

We thank the reviewers and recommenders for the time and attention they devoted to our ms. You will find below the responses to the reviewers and corresponding changes in the new version of the ms now available on the OSF.

Reviewer #1

Has the synthesis of CdSe@C-TPEA been reproduced in other articles (among the > 474 articles that cited the original article)?

To the best of our knowledge, as the time of writing (18.06.2024) there are no studies that reproduce the synthesis of CdSe@C-TPEA nanoprobe. We have in particular checked the 483 articles that cite the original article and none of them has reported the synthesis of CdSe@C-TPEA.

In the original work, the size of the CQDs assessed from HRTEM images is around 5 nm (the number of measurements was not mentioned). Measuring the size of CQDs by HRTEM is very challenging. Hence, I think the variation of maximum 10% (mentioned in the table) might be too strict. What do the authors plan to do if the measurements are not within 10% of the average size value? Similar comments for the Cu²⁺-dependent fluorescence of CdSe@C-TPEA if $r^2 < 0.9$.

We chose this rather strict cut-off because properties of nanoparticles are highly sensitive to particle size. However, we agree with the reviewer that given the limited evidence provided and the difficulty of measuring average particle size by electron microscopy, we should change to 20%, hence accepting an average diameter between 4 and 6 nm. If we cannot obtain particles in this range, we would consider that the synthesis protocol is not reproducible. However, because our main scientific question is about the possibility of detecting cytosolic targets with nanoparticle probes, we will test variations around the synthesis protocol to attempt to obtain nanoparticles within the desired size range. If that were to be the case, we would of course document those attempts and carefully report any deviation to the protocol detailed in our pre-registered report. If those attempts are not successful, we will not be able to proceed with the biological studies and will conclude that the overall study is not reproducible.

For the same reasons, if the copper concentration dependence of the fluorescence properties of the obtained particles (following the protocol or after adjustments as described above) are too different from what was initially reported, we will not be able to proceed with the biological studies and will conclude that the overall study is not reproducible. A value of R^2 larger than 0.9 (and slope larger than 90% of the reported slope) will be indicative that the initial results are reproducible. In line with the comment of the reviewer, we will however proceed with the next stage of the replication, i.e. the biological studies, even if R^2 is equal to or more than 0.8 (or/and the slope larger than 80% of the reported slope). If that were not the case, we will not be able to proceed with the biological studies and will conclude that the overall study is not reproducible. We show below the data replotted from Figure 2a (inset) of Zhu et al. The R^2 value calculated by us from those data is 0.9978 (**Figure 1**). We also show two examples of what “calibration curves” with $R^2 = 0.8$ could look like to illustrate that it could not be used to measure copper concentration reliably in that range. The first graph corresponds to a situation with a large dispersion of measurement (**Figure 2**) whilst the second corresponds to non-linear behaviour in that concentration range (**Figure 3**).

