oc-2021-014048.R1

Name: Peer Review Information for "Real-Time Fast Amyloid Seeding and Translocation (RT-FAST) of α-synuclein with a nanopipette"

First Round of Reviewer Comments

Reviewer: 1

Comments to the Author

The paper describes a detection method for the aggregation of alpha-synuclein using a nanopipette system that is fast, simple, and requires little material to aid the study of neurodegenerative diseases.

The reviewer is convinced that developing methods for studying the aggregation of alpha-synuclein is an important research endeavor. However, we find that the system is quite nuanced, and it difficult to interpret the data with a high degree of confidence (clearly attributing the current signal to the aggregation process). Our suggestion is that the data be analyzed more carefully, and the paper could potentially be submitted after addressing the following comments. Comments are found below to help the writers revise their paper that we hope it is found useful. Perhaps another choice of ss-nanopore might a good strategy in the future to be able to resolve the aggregation dynamics that avoid complications associated with the nanopipette system.

Data interpretation

One of the concerns the reviewer has about the article is the characterization of the translocation events and current signals.

-If you look at figure 1, it seems strange to have some signals with such a long dwell time (90 minWT). These peaks look a lot like artifacts arising in nanopipette systems after long periods of applied potential.

-Translocation event analysis should best be done by a combined analysis of dwell time, amplitude, and frequency, and averaging the data can be misleading in many cases. This would provide a much clearer data analysis. At this point, the data analysis seems disconnected by separating current traces, amplitude analysis, and temporal effects in three different figures.

-The current variance in figure 1f is very large. Is this common for these kinds of experiments? Have you considered applying a shorter pulse more frequently through the measurements?

Have you correlated the amplitude and baseline in absolute terms and as a function of time? The normalization might hide some important information.

-A study of the relationship between potential and translocation parameters would help understand the translocation signal. It would be also valuable for biological understanding refer to early work on α-synuclein conformation and aggregation kinetics seeing Nanoscale 7 (46), 19627-19640,2015; Acta Neuropathologica 142, 87–115 (2021)

Conclusions

The initial conclusion that the peak signals increase when increasing the temperature due to aggregation needs to be backed up with an analysis of the effect of mass transport as a function of temperature. What is the predicted increased rate of aggregation with a temperature increase of 5 degrees?

Data aimed to prove that the detection mechanism is correct would be useful to have in the main paper. The reviewer does not agree with the authors that the data in figure 2 demonstrates that aggregation occurs, rather than it being the detection of the seeds themselves. There appears to be no clear change as a function of time, why is this if the effect is due to the aggregation?

The argument that the signal is somehow amplified in the nanopipette system needs to be explored. Again, relating to the data analysis.

Considering the concentration data in Figure 3f, where is the rest of the data? The effect of the seeding seems to be very small. What is expected?

Other comments:

-It would be helpful to have a scheme showing the aggregation of alpha-synuclein.

-The introduction gets technical quite fast and would benefit from covering alpha-synuclein aggregation, medical application in more detail to aid the reader's understanding of the motivation.

-It would be good to have aggregation data for reference in the main manuscript. This work can be also extended to study the amyloid-inhibitor or co-assembly system. Many researchers will appreciate more for this work. ACS nano 10 (4), 4143-4153, 2016; Chemical communications 48 (2), 191-193, 2012 etc.

Reviewer: 2

Comments to the Author

Referee report ACS Central Science oc-2021-014048

The ability to fully understand the protein aggregation process implied in human diseases remains a challenge at the interface of several disciplines. Moreover, the ability to detect the first aggregation steps of alpha-synuclein implied in the synucleinopathies at low concentrations is a considerable challenge for developing diseases' diagnostic powerful approach.

The manuscript "Real-Time Fast Amyloid Seeding and Translocation (RT-FAST) of α-synuclein with a nanopipette" by Mayer et al. shows the ability to experimentally detect at low concentrations "potential biomarkers" of amyloidogenesis process, using a nanopipette. This device accelerates the kinetic of aggregation and allows detection at the single molecular level with electrical detection. The authors compare WT and variant (A53T) aggregation kinetic by probing the capture rate and the current amplitude evolution as a function of time or monomer protein concentration.

State of the art could be more precisely introduced. The results are original and very interesting for a broad audience but need to be better discussed. See below.

The manuscript form needs some major improvements before publication.

Abstract

- Could the authors start with a general sentence about protein aggregation and human diseases?

- Could the authors define seed for a broad audience (or in the indroduction part)?

