jz-2022-006846.R1

Name: Peer Review Information for "Low-Energy (5 – 20 eV) Electron Induced Single and Double Strand Breaks in Well-Defined DNA Sequences"

First Round of Reviewer Comments

Reviewer: 1

Comments to the Author

In this work the authors performed experiments with origami DNA templates to serve as a platform for biotinylated oligonucleotide target sequences that were exposed to low-energy electrons (LEEs) of specific energies. After LEE irradiation, the biotin label of the remaining intact oligo sequences is visualized by atomic force microscopy (AFM), via binding to streptavidin, and appears as a bright spot in AFM images. The formation of strand breaks (and hence loss of the biotin label) is observed as a decrease in the number these bright spots, following electron irradiation. In previous work, the technique allowed for quantification of single strand breaks in single-stranded oligonucleotide sequences and the determination of the absolute value of the cross section for the process. In the present work, the authors use the same basic principle and type of experiment with different origami targets made of double stranded configurations. i.e., a DNA loop organized such that the double stranded sequence remains closed. This is a considerable advance in the technique that could make it possible to investigate the formation of double strand breaks (DSBs) with the origami method. The versatility in the choice of target sequences and the possibility to irradiate two sequences in a single irradiation experiment provide a direct a comparison of the response of the two target types.

The authors identify for the first time the LEE-energy dependence (5-20 eV) of DSB formation and cross section in a double stranded oligonucleotides. To do this, they bombard with LEEs their novel double stranded structure. In the case of SSBs, maxima in strand break cross sections are found around 7 and 10 eV. For DSBs, they observe a maximum at 10 eV. Apart from the initial observation that LEEs can induce DSBs in plasmid DNA, the present results confirm with a completely different method that LEEs can cause DSBs, via DEA, and hence potentially lethal damage to cells. Considering the importance of the latter in radiobiology and radiotherapy, such a confirmation of the action of LEEs is an important achievement in the field. More generally, the addition of double-stranded target structures is a very interesting and significant addition to the authors previous work with DNA origami, which should help broaden the potential range of experimental systems for future studies. However, and this is perhaps due to choosing a letter format for this manuscript, several issues are unclear and should be addressed in a revision.

1) Arguably the most innovative element in this work is the fabrication of the double stranded target, yet the paper is comparatively silent on this point. On page 7 the authors write:

The correct formation of double stranded DNA extending from the DNA origami platform is confirmed in control experiments reported in the supplementary information (SI, Fig. S1).

This SI information is very interesting and certainly worth including. However, most of the characterisations described (Figure S1) relate to experiments with two complementary sequences of DNA, closely positioned together on a template, which, according to the FRET observations, almost, but not quite, manage to form a length of hybridized, double stranded DNA. Electron impact measurements additionally show that the cross section for strand break damage for this 'failed' double stranded DNA section is indistinguishable from those measured for the isolated complementary strands. This, the authors deduce, means that their construction is not actually double-stranded DNA. Their solution, to link the strands together with a TTTT sequence in a hairpin-like structure is not however characterised in detail (other than by the CS measurements for strand breaks that they report in the paper). So, what additional data demonstrates the formation these hairpin-like structures?

2) Again, with reference to measurements contained in Figure S1, the cross section at 10 eV for strand break damage to the DNA double strand "(5'-Bt-d(CAC)4-Bt / 5'-d(GTG)4-Bt)" is reported. So, if this isn't a typo, for these measurements there were two Bts per double-stranded target? If yes, how do they appear in the AFM image and what impact (if any) does this have on the ability of observe a SSB (or DSB) event and determine absolute cross sections.

3) On page 8 the authors discuss additional control experiments with 5'-d(T(Bt-dT)T2), that were performed to characterize the stability of the single stranded DNA loop. These experiments are highly important in determining the cross section for producing DSBs. Regarding one pathway for removing the Bt label (Figure 2c): while I agree that the removal of the label and a section of DNA could be initiated by two separate SSBs initiated by different electrons, is it not possible, given what is now known on the formation of multiple damage sites by a single LEE, that such excisions could be driven by a single electron? If yes, it is not necessarily true that this pathway would result in a power-law dependency of NSB with increasing F.

