

Chemical and photoinduced interstrand crosslinking of oligo DNA duplexes containing 2'-deoxythioguanosines

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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)

Osman, Onizuka, Yamano and Nagatsugi report the efficient cross-linking of two DNA strands by reacting 6-thioguanine bases to the corresponding disulfide-linked dimers. The TG residues are located in opposite strands of the duplex at neighboring positions. The crosslinking can be induced by oxidation with iodine or by irradiation with 365 nm wavelength UV light. The reactions are quick and quite efficient, as judged by the PAGE gels provided. The authors demonstrate that the reaction can be reversed by reducing reagents, such as DTT, and go to great lengths probing their mechanistic proposal for how the disulfide bond formation comes about. They provide UV-melting points and CD to demonstrate that the DNA duplex remains intact.

This reviewer finds the manuscript interesting and reasonably well done. It has strengths and weaknesses. One of the weaknesses is that the idea of using 6-thioguanines for crosslinking in DNA is not new. What the authors call their designed duplex positions the reactive group different from earlier work (e.g. ref. 50), but the ability of TG and other thionucleosides to undergo photoinduced crosslinking is known. Another weakness is that the study was done on just one DNA duplex sequence. It is not clear what the scope of the method is, and how well it translates into applications.

Other points.

- The deprotection procedure and two-fold HPLC purification are time consuming, and it is not clear why two chromatographic steps are needed. Overall, the synthetic effort appears high, and the authors should comment on this, as well as on the stability of the products.
- The yield of the oligonucleotides should be stated clearly in the text.
- What is the yield of the crosslinking reactions? Figure 7 gives yields, as determined by band ratios, but the earlier Figures do not. HPLC should provide quantitative answers.
- The authors write repeatedly of their designed duplexes. Any duplex prepared from synthetic ODNs is "designed". What is different here to make this an issue?
- What is a "well-designed proximity field"? This and other terminologies used in the paper are unusual, and the paper requires language polishing before it may be printed.
- How sure can the authors be that their crosslinks are not "significantly distorting the B-DNA helix"? A CD signature does not provide detailed structural information. Earlier structural work on other disulfide-containing duplexes show significant distortion. Even modeling, based on existing structures and force field minimization may provide at least a rough estimate of how the interstrand crosslink distorts the DNA.
- The melting transition of the crosslinked duplex (Figure 5b) looks like it should be below and not above 80 °C. The first derivative should be shown.

Overall, an interesting, but not exceptionally strong paper.

Reviewer #2

(Remarks to the Author)

The authors report on the use of 2'-deoxythioguanosine-modified oligonucleotides for interstrand crosslinking using either I2 or photo-induced oxidation. They further show that the crosslinking method features repetitive reversibility.

The study is carried out with care and experiments are well-illustrated and mostly supported through the data. However quite a few concerns can be formulated:

- In the introduction when providing an overview of interstrand crosslinking methodologies, the authors should be more complete and make a distinction between crosslinking between two modified strands and crosslinking of one modified strand to a non-modified target.
- The overview of di-sulfide based methods for interstrand crosslinking is far from complete. Additionally and as a consequence the authors fail to illustrate the main novelty and advantage of their described method.
- Concerning the following sentence:
"To the best of our knowledge, this is the first demonstration of disulfide crosslinking induced by UVA photoirradiation with such a clean reaction profile and remarkable efficiency."
This is a very general statement and it should be specified whether this is applicable to interstrand DNA crosslinking only or broader. What about similar crosslinking in proteins and peptides or polymeric materials?
- The crosslinked duplex should be isolated and correctly characterized by detailed mass analysis. Currently no mass data showing the full construct are available for the crosslinked duplex.
- Concerning the sentence : "The reversible properties of our designed oligo duplex may offer applications in structural dynamics studies and targeted release in biochemical systems." Currently this statement is too vague and more specific examples should be given and illustrated.

Minor remarks:

- Please adapt the title to be grammatically more correct

In conclusion, this is a nice study and well carried out but it remains 'stuck' at the model reaction stage. The authors fail to illustrate and describe the real advantages and application potential of the system.

The 'new' reaction is only illustrated for a simple duplex and it is not clear what the more specific added value is of such crosslinked duplexes per se. The authors are encouraged to exploit the chemistry and demonstrate it to work in different structural and more challenging structural contexts.

Can the strategy be used for reversible stabilisation of secondary structures or for (re)modelling DNA origami tiles ?

Upon including such examples and the experimental illustration thereof, the paper might become suitable for publication in a journal of the standards of Nat Comms Chem.

Reviewer #3

(Remarks to the Author)

Osman et al. report a strategy to cross-link DNA duplexes via disulfide bond formation between 2'-deoxythioguanine residues in (partially) complementary strands. The crosslinking can be induced by treatment with iodine or photoirradiation (365 nm UV light) and can be reversed by DTT. The crosslinked duplex had an increased thermal stability, and the cross-linking reaction can be conducted multiple times, allowing for multiple cycles of cross-linking and cleavage. This approach has potential applications in nanotechnology. Then authors also investigated the crosslinking mechanism (Fig. 6-8).

Overall, the study is well conducted and the paper reads well. It should be published after the following is addressed:

1. This cross-linking strategy involves noncomplementary tG-tG base pair. The authors should discuss how this may interfere with the ability of such probes to recognize target sequences.
2. ESI-HRMS and MS/MS spectra for crosslinked nucleosides should be provided as a figure.
3. It would be interesting to investigate the structure of cross-linked DNA since it includes a noncanonical base pair. Could the authors provide a molecular model?
4. Include Y axis labels in Fig. 4 and label what m/z is being monitored
5. Change "oligo" to "oligodeoxynucleotide"

Reviewer #4

(Remarks to the Author)

This paper of the group of Dr. Onizuka and Dr. Nagatsugi demonstrates the interstrand crosslinking of duplex DNA modified with two 6-thio-2'-deoxyguanosine building blocks. The paper shows efficient and fast crosslinking reactions both via chemical oxidation with I₂ and photoinduced crosslinking with 365 nm UV light. The manuscript overall is well-written with clear diagrams and figures of the experimental results.

Major remarks

1. The authors report on the interstrand crosslinking of DNA using disulfide bond formation. However, disulfide crosslinking in general, and 6-thioguanine (6-TG)-mediated crosslinking in particular, are well-established methods in literature for covalently linking complementary DNA strands, as also cited in the references (e.g. ref 41, 42 and 55). Could the authors provide more details or experimental evidence to clarify how their approach differs from previously published methods?

2. In the gel pictures, for example Figure 3 and 7, faint low-mobility bands are seen in the wells of the single-stranded ODNs, which the authors assign to self-crosslinking (which is also seen in the MALDI TOF spectra of these ODNs). Could the authors elaborate on why these bands appear so high on the gel, given that self-crosslinked duplexes would typically be expected to exhibit similar mobility to the interstrand crosslinked DNA?

3. To investigate the stability of the crosslinked adduct, the authors performed stability studies in the presence of GSH, at biologically relevant GSH concentrations (Figure S3). Since the authors mention that their crosslinking design holds promise for developing biological applications, they should provide more experiments regarding in-cellulo stability of the crosslinked duplex, such as stability tests in cell lysate.

4. MALDI TOF MS spectra for the two synthesized DNA strands are provided in the supplementary information. The authors should also provide a mass analysis of the intact duplex DNA after crosslinking, since mass analysis is only performed on the crosslinked duplex DNA after nuclease digestion.

5. The second part of the paper is focused on elucidating the potential reaction mechanism of the photoinduced crosslinking. In Figure 8, a clear overview is given of a possible reaction scheme together with the key elements involved in the process. Around the end of page 12, H₂O₂ is mentioned as an additional ROS species generated during the reaction. However, this is not shown in the preceding schemes and it is unclear how its role supports the proposed mechanism. Could the authors please clarify how H₂O₂ is involved in the mechanism and where it originates from?

Minor comments

6. On page 12 of the manuscript, around the middle of the page, there is a spelling mistake, 'phot-irradiation' should be replaced by 'photoirradiation'.

7. In the Methods and Materials section, under 'Preparative disulfide crosslink under iodine reaction conditions (large scale)' it is mentioned that 'the oligo concentration was increased to 500 pmol'. However, since 'pmol' refers to the quantity of a substance rather than its concentration, it is advisable to revise this wording for accuracy.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This reviewer is satisfied with the authors' response and the revisions performed.

Reviewer #2

(Remarks to the Author)

The authors have satisfactorily responded to all issues mentioned in the referee reports. They are to be complimented with this revised version that has substantially improved with respect to the first version and now offers a more comprehensive guide for use of the methodology by others.