- The referee disapproves of the amplification used by the authors. The device dot does not allow a real amplification of the number of molecules at the end of the experiment, like the PCR assay. Please find another word.

- What is the justification for choosing the variant (A53T)?

Introduction

- "We have designed … protein amplification assay…."

Please rewrite the sentence without the word "amplification"

- "With a large surface /volume ratio that favors the amyloid sending reaction…."

This sentence is not clear for a non-specialist of the field. Do we expect a conformational change during protein absorption, and what does it mean in terms of statistical conformations? This absorption could also alter the structural elements of the protein. Is it a reversible process?

- Please cite one or two reviews about the principle of RPS and data analysis.

- The paragraph "the solid-state to beta-lactoglobulin" is just a set of citation papers. Could the authors please add one sentence for each citation to explain the main results of the paper in order to understand what state of the art is precisely described before the author's study?

- Please delate amplification at the end of the introduction.

Results and discussion

Major points to discuss

- The dwell times data are not commented on and discussed in the manuscript.

* Figure SI-3: for the control experiments, the dwell times seem to depend on the nanopipettes used; see b and c. Figure SI-5 and figure SI-6: b behavior. How do the authors interpret this phenomenon?

* Did the authors evaluate the diffusion coefficient for the control, the Wt, and variant with the mean dwell time for a specific condition?

* Is it compatible with the expected theoretical translocation time?

* What is the evidence for aggregates translocation?

- The current amplitudes are not discussed in terms of aggregate size, morphology, or the number of monomers composing the aggregates.

Could the authors please try to evaluate the size of the aggregates from the current amplitude in the different conditions?

Minor points

- "The successful coating process is evidenced… density (p5)".

How to be sure that the coating covers all the micropipette's inner surface?

- Figure 2f, why do the authors observe two regimes of the mean current amplitude for the variant?

- Figure 3f, it is difficult to determine the dependency of f versus concentration with only three experimental points. Could the authors add 2 or 3 experimental points to fit the data correctly?

Author's Response to Peer Review Comments:

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Montpellier, January 25, 2022

Object: cover letter

Dear professor Editor,

We thank you for your response and to give us the opportunity to correct our manuscript ID : oc-2021-014048, entitled "Real-Time Fast Amyloid Seeding and Translocation (RT-FAST) of α-synuclein with a nanopipette

We would also like to thank the reviewers their comments which were very useful to improve the quality and readability of our manuscript. In this corrected version, we have were taken into account all the referee's comments. Following their recommendations, we did major changes among them:

(i) Improve the abstract by addind a section dedicated to the medical and the state of art about the amyloid sensing by nanopore.

(ii) Improve the discussion about to the relative current blockade amplitude and the dwell time.

You will find below point-by-point response of all reviewer's queries.

We hope that this new version will meet the high quality criteria required for publication in ACS Central science.We are looking forward to the constructive comments from editorial office and reviewers. We thank you in advance for the time you expend considering our work.

Sincerely,

Sébastien Balme (on behalf all authors)

Reviewer: 1

Recommendation: Reconsider after major revisions noted.

Comments:
The paper describes a detection method for the aggregation of alpha-synuclein using a nanopipette system that is fast, simple, and requires little material to aid the study of neurodegenerative diseases. The reviewer is convinced that developing methods for studying the aggregation of alpha-synuclein is an important research endeavor. However, we find that the system is quite nuanced, and it difficult to interpret the data with a high degree of confidence (clearly attributing the current signal to the aggregation process). Our suggestion is that the data be analyzed more carefully, and the paper could potentially be submitted after addressing the following comments. Comments are found below to help the writers revise their paper that we hope it is found useful. Perhaps another choice of ss-nanopore might a good strategy in the future to be able to resolve the aggregation dynamics that avoid complications associated with the nanopipette system.

Our answer: We thank the referee for the consistent comments.

Data interpretation

One of the concerns the reviewer has about the article is the characterization of the translocation events and current signals.

Our answer: We thank the referee to point out the fact we did not deeply investigate the current blockade parameters in order to deduce some structural information of oligomers during the RT-FAST. This question is relevant because nanopore sensing is usually used to identify biomacromolecules and to obtain information about structural changes, size, sequence, etc. Thus for the amyloid field, the referee could expect to learn more about the different α-synuclein species present in solution as we previously reported for the β-lactoglobulin, tau and Aβ, or other groups for the α-synuclein.