4) From the control experiments (above), the authors have measured a cross section for damage to the loop, which they then subtract then from the σDSB of hpDNA. But are these cross sections really additive? While I am unaware on any work by these authors on the variation of cross sections with strand length, the authors have previously shown in single stranded DNA samples that σSSB is sequence dependent. Does not the sequence around the TTTT loop structure depend on whether the loop is bound to the origami template or within the DNA hairpin? While definitive answers might be beyond the scope of the present article, discussion of these issues is warranted.

5) With reference to Figure 3, why do the NSB values at zero fluence vary so much? Shouldn't they all be the same as they represent base level of strand breaks in an ensemble of unirradiated origami templates? In fact, some more details on the irradiation and AFM analysis procedures would be useful here. How many templates must be monitored to obtain these NSB measurements? Are same templates re-examined after each irradiation? Could such information be included in as supplemental information?

6) The maxima and minima seen in the graph of strand break cross sections do indeed align well with those reported for plasmid samples. The values reported in Fig 3 appear composed of peaks

superimposed on a background. What is the background? Where does it come from? Should we assign to DEA the cross section of the peak and subtract the background, or does the entire absolute value represent the DEA cross section? If so, why?

7) Many references do not appear adequate, particularly on the description of the basic principles and the vast amount of work that has already been done on DNA and LEE attachment, DEA and cross sections. Many times, the authors reference specific work. A look in the literature shows that there are many review articles or book chapters on these topics that would be more instructive to the reader.

Minor:

On page 7 the authors write:

The sequence of the DNA hairpin hpDNA is (5'-d(GTG)4T(Bt-dT)T2(CAC)4) and includes the DNA loop 5'-d(T(Bt-dT)T2) and the dsDNA stem sequence (5'-d(CAC)4 / 5'-d(GTG)4)

Is the change in order of CAC and GTG in the descriptions of the hpDNA and DSDNA, significant?

Supplemental information:

Top of page 3 : The authors write:

“As shown in chapter 4.2 the Bt label is subject to LEE induced DNA”

Does chapter 4.2 = main article?

Figure S1: Note that the caption is incomplete for a) (no mention of iii) or iv). However, these elements are explained elsewhere in this text. Also, why different x-axis scales in c) and d)? This makes it harder to compare the graphs.

Reviewer: 2

Comments to the Author

The authors have shown that DNA origami could be a powerful tool in quantifying dissociative electron attachment (DEA) to DNA caused by low-energy electrons. The following points must be clarified before they can be accepted for publication.

While suggesting a probable pathway for double-strand breaks caused by a single electron, the authors have mentioned that autodetachment of electron from the core-excited resonance would leave the base in an excited state, which can also cause DNA damage. However, autodetachment of the extra-electron should prevent any possible strand breaks that might have occurred. Can the author provide relevant citations for the statement.

Although the authors conclude that the dissociation cross-sections are base sequencedependent, data to support this inference is present only for ssDNA. Can it be claimed as a general statement?

The authors have proposed that it is the base-centered core-excited resonances that lead to strand breaks. However, in addition to core-excited resonances, several studies have suggested the role of shape resonances in DEA to DNA in experiments involving gasphase/micro-solvated oligonucleotides

and other model systems of DNA when the electron energy is below 4 eV*. Sanche and coworkers (J. Phys. Chem. C 2017, 121, 4, 2466–2472) have also suggested the role of shape-resonances in causing double-strand breaks when the extra electron gets autodetaches from the core-excited resonances and forms a shape-resonance centered on a neighboring nucleobase. Also, since the sample is irradiated at an ultra-high vacuum, the role of dipole-bound states in causing strand breaks may be considered. This should be clarified in the revised manuscript.

As a general comment, please improve the language and way of presentation used in the manuscript.

*I need to verify this value.

Author's Response to Peer Review Comments:

Dear Prof. Editor,

Please find attached the revised version of our manuscript entitled “Low-Energy (5 – 20 eV) Electron Induced Single and Double Strand Breaks in Well-Defined DNA Sequences” by Kenny Ebel an Ilko Bald. We gratefully acknowledge the careful and constructive comments by the Reviewers and have taken all comments into account in the revised version. Below you will find a point-by-point response and all the changes to the manuscript are marked in yellow in the enclosed pdf file.