The paper can now be accepted for publication.

Reviewer #4

(Remarks to the Author)

I have reviewed the authors' responses and the revised manuscript. I thank the authors for responding clearly to my questions and adapting the manuscript accordingly. I have no further questions or concerns.

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Point-by-point responses to the reviewers' comments

We sincerely thank the reviewers for the valuable comments and suggestions on the original version of our manuscript. We have carefully considered all feedback and made the necessary revisions accordingly. Point-by-point responses to each comment are provided below. We hope that the revised manuscript meets your expectations and will be suitable for publication in *Communications Chemistry*.

Reviewer #1:

We sincerely thank Reviewer 1 for the valuable comments and suggestions, which have greatly helped us improve the revised manuscript. We have made the necessary corrections accordingly and hope that the revisions meet your satisfaction.

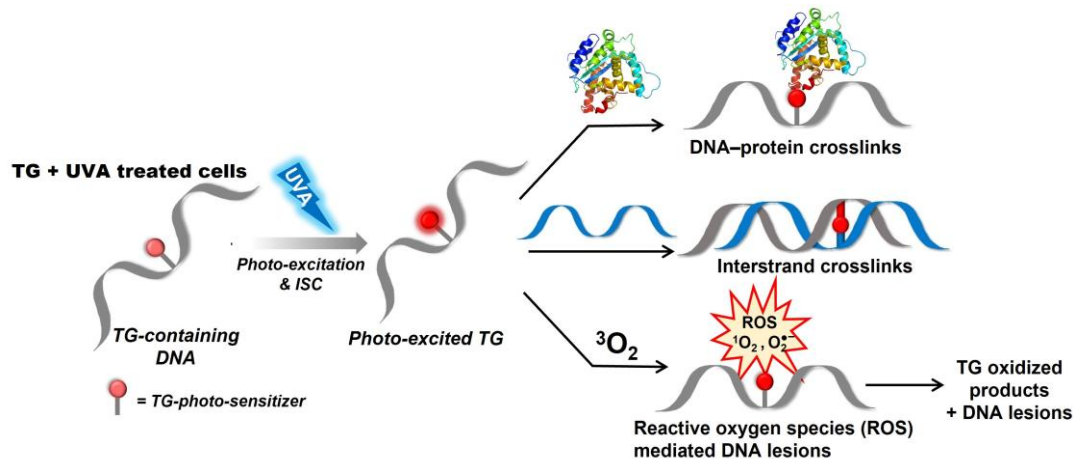
1-1: This reviewer finds the manuscript interesting and reasonably well done. It has strengths and weaknesses. One of the weaknesses is that the idea of using 6-thioguanines for crosslinking in DNA is not new. What the authors call their designed duplex positions the reactive group different from earlier work (e.g., ref. 50), but the ability of TG and other thionucleosides to undergo photoinduced crosslinking is known.

[Answer 1-1] We thank the reviewer for this valuable comment. Indeed, thio-modified nucleobases such as 6-thioguanine (6-TG) are known for their photochemical reactivity. However, to our knowledge, no previous study has clearly demonstrated photoinduced disulfide bond formation between two thio-modified nucleobases within a duplex context. Our study presents three key novelties. First, we show that UVA irradiation of our designed duplex triggers efficient disulfide bond formation between 6-TG residues, in contrast to prior reports involving metal- or chemically induced disulfides or UVA-mediated reactions yielding crosslinks with natural nucleobases, proteins, or oxidative products. Second, the precise arrangement and orientation of TG residues within the duplex provides controlled crosslinking efficiency and reversible formation. Third, we provide a detailed mechanistic elucidation revealing that efficient single-electron transfer to molecular oxygen leads to the pronounced generation of superoxide ($O_2^{\bullet-}$), representing a type I photosensitization pathway not previously reported for 6-TG-containing oligo DNA. Collectively, these findings offer new mechanistic insights into the photochemistry of thionucleosides.

As we agree that the photochemical reactivity of 6-TG has been investigated in various contexts, we have summarized the previously reported photoproducts of 6-TG under UVA irradiation in **Supplemental Review Figure 1**, highlighting its distinct outcomes in cellular DNA, single-stranded and duplex synthetic oligo DNA, and monomeric forms.

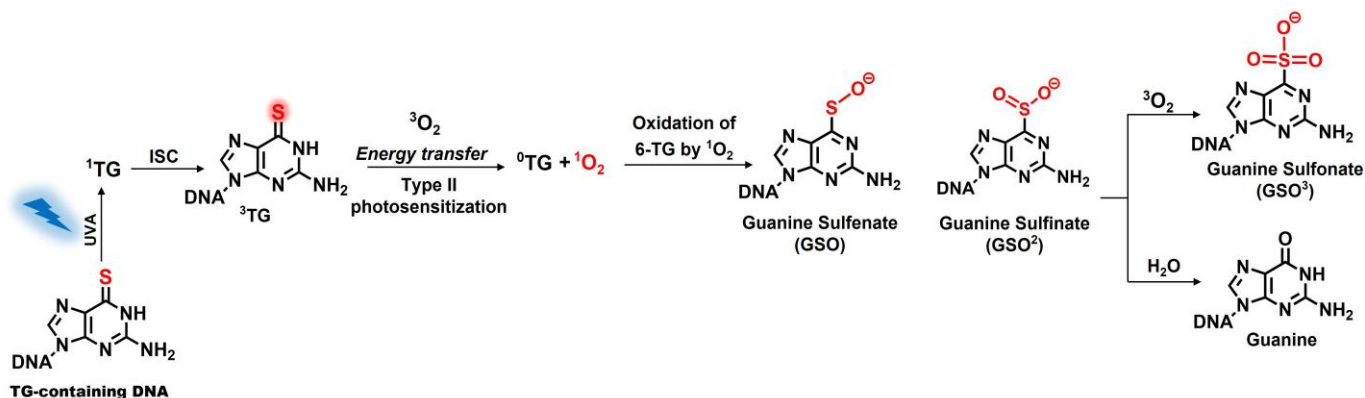
The specific products formed upon UVA irradiation of 6-thioguanine (6-TG) depend strongly on its nucleic acid context—whether it is incorporated into cellular DNA, single-stranded or duplex synthetic oligo DNA, or exists as a monomer.

(a) Photoirradiation of TG incorporated in cellular DNA: the prominent photoproducts are interstrand crosslink with canonical nucleic acids, DNA–protein crosslinks, and ROS-induced lesions.



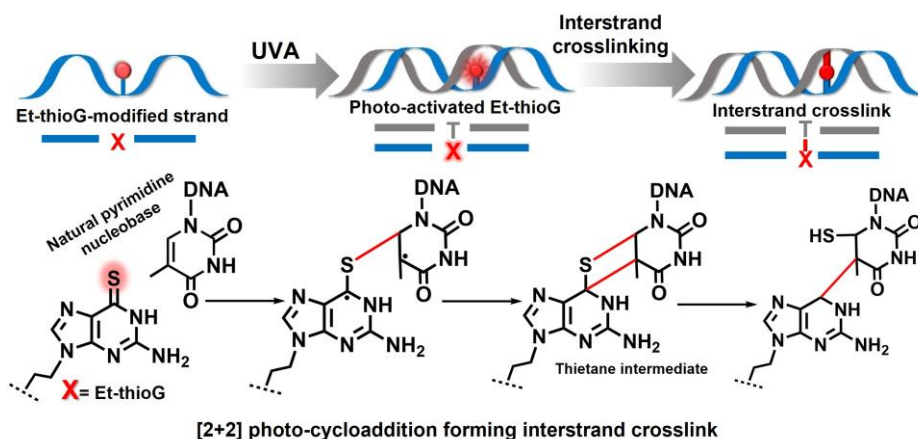
- [1] Favre, A., Saintomé, C., Fourrey, J.-L., Clivio, P. & Laugâa, P. Thionucleobases as intrinsic photoaffinity probes of nucleic acid structure and nucleic acid-protein interactions. *J Photochem Photobiol, B* **42**, 109–124 (1998).
- [2] Brem, R., Daehn, I. & Karran, P. Efficient DNA interstrand crosslinking by 6-thioguanine and UVA radiation. *DNA Repair* **10**, 869–876 (2011).
- [3] Brem, R. & Karran, P. Multiple forms of DNA damage caused by UVA photoactivation of DNA 6-Thioguanine. *Photochem Photobiol* **88**, 5–13 (2012).
- [4] Brem, R., Guven, M. & Karran, P. Oxidatively-generated damage to DNA and proteins mediated by photosensitized UVA. *Free Radic Biol Med* **107**, 101–109 (2017).
- [5] Gueranger, Q., Kia, A., Frith, D. & Karran, P. Crosslinking of DNA repair and replication proteins to DNA in cells treated with 6-thioguanine and UVA. *Nucleic Acids Res* **39**, 5057–5066 (2011).
- [6] Ashwood, B., Pollum, M. & Crespo - Hernández, C. E. Photochemical and photodynamical properties of sulfur-substituted nucleic acid bases. *Photochem Photobiol* **95**, 33–58 (2019).

