

Response to referees of adfm.202112237

Comments from the editor

...[O]nce you have had sufficient time to carefully consider and address all of the concerns raised by the reviewers, we would be willing to consider a new submission based on this work.

Thank you for the opportunity to resubmit. This is our summary of the additional results we present to support publication:

- The lead author ran additional measurements on a quartz crystal microbalance with a graphene-coated sensor to support our response to the comments from reviewer 3.
- We added the transfer curves for the GFETs to the SI and discussed in the main text to address the first comments from reviewer 2.
- We have also added to the SI the source m/z data for the intact protein mass spectrometry.

Statistics: For original research, please check that your manuscript includes a sub-section entitled "Statistical Analysis" at the end of the Experimental Section that fully describes the following information: 1.

Preprocessing of data (e.g., transformation, normalization, evaluation of outliers), 2. Data presentation (e.g., mean \pm SD), 3. Sample size (n) for each statistical analysis, 4. Statistical methods used to assess significant differences with sufficient details (e.g., name of the statistical test including one- or two-sided testing, testing level (i.e., alpha value, P value), if applicable post-hoc test or any alpha adjustment, validity of any assumptions made for the chosen test), 5. Software used for statistical analysis.

Section 4.4.3 (p. 15) was added to describe the data processing, curve fitting, methods for calculating standard deviation, and meaning of the uncertainty values. The software used for the analyses is given there. No tests for significance were used.

Figure legends: Please make sure that all relevant figure legends contain the information on sample size (n), probability (P) value, the specific statistical test for each experiment, data presentation and the meaning of the significance symbol.

The captions of Figs 3, S2, S3, S7 now include information about the data presentation and the meaning of the error bars. The sample size was either given in the caption or, in the case of Figs 3 and S2, in summary Table 1.

Reviewer 1

Comments

This manuscript reports a novel route to non-covalent immobilization of proteins on graphene FETs for detecting nitriles. To achieve this goal, a fusion protein consisting of nitrile reductase is used as the biological recognition element. Overall, the engineering of the sensing surface of electronic devices for performance improvement is an interesting topic.

We thank the referee for taking the time to comment on our submission. We have rewritten several aspects to address some of the referee's misunderstandings. **New text is given in blue.**

It is not very clear what the major advance of this method is compared to the state-of-the-art technologies.

The point of the fusion protein is that researchers would no longer need to modify the sensor surface to make a protein-based biosensor. Immobilisation of bioprobes such as enzymes and antibodies onto graphene use pyrene-NHS (PBASE) linker which is relatively expensive (~£300/g) and involves two-steps immobilisation method to make a functionalised surface. Using PBASE in an industrial scale is not feasible due to its poor scalability and stability of the NHS group.^[1] Here we presented a unique immobilisation route that makes functionalization of the graphene surface a one-step procedure as the recognition element is now also a linker and circumvents this problem.

The abstract now begins “**A new route to single-step, non-covalent immobilization of proteins on graphene...**”. We re-emphasise this point by ending the first sentence of the conclusion with the phrase “**...graphene surface modification is not necessary to attach a biological recognition element.**”

While the authors claim that conventional covalent functionalization on GFET can affect the stability and structural conformation, this seems to be a rather subjective statement.

We do not state that covalent functionalisation affects the stability and structural conformation of the protein. In fact, we wrote, “Conventional covalently mediated immobilization techniques allow for stronger protein attachment methods than non-covalent binding...”. The effect of covalent functionalisation is the decrease in the carrier mobility, and thus the sensitivity, of the graphene transistor.

The point about sp^3 carbon and defects lowering carrier mobility is well-established in the literature. For example, pristine graphene has a carrier mobility of $\sim 10^4 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at room temperature.^[2] Reduced graphene oxide, in which chemical or thermal reduction repairs some of the sp^3 defects, typically gives carrier mobility values four orders of magnitude lower ($< 5 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$),^[3] with the best reported value of $\sim 10^2 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ after 3000 K treatment.^[4]

We clarified the introduction (p3) by writing “Therefore, non-covalent GFET functionalization is preferred in order **to preserve graphene's sp^2 arrangement and consequent high carrier mobility required for high-sensitivity measurements.**^[25]”

Although the affinity between the fusion proteins and nitriles is studied using experimental and theoretical methods, some issues (sensitivity, selectivity, dynamic range, target applications...) in biosensing have been overlooked.

