentration, the Guinier region shifts to lower q values. To me it appears as if it shifts to higher q values. Please clarify. Moreover, what are “high-Z silver ions”?

ASAXS studies: The authors state that in Figure 3 “I-Ag saturates at approximately 1 equivalent and II-Ag over 1.5 equivalents of Ag(I) per base pair”. The authors should indicate this by adding appropriate auxiliary lines to the plot.

NMR studies: The authors state that in Figure 4 “at about 1.5 equivalents, the signals

converged into a single set, which stayed unchanged until the endpoint of the titration".

However, they do not show the NMR spectrum recorded at 1.5 equivalents.

Figure 5: I recommend modifying the x axes of Figure 5c in a way that the DNA sequence is included. This will help the reader to correlate the structural parameters to the respective base pair (step).

Argentophilic interactions: The authors report the average distance between neighboring silver ions and conclude the absence of argentophilic interactions (in the NMR study).

However, Figure 5c shows that for selected base pairs steps, short Ag-Ag interactions are indeed observed. Could there be a sequence-dependence of the Ag-Ag distances?

DFT studies: The authors state that "buckle, propeller, opening, and major groove width parameters exhibit minor variations". However, Table S4 shows that (taking into consideration the standard errors), only the propeller twist differs between experimental and calculated structures.

Experimental section: Please check name of the CD spectrometer. Please check superscript and subscript in the NMR section.

Throughout: Please replace "T-H4" by "T-H3". Please double-check references (ref. 26 on page 12 and ref. 27 on page 15 appear to be incorrect).

Table S1: For ease of comparison, this table should also include a row with the experimental values.

Reviewer #2 (Remarks to the Author):

The authors solved the structure of the DNA-silver complex using various techniques, including UV melting experiments, CD spectra, mass spectra, solution scattering, NMR, and DFT calculations. The most important feature of the structure solved in this study is that silver ions are inserted into all of the Watson-Crick base pairs, and the double helix structure is maintained without significant distortion.

The authors state in the Abstract;

"Herein, we present the solution structures of a B-form DNA and its silver-metallized equivalent, maintaining the same conformation and base pairing by creating silver-modified

Watson-Crick base pairs."

As I comment below, I think the phrase "the same conformation" is somewhat inaccurate. Here, "the binding of Ag does not significantly distort the DNA double helix structure..." or "the binding of Ag does not disturb the base-pairing partners" would be more appropriate.

The authors have evaluated the dependence of duplex I stability on silver ion concentration in UV melting experiments. Figure S1 shows that the melting curve shifts to the high-temperature side depending on the silver ion concentration, and it appears that silver ions stabilize duplex I. However, the melting curves in the presence of silver ions (especially at 0.50 and 0.75 equiv. Ag/bp) do not appear sigmoidal, and the curves are wobbly. Figure S1 was obtained because of the use of relative absorbance. If the absolute absorbance values were used to draw the melting curve, I guess the absorbance would not have changed so much in the presence of Ag. As the authors discuss, the melting curve behavior of duplex I is very different from that of duplex II, so there is no doubt that a lot of silver is bound to duplex I. However, the idea that this is due to the stabilization of duplex I because of the formation of silver-mediated base pairs may be somewhat less convincing.

Related to the above comment, if the authors discuss the stability of silver-mediated base pairs, they would need to show that, for example, X-Ag-T base pairs are more stable than X-T base pairs. There have been several similar experiments, but most studies have compared the stability of mismatch and silver-mediated base pairs (e.g., C-C mismatch and C-Ag-C). As far as I know, the T-Hg-T base pair has been shown to be more stable than the Watson-Crick A-T base pairs.

<https://pubs.acs.org/doi/10.1021/ja056354d>

Similar experiments could probably be made for silver-mediated base pairs. Thus, if such an article exists, citing it would reinforce the discussion.

The authors state the following at the end of their discussion of CD spectral measurements; "However, in the case of the II-Ag system, the CD spectrum showed a significant conformational deviation from duplex II, suggesting the possible formation of non-canonical metallated base pairs (Fig. 1c)."

However, in Figure S1b, the melting curve of duplex II does not change its behavior significantly, even in the presence of Ag, which appears to contradict the results of the CD spectrum.

On page 6, the authors state the following;

"The length of these larger scattering species suggests that these dissolved oligomers are composed of ~4-5 helices linked end-to-end. It is worth mentioning that the formation of polymerized structures derived from discrete silver-mediated base pairs and Ag-DNA rod systems has been previously described and, consequently, should not be ruled out under our experimental conditions."

In the crystal structures of Reference 13 and 14, the bases at the ends of the DNA strand appear to be the glue that holds the multiple double strands together. However, in the NMR structure of I-Ag, there is no base that behaves as a glue. It is curious that I-Ag aggregates along the helix axis in solution without such bases. Under conditions of high Ag concentration, can I-Ag have a structure different from the NMR structure? For example, the two DNA strands forming the double helix shift up and down, resulting in a base acting as a glue at the end of this double helix.