(b) Photo-irradiation of single-stranded oligo DNA containing 6-TG forms various 6-TG oxidation products (guanine sulfenate, sulfinate, and sulfonate) with singlet oxygen ($^1\text{O}_2$) involved as the key reactive species driving these transformations.



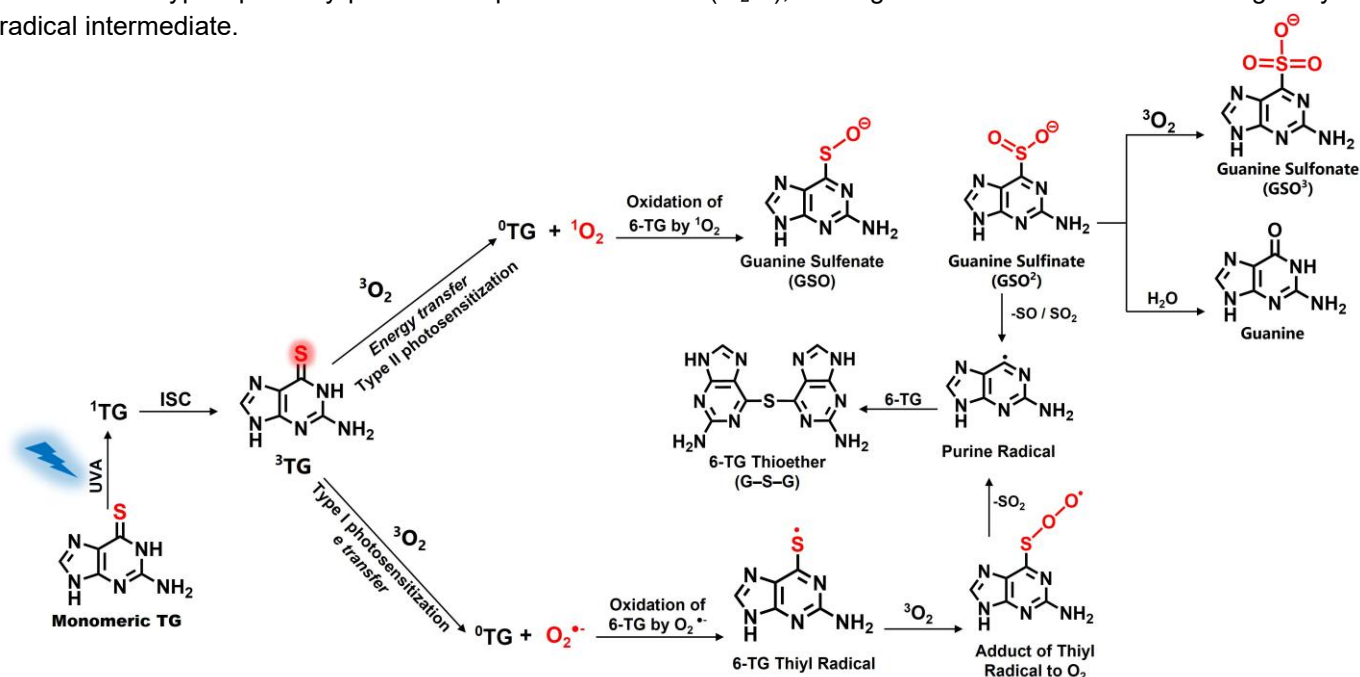
- [1] Zhang, X. et al. Novel DNA lesions generated by the interaction between therapeutic thiopurines and UVA light. *DNA Repair* **6**, 344–354 (2007).
- [2] Ren, X. et al. Guanine sulphinate is a major stable product of photochemical oxidation of DNA 6-thioguanine by UVA irradiation. *Nucleic Acids Res* **38**, 1832–1840 (2010).

(c) Synthetic oligo DNA duplexes containing 6-TG can form interstrand crosslinks with complementary pyrimidine bases via a [2+2] photocycloaddition reaction under UVA irradiation:



- [1] Nishioka, T., Oshiro, I., Onizuka, K., Taniguchi, Y. & Sasaki, S. Efficient thymidine-selective DNA interstrand photo-activated crosslinking by the 6-Thioguanine connected via an ethylene-linker to the 2'-Deoxyribose unit. *Chem Pharm Bull* **64**, 1315–1320 (2016).

(d) Photoirradiation of monomeric 6-TG: primarily undergoes Type II photosensitization, generating singlet oxygen ($^1\text{O}_2$) that yields various photooxidation products (major) and possible G–S–G dimer formation (minor). A less favored Type I pathway produces superoxide radicals ($\text{O}_2^{\bullet-}$), leading G–S–G dimer formation through thiyl radical intermediate.



- [1] Zou, X., Zhao, H., Yu, Y. & Su, H. Formation of Guanine-6-sulfonate from 6-Thioguanine and singlet oxygen: A combined theoretical and experimental study. *J Am Chem Soc* **135**, 4509–4515 (2013).
- [2] Ren, X. et al. Guanine sulphinat is a major stable product of photochemical oxidation of DNA 6-thioguanine by UVA irradiation. *Nucleic Acids Res* **38**, 1832–1840 (2010).
- [3] Euceda, N. et al. Thioguanine restoration through type I photosensitization-superoxide oxidation-glutathione reduction cycles. *Phys Chem Chem Phys* **23**, 5069–5073 (2021).

Supplemental Review Figure 1. Schematic summary of the major photoproducts formed upon UVA irradiation of 6-thioguanine (6-TG) in different contexts: (a) cellular DNA, (b) single-stranded oligo DNA, (c) duplex oligo DNA, (d) monomeric 6-TG.

1-2: Another weakness is that the study was done on just one DNA duplex sequence. It is not clear what the scope of the method is, and how well it translates into applications.

[Answer 1-2] We thank the reviewer for this insightful comment, which helped us improve the revised manuscript. To address this concern, we systematically evaluated the scope and generality of our crosslinking strategy by examining both sequence and orientation dependencies. Specifically, we tested different positions of the 6-TG residues within the duplex — internal versus terminal — while maintaining the original bond orientation, to assess how varying the position within the duplex and electron transfer from adjacent bases affect the outcome. We also investigated the effect of reversing the orientation of the reactive groups. These studies revealed that the orientation and positioning of TG residues within a DNA duplex critically dictate both the efficiency and selectivity of crosslink formation. These detailed findings, now included in the revised manuscript.

The following discussion sections have been added to contextualize these results:

Results and Discussion:

Evaluating the applicability of crosslinking design: sequence and orientation dependency (Page 8 – 12)

- *New figures included: Figure 6, figure 7, and Figure S8 (Supplementary Information).*

Impact of TGs orientation on photoinduced crosslinking efficiency (Page 18 – 19)

- *New figures included: Figure 12 and Figure S14 (Supplementary Information).*

1-3: The deprotection procedure and two-fold HPLC purification are time consuming, and it is not clear why two chromatographic steps are needed. Overall, the synthetic effort appears high, and the authors should comment on this, as well as on the stability of the products.

1-4: The yield of the oligonucleotides should be stated clearly in the text.

[Answer 1-3] We thank the reviewer for this comment. Although the deprotection and two HPLC purification steps increase the synthetic effort, both are essential to obtain highly pure TG-containing oligonucleotides. The first HPLC purification removes truncated sequences and by-products from solid-phase synthesis, while the second, performed after 5'-DMTr deprotection, eliminates residual impurities and self-crosslinked species, yielding oligonucleotides suitable for reliable crosslinking experiments. The TG-containing oligonucleotides remained stable during purification and long-term storage, with only minor self-crosslink formation observed over extended storage. We have addressed this point in the revised manuscript and included the corresponding data in the Supplementary Information, which show the formation of self-crosslinked species over time.