We hope that the manuscript is now suitable for publication in JPCL.

Yours sincerely

Ilko Bald

Reviewer: 1

Recommendation: This paper may be publishable, but major revision is needed; I would like to be invited to review any future revision.

Comment:

In this work the authors performed experiments with origami DNA templates to serve as a platform for biotinylated oligonucleotide target sequences that were exposed to low-energy electrons (LEEs) of specific energies. After LEE irradiation, the biotin label of the remaining intact oligo sequences is visualized by atomic force microscopy (AFM), via binding to streptavidin, and appears as a bright spot in AFM images. The formation of strand breaks (and hence loss of the biotin label) is observed as a decrease in the number these bright spots, following electron irradiation. In previous work, the technique allowed for quantification of single strand breaks in single-stranded oligonucleotide sequences and the determination of the absolute value of the cross section for the process. In the present work, the authors use the same basic principle and type of experiment with different origami targets made of double stranded configurations. i.e., a DNA loop organized such that the double stranded sequence remains closed. This is a considerable advance in the technique that could make it possible to investigate the formation of double strand breaks (DSBs) with the origami method. The versatility in the choice of target sequences and the possibility to irradiate two sequences in a single irradiation experiment provide a direct a comparison of the response of the two target types.

The authors identify for the first time the LEE-energy dependence (5-20 eV) of DSB formation and cross section in a double stranded oligonucleotides. To do this, they bombard with LEEs their novel double stranded structure. In the case of SSBs, maxima in strand break cross sections are found around 7 and 10 eV. For DSBs, they observe a maximum at 10 eV. Apart from the initial observation that LEEs can induce DSBs in plasmid DNA, the present results confirm with a completely different method that LEEs can cause DSBs, via DEA, and hence potentially lethal damage to cells. Considering the importance of the latter in radiobiology and radiotherapy, such a confirmation of the action of LEEs is an important achievement in the field. More generally, the addition of double-stranded target structures is a very interesting and significant addition to the authors previous work with DNA origami, which should help broaden the potential range of experimental systems for future studies. However, and this is perhaps due to choosing a letter format for this manuscript, several issues are unclear and should be addressed in a revision.

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This SI information is very interesting and certainly worth including. However, most of the characterisations described (Figure S1) relate to experiments with two complementary sequences of DNA, closely positioned together on a template, which, according to the FRET observations, almost, but not quite, manage to form a length of hybridized, double stranded DNA. Electron impact measurements additionally show that the cross section for strand break damage for this 'failed' double stranded DNA section is indistinguishable from those measured for the isolated complementary strands. This, the authors deduce, means that their construction is not actually double-stranded DNA. Their solution, to link the strands together with a TTTT sequence in a hairpin-like structure is not however characterised in detail (other than by the CS measurements for strand breaks that they report in the paper). So, what additional data demonstrates the formation these hairpin-like structures?

Response:

It should be emphasized that the double stranded stem sequence is connected to two different staple strands in the DNA origami platform, and it is highly unlikely that one of these strands is completely loose, giving rise to an “open” hairpin structure. To quantify the number of incorrectly formed hairpins, single-molecule FRET experiments could be done, however, this is currently not available for us. We have added the following sentence to the manuscript:

“Furthermore, the stem of the DNA hairpin is attached to two staple strands within the DNA origami platform to make sure that the double strand forms correctly.”

Since the length of the manuscript is limited we are not able to move the section on the FRET measurements into main manuscript.

Comment:

2) Again, with reference to measurements contained in Figure S1, the cross section at 10 eV for strand break damage to the DNA double strand “(5'-Bt-d(CAC)4-Bt / 5'-d(GTG)4-Bt)” is reported. So, if this isn’t a typo, for these measurements there were two Bts per double-stranded target? If yes, how do they appear in the AFM image and what impact (if any) does this have on the ability of observe a SSB (or DSB) event and determine absolute cross sections.