Before answering all specific questions concerning the analysis and interpretation of the $\Delta I/I_0$ and the Δt, we want to provide a general statement on the aggregation kinetics of α-synuclein, which is an intrinsically disordered protein. Our nanopore results are obtained in short-term experiments (2h). We show by TEM and ThT assay that there is no β-sheet fibrils in the sample but only oligomers. Recent literature on α-synuclein aggregation suggests a kinetic model with two types of oligomers, according to their ability to form fibrils. However, each oligomers type is composed by a wide range of kinetic and thermodynamic stabilities (Chem. Sci., 2020, 11,6236). Thus, oligomer species are highly heterogeneous, varying significantly both in conformation and in size (PNAS 2020, 117, 12087-12094). In addition, the majority of oligomers dissociate to monomers rather than to form fibrils (PNAS 2020, 117, 12087-12094 and Nature Chemistry 2020, 12, 445–451). On the other hand, in our experiments, we did not control the conditions to form well-identified oligomers to with the aim to analyses a homogenous sample. Indeed, our goal is to gain the protein misfolding amplification by seeding to detect the presence of seeds at t=0 despite their low concentration. This leads to the detection of a heterogeneous oligomeric sample. That is the main reason why we did not provide any correlation between the current blockade parameters and the aggregate morphology.

However, according to the referee comments, we are aware that a better interpretation of both the $\Delta I/I_0$ and the Δt should be provided, and thus we have added a short description of the current blockade parameters as well as an explanation about the heterogeneity of the sample that limit the interpretation.

-If you look at figure 1, it seems strange to have some signals with such a long dwell time (90 minWT). These peaks look a lot like artifacts arising in nanopipette systems after long periods of applied potential.

Our answer: We thank the referee for this comment. We agree that we observed some events with long dwell time that could be artefacts due to a long period of applied voltage. First, we could like to clarify that in our experiment the voltage is not applied during all the incubation time but only during 10 min. This is followed with 20 min without voltage. We observe a correlation between the total number of events and the long event regardless the incubation time.

-Translocation event analysis should best be done by a combined analysis of dwell time, amplitude, and frequency, and averaging the data can be misleading in many cases. This would provide a much clearer data analysis. At this point, the data analysis seems disconnected by separating current traces, amplitude analysis, and temporal effects in three different figures.

Our answer: As we mention before, we agree with the referee. We would like to stress that the main goal of our work is to demonstrate that the nanopipette approach is suitable to provide information about the presence of the seed at t=0, without waiting for the formation of β-sheet fibrils, which is an improvement compared to the RT-QuIC assay and the detection by ThT fluorescence.

We have averaged the result of several pipettes because the first aggregation step is reversible and follow different pathways. In addition, we have observed, especially for the control and at seed concentration 2 pM, variability in the detection time of

aggregates. Thus, we believe that these points are of great importance and must be taken into account. In addition, the production of multiple nanopipettes results in the variability of the tip diameter and the shank length. As our aim is to provide an analytical method, this variability must be considered. This explains why averaged data from several nanopipettes are provided. Of course, such averaging is not suitable to correlate the current blockade parameter to the aggregate morphology. However, this is not the goal of our work.

We also agree with the second referee, who also point that we did not deeply interpret the amplitude of the current blockade and the dwell time. We have followed the comments and improve the discussion of these parameters according to the recent literature as previously mentioned. However, we make the choice to be cautious concerning the size of aggregates because at this stage co-exist in solution a wide range of oligomers with different lifetime.

Discussion for the control Page 7 : *"Regardless the nanopipette and the incubation time, the mean distribution of the $\Delta I/I_0$ amplitude is mostly below 0.2. However, the low number of events makes it impossible to provide further information on the aggregate size or morphology."*

Discussion for the samples with WT seeds Page 8-9" *Compared to the control, the mean values of $\Delta I/I_0$ are larger and the distribution more spread. This suggests that the detected αS assemblies are larger and heterogeneous. This is supported by the wide range of the dwell time distribution over 2 orders of magnitude at the ms scale. We notice that the ms scale for the dwell time is in good agreement with the literature related to the detection of protein aggregates by nanopipette[20,21,34]. Regardless the sample used, the value of $\Delta I/I_0$ does not increase with time, as it could be expected for the formation of protofibrils. Previous published data using nanopore were reported with an incubation period of several days and the aggregates were withdrawn at the different stages of fibril formation (i.e., monomer, lag phase, elongation phase and plateau)[23,24]. Here, we show that oligomer species are generated during the lag phase (short time scale dynamics), before the formation of fibrils with β-sheet structure. At this stage, the oligomer species are highly heterogeneous in terms of size and conformation[35,36]. Among them, the majority will not convert to fibrils but dissociate to monomers to further re-aggregate[37]. Thus, the observation of a wide range of events is consistent with the presence of transient oligomers in association/dissociation equilibrium during the lag phase, as previously reported for the Aβ peptide[29]*