The following sentences have been added to the revised manuscript:

Results and Discussion:

Testing of disulfide crosslinking under chemical oxidation: (page 4, 2nd paragraph)

“In addition to the single stranded and hetero-crosslinked product bands, faint low-mobility bands were consistently observed by PAGE (Figure 3). These bands disappeared upon DTT treatment (Figure 3c), suggesting the presence of self-crosslinked species formed via disulfide bonds. Such self-crosslinking likely arises from a partial self-complementary base pairing that enables transient duplex formation, thereby

facilitating intermolecular disulfide homo-crosslinking (Figure S3a) through slow air oxidation over time. Denaturing PAGE analysis indicated that these self-crosslinked ODNs migrated more slowly than the fully complementary crosslinked duplex, likely due to highly mismatched crosslinked structures that hinder electrophoretic mobility. Notably, these self-crosslinks were readily reduced by DTT, whereas the presence of a complementary strand selectively promoted hetero-crosslinking with the aid of an excess oxidizing agent (Figure 3c). To better characterize the self-crosslinked species, HPLC was used to analyze each single-stranded ODN solution, which revealed two distinct peaks A and B (**Figure S3b**). Peak B was identified as a self-crosslinked product, as DTT reduction yielded the corresponding single-stranded ODN in PAGE analysis (**Figure S3c**), confirming the presence of reversible disulfide bonds. MALDI-TOF MS analysis of peak B predominantly showed reduced single-stranded ODNs due to the cleavage of disulfide bonds during laser desorption/ionization. However, low-intensity signals corresponding to intact self-crosslinked products were also detected (SI, MALDI-TOF MS)."

Supplementary Information:

Synthesis and purification of oligo DNA containing 2'-deoxythioguanosine (Scheme 2): (Page S6, 2nd paragraph)

"The 2'-deoxythioguanosine-containing ODNs were stable during purification and storage, with only minor self-crosslinking observed over time. Despite high coupling efficiency in solid-phase synthesis, the overall yield after DMTr deprotection was moderate due to the removal of self-crosslinked species during the second HPLC purification, which was necessary to obtain ODNs of sufficient purity for crosslinking studies."

New figures included:

Figure S3. Characterization of self-crosslinked products (Page S9).

[Answer 1-4] We thank the reviewer for this suggestion. Although the solid-phase synthesis proceeded with high coupling efficiency, the overall yield of purified oligonucleotides after DMTr deprotection was moderate, primarily because self-crosslinked species had to be removed during the second HPLC purification step, which is essential to obtain oligonucleotides of sufficient purity for reliable crosslinking experiments. We have now clearly stated this in the revised manuscript by adding the following sentence in the Supplementary Information:

Synthesis and purification of oligo DNA containing 2'-deoxythioguanosine (Scheme 2): (Page S6, 1st paragraph)

"The obtained amounts of the target oligo DNA were as follows: ODN1, 50 nmol (20%); ODN2, 55 nmol (22%); and ODN3, 48 nmol (19%). Yields were calculated based on 0.25 μ mol scale."

1-5: What is the yield of the crosslinking reactions? Figure 7 gives yields, as determined by band ratios, but the earlier Figures do not. HPLC should provide quantitative answers.

[Answer 1-5] We thank the reviewer for this useful suggestion. We have quantified the exact yield of the disulfide crosslinking reaction under both chemical and photo-induced oxidation conditions using HPLC. The corresponding data and discussion have been added to the revised manuscript. Specifically, the following sentences have been included to report these results:

Results and Discussion:

Testing of disulfide crosslinking under chemical oxidation: (page 5, 4th paragraph)

“Next, we quantified the yield of the disulfide crosslinking reaction. To ensure accurate measurement, pre-existing self-crosslinked ODNs were removed prior to the reaction because they reduced the availability of free thiol groups required for efficient crosslinking. HPLC analysis using ODN1 and ODN2 showed that the crosslinked product was obtained with a 95% yield (Figure S5), indicating near-quantitative conversion of single-stranded ODNs to the disulfide-linked product.”

Testing crosslinking potential under photoirradiation: (page 6, 1st paragraph)

“HPLC analysis showed that photoinduced crosslinking between ODN1 and ODN2 achieved a yield of 93% (Figure S5), which indicates that this method efficiently converts single-stranded ODNs into disulfide-linked duplexes, similar to I₂-induced crosslinking.”

Supplementary Information: (page S11)

Figure S5. HPLC quantification of crosslinking yield

1-6: The authors write repeatedly of their designed duplexes. Any duplex prepared from synthetic ODNs is "designed". What is different here to make this an issue?

[Answer 1-6] We thank the reviewer for this comment. We agree that all duplexes prepared from synthetic ODNs are, by definition, “designed.” Accordingly, we have revised the manuscript to remove repetitive use of the term “designed” where it is unnecessary.

1-7: What is a "well-designed proximity field"? This and other terminologies used in the paper are unusual, and the paper requires language polishing before it may be printed.

[Answer 1-7] We thank the reviewer for this comment. We have carefully revised the manuscript to improve language clarity and removed unusual or ambiguous terms, including “well-designed proximity field.”

1-8: How sure can the authors be that their crosslinks are not "significantly distorting the B-DNA helix"? A CD signature does not provide detailed structural information. Earlier structural work on other disulfide-containing duplexes shows significant distortion. Even modeling, based on existing structures and force field minimization may provide at least a rough estimate of how the interstrand crosslink distorts the DNA.

[Answer 1-8] We thank the reviewer for this important comment. In the revised manuscript, we have thoroughly discussed the conformational changes associated with crosslink formation. Molecular modeling shows that only minor conformational adjustments are required to form the interstrand disulfide bond, and the crosslink does not significantly distort the original TG-containing duplex. This is illustrated by the angular changes between pre- and post-crosslinking states.

The modeling figures and corresponding detailed discussion have been added to the revised manuscript under the following sections:

Results and Discussion:

Evaluating the applicability of crosslinking design: sequence and orientation dependency (Page 11, 3rd paragraph)

“To elucidate the origin of the distinct reactivity observed between the two orientations in disulfide crosslinking, molecular modeling was conducted to evaluate the distance and angular parameters of the disulfide-forming TG residues. The interatomic distance between the sulfur atoms of closely positioned TG residues was calculated to be 4.27 Å in the ODN1–ODN2 duplex (original design) (**Figure S8a**) and 4.86 Å in the ODN3–ODN2 duplex (opposite orientation) (**Figure S8b**). Although the sulfur atoms in the original design were in closer proximity, this difference ($\Delta = 0.62$ Å) alone is insufficient to explain the contrasting reactivity outcomes. Instead, structural analysis revealed pronounced differences in the conformational changes and angular alignment associated with crosslink formation.

The bond angles were measured between the C6–S bonds of each TG and the two sulfur atoms (C6–S···S) in the pre-crosslinking state, and between the C6–S–S bonds in the post-crosslinking state. (**Figure S8**). In the original design, the angles decreased modestly from 110.6° and 111.6° to 104.9° and 105.4° (**Figure 7a**), corresponding to angular adjustments (i.e., change required to achieve the crosslinked geometry) of $\Delta = -5.7^\circ$ and -6.2° , respectively. Thus, the initial geometry ($\sim 111.1^\circ$) was already close to the final crosslinked angle ($\sim 105.15^\circ$). This minor reorientation ($\sim 6^\circ$) indicates that the duplex was pre-organized for the crosslinking reaction, requiring only a minor conformational adjustment to reach the transition state, thereby lowering the activation energy for bond formation. Consequently, this design efficiently produced disulfide crosslinks under chemical oxidation and photoirradiation conditions.

In contrast, the opposite-direction design displayed notable angular changes from 93.3° and 87.9° to 106.5° and 106.8°, respectively, after crosslinking (**Figure 7b**). These correspond to significant increases of $\Delta = +13.2^\circ$ and $+18.9^\circ$, respectively. The initial geometry ($\sim 90.6^\circ$) was far from the final disulfide-linked angle ($\sim 106.65^\circ$), which indicates that the duplex is poorly pre-organized for the crosslinking reaction. Consequently, a major conformational adjustment and substantial reorientation ($\sim 16^\circ$) were required prior to bond formation, which resulted in a higher transition energy barrier that was unfavorable for direct disulfide formation. As a result, the reaction was diverted toward unexpected reaction pathways where the slower formation of S–S bonds allowed competing reactions to take place, resulting mainly in DTT-resistant crosslinked products. Under chemical oxidation conditions, disulfide-linked species were formed only as minor products, and no disulfide crosslink formation was observed upon photoirradiation.

Taken together, these results indicate that the near-optimal pre-organization in the original design facilitated efficient and selective disulfide crosslinking, whereas the poor pre-organization in the opposite design required a substantial conformational reorientation that raised the activation barrier and led to inefficient disulfide crosslink formation.

Thus, the efficiency and selectivity of disulfide crosslinking in TG-modified DNA duplexes are governed primarily by the relative orientation of the reactive residues rather than their exact sequence position. Therefore, this

strategy offers a broad scope with respect to sequence context and residue positioning provided that the favorable orientation of TG residues is maintained.”