Response:

This was not a typo, because two Bt are necessary when there is no hairpin loop in order to distinguish DSBs and SSBs within the double strand. In this way, an SSB in the double strand will not lead to a loss of Bt, but only a DSB will. Basically, it is possible to identify the two corresponding Sav molecules, however, they appear “melted” together and it is not always possible to distinguish them in AFM. As a consequence, a missing SAv (or two) will be associated with a DSB. But since this strategy turned out to be unreliable, we have not pursued this further.

Comment:

3) On page 8 the authors discuss additional control experiments with 5'-d(T(Bt-dT)T2), that were performed to characterize the stability of the single stranded DNA loop. These experiments are highly important in determining the cross section for producing DSBs. Regarding one pathway for removing the Bt label (Figure 2c): while I agree that the removal of the label and a section of DNA could be initiated by two separate SSBs initiated by different electrons, is it not possible, given what is now known on the formation of multiple damage sites by a single LEE, that such excisions could be driven by a single electron? If yes, it is not necessarily true that this pathway would result in a power-law dependency of NSB with increasing F.

Response:

The Reviewer is indeed correct that a one electron process is possible for pathway 2c. Therefore, we have modified the sentence in the manuscript:

"Nevertheless, if path c is a two-electron process, this will result in a power-law dependency of NSB with increasing F in the exposure-response curves, which was, however, not observed in the present experiments."

Comment:

4) From the control experiments (above), the authors have measured a cross section for damage to the loop, which they then subtract then from the σDSB of hpDNA. But are these cross sections really additive? While I am unaware on any work by these authors on the variation of cross sections with strand length, the authors have previously shown in single stranded DNA samples that σSSB is sequence dependent. Does not the sequence around the TTTT loop structure depend on whether the loop is bound to the origami template or within the DNA hairpin? While definitive answers might be beyond the scope of the present article, discussion of these issues is warranted.

Response:

This is a very good point. Especially for longer DNA sequences the absolute strand break cross sections must not depend linearly on the strand length (Int. J. Mol. Sci. 2020, 21, 111; doi:10.3390/ijms21010111), and therefore the additivity of the two cross sections for breakage of stem and loop is only a first approximation. The sequence dependence of strand break cross section is most pronounced for specific sequences such as telomeric DNA sequences, therefore we don't expect the cross section of loop damage to change significantly when it is bound to the DNA origami or the stem sequence. Nevertheless, we agree with #reviewer that such issues need to be investigated in more detail in the future. We have added the following sentence to make this clear:

"It needs to be noted that the additivity of cross sections is only a first approximation, because the strand break cross section does not depend linearly on the length of the sequence, and also the environment might have an effect on the strand break cross section."

Furthermore, we have included the data for the complete hairpin damage into the figure 4 in order to present the complete data.

Comment:

5) With reference to Figure 3, why do the NSB values at zero fluence vary so much? Shouldn't they all be the same as they represent base level of strand breaks in an ensemble of unirradiated origami templates? In fact, some more details on the irradiation and AFM analysis procedures would be useful

here. How many templates must be monitored to obtain these NSB measurements? Are same templates re-examined after each irradiation? Could such information be included in as supplemental information?

Response:

This is a good observation. The NSB value at zero fluence represents the binding efficiency of Bt to Sav, which varies from batch to batch, but much less from sample to sample within the same batch. One series of irradiation experiments is always done with the same batch, and therefore the relative changes of NSB provide reliable values for the strand break cross sections, although the level of NSB at zero fluence varies from batch to batch.

We have also added two more sections on the preparation, irradiation and analysis of the irradiated samples into the SI. For each energy we analysed approximately 30.000 DNA origami structures. The DNA origami structures that were irradiated once, are not further used for more irradiation experiments.

Comment:

6) The maxima and minima seen in the graph of strand break cross sections do indeed align well with those reported for plasmid samples. The values reported in Fig 3 appear composed of peaks superimposed on a background. What is the background? Where does it come from? Should we assign to DEA the cross section of the peak and subtract the background, or does the entire absolute value represent the DEA cross section? If so, why?