We have addressed all these points except for selectivity, which is beyond our groups' resources and would form the basis of a fully realised sensing system so is out of scope of the work described in this submission. We include sample calculations for the most relevant system with both modified protein and non-natural substrate but decided against including these values based on proof-of-concept work.

Sensitivity in terms of limit of detection (LoD) can be calculated from fitting data presented in Table 1 (p7) or by using the 95% prediction band calculated using Origin (**Figure R1**), consistent with ICH recommendations.^[5] For example, our test nitrile benzyl cyanide (BnCN), the limit of detection is about 0.12 μM .

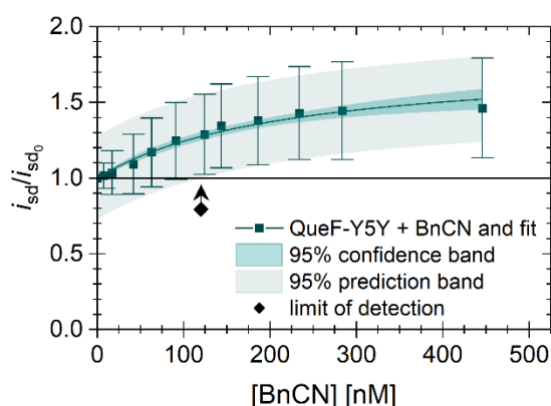


Figure R1. Determination of limit of detection based on the point at which the 95% prediction band is greater than 1.

Sensitivity in terms of response per change in concentration can either be inferred from the dissociation constant, which quantifies analyte binding strength, or from the slope of the line. At the LoD, the slope is $1.47 \times 10^{-3} \text{ nM}^{-1}$. At $[\text{BnCN}] = K_d$, the slope is about $0.9 \times 10^{-3} \text{ nM}^{-1}$. These values are equivalent to current changes in the nA range.

Dynamic range is poorly defined for non-linear sensor responses. If we use the criterion that the top end of the range is when a 20% increase in analyte produces a statistically meaningful change in response (here, based on the 95% confidence interval), then the upper limit would be about 0.25 μM BnCN.

To address target applications, we rewrote the justification paragraph in the introduction (p2):

The development of a sensor capable of accurate and rapid detection of nitriles would be an important tool in agriculture. Volatile nitriles are produced from glucosinolates, predominately found in brassicas, when under herbivore attack.^[6] Benzyl cyanide (BnCN) is a useful proof-of-concept compound for agricultural biosensors because they would be able to detect the plants' natural distress signals to recruit parasitoid species as a secondary defense mechanism.^[7] Nitrile emissions can be used to alert farmers of pest location and allow site-specific pesticide administration, which in turn would lower pesticide usage.^[8] Additionally, nitriles are ubiquitous in industry, such as in the production of textile fibers, synthetic rubbers, and thermoplastic resins;^[9] a nitrile sensor would be useful for environmental monitoring.

The manuscript needs to be thoroughly revised for further consideration by addressing the following issues.

The labels in Figure 1 are inconsistent with those in the figure caption. For example. The schematic representation of an antiparallel β -sheet formation of the Y5Y fusion protein is labeled as 1b in the figure but 1a in the caption. Please double check.

Thank you for spotting that error! Figure 1 and its caption are now consistent in the introduction (p4).

Although the major innovation of this work is in the bio-recognition element part, I feel that it is still important to present a systematic characterization of graphene and the FET devices in the main figures, as

the performance of the system largely depends on the materials and electronics. In addition, Figure S8 is not displayed in the Supporting information.