- *New figures included: figure 7, and Figure S8 (Supplementary Information).*

Impact of TGs orientation on photoinduced crosslinking efficiency (Page 19, 2nd & 3rd paragraph)

“As noted earlier, structural analysis of the two duplex designs using molecular modeling revealed key differences in the orientation and angular alignment of the TG residues. When viewed from the same angle, a striking difference in their relative positioning was apparent. In the original design (ODN1–ODN2 duplex), the TG residues were oriented away from each other rather than directly stacked on top, resulting in larger spatial separation. This observation is consistent with the longer interatomic distances (N1–N1 = 4.76 Å, C2–C2 = 5.76 Å), which suggests reduced overlap of the reactive groups (**Figure S14a, S14b**). In contrast, the opposite orientation (ODN3–ODN2 duplex) caused the TG residues to be more directly stacked, leading to a closer spatial approach. This is consistent with the shorter interatomic distances (N1–N1 = 3.80 Å, C2–C2 = 3.69 Å), which reflect partial overlapping of the residues (**Figure S14a, S14b**).

These structural differences align well with the experimental results of O₂•⁻ generation and can be rationalized within the proposed reaction mechanism. In the original design, the TG residues point in directions that maintain sufficient spatial separation for charge-separated species to persist long enough for TG•⁻ to transfer an electron to O₂, efficiently producing O₂•⁻. In contrast, the opposite orientation (ODN3–ODN2 duplex) has partial overlapping between TG residues, which facilitates rapid charge recombination (**Figure 12b**) that shortens the lifetime of TG•⁻. This limits its ability to transfer an electron to O₂ and thereby results in slower O₂•⁻ generation. Collectively, by taking all structural analyses into account, these findings demonstrate that the orientation and degree of overlap of TG residues within a DNA duplex dictate photoinduced crosslinking. In the original design, the pre-organized duplex structure minimizes the angular adjustments required for crosslinking, while the smaller overlap between TG residues enables effective electron transfer to O₂, leading to efficient O₂•⁻ generation and disulfide crosslinking. In the opposite orientation, the poorly pre-organized duplex structure requires major conformational adjustments, and the closely stacked TG residues accelerate charge recombination, which slows electron transfer to O₂ and delays O₂•⁻ formation. This ultimately reduces the photoinduced reactivity toward crosslinking.”

- *New figures included: Figure S14 (Supplementary Information).*

1-9: The melting transition of the crosslinked duplex (Figure 5b) looks like it should be below and not above 80 °C. The first derivative should be shown.

[Answer 1-9] We thank the reviewer for this valuable suggestion. We have added the first derivative plots of the melting curves for both I₂-induced and photo-induced crosslinked duplexes in the Supplementary Information, which show sharp transitions above 80 °C.

Supplementary Information: (page S12)

Figure S6. First-derivative plots of the melting curves of (a) I₂-induced, (b) photo-induced crosslinked duplexes.

Reviewer #2:

We thank Reviewer 2 for the valuable comments and suggestions, which have helped improve our revised manuscript. The manuscript has been updated accordingly, and we hope the revisions meet your satisfaction.

2-1: In the introduction when providing an overview of interstrand crosslinking methodologies, the authors should be more complete and make a distinction between crosslinking between two modified strands and crosslinking of one modified strand to a non-modified target.

[Answer 2-1] We thank the reviewer for this valuable suggestion. We have revised the Introduction to provide a more comprehensive overview of interstrand crosslinking strategies, clearly distinguishing between crosslinking of a modified strand to an unmodified target and crosslinking between two modified strands. Representative examples from the literature have been added for each strategy to illustrate these approaches.

The following paragraph had been included in the revised manuscript:

Introduction: (Page 1, 3rd paragraph)

“Various strategies have been developed to achieve interstrand crosslinking within nucleic acids using chemically functionalized ODNs. The introduction of reactive moieties into single-stranded probes enables site-specific crosslinking with unmodified complementary targets through diverse chemical reactions. For example, photo-activated crosslinking reactions have been reported using psoralen,^[19–21] carbazole,^[22–25] diaziridine,^[26,27] chloro-aldehyde,^[28] coumarin-modified ODNs,^[29,30] or photocaged nucleobases,^[31] which can be light-activated to generate interstrand crosslinks with complementary DNA strands. Chemically activated groups, such as furan derivatives undergoing oxidation-based crosslinking,^[32,33] have also been applied. Our group has previously developed vinyl-mediated Michael-type crosslinking reactions between a reactive base analog and its complementary natural nucleobase.^[34–37] Other strategies involve modifying one strand to generate electrophilic intermediates that are subsequently attacked by nucleophilic sites on the complementary DNA strand.^[38–42] Alternatively, interstrand crosslinking can be achieved by incorporating reactive groups into each strand of the duplex. Upon hybridization, these groups are brought into close proximity, which allows them to undergo covalent bond formation through well-established chemical reactions such as amide bond formation,^[43] imine formation,^[44–46] click reactions,^[47,48] and photocycloaddition.^{[49–52]”}

2-2: The overview of di-sulfide based methods for interstrand crosslinking is far from complete. Additionally, and as a consequence the authors fail to illustrate the main novelty and advantage of their described method.

[Answer 2-2] We thank the reviewer for this comment. As discussed in our response to Reviewer 1 [Answer 1-1], although disulfide-based interstrand crosslinking methods in nucleic acids have been reported previously, these typically rely on metal-mediated or chemical oxidation processes. In contrast, our study demonstrates that photoirradiation can induce efficient interstrand disulfide crosslinking between two 6-TG residues within a duplex, with precise residue positioning enabling controlled and reversible crosslinking. Furthermore, we provide detailed mechanistic insights showing that the reaction proceeds via a type I photosensitization

pathway—an unreported mechanism for 6-TG-containing oligonucleotides—thereby underscoring the novelty of our work over existing methods. We have summarized the previously reported photoproducts of 6-TG under UVA irradiation in the Supplemental Review Figure 1.

As advantages, our study establishes a clear design principle supported by molecular modeling and mechanistic analyses, validating the proximity-driven and photoinduced nature of the reaction. Collectively, these findings provide a strong foundation for the rational design of controllable and reversible nucleic acid crosslinking strategies, with potential to inspire future research in nucleic acid nanotechnology and dynamic structural probing of nucleic acid assemblies. The demonstrated reversible redox switching further expands these possibilities, enabling environmentally triggered disulfide cleavage and controlled release within complex nucleic acid architectures.

To emphasize the novelty and advantage of our approach over existing methods, we have added the following sentences in the revised manuscript:

Conclusion: (Page 22, 3rd & 4th paragraph)

“Furthermore, this redox-responsive switching between crosslinked and noncrosslinked states can be extended to more structurally complex nucleic acid constructs. Such reversible redox responsiveness holds promise for developing dynamic DNA-based systems for nanotechnology, drug delivery, and biotechnological applications. The design principles established here, together with the insights from molecular modeling and mechanistic elucidation of the reaction mechanism, provide a valuable foundation for the rational design of nucleic acid crosslinking strategies. Beyond the validation of proximity-driven crosslinking and photoinduced reactivity, these findings highlight the potential of thioguanine-mediated chemistry as a versatile platform for dynamic and reversible covalent modifications of nucleic acids. Such advances are expected to broaden the scope of nucleic acid chemistry and facilitate future applications of chemically and photoinduced crosslinking.”

2-3: Concerning the following sentence:

“To the best of our knowledge, this is the first demonstration of disulfide crosslinking induced by UVA photoirradiation with such a clean reaction profile and remarkable efficiency.”

This is a very general statement, and it should be specified whether this is applicable to interstrand DNA crosslinking only or broader. What about similar crosslinking in proteins and peptides or polymeric materials?

[Answer 2-3] We thank the reviewer for this valuable comment. We agree that our original statement was too broad and have revised it to clarify the scope of our claim. Our study specifically establishes photoinduced disulfide crosslinking within nucleic acid systems, as no previous work has shown photoinduced disulfide bond formation between two 6-TG residues in an oligonucleotide duplex with such a clean reaction profile and high yield and efficiency. Since disulfide chemistry in proteins, peptides, and polymeric materials occurs under fundamentally different chemical environments and reaction mechanisms, direct comparison is not relevant. **This sentence has been revised accordingly in the revised manuscript to avoid overgeneralization and to accurately emphasize that our findings apply to interstrand DNA crosslinking:**

Results and Discussion:

Testing crosslinking potential under photoirradiation: (previous page 7, 2nd paragraph)

“To the best of our knowledge, this is the first demonstration of disulfide crosslinking induced by UVA photoirradiation with such a clean reaction profile and remarkable efficiency.”