Discussion for the samples with A53T seed *"This suggests that the A53T seeds induce the formation of larger and more heterogeneous aggregates than the WT. The oligomerization of A53T was reported to consume more rapidly the monomer than the WT[41]. On the other hand, the cross-seeding of αS monomers by A53T seed was found to accelerate the elongation process[42] as well as to modify the properties of oligomer species[43]. Thus, it is plausible that the cross-seeding by A53T produced different populations of oligomeric species compared to the WT during the lag phase."*

-The current variance in figure 1f is very large. Is this common for these kinds of experiments? Have you considered applying a shorter pulse more frequently through the measurements? Have you correlated the amplitude and baseline in absolute terms and as a function of time? The normalization might hide some important information.

Our answer: The referee is right. However, the current trace shown in figure 1f is the result of different nanopipettes, and explains the observed difference. We have added the information in the caption.
We also agree that the normalization can hide information such as the volume or the morphology of the aggregate. Nevertheless, this type of analysis is out of the scope of this paper. The normalization is used here to extract information about the presence of larger aggregates compared to the control (qualitative information) and the capture rate. The complexity of the reaction and the fact that the aggregation does not occur for all experiments forces us to average the result of several nanopipettes. Thus, according to the variability of the pipette, the most suitable way to compare the results is the normalization.

-A study of the relationship between potential and translocation parameters would help understand the translocation signal. It would be also valuable for biological understanding refer to early work on α-synuclein conformation and aggregation kinetics seeing Nanoscale 7 (46), 19627-19640,2015; Acta Neuropathologica 142, 87–115 (2021)

Our answer: We agree that studying the influence of the applied voltage can provide interesting information on the translocation process. However, our approach has another goal. Indeed, a deep investigation of the translocation process makes sense only if the translocating object is well defined. In our experiment, the sample is composed of numerous species of co-existing oligomers. Thus, we have focused in one selected voltage, which is the best compromise between a good signal/noise ratio and signal stability.

Conclusions
The initial conclusion that the peak signals increase when increasing the temperature due to aggregation needs to be backed up with an analysis of the effect of mass transport as a function of temperature. What is the predicted increased rate of aggregation with a temperature increase of 5 degrees?

Our answer: The referee is right concerning the temperature influence the mass transport and the capture rate. Basically, assuming that the capture rate (f) is mainly driven by a diffusion process. As commonly described for proteins, f=2πDr*, where D is the diffusion coefficient and r* the capture radius. We assume (i) that the species are similar according to the similar distribution of $\Delta I/I_0$ and (ii) capture radius of the nanopore are similar. The increase of 5°C influence the diffusion coefficient temperature and the viscosity of the media. We could thus expect an increase in the frequency of a factor 1.13. Experimentally, the increase of 5°C, increase the capture rate of a factor > 4.

We add a comment in the main text page *". A rise in the incubation temperature, significantly increases the capture rate (f>0.1 after 30 min). In addition, the amplitudes of $\Delta I/I_0$ are comparable to those obtained at 25 °C suggesting that the distribution of the aggregate size is similar. Since the capture rate is mainly due to a diffusion process[28] $f = 2\pi Dcr^*$ (D is the diffusion coefficient, C is the concentration and r^* the capture radius), the expected ratio is $f_{T=30°C}/f_{T=25°C} \approx 1.13$, for similar capture radius and aggregate size. The experimental result shows that the ratio $f_{T=30°C}/f_{T=25°C} > 4$, suggesting that more aggregates are formed in the nanopipette reservoir due to temperature-accelerated kinetics."*

Data aimed to prove that the detection mechanism is correct would be useful to have in the main paper. The reviewer does not agree with the authors that the data in figure 2 demonstrates that aggregation occurs, rather than it being the detection of the seeds themselves. There appears to be no clear change as a function of time, why is this if the effect is due to the aggregation?

Our answer: We thank the referee for the comment. We do not agree that our results do not demonstrate the seeding. We detect events at t=0. As explained, the seeded-aggregation process takes place during the detection time, which is 10 min. The aggregation is clearly demonstrated in figure 3a and 3b, where the minimum values of capture rate at t=0 are observed for both seed WT and A53T. This is confirmed in figure 3c and 3d, showing a time shift for the detection of aggregates with seed concentration.