Response:

According to previous measurements using plasmid DNA the cross sections for DSBs should go down to zero at 14 eV and below 6 eV, while the cross sections for SSBs in this energy regime are non-zero (see e.g. J. Am. Chem. Soc. 2003, 125, 4467-4477.), most likely because of the many different damage pathways possible at the respective electron energy. However, in our experiments, the electron energy resolution is comparatively poor compared to the data reported by the Sanche group, which we assume is the reason that the cross sections for DSBs don't drop to zero. We currently install a new electron monochromator with significantly improved energy resolution, which will be used to verify this hypothesis in the future. We have modified one sentence in the manuscript that now reads:

"Due to the electron energy distribution in the DNA origami experiments the DSB yield is still non-zero in this energy regime and the smallest σ_{DSB} for dsDNA of $(0.60 \pm 0.29) \cdot 10^{-15}$ cm² is observed."

Comment:

7) Many references do not appear adequate, particularly on the description of the basic principles and the vast amount of work that has already been done on DNA and LEE attachment, DEA and cross sections. Many times, the authors reference specific work. A look in the literature shows that there are many review articles or book chapters on these topics that would be more instructive to the reader.

Response:

We have included now references to review articles that summarize the previous work on DNA damage be LEEs.

Minor comments:

On page 7 the authors write:

The sequence of the DNA hairpin hpDNA is (5'-d(GTG)4T(Bt-dT)T2(CAC)4) and includes the DNA loop 5'-d(T(Bt-dT)T2) and the dsDNA stem sequence (5'-d(CAC)4 / 5'-d(GTG)4)

Is the change in order of CAC and GTG in the descriptions of the hpDNA and DSDNA, significant?

Supplemental information:

Top of page 3 : The authors write:

"As shown in chapter 4.2 the Bt label is subject to LEE induced DNA"

Does chapter 4.2 = main article?

Figure S1: Note that the caption is incomplete for a) (no mention of iii) or iv). However, these elements are explained elsewhere in this text. Also, why different x-axis scales in c) and d)? This makes it harder to compare the graphs.

Response:

We have corrected all the above-mentioned issues.

Reviewer 2:

Comment:

The authors have shown that DNA origami could be a powerful tool in quantifying dissociative electron attachment (DEA) to DNA caused by low-energy electrons. The following points must be clarified before they can be accepted for publication.

While suggesting a probable pathway for double-strand breaks caused by a single electron, the authors have mentioned that autodetachment of electron from the core-excited resonance would leave the base in an excited state, which can also cause DNA damage. However, autodetachment of the extra-electron should prevent any possible strand breaks that might have occurred. Can the author provide relevant citations for the statement.

Response:

Autodetachment can result in an excited state, which might be dissociative. Then, a strand break can occur, as is reported in reference 31 of the revised manuscript. The process is described in the manuscript based on the ref. 31:

"The latter leaves the base in an electronically excited state resulting in a separation of the additional electron and the electronic excitation. Both can cause a damage on each of the complementary DNA single strands. The initial base stays in a dissociative state leading to C-O bond scission within the same strand, while the additional electron transfers to the phosphate group on the opposite strand causing rupture of the C-O bond via DEA."

Comment:

Although the authors conclude that the dissociation cross-sections are base sequence-dependent, data to support this inference is present only for ssDNA. Can it be claimed as a general statement?

Response:

Currently, we have not investigated the sequence dependence for dsDNA, but it will be done in the future. Based on our current knowledge it is likely that there is a slight sequence dependence also for DSBs, but we can't draw a general conclusion yet.

Comment:

The authors have proposed that it is the base-centered core-excited resonances that lead to strand breaks. However, in addition to core-excited resonances, several studies have suggested the role of shape resonances in DEA to DNA in experiments involving gas-phase/micro-solvated oligonucleotides and other model systems of DNA when the electron energy is below 4 eV. Sanche and coworkers (J. Phys. Chem. C 2017, 121, 4, 2466–2472) have also suggested the role of shape-resonances in causing double-strand breaks when the extra electron gets autodetaches from the core-excited resonances and forms a shape-resonance centered on a neighboring nucleobase. Also, since the sample is irradiated at an ultra-high vacuum, the role of dipole-bound states in causing strand breaks may be considered. This should be clarified in the revised manuscript.