The T-H4 on page 8, line 9, is supposed to be T-H3.

At the end of page 9, the authors state;

"the number of signals in ^1H NMR spectra increased throughout the course of titration and did not converge to a single well-defined structure by ten equivalents AgI ions per base pair (Fig. S29). These results are consistent with previous observations that AgI ions tend to promote the formation of non-canonical base pairs in DNA, such as C-AgI-C, T-AgI-T, or G-AgI-G homodimers, while the standard metallized Watson-Crick A-T and G-C are disfavored."

Is it possible that this experimental result shows not the formation of metallized non-canonical base pairs such as C-Ag-C, but rather the formation of several different structures? In any case, as with the CD spectra, the results of this experiment appear to be inconsistent with the fact that the melting curve of duplex II does not change significantly in the presence of Ag.

Related to my first comment, on Page 11 the authors say that I-Ag is similar to B-type DNA. However, Figure 5a shows that it may be more like A-type since the base pairs are not on the helix axis. I suggest adding a graph of "Shift" values as a structural parameter to Figure 5c. Then, it should be easy to see that I-Ag is similar to A-type. On the other hand, the DFT calculated structure shown in Figure 6b appears to be slightly similar to B-type DNA.

On Page 12, the authors state the following;

"The average distance between consecutive silver ions along the structure is $3.8 \pm 0.3 \text{ \AA}$, which is greater than the sum of ionic radii of silver ions (3.44 \AA), suggesting the absence of argentophilic interactions. This last observation is similar to the only other reported high-resolution NMR structure of a DNA fragment containing consecutive silver ion-mediated base pairs but different to X-ray structures."

Which NMR structure are the authors referring to? The authors need to provide a citation. It would be easier to understand if they also provided a citation for the X-ray structures.

In addition, what is the reason for the longer distance between silver ions? Wouldn't the distance between silver ions be longer when a pyrimidine-purine base pair is present next to a purine-pyrimidine base pair, and conversely, the distance between silver ions would be relatively shorter at locations where purine-pyrimidine base pairs are arranged in succession?

The above comments also respond to the authors' discussion on Page 13.

On Page 12, the authors state the following;

"To our knowledge, no comprehensive DFT study has been conducted on an entire metal-DNA system with twelve consecutive metal-modified base pairs."

The following papers would need to be cited here.

<https://pubs.acs.org/doi/10.1021/acs.jpcclett.8b02851>

In conclusion, the authors state the following;

"Importantly, our study also revealed that the structural framework of duplex I dictate the helical organization of silver ions along the central axis, in contrast to prior X-ray diffraction findings."

This feature of the silver ions winding around the helix is the reason why I believe this DNA is A-type, as I commented above.

Reviewer #3 (Remarks to the Author):

The authors present the solution structures of a B-form DNA (DNA molecules modified with 7-deazapurine) and its silver-metalized equivalent, maintaining the same conformation and base pairing by creating silver-modified Watson-Crick base pairs. They found that, unlike the Ag straight linear chain in the documented X-ray structures of Ag-DNA systems, the Ag

atoms adhere to a helical arrangement dictated by the underlying DNA structure in the new structures.

The results seem interesting, but I can not recommend the manuscript for publishing in Nature Communication for three reasons.

- 1) I am not sure if the novelty of this work meets the journal's high requirements.
- 2) The authors claim that "I-Ag remained more similar to duplex I, with reduced intensity, suggesting a close relationship between their conformations (Fig 2b). "In my opinion, these two CD spectra are not similar; the positive 280nm peak in the duplex I disappears in the I-Ag. Instead, I-Ag has a negative CD in that region. The experiment is not sufficient to give the conclusion.
- 3) The author further used DFT to study the atomic structure of I-Ag and found it is in good agreement with the NMR structure. For the DFT calculation, the optimized structures are usually close to the initial structures. If the initial structure has a spiral Ag chain, the structure optimization will most likely obtain a similar structure. I suggest doing molecular dynamics (ex QM/MM) to check the stability.

Reviewer #4 (Remarks to the Author):

I was asked to comment on the SAXS data of this work on silver modified DMA duplexes. Unfortunately I have to say that these SAXS data are of rather poor quality both in view of intensity (about one order of magnitude only) as well as in q-range covered (limited by background at high q but also low instrument performance at small q). This seems to be an unlucky combination of working at low concentrations on the one hand, but maybe also low amount of sample in the beam, especially at high energies(25 keV for ASAXS). Since the sample cell / capillary diameter is not mentioned (or I did not find it) it remains unclear how the sample was measured. In response, all of the analysis is really not so exciting and hardly surpasses a Guinier analysis. This is all in huge contrast to the reference 14, which shows how atomic resolution was obtained for their reference system (duplex II with Ag). The

authors also refer to other references, which make use of wide angle data, which are here buried in the background. After this general complains, some detailed comments on the data.

1) For the bare duplex, the minimum q -range seems appropriate, since there is a clear plateau. With addition of Ag, the plateau is not reached, intensity keeps rising. Why did you not try to go to smaller q to understand the nature of this intensity increase towards low q ? This should be possible at lab sources optimized for SAXS. Maybe the intensity rise is due to aggregation, maybe due to filament formation, difficult to say without data.