The argument that the signal is somehow amplified in the nanopipette system needs to be explored. Again, relating to the data analysis.

Our answer: We think that there is a misunderstanding with the term "amplification". The signal is not amplified in the nanopipette. The presence of seeds amplifies the protein misfolding process thus producing detectable aggregates by the nanopipette.

Considering the concentration data in Figure 3f, where is the rest of the data? The effect of the seeding seems to be very small. What is expected?

Our answer: The figure 3f shows the dependence of the capture rate with the initial seed concentration. Full experimental data are given in supporting information section. In figure 3f, we show the results of the mean capture rate, from 3 independent nanopipettes, for 3 different concentrations of seeds and the control. In other words, 24 experiments are summarized in this figure. We can see a clear effect of the seeding process compared to the control (sample without seeds, in violet dash line).

We notice that we mentioned that *"This probes that RT-FAST assay is able to provide a quantitative information on seed concentration based only on the capture rate."*. **We did not fit the data to propose a quantitative method to measure the seed concentration. This work is under progress and requires several technical improvements.**

Other comments:

-It would be helpful to have a scheme showing the aggregation of alpha-synuclein.

Our Answer: We have added a scheme showing the aggregation of α-synuclein in figure 1f

-The introduction gets technical quite fast and would benefit from covering alpha-synuclein aggregation, medical application in more detail to aid the reader's understanding of the motivation.

Our answer: We thank the referee for the comment. We have added a general introduction about the protein aggregation and human disease. See Page 2 : *"The aberrant aggregation of intrinsic-disordered proteins into highly ordered structures rich in β-sheets, called amyloids, is involved in age-related diseases. Among them, the α-synuclein (αS), a neuronal pre-synaptic protein, is involved in the development of Parkinson's disease (PD)[1]. Its aggregated forms disrupt the functioning and survival of neurons and represent the main markers of the pathology[2]. Indeed, certain aggregate structures (e.g., soluble oligomers) may be present decades before the onset of the first major motor disorders. Today, the accuracy of clinical (or radiological) diagnosis improves with the progression of the disease[3]. As a result, these techniques often do not allow the diagnosis at early stages and are not able to distinguish PD from other*

synucleinopathies, such as Lewy body or multiple system atrophy[4]. For all these reasons, developing analytical methods for the detection and quantification of αS assemblies in biofluids is an important challenge to propose early diagnosis of synucleinopathies."

-It would be good to have aggregation data for reference in the main manuscript. This work can be also extended to study the amyloid-inhibitor or co-assembly system. Many researchers will appreciate more for this work. ACS nano 10 (4), 4143-4153, 2016; Chemical communications 48 (2), 191-193, 2012 etc.

Our answer: We have mentioned that the RT-FAST could be extended to investigate the effects of amyloid inhibitors or co-assemblies and include the references. See Page : *"Besides that, the RT-FAST is also a promise tool for fundamental investigations of the protein aggregation process including the impact of the cross-seeding[39,40], co-assembly[41,42] or inhibitor[43,44], leading to future advances in the understanding of the lag phase of amyloidogenesis."*

Reviewer: 2

Referee report ACS Central Science oc-2021-014048
The ability to fully understand the protein aggregation process implied in human diseases remains a challenge at the interface of several disciplines. Moreover, the ability to detect the first aggregation steps of alpha-synuclein implied in the synucleinopathies at low concentrations is a considerable challenge for developing diseases' diagnostic powerful approach. The manuscript "Real-Time Fast Amyloid Seeding and Translocation (RT-FAST) of α-synuclein with a nanopipette" by Mayer et al. shows the ability to experimentally detect at low concentrations "potential biomarkers" of amyloidogenesis process, using a nanopipette. This device accelerates the kinetic of aggregation and allows detection at the single molecular level with electrical detection. The authors compare WT and variant (A53T) aggregation kinetic by probing the capture rate and the current amplitude evolution as a function of time or monomer protein concentration. State of the art could be more precisely introduced. The results are original and very interesting for a broad audience but need to be better discussed. See below. The manuscript form needs some major improvements before publication.

Abstract
- Could the authors start with a general sentence about protein aggregation and human diseases?