Response:

The reviewer is correct for processes below 5 eV, which, however, are not considered here. In the future we will use a new electron monochromator that will allow for irradiations below 5 eV, where shape resonances and also dipole-bound states become relevant. However, I assume that in the condensed phase the dipole-bound states will have a too short lifetime to be relevant for strand breakages, even in UHV.

Comment:

As a general comment, please improve the language and way of presentation used in the manuscript.

Response:

We have carefully checked the manuscript to improve the language and presentation.

jz-2022-006846.R2

Name: Peer Review Information for "Low-Energy (5 – 20 eV) Electron Induced Single and Double Strand Breaks in Well-Defined DNA Sequences"

Second Round of Reviewer Comments

Reviewer: 1

Comments to the Author

I think that at this stage the paper should be considered acceptable for publication after the authors have taken into consideration the following comments and made corresponding changes in their manuscript.

The answer given to comments 1 and 2 is fine. I had not realised that the hairpin was attached to the template at both ends.

Regarding my previous comment 3, concerning the possibility that pathway c in Fig.2 could be driven 1 and not 2 electrons, the authors have on page 6 modified the following sentence

“Nevertheless, if path c is a two-electron process, this will result in a power-law-dependency of NSB with increasing F in the exposure-response curves, which was, however, not observed in the present experiments.”

Unfortunately, the authors did not delete the original version of the sentence which is immediately before this quote. Moreover, I don’t really think that their modification is sufficient. I suggest they add/modify the following:

Possible loss of Bt can occur due to the loss of the thymine base to which the Bt marker is bound (Figure 2a), LEE induced damage to the Bt label (Figure 2b) or damage of the DNA loop involving two SSBs (Figure 2c) that may be initiated by either 1 or 2 incident electrons.

I have some doubt concerning the power-law-dependency of NSB with increasing F in the exposure-response. This is true for an infinite number of targets, which is not the case in these measurements. With a finite number of targets, as they are being depleted with increasing fluence the exposure-response curve saturates exponentially.

The response to comment 4 is satisfactory.

Regarding comment 5, the new details added to the SI are most welcome and give a clearer indication of the complexity of these measurements. However, it occurs to this reviewer, that if the binding efficiency of Bt to Sav can vary from batch to batch (batch of origami templates, Bt marker or Sv agent?) then could this represent a change in sensitivity that could compromise comparisons between batches (and thus damage yields and electron energies)?

I am only partly satisfied with the answer to comment 6 that the background DSBs is due to poor electron beam resolution. It certainly contributes, but how poor can you get? A one-eV resolution would be sufficient to resolve the DEA peaks leaving a much lower background and certainly not an essentially flat signal from 5 to 8.5 eV. Could it be that inelastically backscattered electrons from the template further interact with DNA to smear out the structures, making them less visible and adding background DSBs? Perhaps, the is why they do not see the 6-eV resonance that is also present in the yield function of DSBs from plasmids. I would like to note that the authors report old cross section (CS) measurements with plasmids, recorded when the technique was not as well developed as today. In recent investigations, the ratio of the CS for double strand breaks to that for single strand breaks was found to be about 0.1.

Reviewer: 2

Comments to the Author

I am satisfied with the reply to the questions raised.

Author's Response to Peer Review Comments:

Dear Prof. Editor,

Thank you very much for sending as the comments of the Reviewers, which are gratefully acknowledged. Please find attached the revised version of our manuscript, in which all comments have been considered. Below you will find a point-by-point response and all the changes to the manuscript are marked in yellow in the enclosed pdf file.

We hope that the manuscript is now suitable for publication in JPCL.

Yours sincerely

Ilko Bald

Reviewer 1

Recommendation: This paper is publishable subject to minor revisions noted. Further review is not needed. I think that at this stage the paper should be considered acceptable for publication after the authors have taken into consideration the following comments and made corresponding changes in their manuscript.

Comment:

The answer given to comments 1 and 2 is fine. I had not realised that the hairpin was attached to the template at both ends.