Thank you for spotting the omission of the GFET Raman spectrum. We did not notice it had not been included in the PDF. It now appears as **Figure S9**. **Section 4.4.1** now also includes details of the Raman characterisation and its interpretation:

Raman analysis of the GFET surface (**Figure S9**, Isoplane 320, Teledyne Princeton Instruments, 532 nm excitation) showed characteristics of bilayer graphene ($I_{2D}/I_G = 1.3$, $\nu_{2D} - \nu_G = 1082 \text{ cm}^{-1}$) with a crystallite size (L_a) of ca. $0.11 \mu\text{m}$ ($I_D/I_G = 0.17$).^[10]

The graphene was also assessed by AFM (**Figure S4**) and by studying its transfer curves before and after measurements (**Figure S3**). The transfer curves indicate the position of the GFETs' charge neutral points (CNPs) and how they change with the addition of the QueF. These changes to CNP are discussed in the SI (**Section S3.2**) and in **Section 2.1** of the main text (p7) where we added:

The polarity and amplitude of the GFET signal depend on the gate voltage (V_g) relative to the charge neutral point (CNP). For QueF-Y5Y samples, preQ₀ shows p-type doping as the CNP increases which can be attributed in part to the reduction of the nitrile to amine while BnCN shows an opposite trend except at a potentially denaturing 1 M concentration (**Figure S3**).

The authors claim that conventional covalently mediate immobilization will disrupt the electronic structure and affect the sensitivity. What level of sensitivity is needed for nitrile sensing, and is that impossible to achieve using the conventional immobilization method? The authors should collect data and compare the performance to that of the sensor reported here to support this claim.

We address the comment about the requisite level of sensitivity at two other points in our response to this reviewer's comments. With respect to comparisons, there is already scientific literature offering systematic comparisons of conventional immobilisation techniques of graphene.^[11] We selected direct adsorption of the protein onto the surface of graphene because it is the simplest route for single-step functionalisation and therefore the best comparison. Therefore, adding controls with non-covalent linkages between the graphene through one (more more!) of the QueF monomer's six lysine side chains is beyond the scope of this work and would offer marginal benefit.

The authors should include some details in the sensing mechanism - it is mentioned that "Most of the stages cause an electrochemical shift in the system that can be translated into an i_{sd} change from GFET." What is exactly happening that leads to a shift in i_{sd} ? Why does QueF-Y5Y show i_{sd} response in opposite directions for the two nitriles studied here?

We clarified our interpretation of the origin of the response the discussion of the DFT results (**Section 2.2**, p8–9) and protein mass spectroscopy results (**Section 2.3**, p10).

In the introduction (p4) we added:

The charge around the active site differs in three of the four intermediates, which could be translated into a change in source-drain current (i_{sd}) from GFET:^[12] **RS-1** to **RS-2** takes two protons from the solvent, and the **RS-2** to **RS-3** and **RS-4** to **RS-1** steps both release NADP⁺.

In Section 2.2, we added:

This charge disparity can be used to explain shift in i_{sd} . For BnCN, an increase in i_{sd} is seen indicating a positive shift in CNP due to positively charged doping attributed to the 2H^+ taken from the solvent.

For preQ₀ an opposite trend is observed and that can be attributed to the dissociation of the positively charged preQ₁ amine and the subsequent hydride transfer in **RS-4**.

In Section 2.3, we added:

Protein mass spectrometry of combinations of QueF, the two nitriles, and NADPH (**Figure 5**) shows that QueF forms a 1:1 stoichiometric complex with its natural preQ₀ substrate in the absence of NADPH, suggesting all the protein molecules have formed a stable intermediate. The addition of NADPH causes the original protein signal to reappear. The presence of a higher-mass adduct also appears that could be attributed to a protein species with multiple NADPH or preQ₀ molecules stably bound.

Non-covalent interaction is usually less stable in liquid environment. Is there any performance degradation over time? If so, is that because of the dissociation of the bio-recognition elements, degradation of enzymatic activities, or the electrochemical corrosion of the electronics?

The stability of the fabricated GFETs was assessed using quartz-crystal microbalance (QCM) measurements. The results and discussion related to these measurements begins in the middle of p11 in **Section 2.3**. QueF-Y5Y adsorbed on CVD graphene and kept in buffer show the enzyme was stably bound in monomer configuration for at least 12 h. The experimental details are given in Section 4.5 (p15) with supporting data in **Section S8**. The QCM results are consistent with the static light scattering analysis of QueF shown in **Section S6**: at 25°C, QueF remains stable and does not aggregate for at least 72 h (**Section S6**). We do not expect that 1 mM potassium phosphate pH 7 (*I* = 2.1 mM) to be especially corrosive to CVD graphene, but we did not test this.

