PCI-RR

Revision round #1

Decision for round #1: Revision needed

Revision needed

I am a co-recommender of this manuscript with Emily. First of all, we are very pleased to see your submission from this new research field! I have seen a variety of manuscripts in the PCI RR, and here I would like to comment on to help you proceed smoothly toward the peer review process.

I encourage you to take another closer look at the Guide for Authors for more information about Registered Reports and the PCI RR system. The “criteria" in 2. Submission requirements are particularly important.

As you may already know, to proceed with peer review in this system, a solid background for the hypothesis is necessary, and it must be clear in advance which experimental results will be required to support the hypothesis. Your protocol appears to present multiple hypotheses. The PCI RR provides a study design template that summarizes each hypothesis. Your table is somewhat different from this, but I think it would be relatively easy to modify it into our study design template. I believe it should at least be revised before undergoing peer review for this submission. Whenever possible, what is written here should also be explained in the text. Then, after revising the study design template and related materials, have Emily scrutinize them for content.

We thank you for the comments. We have tried very hard to use the PCI RR study design template but we did struggle considerably because this approach to experimental design is very far from the practice in that field. To illustrate some of our struggles, we can consider one of Emily’s point below and our response to it. Emily suggested adding an additional cytotoxicity assay. We agreed with the suggestion and therefore took it on board, but this means we have now three techniques to test the same hypothesis. Specifying in details in advance all the possible outcomes from those different measurements and the conclusions we would draw from them is rather challenging… and this is just for one small aspect of the study. We hope that we have achieved overall some reasonable middle ground between the ideal of preregistration and the reality of such a complex multidimensional study.

Looking forward to seeing the revised manuscript again.

Yuki Yamada

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I am very pleased to be a co-recommender on this interesting replication study. I have below some suggestions for revision before sending to review.

We thank you for your interest in our replication study and for your constructive comments below.

In keeping with the submission criteria and as mentioned by Yuki, the full study design table needs to be included. In addition to this, the structure of the article needs to be similar to that of a final article and therefore some amendments to the manuscript structure would be useful at this stage to fit with this format.

We thank you for the comment on the study design table and have done our best to improve it; see response to Yuki above.

The authors have provided an overview of the research question/rationale and three key aims for this replication study, as well as an in-depth outline of methods and protocols both in line with the original study and also with additional controls where necessary.

With reference to the assessment of materials on cell viability (Assessing cytotoxicity of CdSe@C-TPEA in Hela cells, 1.14.1. MTT cytotoxicity assay and 1.14.2. Alamar Blue assay)

To measure cytotoxicity of the probes, the authors of the original article used the MTT assay. Given the limitations and pitfalls recently reviewed, in addition to the MTT assay, the authors plan to assess cytotoxicity with the more sensitive Alamar Blue assay. I would suggest it may also be important to include a cytotoxicity assay which isn't impacted by cellular metabolism as a read out, e.g., SRB assay. Also, it might be valuable to include the HepG2 cells in this experimental section too, as it is not known if these cells would be more sensitive to the nanoparticles and therefore to have a cytotoxicity readout would be useful as these are including in the sensing experiments.

We thank you for the comment and agree that adding an additional cytotoxicity assay i.e., SRB assay, that is not dependent on metabolites adds a second level of understanding. Accordingly, we have added it to section 14. We also agree that the addition of the cytotoxicity tests for HEPG2 cell line is crucial and have also added it to section 14.

Can the authors comment on the number of repeats to be carried out and statistical analysis

For the MTT assay we plan to have triplicate wells for each sample condition as an internal control and to have 3 independent replicates of each test. As for the statistical test, ANOVA testing will be carried out.

The authors note that typically ~1-2% of nanoparticles are reported to escape. They plan to test whether the localization of the probes is in line with this expectation or whether instead a high proportion of nanoparticles has escaped endosomes, by studying intracellular localization through several different microscopy experiments.

Regarding the plans for fixed cell immunofluorescence with endosomal and lysosomal markers (1.15.2. Immunofluorescence Imaging with CdSe@C-TPEA in HeLa cells using markers (EEA1, LAMP-1, LysoTracker®)

• How will the percentage of nanoparticles (escaped or in endosomes/ lysosomes) be calculated?

- **The cells will be incubated for 2 hours, how will the total amount of particle internalised be assessed (e.g. what about the material remaining in the culture media?)**

• What analysis will be carried out/what statistics will be used?
• How many experimental repeats will be carried out?
• If co-localisation with endosomes/ lysosomes will be assessed, what software will be used (e.g. Image J or commercial software). Will any correlation analysis be carried out?
• The authors state that at least 30 cells will be used per experiment, is this enough cells? how will bias be removed from the analysis in the selection of cells (e.g. will this be automated / or done blind?)

Thank you for these comments about 1) the quantification of uptake and, 2) endosomal escape.

Regarding uptake, we have added a simple experiment quantifying the fluorescence in the culture medium. The other experiments on uptake (testing with incucyte 15.2, Immunofluorescence with endocytic and lysosomal markers and measuring correlation coefficients) aim at providing a descriptive background and a context but they will not be used to test a specific hypothesis. They are not essential to analyse and interpret the intracellular-copper sensing results which are the focus of this study.

On the contrary, as explained in the introduction, the questions of endosomal escape is crucial. To assess quantitatively endosomal escape, we plan to use electron microscopy. Additional details of the approach are now provided.