2) All SAXS data show a giant peak at $q=2 \text{ \AA}^{-1}$. Given that buffer was subtracted, what is the nature of this peak?

3) Related to 2, which buffer do you subtract, buffer with Ag or buffer without Ag? Does it make a difference? It is clear what to use?

3) Most of the labels in the SI are unreadable.

Overall, I think these SAXS data lack impact. On the other hand, the authors clearly know how to analyze these data (if they were good enough), so I would like to encourage more measurements at higher concentrations and better instrument settings and/or try to crystallize as in Ref 14.

Point-by-point response to the reviewers' comments, reproduced verbatim

Reviewer #1

1.1. UV melting: Why are the melting curves reported up to 0.75 equiv. of Ag(I) only? For all other titrations, data are also reported for stoichiometric conditions and for excess Ag(I). Moreover, why are different experimental conditions (salt concentration) used for the melting studies compared to the CD-spectroscopic studies?

We appreciate the reviewer's feedback, and for data consistency and easy comparison, we conducted new UV-melting curves at the relevant equivalents using the same salt and pH conditions as those utilized for the CD, SAXS and NMR data. We have also corrected an error (pH value) that was detected in the CD conditions.

Moreover, we have included and analyzed new CD-melting experiments on the duplexes with and without Ag(I) ions to enhance our understanding of the denaturing process of the duplexes in the presence of Ag(I) ions.

We initially excluded the curve for 1 equiv. of Ag(I) from our graph due to a flat UV-melting curve at this stoichiometry that led to noisy normalized data. However, in our new graphs, we have included all stoichiometric points and plotted the variation of absolute UV absorption (instead of normalized data) for a clearer understanding of the melting process.

We have also revised the main text based on this new data.

1.2. ESI-MS: Why are no ESI-MS studies reported for the reference duplex?

We appreciate this comment and the ESI-MS results for the oligonucleotides have now been added to the supporting information along with the HPLC chromatogram for ODN1 (Figure S30-S32).

1.3. SAXS: The authors report that with increasing Ag(I) concentration, the Guinier region shifts to lower q values. To me it appears as if it shifts to higher q values. Please clarify. Moreover, what are "high-Z silver ions"?

The reviewer is absolutely correct regarding the shift to higher q-values. We have completely revamped the SAXS section, see answer to 4.1 (reviewer 4).

Also, we understand the confusion and, consequently, we have removed the term "high-Z". It just meant heavier scatterers than the DNA.

1.4. ASAXS studies: The authors state that in Figure 3 "I-Ag saturates at approximately 1 equivalent and II-Ag over 1.5 equivalents of Ag(I) per base pair". The authors should indicate this by adding appropriate auxiliary lines to the plot.

We appreciate the comment from the reviewer and added auxiliary plots to the graphs and rewritten the sentence to: "However, I-Ag saturates at approximately 1 equivalent and II-Ag does not appear to saturate throughout the duration of the experiment".

1.5. NMR studies: The authors state that in Figure 4 "at about 1.5 equivalents, the signals converged into a single set, which stayed unchanged until the endpoint of the titration". However, they do not show the NMR spectrum recorded at 1.5 equivalents.

The requested titration point has been added to the figure. Additionally, a graph of relative intensities of methyl groups belonging to the starting and end structures through the course of the titration has been added.

1.6. Figure 5: I recommend modifying the x axes of Figure 5c in a way that the DNA sequence is included. This will help the reader to correlate the structural parameters to the respective base pair (step).

Thank you for your suggestion regarding Figure 5c. We have implemented the modification by including the DNA sequence on the x-axis.

1.7. Argentophilic interactions: The authors report the average distance between neighboring silver ions and conclude the absence of argentophilic interactions (in the NMR study). However, Figure 5c shows that for selected base pairs steps, short Ag-Ag interactions are indeed observed. Could there be a sequence-dependence of the Ag-Ag distances?

We appreciate the observation regarding argentophilic interactions in our study. Indeed, while our NMR study suggests an absence of such interactions on average, Figure 5c reveals short Ag-Ag interactions for selected base pair steps. This discrepancy may indeed indicate a sequence-dependence of Ag-Ag distances.

There is step-to-step variation in the distances between the imino protons in base pairs in canonical DNA dictated by the differences in purine versus pyrimidine geometries, as well as small differences in AT versus GC base pair geometries, stacking force and sterical conflict optimization. All of these effects will also determine the structure of the present system. It could be argued that it is a reflection of the fact that the interactions of the DNA nucleotides are the primary drivers of structure properties in the presented system. The fact that it is apparently possible to have Ag atoms in sufficient proximity to form argentophilic interactions under the control of the scaffold is relevant since it should influence the electronic properties of the system, and the reflection is included in the discussion of the results.

1.8. DFT studies: The authors state that "buckle, propeller, opening, and major groove width parameters exhibit minor variations". However, Table S4 shows that (taking into consideration the standard errors), only the propeller twist differs between experimental and calculated structures.