What is the rationale for selecting the analyte concentrations used for this study (10 to 500 nM)? Is there a specific application scenario that the authors are targeting at, and if so, are the concentrations used here relevant? What is the limit of detection of this sensor? The authors give an example in Introduction that BnCN is a volatile organic compound given off by some brassicas when under herbivore attack to recruit parasitoid species as a secondary defense mechanism. However, in this case, chemicals will be released to air instead of to solution. Can the authors elaborate on how the reported sensor system can be used to monitor this process?

The lower concentration was chosen by chance. The higher concentration was set based on the point at which little change in response could be detected.

As noted above, we amended our description of the use case as a nitrile sensor and provided a LoD of about 0.12 μM.

We added **Section 2.4** to acknowledge what we still do not know about the feasibility of this GFET element as a sensor in a field as well as providing some back-of-the-envelope calculations about whether we are near what would be useful. The first paragraph of this future work section captures these points:

The release rate of BnCN from infected brassicas has so far only been presented as relative concentrations, but volatile releases of ~0.1 μmol per square centimeter of leaf area are typical over 24 h following insect infestation.^[13] Converting this value into a local concentration requires assumptions about vapor diffusion and convection near the leaf surface and the amount of leaf area under attack, but detectable nanomolar concentrations are plausible. This study did not examine the effect of possible interferences or selectivity of GFETs using QueF-Y5Y, but these measurements would be essential to translate this research into practice.

It seems that the reductase is sensitive to multiple nitrile species. In that case, how can the selectivity issue be addressed during practical applications? Does the sensor respond to other interferents in addition to nitriles?

QueF is naturally selective in its reduction mechanism to its natural preQ₀ substrate^[14] and difficult to expand its substrate scope. This is, in part, due to the induced fit mechanism initiated by the nitrile and several docking residues that lining the entry into the active site.^[15] However it is known that the Cys-Asp-His catalytic triad utilised by QueFs active site has reactivity towards various nitriles.^[16] We hypothesise that the residues that orient preQ₀ could be mutated to add selectivity. However, we did not test QueF's selectivity to nitriles or resistance to interferent.

What do error bars represent in the I_{sd}/I_{sd0} - concentration curve? Is that the deviation among three different devices, or the deviation among three set of test results on the same device? If the latter, during what time point after adding nitrile do the authors record the data? A related question is: is there a large sample-to-sample variation among different devices prepared under the same condition?

Error bars represent deviation among different graphene channels on the same device and so all the time points are collected simultaneously. The time points in the i_{sd}/i_{sd0} vs. concentration curve were taken 5 min. after each nitrile addition. The captions to **Figure 3** and **Table 1** have been amended to make this clear.

Transfer curves were taken before the start of each experiment, and they had showed similar trends and CNP value suggesting small sample-to-sample variation (**Section S3.2**).

Is the sensor reusable or only for one time use?

The sensor single use. The GFET does not return to its initial i_{sd} when washed indicating strong binding of substrate to protein. We added this sentence to **Section 2.4** (p13) to make this explicit: "The sensors are likely to be single use because of the strong binding of nitriles to the recognition element."

The raw data in the I-t curve seems to be quite noisy. The authors should provide details about the denoising steps.

After ~30 min. equilibration, typical RMS error on i_{sd} was 1 nA. There was no denoising. We added more details on the data collection to **Section 4.4.2** (p14). The relatively wide standard deviation on the plots in Figure 3 is a result of the replica measurements.

Reviewer's responses to questions

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

Reviewer #1: Moderate - Top 50% in the subject area

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

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

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

Reviewer #1:

**Clarity*

**Manuscript structure*

**Display items*

Thank you, we hope that our responses, clarifications, and additions to the manuscript raise your estimation of the potential impact and novelty of the work we report.

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

Reviewer #1: Partially

We anticipate that our amendments lead you to the same conclusions as we reached based on our data and analyses.

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 fabricated a nitrile sensor based on nitrile reductase QueF with dodecapeptide fused on graphene surface. The results suggest an alternative non-covalent route for graphene biointerfacing. The manuscript is well written and the work is interesting. However, I do have concerns regarding the performance of GFET and the data analysis of the measurements in real time. Therefore, a revision is still needed before the manuscript can be accepted for publication.

Thank you for taking the time to critique this submission and for your compliments. The additional GFET and QCM measurements run by the first author should address your concerns, as we describe below.