↓

Elucidating photoinduced crosslinking reaction mechanism: (page 13, 2nd paragraph)

“Despite the well-known and diverse photochemical reactivity of 6-TG, no previous study, to our knowledge, has clearly demonstrated photoinduced disulfide bond formation between two 6-TG nucleobases incorporated within an oligo DNA duplex.”

2-4: The crosslinked duplex should be isolated and correctly characterized by detailed mass analysis. Currently no mass data showing the full construct are available for the crosslinked duplex.

[Answer 2-4] We thank the reviewer for this comment. The disulfide bond in the crosslinked duplex can be cleaved during laser desorption/ionization, and therefore MALDI-TOF MS data were not initially included, as they may not have fully characterize the crosslink product. To provide evidence for this, and to respond to this comment, we have now measured the MALDI-TOF MS of the isolated disulfide-crosslinked duplex, and the corresponding data, along with an explanation of this observation, have been added to the revised manuscript with the following sentences:

Results and Discussion:

Testing of disulfide crosslinking under chemical oxidation: (page 5, 2nd paragraph)

“The HPLC-purified hetero-crosslinked product was similarly analyzed by MALDI-TOF MS. As observed for the self-crosslinked species, the spectrum mainly showed peaks corresponding to the reduced single strands (ODN1 and ODN2) with a low-intensity peak corresponding to the intact crosslinked duplex ([M-H]⁻ calcd for disulfide-crosslinked duplex: 7320.11, found: 7320.32).”

Testing crosslinking potential under photoirradiation: (page 6, 2nd paragraph)

“MALDI-TOF MS analysis of the HPLC-purified hetero-crosslinked product obtained after photoirradiation revealed peaks corresponding to the reduced single strands (ODN1 and ODN2), along with a low-intensity peak corresponding to the intact crosslinked duplex (SI, MALDI-TOF MS).”

2-5: Concerning the sentence : “The reversible properties of our designed oligo duplex may offer applications in structural dynamics studies and targeted release in biochemical systems.” Currently this statement is too vague and more specific examples should be given and illustrated.

[Answer 2-5] We thank the reviewer for this comment. We agree that the original statement was too vague and have revised it to include a specific example. To demonstrate the applicability of our crosslinking strategy in these potential applications, we applied it to a linear DNA strand with TG residues near the 3' and 5' ends, enabling intramolecular disulfide crosslinking and a “locked” stem-loop structure formation. This reversible

redox-responsive system allows repeated switching between crosslinked and linear forms, providing a controllable platform for studying nucleic acid structural dynamics. Furthermore, the disulfide bond can be selectively cleaved in a reducing intracellular environment, enabling triggered release of the linear construct, and illustrating potential applications in targeted delivery and functional modulation of nucleic acid constructs in biochemical systems.

The data illustrating this example have been added to the revised manuscript as follows:

Results and Discussion:

Repetitive reversibility of crosslinking reaction: (page 21, 1st & 2nd paragraph)

“To evaluate the biological relevance of this system, we examined the stability of disulfide-crosslinked duplexes in cell lysates. The disulfide crosslinks were rapidly cleaved within 1 min upon incubation in the lysate (**Figure S16**), demonstrating that this system responds efficiently to intracellular reducing environments under physiologically relevant conditions. This reversible property could enable applications in structural dynamics studies as well as in targeted release within biochemical systems.^[66,110]

To expand this crosslinking strategy beyond simple DNA duplexes, we examined its applicability to structurally diverse nucleic acid systems. A 72-mer linear DNA strand was designed with TG residues incorporated into the complementary regions near the 5' and 3' ends, along with two poly(T)₁₄ flexible spacers to facilitate strand folding (**Figure S17a**). This design brings the TG residues near the 5' and 3' ends into proximity, enabling intramolecular disulfide bond formation under oxidative conditions and resulting in a disulfide-crosslinked stem-loop structure (**Figure S17a**). Both chemical oxidation and photoirradiation conditions efficiently promoted crosslinking, and subsequent treatment with reducing agents restored the linear form (**Figure S17b, S17c**). Reversibility was confirmed through repeated redox cycling: sequential I₂-mediated oxidation and DTT reduction allowed multiple cycles of bond formation and cleavage (**Figure S17b**), whereas photoirradiation–DTT cycles showed reduced efficiency after the first cycle, likely due to partial hydrolysis of TG residues (**Figure S17c**). This reversible redox-responsive switching offers promising applications because disulfide cleavage can be precisely triggered by the intracellular environment to restore the linear construct and enable controlled intracellular release. Importantly, these results demonstrate that disulfide-based crosslinking can be applied beyond simple duplexes to more structurally complex nucleic acid architectures.”

Supplementary Information: (page S19 – S20)

Figure S16. Stability of crosslink products in cell lysate.

Figure S17. Intramolecular disulfide crosslinking, forming a stem-loop structure.

2-6: Please adapt the title to be grammatically more correct

[Answer 2-6] We thank the reviewer for this comment. We have revised the title to ensure it is grammatically correct in the revised manuscript.

Chemical and photoinduced interstrand crosslinking using oligo DNA duplex functionalized with 2'-deoxythioguanosines.

↓

Chemical and photoinduced interstrand crosslinking of oligo DNA duplexes containing 2'-deoxythioguanosines.

2-7: This is a nice study and well carried out, but it remains 'stuck' at the model reaction stage. The authors fail to illustrate and describe the real advantages and application potential of the system. The 'new' reaction is only illustrated for a simple duplex, and it is not clear what the more specific added value is of such crosslinked duplexes per se. The authors are encouraged to exploit the chemistry and demonstrate it to work in different structural and more challenging structural contexts. Can the strategy be used for reversible stabilization of secondary structures or for (re)modelling DNA origami tiles ?

[Answer 2-7] We thank the reviewer for suggestion. To address this concern, as described in [Answer 2-5], we have expanded our study beyond a simple duplex by investigating structurally diverse nucleic acid system. Specifically, we designed a DNA strand incorporating TG residues using the same design strategy as the original duplex, which enables intramolecular disulfide crosslinking, resulting in the formation of a crosslinked stem-loop structure, proving an example that, this crosslinking can be applied beyond simple duplexes to more structurally complex nucleic acid architectures. This example illustrates reversible stabilization of a secondary structure, as the disulfide bond maintains the stem-loop in a defined conformation, while reduction restores the linear form.

The detailed design, experimental results, and corresponding discussion have been added to the revised manuscript with the following sentences:

Results and Discussion:

Repetitive reversibility of crosslinking reaction: (page 21, 2nd paragraph)

“To expand this crosslinking strategy beyond simple DNA duplexes, we examined its applicability to structurally diverse nucleic acid systems. A 72-mer linear DNA strand was designed with TG residues incorporated into the complementary regions near the 5' and 3' ends, along with two poly(T)₁₄ flexible spacers to facilitate strand folding (**Figure S17a**). This design brings the TG residues near the 5' and 3' ends into proximity, enabling intramolecular disulfide bond formation under oxidative conditions and resulting in a disulfide-crosslinked stem-loop structure (**Figure S17a**). Both chemical oxidation and photoirradiation conditions efficiently promoted crosslinking, and subsequent treatment with reducing agents restored the linear form (**Figure S17b, S17c**). Reversibility was confirmed through repeated redox cycling: sequential I₂-mediated oxidation and DTT reduction allowed multiple cycles of bond formation and cleavage (**Figure S17b**), whereas photoirradiation–DTT cycles showed reduced efficiency after the first cycle, likely due to partial hydrolysis of TG residues (**Figure S17c**). This reversible redox-responsive switching offers promising applications because disulfide cleavage can be precisely triggered by the intracellular environment to restore the linear construct and enable controlled intracellular release. Importantly, these results demonstrate that disulfide-based crosslinking can be applied beyond simple duplexes to more structurally complex nucleic acid architectures.”

Supplementary Information: (page S20)

Figure S17. Intramolecular disulfide crosslinking, forming a stem-loop structure.

Reviewer #3:

We thank Reviewer 3 for the valuable comments and suggestions, which have helped improve our revised manuscript. The manuscript has been updated accordingly, and we hope the revisions meet your satisfaction.

3-1: This cross-linking strategy involves noncomplementary tG-tG base pair. The authors should discuss how this may interfere with the ability of such probes to recognize target sequences.