We appreciate this meticulous observation and rephrase this sentence in the main text: "In addition, the parameters of buckle, opening, and major groove width appear to show minor variations considering the standard errors. However, the propeller angle ($-8^{\circ} \pm 9$) appears to be more distinct from that revealed in the NMR ($-21^{\circ} \pm 4$)."

1.9. Experimental section: Please check name of the CD spectrometer.

Thank you for bringing attention to the error in the name of the CD spectrometer. We have corrected this oversight and revised the name accordingly.

1.10. Please check superscript and subscript in the NMR section.

Thanks, we have checked them.

1.11. Throughout: Please replace "T-H4" by "T-H3". Please double-check references (ref. 26 on page 12 and ref. 27 on page 15 appear to be incorrect).

Thank you for bringing attention to his mistake, we have corrected it.

1.12. Table S1: For ease of comparison, this table should also include a row with the experimental values.

We acknowledge the reviewer's concern. However, we did not calculate the melting temperature due to the coexistence of different metallized and unmetallized duplexes during the titration experiment (as revealed by the NMR experiments). We believe that providing melting temperatures under these conditions could be misleading. Therefore, our primary focus at this stage was not to report the exact melting temperature of the systems but rather to demonstrate the thermal stabilization of the duplexes resulting from the presence of silver ions in solution. We have revised the main text to incorporate this clarification.

Reviewer #2

2.1 The authors state in the Abstract;

"Herein, we present the solution structures of a B-form DNA and its silver-metallized equivalent, maintaining the same conformation and base pairing by creating silver-modified Watson-Crick base pairs."

As I comment below, I think the phrase "the same conformation" is somewhat inaccurate. Here, "the binding of Ag does not significantly distort the DNA double helix structure..." or "the binding of Ag does not disturb the base-pairing partners" would be more appropriate.

We appreciate the comment and we have rephrased the sentence: "In this study, we present the solution structure of a DNA duplex that can be transformed into its metallized equivalent while retaining the natural base pairing arrangement through the creation of silver-modified Watson-Crick base pairs. Unlike previously documented X-ray structures of Ag-DNA systems, our research demonstrates the feasibility of preserving the intrinsic DNA self-assembly while incorporating AgI into the double helix, illustrating that the binding of silver does not disrupt the canonical base-pairing organization."

2.2. "The authors have evaluated the dependence of duplex I stability on silver ion concentration in UV melting experiments. Figure S1 shows that the melting curve shifts to the high-temperature side depending on the silver ion concentration, and it appears that silver ions stabilize duplex I. However, the melting curves in the presence of silver ions (especially at 0.50 and 0.75 equiv. Ag/bp) do not appear sigmoidal, and the curves are wobbly. Figure S1 was obtained because of the use of relative absorbance. If the absolute absorbance values were used to draw the melting curve, I guess the absorbance would not have changed so much in the presence of Ag. As the authors discuss, the melting curve behavior of duplex I is very different from that of duplex II, so there is no doubt that a lot of silver is bound to duplex I. However, the idea that this is due to the stabilization of duplex I because of the formation of silver-mediated base pairs may be somewhat less convincing".

We appreciate the reviewer's comments on the UV melting experiments. We have registered new UV-melting experiments using the same salt concentration and pH as the CD, SAXS, NMR studies. Moreover, we have now included the UV-melting curves that illustrate absolute UV absorption variation (instead of normalized values) for a clearer comprehension of the melting process. Also, we have conducted additional comprehensive CD-melting experiments to enhance our understanding of the denaturing process of the duplexes in the presence of Ag(I) ions.

The primary conclusion drawn from the melting/denaturing experiments is the thermal stabilization of the duplexes in the presence of silver ions in solution, as opposed to the duplexes formed solely by hydrogen bonds in the absence of metal ions. The relationship between the thermal stabilization and the formation of silver-mediated base pairs is firmly established in the literature. There are numerous studies on metallized DNA duplexes support this phenomenon (some examples: Chem. Eur. J. 2017, 23, 17166-17178, DOI:10.1002/chem.201703518 // Chem. Eur. J. 2018, 24, 8883–8892, DOI:10.1002/chem.201801273). Moreover, our study shares important similarities with the work published in Ref.14 (Nature Chemistry 2017, DOI: 10.1038/NCHEM.2808). This study features a twelve-mer duplex with metal-mediated base pairs, demonstrating similar melting behaviour to our observations.

We have revised the UV-melting section to clarify our findings. It's essential to note that our claim regarding the formation of silver-metallized base pairs is not solely

dependent on melting/denaturing duplex experiments. Our study also present NMR data, along with other supporting experimental evidence, collectively providing unambiguous confirmation of the formation of metal-modified base pairs.

2.3. Related to the above comment, if the authors discuss the stability of silver-mediated base pairs, they would need to show that, for example, X-Ag-T base pairs are more stable than X-T base pairs. There have been several similar experiments, but most studies have compared the stability of mismatch and silver-mediated base pairs (e.g., C-C mismatch and C-Ag-C). As far as I know, the T-Hg-T base pair has been shown to be more stable than the Watson-Crick A-T base pairs.