In Figure S3, normalized i_{sd} (i_{sd}/i_{sd_0}) was given to evaluated the shift in charge neutrality point (CNP) of the GFET. What about the hysteresis, the distribution of the V_{CNP} /carrier mobilities, and the long-term stability of the fabricated GFETs? In my opinion, these evidences and analysis are essential for evaluating the performance and reliability of the biosensors. For example, the authors kept the gate voltage at 500 mV throughout all experiments, but the sensing response (i_{sd}/i_{sd_0}) might be zero if it is near the V_{CNP} of the GFET. Also, for real-time measurements, the authors are suggested to present the shift of V_{CNP} (instead of the i_{sd}/i_{sd_0}), which is intrinsic to the GFET and more reliable for compassion (see DOI:10.1002/adma.201603610, DOI:10.1021/acs.nanolett.7b04466).

These are good points. We included more comprehensive (and comprehensible) plots of the transfer characteristics of the QueF-Y5Y sensors as a revised **Figure S3**. In **Section S3.2**, which includes that figure, we amended the text to read “Initial transfer characteristics of chip showed similar CNP and so to maintain consistency between experiments, the gate voltage was kept at a constant 500 mV throughout all experiments. Choosing appropriate V_g requires a compromise between noise and signal and so 500 mV provided highest transconductance while being close to the neutrality point.”

We recognise that V_g could be optimised in future, so we also added this paragraph to discussion on future work (**Section 2.4**):

V_g was held close to the CNP for maximum transconductance and the substrate doping could have shifted V_g towards the CNP.^[12,17] This shift may have occurred with preQ₀, consistent with the GFET's low response after 135 nM substrate addition. While V_g was held at 500 mV for consistency, the limit of detection therefore could be increased by optimizing V_g for the specific nitrile to be detected.

Although transfer curves provide a deeper understanding of doping and charrier mobility of the graphene. They do not give sufficient insight into the kinetics of protein–substrate binding/dissociation which was a major interest for us. We have, however, used transfer curves to show good device performance before each experiment and a final transfer curve for analysing overall polarity shift at the end and not as a full sensing metric (**Section S3.2**).

We can partly address comments about the stability of the fabricated GFETs based on quartz-crystal microbalance (QCM) measurements that the first author ran in response to the referees' comments. The results and discussion related to these measurements begins in the middle of p11 in **Section 2.3**. QueF-Y5Y adsorbed on CVD graphene and kept in buffer show the enzyme was stably bound in monomer configuration for at least 12 h. The experimental details are given in **Section 4.5** (p15) with supporting data in **Section S8**. The QCM results are consistent with the static light scattering analysis of QueF shown in **Section S6**: at 25°C, QueF remains stable and does not aggregate for at least 72 h (**Section S6**). We do not expect that 1 mM potassium phosphate pH 7 ($I = 2.1$ mM) to be especially corrosive to CVD graphene, but we did not test this.

Thank you pointing out the work from the Leiden groups. We included both references in the manuscript as refs 25 and 46. We also corrected the second one.

The authors claimed that PBASE binds to any surface amine groups and can affect the stability and structural conformation of the protein, and the fusion protein method with dodecapeptide is beneficiary in these aspects. However, for single-step routes to GFET modification, direct adsorption of the protein onto the surface of graphene cannot be ruled out.

These are valid points. Direct adsorption is simplest method, which is why we used it as a point of comparison. For QueF, there was not a statistically significant response of the WT protein to either analyte. QCM measurements shown in **Figure 6** (p12) indicated that the WT protein formed a stiffer, possibly denatured, layer, consistent with the GFET responses.

The formation of monomers rather than dimers was an unexpected benefit. For proteins like QueF or many lectins that form dimers, tetramers, etc. more of the protein may be too far away from the graphene surface to contribute to the GFET response.

In addition, the bioengineering of the Y5Y tag as an extension to the protein, is complicated and might be a limitation for practice application.

The complexity of the biochemical transformations is subjective. We acknowledge that for laboratory-scale studies the method is more complex than simple esterification. On a larger scale, Y5Y-modified biological recognition elements could easily be overexpressed in cell culture, in the same way that custom monoclonal antibodies are routinely manufactured, and produce more reliable GFET biosensors.