[Answer 3-1] We thank the reviewer for this comment. The reviewer's concern appears to arise from the assumption that our crosslinking strategy involves a complementary tG–tG base pair. However, in our design, the two tG residues are not positioned opposite each other as mismatched bases, instead, they are incorporated at noncomplementary positions, offset by one base, each forming a base pair with cytosine (tG–C) on the opposite strand.

3-2: ESI-HRMS and MS/MS spectra for crosslinked nucleosides should be provided as a figure.

[Answer 3-2] We thank the reviewer for this comment. In the original manuscript, the ESI-HRMS spectrum of the photoinduced crosslinked nucleosides obtained after enzymatic digestion was provided in the Supporting Information. In the revised manuscript, we have now additionally included the ESI-HRMS data for the I₂-induced crosslinked nucleosides. These new data are presented in the revised Supporting Information to provide clearer structural evidence for the crosslinked products.

3-3: It would be interesting to investigate the structure of cross-linked DNA since it includes a noncanonical base pair. Could the authors provide a molecular model?

[Answer 3-3] We thank the reviewer for this insightful comment. In the revised manuscript, we have included detailed molecular modeling of the crosslinked DNA along with a thorough discussion of the associated conformational changes. The modeling highlights the orientation and angular alignment of the 6-TG bases and provides insight into the structural consequences associated with the crosslinking.

The modeling figures and corresponding detailed discussion have been added to the revised manuscript under the following sections:

Results and Discussion:

Evaluating the applicability of crosslinking design: sequence and orientation dependency (Page 8 – 12)

- *New figures included: figure 7, and Figure S8 (Supplementary Information).*

Impact of TGs orientation on photoinduced crosslinking efficiency (Page 18 – 19)

- *New figures included: Figure S14 (Supplementary Information).*

3-4: Include Y axis labels in Fig. 4 and label what m/z is being monitored

[Answer 3-4] We thank the reviewer for this comment. We agree that the Y-axis label in the HPLC chromatograms should be clearly indicated. Figure 4 shows the HPLC analysis of the hydrolysates obtained

from digested crosslinked duplexes, in which disulfide-crosslinked guanosine nucleosides were isolated and subsequently analyzed by ESI-MS analysis. Therefore, the Y-axis represents the HPLC detector signal (intensity, μV), not m/z . Accordingly, we have added the Y-axis units and measurement range in both the figure caption and the Materials and Methods section to clearly specify what is being monitored.

Results and Discussion:

Testing crosslinking potential under photoirradiation: (page 7, Figure 4 caption)

Figure 4. Nuclease digestion analysis confirming the structure of the crosslinked product. (a) Schematic representation of enzymatic digestion experiment for I_2 -mediated and photoinduced crosslinked products. The CL product (500 pmol) was incubated with a 10 $\times$ nucleoside digestion mix reaction buffer and 1 $\times$ nucleoside digestion mix at 37 °C for 3 h. **(b)** HPLC analysis followed by electrospray ionization–mass spectrometry (ESI-MS) of hydrolysates obtained from digested crosslinked duplexes, confirming the mass of disulfide-crosslinked guanosine nucleosides. **The chromatograms show retention time (X-axis) from 0–25 min, and the peaks correspond to detector response (intensity, Y-axis) monitored at 254 nm, with an approximate signal range of 0–450,000 μV .**

3-5: Change "oligo" to "oligodeoxynucleotide"

[Answer 3-5] We thank the reviewer for this comment. Since our manuscript specifically deals with DNA sequences, we have replaced all instances of “oligonucleotide” with the more precise term “oligodeoxynucleotide” or “oligo DNA” throughout the manuscript to ensure clarity and accuracy.

Reviewer #4:

We thank Reviewer 4 for the valuable comments and suggestions, which have helped improve our revised manuscript. The manuscript has been updated accordingly, and we hope the revisions meet your satisfaction.

4-1: The authors report on the interstrand crosslinking of DNA using disulfide bond formation. However, disulfide crosslinking in general, and 6-thioguanine (6-TG)-mediated crosslinking in particular, are well-established methods in literature for covalently linking complementary DNA strands, as also cited in the references (e.g. ref 41, 42 and 55). Could the authors provide more details or experimental evidence to clarify how their approach differs from previously published methods?

[Answer 4-1] We thank the reviewer for this comment. As discussed in our response to reviewer 1 [Answer 1-1] and reviewer 2 [Answer 2-2], while disulfide and 6-TG-mediated crosslinking have been reported, our approach is distinct in several key aspects. In our study, UVA irradiation triggers efficient disulfide formation between 6-TG residues within a duplex context. This contrasts with earlier metal- or chemically induced reactions or UVA-driven processes, which often produce uncontrolled crosslinks or oxidative products. In addition, our study defines a clear design strategy, supported by experimental findings, molecular modelling, and mechanistic analysis.

Compared to previous studies that reviewer highlighted (Wolfrum et al. (2019) and Faiad et al. (2024), they rely on thiol-functionalized DNA strands or site-specific chemical modifications, our design introduces minimal structural alteration to the native duplex structure, using only the sulfur atom of 6-TG to enable both chemical and photoinduced disulfide formation. This approach eliminates the need for external thiol handles or complex chemical derivatization, offering a simpler and more intrinsic strategy for nucleic acid crosslinking.

Unlike Brem et al. (2011), where 6-TG and UVA irradiation produced low yields of various oxidative crosslinks with uncharacterized structures through undefined pathways, that study primarily aimed to demonstrate the existence of interstrand crosslinks and their biological implications. For reference, Supplemental Review Figure 1 summarizes all previously reported photoproducts of 6-TG under UVA irradiation, highlighting the distinct reaction outcomes observed in cellular DNA, single-stranded and duplex synthetic oligo DNA, and monomeric forms. In contrast, our work establishes a precisely controlled, highly efficient, and selective disulfide bond formation between two 6-TG residues within a well-defined duplex architecture, yielding a structurally characterized and mechanistically elucidated product. Furthermore, our system enables reversible redox switching between crosslinked and noncrosslinked states, and offers potential applications in DNA nanotechnology, structural dynamics studies, and controlled release systems.

We have expanded the discussion to provide additional context for these results and to clarify how our approach differs from previously reported methodologies:

Results and Discussion:

Evaluating the applicability of crosslinking design: sequence and orientation dependency (Page 8 – 12)

- *New figures included: Figure 6, figure 7, and Figure S8 (Supplementary Information).*

Impact of TGs orientation on photoinduced crosslinking efficiency (Page 18 – 19)

- *New figures included: Figure 12 and Figure S14 (Supplementary Information).*

Repetitive reversibility of crosslinking reaction: (page 21, 2nd paragraph)

“To expand this crosslinking strategy beyond simple DNA duplexes, we examined its applicability to structurally diverse nucleic acid systems. A 72-mer linear DNA strand was designed with TG residues incorporated into the complementary regions near the 5' and 3' ends, along with two poly(T)₁₄ flexible spacers to facilitate strand folding (**Figure S17a**). This design brings the TG residues near the 5' and 3' ends into proximity, enabling intramolecular disulfide bond formation under oxidative conditions and resulting in a disulfide-crosslinked stem-loop structure (**Figure S17a**). Both chemical oxidation and photoirradiation conditions efficiently promoted crosslinking, and subsequent treatment with reducing agents restored the linear form (**Figure S17b, S17c**). Reversibility was confirmed through repeated redox cycling: sequential I₂-mediated oxidation and DTT reduction allowed multiple cycles of bond formation and cleavage (**Figure S17b**), whereas photoirradiation–DTT cycles showed reduced efficiency after the first cycle, likely due to partial hydrolysis of TG residues (**Figure S17c**). This reversible redox-responsive switching offers promising applications because disulfide cleavage can be precisely triggered by the intracellular environment to restore the linear construct and enable controlled intracellular release. Importantly, these results demonstrate that disulfide-based crosslinking can be applied beyond simple duplexes to more structurally complex nucleic acid architectures.”

Supplementary Information: (page S20)

Figure S17. Intramolecular disulfide crosslinking, forming a stem-loop structure.

4-2: In the gel pictures, for example Figure 3 and 7, faint low-mobility bands are seen in the wells of the single-stranded ODNs, which the authors assign to self-crosslinking (which is also seen in the MALDI TOF spectra of these ODNs). Could the authors elaborate on why these bands appear so high on the gel, given that self-crosslinked duplexes would typically be expected to exhibit similar mobility to the interstrand crosslinked DNA?

[Answer 4-2] We thank the reviewer for this valuable comment. To address this point, we performed additional experiments to further characterize the faint low-mobility bands observed in the single-stranded TG-containing ODNs. HPLC isolation of these species, followed by PAGE and MALDI-TOF MS analyses, confirmed that these bands correspond to self-crosslinked products formed via disulfide bond formation.