Regarding my previous comment 3, concerning the possibility that pathway c in Fig.2 could be driven 1 and not 2 electrons, the authors have on page 6 modified the following sentence "Nevertheless, if path c is a two-electron process, this will result in a power-law-dependency of NSB with increasing F in the exposure-response curves, which was, however, not observed in the present experiments." Unfortunately, the authors did not delete the original version of the sentence which is immediately before this quote. Moreover, I don't really think that their modification is sufficient. I suggest they add/modify the following:
Possible loss of Bt can occur due to the loss of the thymine base to which the Bt marker is bound (Figure 2a), LEE induced damage to the Bt label (Figure 2b) or damage of the DNA loop involving two SSBs (Figure 2c) that may be initiated by either 1 or 2 incident electrons.

Response:

We changed the text as requested, i.e. removed the original sentence and added the following text:

"Possible loss of Bt can occur due to the loss of the thymine base to which the Bt marker is bound (Figure 2a), LEE induced damage to the Bt label (Figure 2b) or damage of the DNA loop involving two SSBs (Figure 2c) that may be initiated by either 1 or 2 incident electrons."

Comment:

I have some doubt concerning the power-law-dependency of NSB with increasing F in the exposure-response. This is true for an infinite number of targets, which is not the case in these measurements. With a finite number of targets, as they are being depleted with increasing fluence the exposure-response curve saturates exponentially.

Response:

This is basically correct for a sufficiently high fluence, at which we indeed observed an exponential saturation in previous work. In the present experiments we have avoided this fluence regime and worked at such a low fluence that the exposure-response curve is linear and two-electron processes become very unlikely. We have added a respective sentence to the experimental details provided in the SI.

Comment:

The response to comment 4 is satisfactory.
Regarding comment 5, the new details added to the SI are most welcome and give a clearer indication of the complexity of these measurements. However, it occurs to this reviewer, that if the binding efficiency of Bt to Sav can vary from batch to batch (batch of origami templates, Bt marker or Sv agent?) then could this represent a change in sensitivity that could compromise comparisons between batches (and thus damage yields and electron energies)?

Response:

Possible contributions to the batch-to-batch variation are indeed the quality of the SAv (presumably the major factor) and the quality of target strands (e.g. the yield of successfully coupled Bt). The DNA origami structure itself is not very error-prone and is not expected to contribute significantly. At this point it should be made clear that the irradiation experiments and the analysis of samples is very time-consuming and took place within two or three years. Within this time the stock solutions of all compounds changed several times. Nevertheless, the determination of the strand break cross sections only requires the determination of the slope, i.e. relative changes with respect to the control sample. Therefore, we made sure, that within one series of irradiations all the sample conditions were the same. Under these conditions the sensitivity should not be compromised. We have this information to the experimental details provided in the SI.

Comment:
I am only partly satisfied with the answer to comment 6 that the background DSBs is due to poor electron beam resolution. It certainly contributes, but how poor can you get? A one-eV resolution would be sufficient to resolve the DEA peaks leaving a much lower background and certainly not an essentially flat signal from 5 to 8.5 eV. Could it be that inelastically backscattered electrons from the template further interact with DNA to smear out the structures, making them less visible and adding background DSBs? Perhaps, the is why they do not see the 6-eV resonance that is also present in the yield function of DSBs from plasmids. I would like to note that the authors report old cross section (CS) measurements with plasmids, recorded when the technique was not as well developed as today. In recent investigations, the ratio of the CS for double strand breaks to that for single strand breaks was found to be about 0.1.

Response:

These are all very good points. We cannot completely rule out the possibility of backscattered electrons, but we don't think that our experiments provide a higher "background" than the results obtained by plasmid DNA irradiation. The flat signal from 5 to 8.5 eV mentioned by the Reviewer might be due to a low number of measurement points (in fact we have used 5, 7 and 8.4 eV). In order to properly investigate the presence of a 6-eV resonance, we will cover this energy regime with a

higher resolution electron gun in the near future. We have now included more up-to-date cross-sectional values as suggested by the Reviewer and refer to them in the main text.