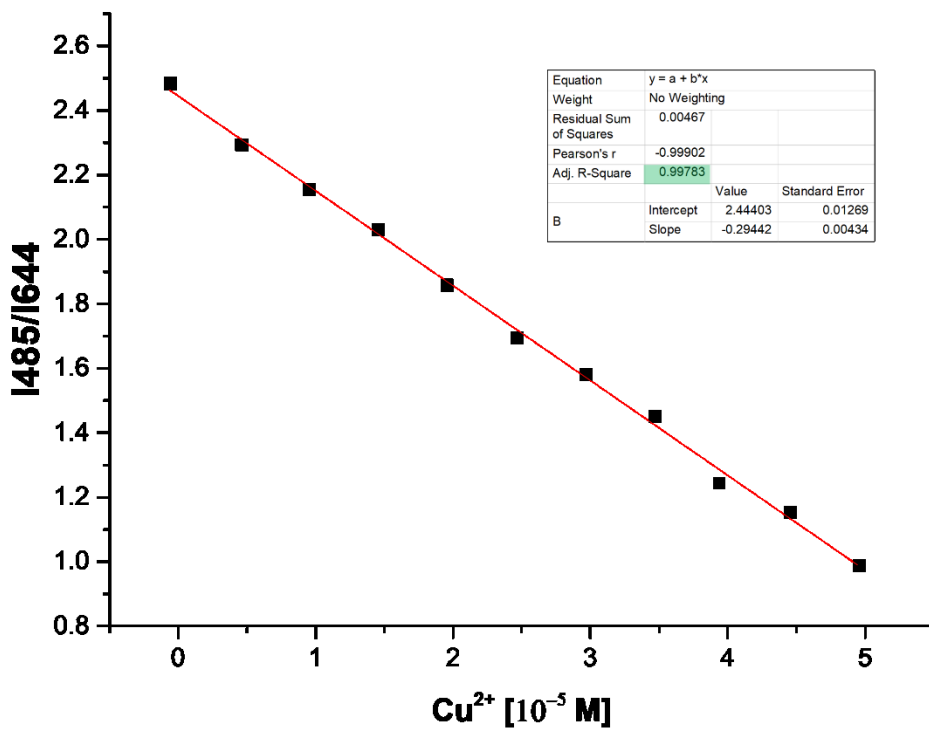


Figure 1: Plot of I_{485}/I_{644} as a function of Cu^{2+} concentration. Data replotted from Figure 2a (inset) of Zhu et al.

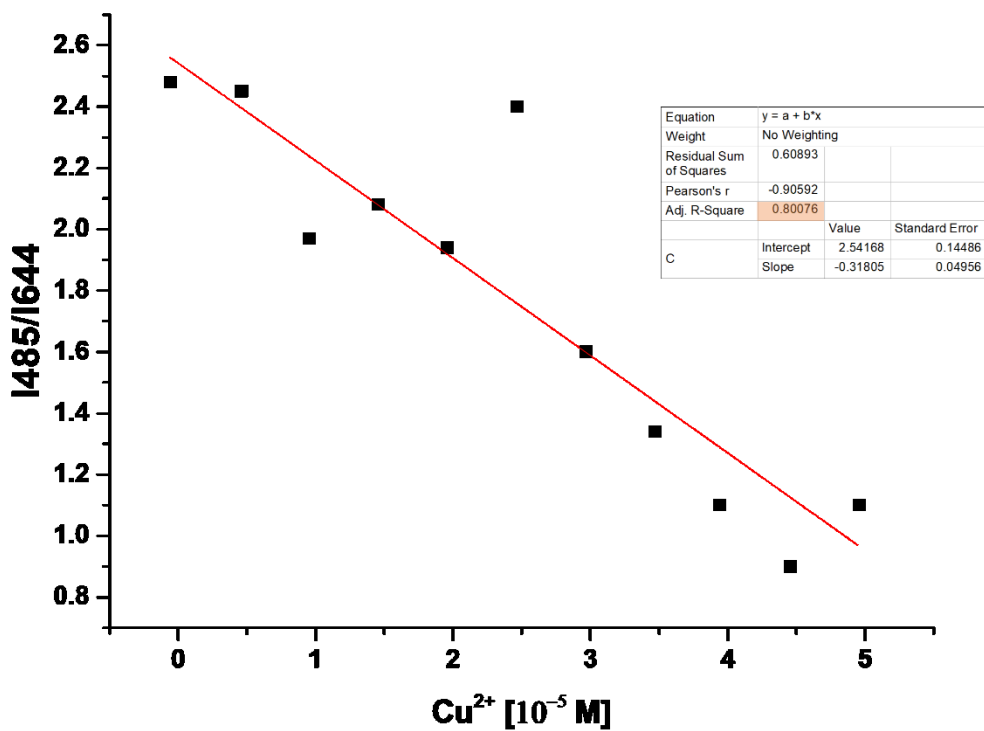


Figure 2: Calibration curve of I_{486}/I_{644} as a function of Cu^{2+} concentration with $R^2 = 0.8$. This plot shows a large dispersion of measurements, indicating significant variability and poor reliability for accurately measuring Cu^{2+} concentration in this range.