<https://pubs.acs.org/doi/10.1021/ja056354d>

Similar experiments could probably be made for silver-mediated base pairs. Thus, if such an article exists, citing it would reinforce the discussion.

We appreciate the reviewer's concern. Prior to elucidating the solution structure of the Ag-DNA presented in this work, we carried out studies on the formation and stability of the X-T and Y-C base pairs, both before and after the addition of Ag(I) ions. These studies have been published in "Angew. Chem. Int. Ed. 2016, DOI: 10.1002/anie.201600924" (ref. 16) and "Chem. Eur. J. 2018, DOI: 10.1002/chem.201705131" (ref. 15). In these studies, we demonstrated the higher stability of these silver-metallized base pairs involving the use of 7-deazapurines.

It is important to note that the stability of the metallized X-T or Y-C base pairs cannot be effectively studied when they are included within a canonical sequence. This is because natural A-T and G-C base pairs also offer similar binding pattern and will compete for the binding of the Ag(I) ions. Our preliminary studies (not published) revealed that mixing the 7-deazapurines with canonical purine bases in the same duplex often led to precipitation events. However, this was not observed when substituting all purine bases with 7-deazapurine bases. Therefore, our studies employed duplexes with the corresponding base pairs, modified or canonical, drawing conclusions through comparison between them.

2.4. The authors state the following at the end of their discussion of CD spectral measurements;

"However, in the case of the II-Ag system, the CD spectrum showed a significant conformational deviation from duplex II, suggesting the possible formation of non-canonical metallated base pairs (Fig. 1c)."

However, in Figure S1b, the melting curve of duplex II does not change its behavior significantly, even in the presence of Ag, which appears to contradict the results of the CD spectrum.

We appreciate the reviewer's comments on this matter. As mentioned before (answer 2.2), we have registered new UV-melting experiments using the same salt

concentration and pH as the CD, SAXS and NMR studies. The new UV-melting curves are presented as UV absorption variances of absolute values, and they display important changes in the melting behaviour of duplex II upon adding Ag(I) ions, thus supporting their binding to the duplex. These new results align with the CD melting and titration results. We have revised the melting/denature section for better understanding.

2.5. On page 6, comment: "the authors state the following; The length of these larger scattering species suggests that these dissolved oligomers are composed of ~4-5 helices linked end-to-end. It is worth mentioning that the formation of polymerized structures derived from discrete silver-mediated base pairs and Ag-DNA rod systems has been previously described and, consequently, should not be ruled out under our experimental conditions".

In the crystal structures of Reference 13 and 14, the bases at the ends of the DNA strand appear to be the glue that holds the multiple double strands together. However, in the NMR structure of I-Ag, there is no base that behaves as a glue. It is curious that I-Ag aggregates along the helix axis in solution without such bases. Under conditions of high Ag concentration, can I-Ag have a structure different from the NMR structure? For example, the two DNA strands forming the double helix shift up and down, resulting in a base acting as a glue at the end of this double helix.

We appreciate the comment. Our duplex has not been designed to have a hanging base that could act as a glue as reported in references 13 and 14, and the formation of longer structures was unexpected in our case.

However, during the revision of our SAXS studies following reviewers' comments and suggestions, we found out that the initial in-bench SAXS experiments were not conducted correctly as planned, and a significant excess of Ag(I) was employed instead of expected stoichiometric amounts (see answer to 4.4, Reviewer 4). Consequently, we have then conducted new SAXS experiments using 1.1 equivalent of Ag(I) pair base pair, corresponding to saturation point as revealed by CD titration experiments. Additionally, we have expanded the SAXS experiment to cover smaller "q values" to offer further insights into the solution behaviour of the duplex I-Ag system. The new SAXS results have been explained in the main text and are consistent with other experimental data, confirming the formation of a metallized duplex I with the expected size dimensions, without the formation of larger species.

2.6. The T-H4 on page 8, line 9, is supposed to be T-H3.

Fixed the T-H4 to T-H3

2.7. At the end of page 9, the authors state;

"the number of signals in ¹H NMR spectra increased throughout the course of titration and did not converge to a single well-defined structure by ten equivalents AgI ions per base pair (Fig. S29). These results are consistent with previous observations that AgI ions tend to promote the formation of non-canonical base pairs in DNA, such as C-AgI-C, T-AgI-T, or G-AgI-G homodimers, while the standard metallized Watson-Crick A-T and G-C are disfavored."

Is it possible that this experimental result shows not the formation of metallized non-canonical base pairs such as C-Ag-C, but rather the formation of several different structures? In any case, as with the CD spectra, the results of this experiment appear to be inconsistent with the fact that the melting curve of duplex II does not change significantly in the presence of Ag.