Blind imaging will be done for the electron microscopy. Furthermore the image analysis to ascribe whether individual particles are within endosome or not will be done by researchers independent from the replication project. Although this will not be the case for the immunofluorescence, we plan to share all our data thus enabling independent re-analysis.

Regarding the plans for live cell fluorescence Imaging with (1.15.3. CdSe@C-TPEA in HeLa cells using marker CellLight™ Early Endosomes-RFP BacMam 2.0, CellLight™ Late Endosomes-RFP BacMam 2.0, LysoTracker®)

• What analysis will be carried out on the Incucyte®?
• How will this data be quantified/ represented?
• How many repeats will be carried out?

We plan to use the cell-by-cell analysis on the Incucyte. More details on the image acquisition, analysis, and representation have been added in the text (section 1.15.2). We plan to have triplicate wells for each sample condition as an internal control and to have 3 independent replicates of each test.

The experimental plans: 1.17. Intracellular Cu quantification in Hela and HepG2/C3a cell lines using inductively coupled plasma mass spectrometry (ICP-MS)

• Can the authors comment on the quantification by ICP-MS, how the data will be analysed and number of conditions and repeats etc?

After further research and thorough discussions we have decided to remove ICP-MS from the experimental plan as we will not be able to distinguish Cu^{+} from Cu^{2+}, using this technique, and will not be able to get any conclusive results on the intracellular Cu^{2+} concentration to correlate with the signal we achieve with the probe.

Minor experimental suggestions and comments (if applicable but not necessary for replication study)

• The authors from the original paper (Zhu et al) incubate HeLa cells with the probe and phorbol-12,13-dibutyrate PDBu - a compound they state is known to increase the endocytic activity. In the replication study this PDBu is therefore also included in the experimental plans. Is there any rationale for including another set of experimental conditions without the PDBu? This may be beyond the scope of the replication study and is not necessary for assessment of the aims outlined in the

paper introduction, however inclusion may impact the overall conclusion on endosomal escape of the particles if endocytosis is affected by this compound.

We thank you for the question, and would like to clarify our rationale. We think that testing the probe without PDBu will give us a better understanding of the cells' ability to endocytose the probe without any external agent, especially since there are future prospects for its use to sense copper in true biological settings. We also would like to control that PDBu does not have any secondary effect that might affect endosomal escape.

Also, does PDBu have comparable impact on endocytic activity in HepG2 cells as HeLa cells?

Studies have been carried out in Hep G2 cells that were treated with Phorbol esters (including PDBu) and have shown that PDBu impacts cell surface receptors recycling rate, hence modulating receptor-mediated endocytosis (Fallon, R. J., & Schwartz, A. L. (1988). Journal of Biological Chemistry, 263(26)) . However, we haven't found any direct evidence for PDBu dependent increase in endocytosis in Hep G2 cells

• Would also recommend STR profiling of cells at least once during the study as well as mycoplasma testing

We thank you for the recommendation we have planned to purchase an authenticated Hela cell line vial from the European cell collection bank and have planned routine mycoplasma testing (section 1.13). As for the established Hep G2 cell line that we will acquire from a collaborator, we will authenticate them before starting our experiments, and we have included a section on this in our registered report. We also plan to authenticate our cell lines by STR profiling at the end of our experiments.

• If technically possible (I don't know if this would be and it is likely to be beyond the scope of this study) but including a non-microscopy-based quantification of probes would be useful, e.g. performing cellular fractionation to separate membrane bound vesicles from the cytoplasm then carrying out e.g mass spec (if possible) to provide a more robust quantitative measure of probe present

We thank you for this suggestion. We might explore other options to quantify our probes at a different stage outside of the context of our replication study due to time and technical constraints.

In addition to your comments, we have obtained privately additional reviews from Laurence Motte (Université Sorbonne Paris Nord, LVTS) as well as Jean Marc Verbavatz and René-Marc Mège (Institut Jacques

Monod). We also have a public review, *via* **PubPeer, of Cécilia Menard-Moyon (CNRS, IBMC, Strasbourg). We detail below additional improvement to the paper made thanks to these comments.**

- We have added a better description of the Zhu et al article under study. (See introduction).
- We have better described the selection process of the articles for our replication project. (See introduction)
- We have removed the experiment testing photostability because the lack of experimental details in Zhu et al does not enable an adequate comparison.
- We have corrected a lack of consistency regarding tests performed with one of the two cell lines, and have decided to add HepG2/C3a +/- ATP7B cell line testing to all experiments. (See sections 1.14-1.15)
- We have made significant changes to the protocol in which we plan to use the live image analysis system, Incucyte based on the limitation of the apparatus. (See section 1.15.2)
- We have removed lysotracker from the immunofluorescence experiments and will only use LAMP-2 which should allow us to locate late endosomes and lysosomes.(See section 1.15.3)
- We have also added more details on the co-localization analysis of the immunofluorescence images. (See section 1.15.4)
- We have removed the pelleting step before fixation of cells in the TEM protocol and plan to fix cells directly in the dish. (See section 1.15.5)
- We have also added a point on measuring nanoparticles suspended in the culture medium (mentioned above). (See section 1.15.5)
- We had also changed the number of cells seeded for HepG2/C3a as we realized there was a mistake. (See section 1.16.2)