Our answer: We thank the referee for the comment. We have added a general introduction about the protein aggregation and human disease page 2. *"The aberrant aggregation of intrinsic-disordered proteins into highly ordered structures rich in β-sheets, called amyloids, is involved in age-related diseases. Among them, the α-synuclein (αS), a neuronal pre-synaptic protein, is involved in the development of Parkinson's disease (PD)[1]. Its aggregated forms disrupt the functioning and survival of neurons and represent the main markers of the pathology[2]. Indeed, certain aggregate structures (e.g., soluble oligomers) may be present decades before the onset of the first major motor disorders. Today, the accuracy of clinical (or radiological) diagnosis improves with the progression of the disease[3]. As a result, these techniques often do not allow the diagnosis at early stages and are not able to distinguish PD from other synucleinopathies, such as Lewy body or multiple system atrophy[4]. For all these reasons, developing analytical methods for the detection and quantification of αS assemblies in biofluids is an important challenge to propose early diagnosis of synucleinopathies."*

- Could the authors define seed for a broad audience (or in the indroduction part)?

Our answer: We have followed the referee comment and defined the seed as follows (See Page 2): *"The seeds that are performed αS aggregates induce misfolding of natives αS to form amyloid-type aggregates that bind to the fluorescence reporter thioflavin T (ThT)"*

- The referee disapproves of the amplification used by the authors. The device dot does not allow a real amplification of the number of molecules at the end of the experiment, like the PCR assay. Please find another word.

Our answer: The referee is right. The term "amplification" is not directly comparable to the PCR approach. We replace the term "seed amplification" by "protein misfolding amplification" , which is used in amyloid seeding approaches, as defined by Soto et al. in Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature, (2001), 411, 810-813"

- What is the justification for choosing the variant (A53T)?

Our answer: As we explained in the manuscript, we have chosen this protein variant to enhance the aggregation process. It has also a clinical interest since this genetic mutation is associated to early-onset Parkinson disease (*Science*, 1998;276(5321), 2045–2047). We have added the sentence and the reference (See Page 9) : *"This mutation is associated with early-onset Parkinson's disease[34]."*

Introduction
- "We have designed ... protein amplification assay...." Please rewrite the sentence without the word "amplification"

Our answer: As mentioned before we have modified the term "protein amplification" by "protein misfolding amplification" as defined by Soto et al. Nature, 411, 810-813

- "With a large surface /volume ratio that favors the amyloid sending reaction...." This sentence is not clear for a non-specialist of the field. Do we expect a conformational change during protein absorption, and what does it mean in terms of statistical conformations? This absorption could also alter the structural elements of the protein. Is it a reversible process?

Our answer: We thank the referee for this comment. The catalytic role of the surface on α-synuclein or Aβ peptide aggregation is often reported as shown the recent review of Grigolato, F. and Arosio, P. (*Biophysical chemistry* 2021, *270*, 106533). However, as it was mentioned in the conclusion section of this review, the exact molecular mechanism of surface effects on aggregation kinetics is still unrevealed. Thus, in our manuscript we cannot include further mechanistic details.

- Please cite one or two reviews about the principle of RPSand data analysis .

Our answer: We thank the referee for this comment. We have added the 2 following references on RPS (Makra,et al. Electrochemistry Communications, 43, 55–59.) and data analysis (Wen, C.et al. *ACS sensors*, *6*, 3536-3555.)

- The paragraph "the solid-state to beta-lactoglobulin" is just a set of citation papers. Could the authors please add one sentence for each citation to explain the main results of the paper in order to understand what state of the art is precisely described before the author's study?

Our answer: We thank the referee for this comment. We have followed his/her suggestion and provide the main result of each reference given in the paragraph (see page :

"The solid-state nanopore were recently considered investigating protein assemblies[11,18]. The quartz nanopipettes were used to detect lysozyme[19] and prion[20] assemblies based on the enhancement of current blockade amplitude. In order to improve the detection of the assemblies, a crowded media was considered to discriminate αS fibrils before and after sonication [21]. The silicon nitride nanopores (SiN) were used to detect various proteins. For the αS, four oligomeric species were identified after 3 days of incubation[22]. Using a lipid coated nanopore, Aβ peptide oligomers and fibrils were detected after several days of incubation[23]. The common point between these two previous works is that amyloid samples were withdrawn at various time intervals corresponding to different aggregation steps (i.e., monomer, lag phase, elongation phase and the plateau). These studies also showed the importance of the nanopore coating using phospholipid or tween20 to prevent its fouling. The SiN nanopore was found suitable to characterize the morphology of lysozyme, β-lactoglobulin, and BSA[24,25] aggregates. Polymer nanopores obtained by track-etched method on polyethylene terephthalate film were also used to differentiate different protein species (oligomers and protofibrils) during the aggregation process of β-lactoglobulin[26], as well as to discriminate calibrated fibrils of the Aβ-peptide [27]. Another study points out the importance of conformational rearrangements and protomer exchanges during the self-assembly of Aβ peptide [28]. The evidence of auto-fragmentation processes was demonstrated during the aggregation of tau induced by heparin[29]. The track etched nanopore allowed to study the seed-induced re-aggregation after protease digestion of β-lactoglobulin[30]."