Yes, we believe it is both. The listed non-canonical base pairs are generally known as the most stable silver mediated base pairs (C-Ag-C in particular) and their formation drives the structuring of Ag-DNA oligonucleotides for the cases where detailed structural information is available in standard DNA. Our expectation is that the preference for non-canonical pairing of the silver mediated base pairs will cause the breakup of the original canonical pattern, at the cost of leaving some bases exposed or forming suboptimal base pairs. The sum energy effect might not be overwhelmingly stabilizing, and it is likely that several arrangements with comparable energy exist, leading to an ensemble of structures. It is also likely that exposed bases will form interactions with oligonucleotides outside the duplex leading to aggregation. The text in the article has been corrected to express this more clearly.

The UV-melting experiment has been conducted under conditions now aligned with those of the CD experiments. Furthermore, new comprehensive CD-melting experiments have been conducted and integrated into the manuscript. The new data clearly shows that the Ag(I) ions bind to both duplexes I and II, albeit resulting in different conformational consequences.

2.8. Related to my first comment, on Page 11 the authors say that I-Ag is similar to B-type DNA. However, Figure 5a shows that it may be more like A-type since the base pairs are not on the helix axis. I suggest adding a graph of "Shift" values as a structural parameter to Figure 5c. Then, it should be easy to see that I-Ag is similar to A-type. On the other hand, the DFT calculated structure shown in Figure 6b appears to be slightly similar to B-type DNA.

A graph of shift values was added to the figure. The average shift is $-2.7 \text{ \AA} \pm 0.3 \text{ \AA}$ for I-Ag, intermediate between B-type average values (0.8 \AA) than A-type (-4.1 \AA), but indeed closer to A-type structure. The shift is also greater in magnitude compared to

duplex I, possibly an adjustment related to the accommodation of the silver ion, along with the increase in the opening of the base pairs. The structure of duplex I also has increased larger shift compared to B-type DNA, so some of the effect might be related to the absence of nitrogen at position 7, and the related changes in electrostatic and steric interactions in the major groove.

Other structural parameters related to backbone structure, such as helical rise, and distance between consecutive phosphorous atoms, are closely aligned to typical values for B-type duplexes, leading us to assign the structure to that general family. A graph of the P-P distances was also added to the figure, and typical A-type values are indicated in the graphs alongside B-type values in the graphs.

Nevertheless, we have taken the reviewer's comment into consideration and removed the statement "...B-form structure" from the title of the manuscript. Instead, we now refer to the "...solution structure". Our understanding of the B-form is then retained for discussion throughout the manuscript.

2.9. On Page 12, the authors state the following;

"The average distance between consecutive silver ions along the structure is 3.8 ± 0.3 Å, which is greater than the sum of ionic radii of silver ions (3.44 Å), suggesting the absence of argentophilic interactions. This last observation is similar to the only other reported high-resolution NMR structure of a DNA fragment containing consecutive silver ion-mediated base pairs but different to X-ray structures."

Which NMR structure are the authors referring to? The authors need to provide a citation. It would be easier to understand if they also provided a citation for the X-ray structures.

In addition, what is the reason for the longer distance between silver ions? Wouldn't the distance between silver ions be longer when a pyrimidine-purine base pair is present next to a purine-pyrimidine base pair, and conversely, the distance between silver ions would be relatively shorter at locations where purine-pyrimidine base pairs are arranged in succession?

The above comments also respond to the authors' discussion on Page 13.

(Answer to 2.9 & 2.11) References are added. The distances depend on the sequence, and have some relations to patterns seen in A-type DNA models, possibly because of the similar shift and slide parameters. More discussion is already included above.

2.10. On Page 12, the authors state the following;

"To our knowledge, no comprehensive DFT study has been conducted on an entire metal-DNA system with twelve consecutive metal-modified base pairs."

The following papers would need to be cited here.

The work referred to by the referee pertains to an experimental study of "parallel-stranded structures consisting of up to "20 [G–Ag(I)–G]", where the theoretical calculations only extend to fragments of six units ([dG6]2•Ag6 duplex and [dC6]2•Ag6 duplex using DFT with M06-2X functional). In order to acknowledge this work, we have rephrased our statement to: "Previous theoretical DFT (M06-2X) investigation in this area have been limited to fragments of up to six units in size (Ref. 21).

2.11. In conclusion, the authors state the following;

"Importantly, our study also revealed that the structural framework of duplex I dictate the helical organization of silver ions along the central axis, in contrast to prior X-ray diffraction findings."

This feature of the silver ions winding around the helix is the reason why I believe this DNA is A-type, as I commented above.

(Answer to 2.9 & 2.11) References are added. The distances depend on the sequence and have some relation to patterns seen in A-type DNA models, possibly because of the similar shift and slide parameters. More discussion is already included above.

Reviewer #3:

The results seem interesting, but I can not recommend the manuscript for publishing in Nature Communication for three reasons.

3.1. I am not sure if the novelty of this work meets the journal's high requirements.

We respect the reviewer's perspective on the novelty of our work. However, we would like to underscore the significance of our findings in addressing a long-standing pursuit in metal-DNA research. The formation of metallized DNA double helices, where metal ions are inserted into the central axis while adhering to the Watson-Crick pattern, has been a coveted objective in the scientific community.