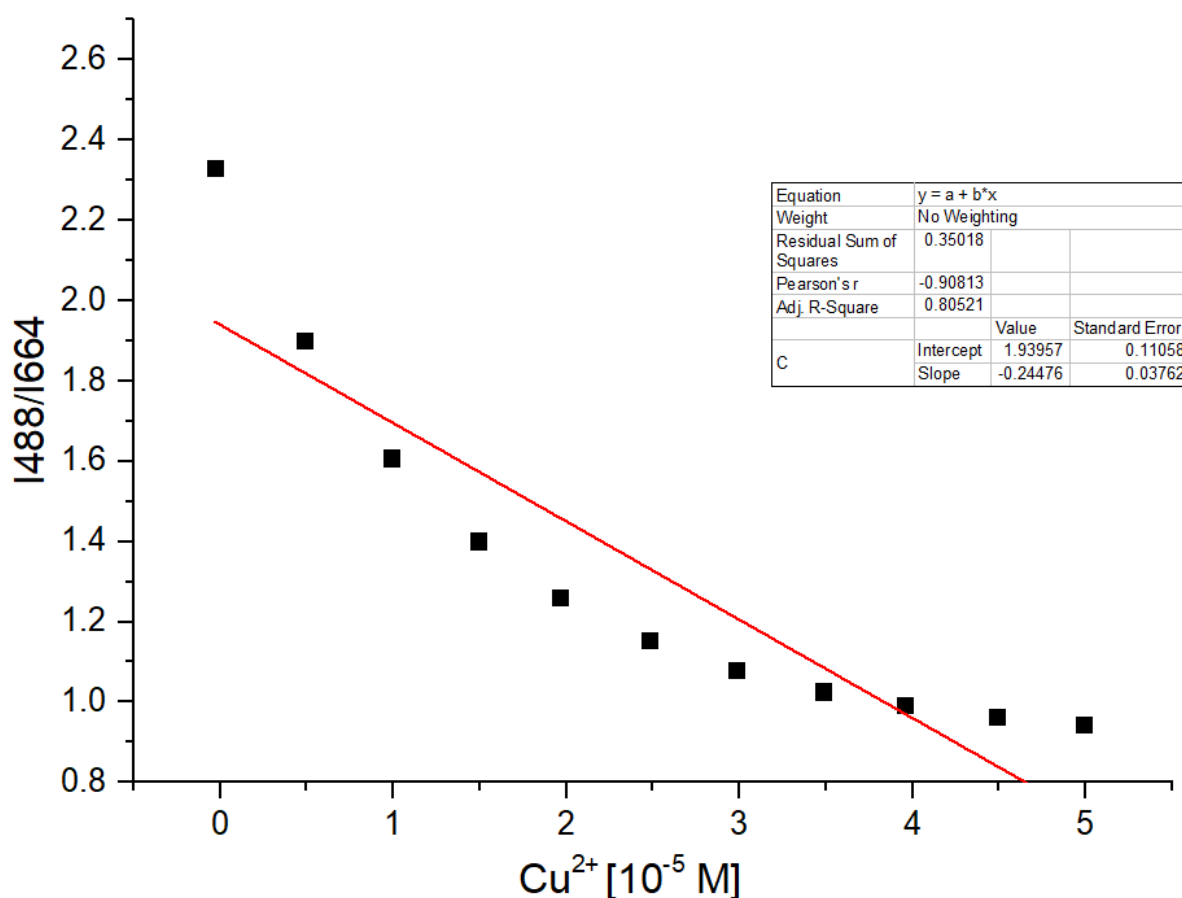


Figure 3: Calibration curve of I_{486}/I_{644} as a function of Cu^{2+} concentration with $R^2 = 0.8$. The plot exhibits non-linear behaviour within this concentration range, demonstrating that the curve is not suitable for reliable copper concentration measurements due to the poor fit of the data.

How did the author chose the value of 20% as “threshold” to state that a high proportion of CdSe@C-TPEA escape endosomes?

We acknowledge that this threshold value is somewhat arbitrary. It is more a marker for discussion than an absolute threshold triggering actions or conclusions. It corresponds to roughly ten times the value reported for optimised systems such as lipid nanoparticles (as mentioned in our MS) so we argue that it is correct to describe it as “a high proportion”, not in absolute terms, but in comparison to what we would expect based on those careful studies of endosomal escape. Furthermore, we would also argue that less than 20% endosomal escape would make sensing of cytosolic targets particularly challenging since the signal would be dominated by the 80% of the probes residing in endosomal compartments.