- Please delate amplification at the end of the introduction.

Our answer: As discussed before "seed amplification" was replaced by "protein misfolding amplification."

Results and discussion
Major points to discuss

Our answer: We thank the referee to point out the fact we did not deeply investigate the current blockade parameters in order to deduce some structural information of oligomers during the RT-FAST. This question is relevant because nanopore sensing is usually used to identify biomacromolecules and to obtain information about structural changes, size, sequence, etc. Thus for the amyloid field, the referee could expect to learn more about the different α-synuclein species present in solution as we previously reported for the β-lactoglobulin, tau and Aβ, or other groups for the α-synuclein.

Before answering all specific questions concerning the analysis and interpretation of the $\Delta I/I_0$ and the Δt, we want to provide a general statement on the aggregation kinetics of α-synuclein, which is an intrinsically disordered protein. Our nanopore results are obtained in short-term experiments (2h). We show by TEM and ThT assay that there is no β-sheet fibrils in the sample but only oligomers. Recent literature on α-synuclein aggregation suggests a kinetic model with two types of oligomers, according to their ability to form fibrils. However, each oligomers type is composed by a wide range of kinetic and thermodynamic stabilities (Chem. Sci., 2020, 11,6236). Thus, oligomer species are highly heterogeneous, varying significantly both in conformation and in size (PNAS 2020, 117, 12087-12094). In addition, the majority of oligomers dissociate to monomers rather than to form fibrils (PNAS 2020, 117, 12087-12094 and Nature Chemistry 2020, 12, 445–451). On the other hand, in our experiments, we did not control the conditions to form well-identified oligomers to with the aim to analyses a homogenous sample. Indeed, our goal is to gain the protein misfolding amplification by seeding to detect the presence of seeds at t=0 despite their low concentration. This leads to the detection of a heterogeneous oligomeric sample. That is the main reason why we did not provide any correlation between the current blockade parameters and the aggregate morphology.

However, according to the referee comments, we are aware that a better interpretation of both the $\Delta I/I_0$ and the Δt should be provided, and thus we have added a short description of the current blockade parameters as well as an explanation about the heterogeneity of the sample that limit the interpretation.

- The dwell times data are not commented on and discussed in the manuscript.
* Figure SI-3: for the control experiments, the dwell times seem to depend on the nanopipettes used; see b and c. Figure SI-5 and figure SI-6: b behavior. How do the authors interpret this phenomenon?

Our answer: The referee is right. For the control experiments, the dwell time seem to depend on the nanopipette. However, we noticed that the number of events detected for the control is low (lower than 20 in most cases) and thus it is not possible to interpret the dwell time. Concerning the Figure SI-5 and SI-6, in relation to the seed experiments, the number of events is significant > 100 in most cases. However, as previously mentioned, the heterogeneity of the aggregate during the lag phase does not allow us to explore deeper the correlation between aggregate and current blockade parameter.

* Did the authors evaluate the diffusion coefficient for the control, the Wt, and variant with the mean dwell time for a specific condition?

Our answer: As previously mentioned, the heterogeneity of the oligomer cannot allow to provide relevant information about the diffusion constant. Indeed, the evaluation of the diffusion constant from the capture rate requires to know the concentration and the capture rate f=2πDCr* (where D is the diffusion coefficient, r* the capture radius and C the concentration that is not known.
From the dwell time we can extract D

$$\mu = \frac{QD}{k_B T}$$

However, the aggregate charge Q cannot be known due to the heterogeneity of the sample. This makes it impossible to obtain the diffusion coefficient of aggregates.

* Is it compatible with the expected theoretical translocation time?
* What is the evidence for aggregates translocation?

Our answer: We observe a dwell time ranging from ms to 100 ms that depends on the presence of the seed and the incubation time. We cannot determine a theoretical value for Δt due to the heterogeneity of the sample. However, the ms time scale is in good agreement with previous studies to detect prion protein (U. Keyser group : *ACS Nano* 2013, 7, 5, 4129–4134), Aβ peptide (Y. Long group *Chem. Sci.*, 2019,10, 10728-10732) and lysozyme (J. Edel group *Anal. Chem.* 2019, 91, 10, 6880–6886) using nanopipettes. We did not observe bumping events since the expected dwell time should be µs scale as shown in ref *Chem. Sci.*, 2019,10, 10728-10732. In addition, if aggregates are not able to pass through the nanopore, we should observe a nanopore clogging. For these reasons, we believe that the dwell time that we observe is relevant.

