Review of: "Effects of the SARS-CoV-2 Spike protein on in vitro aggregation of alpha synuclein- probable molecular interactions and clinical implications"

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Potential competing interests: No potential competing interests to declare.

Overall, this manuscript presents interesting preliminary results regarding the interaction between the SARS-CoV-2 Spike protein and the Parkinson's disease-associated, amyloidogenic alpha-synuclein protein. The manuscript would benefit from some revision and further analyses, as described below.

In Section 1, the authors should consider adding a reference to the recent article "SARS-CoV-2 drives NLRP3 inflammasome activation in human microglia through spike protein" by Albornoz *et al*. Additionally, referencing the study "Interactions between SARS-CoV-2 N-Protein and α-Synuclein Accelerate Amyloid Formation" by Semerdzhiev *et al* here would provide further background information for the rationale behind the present study.

Key information is missing from the description of the Thioflavin T aggregation assay in Section 2.2:

- The original publication cited in the Methods description for the Thioflavin T assay used Thioflavin T concentrations of 5 μM and 10 μM depending on the experiment. Please specify the concentration of Thioflavin T used for the assays in the present study.
- Additionally, the concentrations of each protein incubated for each aggregate are reported here, but the concentration or volume of the aliquots taken from these samples to use for the Thioflavin T assay solution are not reported. Please include these details.
- It may be beneficial to add a description of the whole solution composition (protein concentration, Thioflavin T concentration, and diluent or buffer) for the assay mixtures that were measured spectrofluorometrically.

Section 3.1 would benefit from further analysis; for example, descriptions of the key interacting residues and interaction types, particularly if there are any previously reported residues of interest in either protein that were found to be interactors here. The data showing interface residues should also be moved from the Supplementary File to the main text to support this.

Section 3.2 is interesting but raises points for further investigation. The results of statistical analysis should be included in the main text rather than the Supplementary File. A more detailed comparison of the endpoint aggregation state of alpha-synuclein on its own, compared to alpha-synuclein in the presence of Spike protein, should be included.

Section 4 references the study "Interactions between SARS-CoV-2 N-Protein and α-Synuclein Accelerate Amyloid Formation" by Semerdzhiev *et al* and outlines the substantial difference in Spike protein concentration used between the

two studies as the basis for the discrepancy in aggregation results. Some mention of the justification for the Spike concentration used in the present study would be beneficial.

In Section 4, the authors mention that the more rapid aggregation of alpha-synuclein in the presence of Spike protein in comparison to the lag phase observed in alpha-synuclein-only aggregation may represent the formation of multiple nucleation centers, accelerating the aggregation process. As both conditions resulted in similar endpoint fluorescence levels, it would be helpful to raise points for the potential pathophysiological implications of accelerated alpha-synuclein aggregation. For example, do the authors believe this would lead to earlier development of disease pathology? Are there further experiments suggested to characterise the effects of this accelerated aggregation?

In general, the manuscript would strongly benefit from further biophysical characterisation*in vitro*, such as microscale thermophoresis or circular dichroism for analysis of direct protein binding and the resulting aggregation states, respectively. Additionally, considering the context of clinical implications mentioned in the title, some preliminary cell culture studies would be beneficial to highlight the physiological relevance of aggregate formation in response to Spike protein.