The authors propose to use quinine sulfate as a standard to assess the quantum yield of the CQDs, while rhodamine B was used in the original work, in order to more precisely determine the quantum

yield. In parallel, it would be also useful to use rhodamine B to see if the quantum yield is in agreement with the value reported in the original article.

We thank the reviewer for her valuable feedback. In a response, we will use both quinine sulfate and rhodamine B to assess the QY of the CQDs.

The NMR spectroscopy confirmed the structure of all synthesized molecules. Do the authors know what is the broad peak at around 1.75 ppm in the NMR spectrum of CPD1? It does not seem to correspond to a solvent residual peak, does it?

We would like to thank the reviewer for pointing this out. After careful discussion with our collaborator, we do think that this peak around 1.75 ppm could be ascribed to the downfield chemical shift of H₂O (normally at around 1.56 ppm) which takes place in the presence of hydrogen bond acceptor such as the pyridine moieties, previous work has also found similar behaviour¹.

¹Fulmer et al, 2010. NMR chemical shifts of trace impurities: common laboratory solvents, organics, and gases in deuterated solvents relevant to the organometallic chemist. *Organometallics* 29, no. 9 (2010): 2176-2179.

Do the authors plan to study the photostability of CdSe@C-TPEA?

We thank the reviewer for this suggestion. It was initially our intention to replicate Figure S9 but the lack of experimental details made it impossible. Instead, following this suggestion, we have decided to add a condition in the section 1.14.2 as lack of photostability could impact the interpretation of the kinetics of uptake:

“The eventual lack of photostability of CdSe@C-TPEA could affect the interpretation of the results. To account for this possibility, we will also measure the fluorescence of CdSe@C-TPEA particles irreversibly fixed to microscope slides and subjected to similar imaging conditions to the cells.”

List of minor corrections to improve the readability of the manuscript:

- Some abbreviations are not defined.
- BOC should be corrected to Boc.
- Scheme 2, 4 & 5: add the solvent in the conditions below the arrow (even if it is mentioned in the protocols), otherwise indicating “Reflux” is meaningless.
- Section 1.9: correct “were added” to “will be added”. Correct “Cus” by “Cys” and also “Phy” by “Phe” (“Phy” appears in the x axis of Fig. 3b in the original paper, but I guess it was a mistake too). Correct “Iso” by “Ile” (the three letter code of isoleucine is Ile and not Iso).
- Add the exact reference in the section 1.14.4.

We do thank the reviewer for pointing out these minor changes. We took all the changes into consideration and edited the manuscript with all modifications indicated in Track Changes and highlighted in yellow.

Reviewer #2

To correlate results across different assays, the use of FACS is proposed to validate viability results, understand cell death pathways, dynamics, and enhance experimental reproducibility, generating high-quality data with established protocols. [2]

In addition to the planned characterization methods, it is proposed to include Differential Centrifugal Sedimentation (DCS) for high-resolution nanoparticle analysis in relevant biological fluids used for in vitro analysis. This will assess the overall stability of the dispersions and exclude the formation of aggregates that can significantly impact biological assessment outcomes for ultra-small nanoparticles. [3] Nanoparticle Tracking Analysis (NTA) is also suggested for reproducible nanoparticle concentration measurements, which are important for dosing of the particles in biological experiments.

In this instance, we will rely on dynamic light scattering but we thank the reviewer for her valuable feedback and we will consider adding those analyses for future replications.

This study holds a high hope to advance our understanding of the reproducibility in field of nanomedicine by addressing challenges in adapting synthesis to reduce batch-to-batch variability, validating physico-chemical characterization tools, and ensuring the reliability of cell viability tests. It will identify best practices in operative procedures, create opportunities for standardizing measurements and in vitro assessment approaches, and ultimately guide new commercial opportunities, regulatory requirements, and wider applications in future clinical trials.

We do thank the reviewer for her constructive feedback.

ADDITIONAL POINT NOT RAISED BY REVIEWERS

FTIR: In the course of this review, we noticed that we had listed the use of FTIR twice, employing different instruments. In this version, we have improved consistency and clarified our intention to use a single FTIR instrument that fits the requirements of our study.