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

Thank you.

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

Reviewer #2:

*References

*Other

We have cleaned up the errors in the reference list such as missing journal information, swapping authors' first and last names, and selecting the wrong reference type (e.g., book for journal).

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

Reviewer #2: Partially

As we wrote in response to Reviewer #1, we expect that our amendments lead you to the same conclusions as we reached based on our data and analyses.

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

Reviewer 3

Comments

The manuscript by Mohamed et al. describes a non-covalent protein immobilization method for the construction of GFET-based nitrile sensors. Overall the design is interesting and it has potential prospects in the field of bioanalysis and bioelectronics, if real chip-scale devices could finally be fabricated. However, from my point of view, the characterizations of the adsorption layer as well as the GFET devices are still preliminary, which makes me reluctant to support its publication at this stage.

Thank you for taking the time to critique this submission. As we noted for Reviewer 2, additional GFET and QCM measurements run by the first author address your comments.

My concerns are as follows:

Based on my experience, mechanically exfoliated samples often have a large variation of thicknesses, especially when it was only repeated twice. On this basis, was there an intended selection of samples/areas when the AFM was run, or AFM was done all over the wafer? The enzyme was immobilized on exfoliated graphite. Could it fully depict the situation of "on graphene"? CVD graphene was mainly for the device. Any difference of the immobilization process when it was monolayer or multilayer? How will it influence the electrical test?

To address this, we have now carried out QCM to quantify areal mass density and viscoelastic changes upon the two enzyme variants binding to CVD-graphene doped QCM chip (**Section 2.4, p11**). This has now been used to verify binding orientation of these enzyme variants onto the graphene surface.

Moreover, AFM was carried out multiple times on different flakes. They had shown similar height distribution and orientation however only the most suitable ones were presented here.

The same immobilisation process was used for QueF-Y5Y and QueF-WT variant.

AFM itself seems to be not capable of fully characterizing the assembled layers. How to define a monolayered assembly? It looks like a full coverage, but what's the thickness of a monolayer? What's the distribution of oligopeptide on the surface? Strongly suggest a few other advanced techniques are used to provide a more detailed and convincing results.

We validated the assembly using graphene-coated QCM sensors. The technique shows how adlayers form and evolve with ~ 1 s resolution,^[18] Initially, the QueF-Y5Y variant forms a multilayer that rinses off, then forms a single, stable, saturated layer. We have removed the term 'monolayer' because random rather than ordered adsorption is likely. We present and discuss our evidence for this from p11 of

Section 2.3:

The binding orientation was deduced from estimates of the areal mass density based on two proposed surface orientations. The crystal structure of a QueF monomer (PDB: 4GHM) shows $3.7 \times 6.6 \times 3.2$ nm dimensions which translates to a theoretical monomer binding density of 299 ng cm^{-2} where the long axis is parallel to the surface. QCM-D mass binding analysis suggests that QueF-Y5Y takes roughly 2 h to form stable layer of QueF monomer (**Figure 6a**) and this areal mass density correlates to a thickness of ~ 2.2 nm which suggest monomer conformation and aligns with AFM analysis done on exfoliated graphene (**Figure S4**). QueF-WT suggests binding as a multilayer as the density is too high to suggest binding as monomer nor dimer. QCM measures the wet mass of adsorbed proteins which can increase the estimated mass,^[19] and the packing of the protein on the surface will be less than its maximum, which would lower the estimated mass and may explain initial value for QueF-WT. However, after ~ 8 h, QueF-WT appears to change its surface orientation to

match QueF-Y5Y (Figure S8). QueF-Y5Y, in contrast, appears stably bound for at least 12 h. Both enzymes are stably bound to graphene and do not wash off.

Reviewer's responses to questions

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

Reviewer #3: Considerable - Top 30% in the subject area

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

Reviewer #3: Considerable - Top 30% in the subject area

Thank you, we hope our revisions raise your estimation of the importance and novelty of the work.

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

Reviewer #3: (No Response)

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

Reviewer #3: Partially

As we wrote in response to the other two reviewers, we now expect that our amendments lead you to the same conclusions as we reached based on our data and analyses.

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

Reviewer #3: 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 #3: Considerable - Top 30% in the subject area

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