We have added a sentence (see Page 8) *: "This is supported by the wide range of the dwell time distribution over 2 orders of magnitude at the ms scale. We notice that the ms scale for the dwell time is in good agreement with the literature related to the detection of protein aggregates by nanopipette[20,21,34].."*

- The current amplitudes are not discussed in terms of aggregate size, morphology, or the number of monomers composing the aggregates. Could the authors please try to evaluate the size of the aggregates from the current amplitude in the different conditions?

Our answer: We agree with the referee comments. We did not discuss the aggregate size because the oligomeric species are highly heterogeneous. We could only provide a means volume but it does not make sense given the large distribution of the relative current blockade. However, according to the referee comments we have improved the discussion about the dwell time and the relative current blockade.

Discussion for the control (Page 7): *"Regardless the nanopipette and the incubation time, the mean distribution of the $\Delta I/I_0$ amplitude is mostly below 0.2. However, the low number of events makes it impossible to provide further information on the aggregate size or morphology."*

Discussion for the samples with WT seeds (Page 8-9): *" Compared to the control, the mean values of $\Delta I/I_0$ are larger and the distribution more spread. This suggests that the detected αS assemblies are larger and heterogeneous. This is supported by the wide range of the dwell time distribution over 2 orders of magnitude at the ms scale. We notice that the ms scale for the dwell time is in good agreement with the literature related to the detection of protein aggregates by nanopipette[20,21,34]. Regardless the sample used, the value of $\Delta I/I_0$ does not increase with time, as it could be expected for the formation of protofibrils. Previous published data using nanopore were reported with an incubation period of several days and the aggregates were withdrawn at the different stages of fibril formation (i.e., monomer, lag phase, elongation phase and plateau)[23,24]. Here, we show that oligomer species are generated during the lag phase (short time scale dynamics), before the formation of fibrils with β-sheet structure. At this stage, the oligomer species are highly heterogeneous in terms of size and conformation[35,36]. Among them, the majority will not convert to fibrils but dissociate to monomers to further re-aggregate[37]. Thus, the observation of a wide range of events is consistent with the presence of transient oligomers in association/dissociation equilibrium during the lag phase, as previously reported for the Aβ peptide[29]*

Discussion for the samples with A53T seed (Page 9-10): *"This suggests that the A53T seeds induce the formation of larger and more heterogeneous aggregates than the WT. The oligomerization of A53T was reported to consume more rapidly the monomer than the WT[41]. On the other hand, the cross-seeding of αS monomers by A53T seed was found to accelerate the elongation process[42] as well as*

to modify the properties of oligomer species[43]. Thus, it is plausible that the cross-seeding by A53T produced different populations of oligomeric species compared to the WT during the lag phase."

Minor points

- "The successful coating process is evidenced... density (p5)". How to be sure that the coating covers all the micropipette's inner surface?

Our answer: We thank the referee for this comment. Actually, there is no method to precisely obtain the surface density of L-DOPA coating. Indeed, such information for a single nanopore can only be obtained by a measurement of the number of active chemical sites before and after grafting and assuming a cylindrical geometry as well as an infinite length of the nanopore, as our group have previously shown for the track-etched nanopore (see *Nanomaterials* 2021, *11*, 244 and Scientific Reports, 2015, 5, 10135)

- Figure 2f, why do the authors observe two regimes of the mean current amplitude for the variant?

Our answer: We observe for the variant a fluctuation in the amplitude of the current blockade. Such fluctuation was also reported for the aβ peptide aggregation during the lag phase (Meyer et al. *Chemosphere*, 2021, p. 132733). This also occurs for the WT and is likely due to the aggregation/dissociation process. We guess that this does not correspond to two distinct regimes.

- Figure 3f, it is difficult to determine the dependency of f versus concentration with only three experimental points. Could the authors add 2 or 3 experimental points to fit the data correctly?

Our answer: In figure 3f, we show the dependence of the capture rate with the initial seed concentration. We report, for 3 seed concentrations and the control, the mean capture rate from 3 independent nanopipettes. Basically, at mentioned before, 24 experiments are summarized in this figure. We did not fit the data to propose a quantitative method to measure the seed concentration. This work is under progress and requires improving several technical aspects at this stage.