Our study presents a unique approach towards achieving this objective, providing not only a high-resolution solution structure but also a comprehensive analysis of the system's behavior in solution and the organization of metal ions within it. Notably, while a few double helix solid structures incorporating continuous array of metal ions have been reported in prestigious journals such as Nature Chemistry (ref. 14) and Angewandte Chemie (ref. 13), these structures do not adhere to the natural base pairing of DNA. Furthermore, our work distinguishes itself by delving into solution studies, unlike previous reports that focused on solid-state structures. This aspect is

particularly significant as it prompts speculation regarding the existence of such structures in solution versus solely in the solid state.

Additionally, while other solution studies have explored DNA containing metal ions within specific locations, they have been limited in scope and have not involved the metallization of Watson-Crick base pairs. Thus, we believe our work represents a significant advancement in the field, offering novel insights into the metallization of DNA double helices and paving the way for further exploration in this area.

3.2. The authors claim that "I-Ag remained more similar to duplex I, with reduced intensity, suggesting a close relationship between their conformations (Fig 2b). "In my opinion, these two CD spectra are not similar; the positive 280nm peak in the duplex I disappears in the I-Ag. Instead, I-Ag has a negative CD in that region. The experiment is not sufficient to give the conclusion.

We appreciate the feedback provided by the reviewer, but we respectfully disagree with the assessment made. Upon comparing the CD spectra of duplex I and I-Ag in the presence of one equivalent of Ag(I) ions, it is evident that differences arise, as expected due to the metallization process within the duplex, which results in structural widening of the helix (given that Ag ions are larger than protons).

However, it's important to note that despite these differences, the overall profile of the spectra remains comparable, with both spectra exhibiting a decline around 245 nm and an ascent around 280 nm, indicating closely superimposable profiles, with the main distinction being the reduction in intensity. While we acknowledge an opposite trend observed at 300nm, we contend that the fundamental profile of the spectra remains consistent, indicating a close relationship between the conformations of duplex I and I-Ag.

Furthermore, comparing the spectra of reference oligo II in the presence of Ag reveals more pronounced differences (complete inversion with respect to each other, resembling mirror images), emphasizing a distinct metallization pattern between duplex I and II, resulting in markedly different structures. This comparison underscores the unique characteristics of each metallized duplex and further supports our conclusion regarding some similarity between the conformations of duplex I and I-Ag.

Importantly, the NMR structure provided in our study further supports our CD conclusions. The NMR and DFT data offer detailed insights into the three-dimensional structure of the metallized duplex, corroborating the observations made from the CD spectra analysis. This additional experimental evidence strengthens the validity of our findings and reinforces the conclusion regarding the similarity between the conformations of duplex I and I-Ag.

3.3. The author further used DFT to study the atomic structure of I-Ag and found it is in good agreement with the NMR structure. For the DFT calculation, the optimized

structures are usually close to the initial structures. If the initial structure has a spiral Ag chain, the structure optimization will most likely obtain a similar structure. I suggest doing molecular dynamics (ex QM/MM) to check the stability.

We thanks to referee for the insightful comment regarding our use of DFT in studying the atomic structure of the compound studied. We also appreciate the suggestion to employ molecular dynamics (specifically QM/MM) to verify the stability of the I-Ag.

In response to this suggestion, we would like to highlight an essential aspect of our findings. It should be noticed that although the optimized structures would predominantly exhibit a "spiral Ag chain" configuration, we also observed that the final structure has some sections that deviate from this pattern. Specifically, three silver atoms are arranged almost linearly, with an angle of approximately 173° between them. This observation suggests that all atoms, including silver, had sufficient freedom of movement during the geometric optimization process to adopt the most stable linear arrangement for Ag atoms.

Furthermore, we would like to address why we have chosen not to pursue QM/MM methodologies. Firstly, we have reservations regarding the accuracy of MM methods, which could introduce inaccuracies in our calculations. Additionally, our preliminary attempts at molecular dynamics using force fields incorporating silver parameters yielded unsuccessful results in accurately reproducing the experimentally determined NMR geometries. Notably, these experimental geometries are precise for all atoms except silver, indicating the incompatibility of entirely linear silver arrangements with the DFT results.

Therefore, based on our findings and the limitations of alternative methodologies, we believe that our approach using DFT provides valuable insights into the atomic structure of I-Ag. In this work, it is essential to consider the important experimental results obtained by NMR, providing a validated structure to corroborate the optimized structure found by computational analysis.

Reviewer #4:

I was asked to comment on the SAXS data of this work on silver modified DMA duplexes. Unfortunately I have to say that these SAXS data are of rather poor quality both in view of intensity (about one order of magnitude only) as well as in q-range covered (limited by background at high q but also low instrument performance at small q). This seems to be an unlucky combination of working at low concentrations on the one hand, but maybe also low amount of sample in the beam, especially at high energies (25 keV for ASAXS). Since the sample cell / capillary diameter is not mentioned (or I did not find it) it remains unclear how the sample was measured. In response, all of the analysis is really not so exciting and hardly surpasses a Guinier analysis. This is all

in huge contrast to the reference 14, which shows how atomic resolution was obtained for their reference system (duplex II with Ag). The authors also refer to other references, which make use of wide angle data, which are here buried in the background. After this general complains, some detailed comments on the data.