We have revised the manuscript to include the relevant data and clarify the difference in electrophoretic mobility between the self-crosslinked and hetero-crosslinked duplex, by adding following sentences:

Results and Discussion:

Testing of disulfide crosslinking under chemical oxidation: (page 4, 2nd paragraph)

"In addition to the single stranded and hetero-crosslinked product bands, faint low-mobility bands were consistently observed by PAGE (Figure 3). These bands disappeared upon DTT treatment (Figure 3c), suggesting the presence of self-crosslinked species formed via disulfide bonds. Such self-crosslinking likely arises from a partial self-complementary base pairing that enables transient duplex formation, thereby facilitating intermolecular disulfide homo-crosslinking (Figure S3a) through slow air oxidation over time. Denaturing PAGE analysis indicated that these self-crosslinked ODNs migrated more slowly than the fully complementary crosslinked duplex, likely due to highly mismatched crosslinked structures that hinder electrophoretic mobility. Notably, these self-crosslinks were readily reduced by DTT, whereas the presence of a complementary strand selectively promoted hetero-crosslinking with the aid of an excess oxidizing agent (Figure 3c). To better characterize the self-crosslinked species, HPLC was used to analyze each single-stranded ODN solution, which revealed two distinct peaks A and B (**Figure S3b**). Peak B was identified as a self-crosslinked product, as DTT reduction yielded the corresponding single-stranded ODN in PAGE analysis (**Figure S3c**), confirming the presence of reversible disulfide bonds. MALDI-TOF MS analysis of peak B predominantly showed reduced single-stranded ODNs due to the cleavage of disulfide bonds during laser desorption/ionization. However, low-intensity signals corresponding to intact self-crosslinked products were also detected (SI, MALDI-TOF MS)."

New figures included:

Figure S3. Characterization of self-crosslinked products (Page S9).

4-3: To investigate the stability of the crosslinked adduct, the authors performed stability studies in the presence of GSH, at biologically relevant GSH concentrations (Figure S3). Since the authors mention that their crosslinking design holds promise for developing biological applications, they should provide

more experiments regarding in-cellulo stability of the crosslinked duplex, such as stability tests in cell lysate.

[Answer 4-3] We thank the reviewer for this valuable suggestion. In response to this comment, we evaluated the stability of the disulfide-crosslinked duplex in cell lysates to further assess the biological relevance of our crosslinking design. The corresponding experimental data and discussion have been included in the revised manuscript with the following added sentences:

Results and Discussion:

Repetitive reversibility of crosslinking reaction: (page 21, 1st paragraph)

“To evaluate the biological relevance of this system, we examined the stability of disulfide-crosslinked duplexes in cell lysates. The disulfide crosslinks were rapidly cleaved within 1 min upon incubation in the lysate (**Figure S16**), demonstrating that this system responds efficiently to intracellular reducing environments under physiologically relevant conditions. This reversible property could enable applications in structural dynamics studies as well as in targeted release within biochemical systems.^{[66,110]”}

Supplementary Information: (page S19 – S20)

Figure S16. Stability of crosslink products in cell lysate.

4-4: MALDI TOF MS spectra for the two synthesized DNA strands are provided in the supplementary information. The authors should also provide a mass analysis of the intact duplex DNA after crosslinking since mass analysis is only performed on the crosslinked duplex DNA after nuclease digestion.

[Answer 4-4] We thank the reviewer for this comment. Because the disulfide bond in the crosslinked duplex can be cleaved during laser desorption/ionization, MALDI-TOF MS data were not initially included, as they might not accurately reflect the intact crosslink. To provide this, we have now measured the MALDI-TOF MS of the isolated disulfide-crosslinked duplex. The resulting data, along with an explanation of the observed disulfide bond cleavage, have been added to the revised manuscript with the following sentences:

Results and Discussion:

Testing of disulfide crosslinking under chemical oxidation: (page 5, 2nd paragraph)

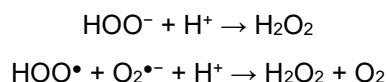
“The HPLC-purified hetero-crosslinked product was similarly analyzed by MALDI-TOF MS. As observed for the self-crosslinked species, the spectrum mainly showed peaks corresponding to the reduced single strands (ODN1 and ODN2) with a low-intensity peak corresponding to the intact crosslinked duplex ([M-H]⁻ calcd for disulfide-crosslinked duplex: 7320.11, found: 7320.32).”

Testing crosslinking potential under photoirradiation: (page 6, 2nd paragraph)

“MALDI-TOF MS analysis of the HPLC-purified hetero-crosslinked product obtained after photoirradiation revealed peaks corresponding to the reduced single strands (ODN1 and ODN2), along with a low-intensity peak corresponding to the intact crosslinked duplex (SI, MALDI-TOF MS).”

4-5: The second part of the paper is focused on elucidating the potential reaction mechanism of the photoinduced crosslinking. In Figure 8, a clear overview is given of a possible reaction scheme together with the key elements involved in the process. Around the end of page 12, H₂O₂ is mentioned as an additional ROS species generated during the reaction. However, this is not shown in the preceding schemes and it is unclear how its role supports the proposed mechanism. Could the authors please clarify how H₂O₂ is involved in the mechanism and where it originates from?

[Answer 4-5] We thank the reviewer for pointing out this lack of explanation. As discussed in the original version of our manuscript, the proposed photoinduced crosslinking mechanism is initiated by a single electron transfer (SET) process, leading to charge separation and formation of radical species—specifically, a radical anion (TG^{•-}) and a radical cation (TG^{•+}). Under air-saturated conditions, TG^{•-} transfers an electron to molecular oxygen (³O₂), generating superoxide (O₂^{•-}), while TG^{•+} undergoes deprotonation to form a thiyl radical (TG[•]). The thiyl radical then reacts with ⁰TG through homolytic cleavage, forming a disulfide-crosslinked adduct bearing a carbon-centered radical. This intermediate can further incorporate oxygen from ³O₂ or O₂^{•-} generating a peroxy anion (–OO⁻) or peroxy radical (–OO[•]), which upon deprotonation yield the stable disulfide-crosslinked structure within the DNA duplex along with hydroperoxide byproducts (HOO[•] or HOO⁻). Formation of H₂O₂ may occur as a secondary process through either protonation of HOO⁻ or recombination of HOO[•] with O₂^{•-}, as shown below:



Thus, H₂O₂ is not directly involved in the crosslinking reaction itself but arises as a subsequent byproduct formed from radical intermediates generated during the photoinduced process.

To clarify this point, we have revised the manuscript by adding the following sentence:

Results and Discussion:

Elucidating photoinduced crosslinking reaction mechanism: (previous page 12, 4th paragraph)

“Further supporting our proposed mechanism, we detected hydrogen peroxide (H₂O₂) in the photoirradiated duplex reaction mixture using the OxiVision™ Green sensor, an H₂O₂-selective fluorescent probe. A strong fluorescence response was observed when the probe was incubated with a 10 s photoirradiated duplex solution compared to the nonirradiated control (Figure S9), confirming H₂O₂ generation upon photoirradiation.”

↓

Elucidating photoinduced crosslinking reaction mechanism: (page 17, 4th paragraph)

Our proposed mechanism is further supported by the likely formation of hydrogen peroxide (H₂O₂) as a secondary byproduct. This H₂O₂ could originate from species generated during the photoinduced reaction, either through protonation of the hydroperoxide anion (HOO⁻) or recombination of a hydroperoxyl radical (HOO[•]) with O₂^{•-}. To confirm its presence, we used the OxiVision™ Green sensor, an H₂O₂-selective fluorescent probe. A strong fluorescence response was observed when the probe was incubated with a 10 s photoirradiated duplex solution compared to the nonirradiated control (**Figure S13**), confirming H₂O₂ generation upon photoirradiation.

4-6: On page 12 of the manuscript, around the middle of the page, there is a spelling mistake, ‘phot-irradiation’ should be replaced by ‘photoirradiation’.

[Answer 4-6] We thank the reviewer for pointing out this typographical error. We have corrected “phot-irradiation” to “photoirradiation” in the revised manuscript.

4-7: In the Methods and Materials section, under ‘Preparative disulfide crosslink under iodine reaction conditions (large scale)’ it is mentioned that ‘the oligo concentration was increased to 500 pmol’. However, since ‘pmol’ refers to the quantity of a substance rather than its concentration, it is advisable to revise this wording for accuracy.

[Answer 4-7] We thank the reviewer for this comment. We have revised the wording in the Materials and Methods section to accurately describe the oligonucleotide amount, ensuring that it correctly reflects the quantity of material used.