

Response to referees of adfm.202207669

Reviewer 1

Comments

Although the authors mentioned that the selectivity is beyond the scope of this work, it is an important performance metric for biosensors and should be properly addressed.

We thank the referee for taking the time to comment on our resubmission. [New text is given in blue.](#)

We completely agree that selectivity is essential for a device like ours to be used as a biosensor.

We considered two strategies to address this repeated comment when planning these responses and revisions.

First, we considered DFT analysis of other nitrile substrates to match those of preQ₀ and benzyl cyanide (BnCN). Our computational corresponding authors, Sam de Visser, stated that “repeating it with another substrate would take several months for the full cycle”. That strategy offered marginal benefit; a proper analysis could take a year and would merit separate publication.

Second, we considered additional GFET runs. The original data were collected in the lab of our Japanese corresponding author, Yuhei Hayamizu. Arranging the shipment of proteins and running the experiments was not feasible in the revision timeframe. Our own attempts to make or buy additional GFETs have been stymied by Brexit-related shipping delays.

Therefore, not only are these additional investigations for this submission out of scope, but they were impossible to achieve in the revision timeframe.

The high selectivity of QueF to its natural substrate is known, and it is difficult to increase its substrate scope.^[1] It is remarkable that we see a response from BnCN at all. The active site of QueF employs a well-characterised Cys–His–Asp catalytic triad already known to bind to nitriles.^[2,3] Therefore we expect larger nitriles to be excluded without engineering the channel to the active site. Our evidence to support this hypothesis is not developed enough for us to make this claim now.

To reflect what we have evidence for with respect to specificity, which is linked to selectivity, we added this paragraph to the results section on p5:

[The limit of detection \(LoD\) was calculated from the point at which the 95% prediction band no longer crossed \$y=1\$. For QueF-Y5Y, the LoD is \$\sim 0.35 \mu\text{M}\$ for BnCN and about 10 nM for preQ₀, consistent with the less specific interaction between BnCN and the enzyme. The response is \$\(8.9 \pm 2.4\) \times 10^{-4} \text{ nM}^{-1}\$ for BnCN and \$\(-55.2 \pm 6.9\) \times 10^{-4} \text{ nM}^{-1}\$ for preQ₀ when then analyte concentration equals \$K_d\$.](#)

We had already begun to address selectivity with the demonstration of the difference in response between the natural substrate and our target one to provide enough evidence that we should systematically study the recognition element for selectivity as part of a separate PhD or postdoctoral project.

It is true that covalent functionalization can cause defects to GFETs and may compromise the sensitivity sometimes. However, the relevance of this conclusion to the present work is not clear, which ties back to my

previous question that has not been addressed in the revised manuscript: "What level of sensitivity is needed for nitrile sensing, and is that impossible to achieve using the conventional immobilization method?"

No one knows what level of sensitivity is required yet! We have an ongoing project with two agricultural botany institutes, Rothamsted Research and NIAB, aiming to answer this question. To date, the answer is complex because it involves not only signalling from damage, but also from stress from drought or heat, plant variety, and soil composition.

We addressed the likely levels of BnCN release, but the referee is correct that we did not address whether this method makes detection possible where it was previously impossible. We do not know, but it is likely that any non-covalent immobilisation with PBASE will provide similar sensitivity while covalent immobilisation will lower the GFET's sensitivity. Béraud et al. summarise these effects in their critical review published in 2021.^[4]

Conventional immobilisation involving simple adsorption will certainly not achieve the required sensitivity, as shown in the controls without the Y5Y tag (Figure 3), controls without a biological recognition element (Figure S2), and controls with another self-assembling peptide (R3RC, Section S7) that suggest the protein forms a non-functional, passivating layer.

We added this clarifying text after Figure S2 (ppS-6 & S-7):

Controls on bare graphene surface with the two nitriles showed negligible response at low nitrile concentrations however at high concentrations, there is a decrease in i_{sd} corresponding to decrease in CNP due to an increase in electron density of graphene. For QueF-Y5Y, BnCN shows an increase in i_{sd} and therefore the observed current change is not caused by nitrile-graphene interaction. For preQ₀, a similar trend in i_{sd} is observed between the QueF-Y5Y functionalized surface and the bare graphene surface and the main difference can be observed only at low concentrations where the QueF-Y5Y has a much stronger response to preQ₀ before saturation. A change in i_{sd} was not observed for the QueF-WT functionalized surface suggesting it may also work as a passivation layer like the R3RC tag (Section S7).

We also clarified that we did not necessarily expect this method to be better than PBASE by softening the tone of the sentence at the end of a paragraph on p3 the introduction from "However, PBASE can bind to any surface amine group,^[27] which can affect the stability and structural conformation of the protein." to "However, PBASE can bind to any surface amine group,^[27] which can move the recognition element's binding site away from the surface, attach the proteins in a more than one orientation, and decrease analyte accessibility."

Reviewer's responses to questions

Please rate the importance compared to published work in this subject area.

Reviewer #1: no response

Please rate the novelty compared to published work in this subject area.

Reviewer #1: no response

Which aspects of scholarly presentation require improvement (if any)?

Reviewer #1: no response

Do the methods, data and analysis (including statistical analysis where applicable) adequately test the hypothesis and support the conclusions?

Reviewer #1: Partially

We understand that we are unable to provide experimental evidence to address all the comments around sensitivity. We hope that you and the editor agree that these measurements are substantial enough to form a separate publication after many months of laboratory work.

Are the methods, data and analysis described in sufficient detail to be reproduced?

Reviewer #1: Yes

What do you anticipate your overall rating (a mean of importance, novelty and scholarly presentation) would be if the requested revisions are adequately addressed?

Reviewer #1: Considerable - Top 30% in the subject area

Reviewer 2

Comments

The authors successfully addressed my concerns about graphene samples and the characterization of the assembled layers. Now I support its acceptance for publication.

Thank you for taking the time to comment on our resubmission.

Reviewer's responses to questions

Please rate the importance compared to published work in this subject area.

Reviewer #2: High - Top 15% in the subject area

Please rate the novelty compared to published work in this subject area.

Reviewer #2: High - Top 15% in the subject area

Which aspects of scholarly presentation require improvement (if any)?

Reviewer #2: no response

Do the methods, data and analysis (including statistical analysis where applicable) adequately test the hypothesis and support the conclusions?

Reviewer #2: Yes

Are the methods, data and analysis described in sufficient detail to be reproduced?

Reviewer #2: Yes

What do you anticipate your overall rating (a mean of importance, novelty and scholarly presentation) would be if the requested revisions are adequately addressed?

Reviewer #2: High - Top 15% in the subject area

Thank you.

References cited in these responses

- [1] B. Wilding, M. Winkler, B. Petschacher, R. Kratzer, S. Egger, G. Steinkellner, A. Lyskowski, B. Nidetzky, K. Gruber, N. Klempier, *Chem. - A Eur. J.* **2013**, *19*, 7007.
- [2] C. Pitchumani Violet Mary, R. Shankar, S. Vijayakumar, *J. Biomol. Struct. Dyn.* **2018**, *36*, 634.
- [3] G. Dodson, A. Wlodawer, *Trends Biochem. Sci.* **1998**, *23*, 347.
- [4] A. Béraud, M. Sauvage, C. M. Bazán, M. Tie, A. Bencherif, D. Bouilly, *Analyst* **2021**, *146*, 403.