4.1. For the bare duplex, the minimum q-range seems appropriate, since there is a clear plateau. With addition of Ag, the plateau is not reached, intensity keeps rising. Why did you not try to go to smaller q to understand the nature of this intensity increase towards low q? This should be possible at lab sources optimized for SAXS. Maybe the intensity rise is due to aggregation, maybe due to filament formation, difficult to say without data.

We thank the reviewer for this comment, which prompted us to reevaluate our experiments, and in the process, we identified significant errors. In our efforts to revisit this data and collect data to lower q-value (we could only get down to 0.01 reciprocal angstroms, but it turns out this is enough), we unfortunately found an error in the student's preparation of the samples. The student miscalculated by an order of magnitude, so the originally presented data had a significant excess of stoichiometric Ag! We have replaced figure 2 with the new collected data and shifted the analysis and discussions accordingly. We apologize for this error; it was collected during the beginning of return-to-work during pandemic, so the student did not have a lot of direct oversight. However, we are grateful to have found this error, thanks to reviewer 4.

4.2. All SAXS data show a giant peak at $q=2 \text{ \AA}^{-1}$. Given that buffer was subtracted, what is the nature of this peak?

We have learnt that background subtraction is never perfect. When dissolving an analyte in the solvent, solvation spheres are created, along with ion association, etc, thus changing the background. Therefore, it is impossible to create a perfect background for subtraction-it simply does not exist. Because we have had the fortune of a wide-angle extension on our benchtop SAXS, we have observed this for a decade now in studying relatively small molecules or light scatterers, which represents the majority of the solutions we study. Only very strongly scattering species (based on elemental composition and size) will overwhelm solvent scattering, so that these sorts of peaks are not observed. Also the scattering signal from both the DNA samples either in SAXS or ASAXS data were mainly observed and analyzed below 0.35 \AA^{-1} . Although the perfect background subtraction could not be achieved, the peaks observed at 2 \AA^{-1} due to background are of least consequence to our results.

4.3. Related to 2, which buffer do you subtract, buffer with Ag or buffer without Ag? Does it make a difference? It it clear what to use?

We have done both and gotten similar results. The presented data has subtraction of the buffer.

4.4. Most of the labels in the SI are unreadable.

We apologize for this, and we have now included an expanded images of the labels for each graph.

Overall, I think these SAXS data lack impact. On the other hand, the authors clearly know how to analyze these data (if they were good enough), so I would like to encourage more measurements at higher concentrations and better instrument settings and/or try to crystallize as in Ref 14.

It is imperative to underscore that the SAXS, despite their limitations, contribute significantly to our understanding of the solution behaviour of the systems under study. Our intention is not to furnish conclusive structural details of the I-Ag system solely from the SAXS/ASAXS data. In addition, ASAXS played an important set of data to further demonstrate the positioning of Ag(I) ions within the double helix. On the other hand, the NMR data provide robust and detailed insights into the Ag-DNA structure, which is supplemented by additional solution studies detailed in the manuscript.

We acknowledge the suggestion regarding the crystallization of our systems, and indeed, we have made concerted efforts in this regard. Nevertheless, while our endeavours to crystallize the I-Ag system persist, it is paramount to recognize that solution structures often diverge significantly from solid-state structures. Indeed, it is plausible that distinct species may coexist in solution and solid state within a metallized DNA sample. Hence, our data are instrumental in elucidating the molecule's behaviour in solution, offering insights that solid-state structures alone cannot afford.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have performed several additional experiments, are now using identical experimental conditions for the various spectroscopic techniques, and have corrected some flawed datasets. As a result, the manuscript was improved significantly, and publication is recommended.

I merely have one minor request: Regarding my initial recommendation that Table S1 should also include a row with the experimental values (for ease of comparison), the authors responded that they did not determine experimentally the melting temperatures. However, Table S1 lists the theoretical masses of the mass spectra, so the lacking experimental data are the experimental masses from the spectra. I still believe that compiling these values in the table would improve the manuscript even further.

Reviewer #2 (Remarks to the Author):

The authors have answered the questions I have raised and have appropriately added new data and references.

Reviewer #3 (Remarks to the Author):

The authors have added a considerable amount of new data, and the revised manuscript better shows the novelty of the work. Although I am not entirely convinced by the authors' reply regarding the CD, the conclusion appears to be supported by the NMR and SAXS. I am not an expert on NMR and SAXS. Therefore, I would recommend the work be accepted if the other reviewers think the NMR and SAXS sufficiently support the conclusion.

Reviewer #4 (Remarks to the Author):

Thank you for repeating the SAXS measurement. The new data in Fig 2 look much more convincing.

Since the authors have addressed all my comments, I have no further requests for changes.