

Reply to Comment on ‘Can Nano-impacts Detect Single-Enzyme Activity?’ Theoretical Considerations and an Experimental Study of Catalase Impacts’

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We thank Sekretaryova *et al.* for their thoughtful paper which is a useful contribution to the on-going debate^{1–4} about the possibility of electrochemical single enzyme detection.

We are surprised to read that our paper⁴ is described as being critical of their initial paper: This was not our intention and, if carefully read, in our opinion does not read as such. On the contrary, we used the reported observations from Sekretaryova *et al.* in conjunction with our own new data to pose and partially answer the question defined in the title of our paper, ‘Can nano-impacts detect single-enzyme activity?’ and indeed the paper was rigorously refereed on that basis.

At the heart of the question lies the mechanism by which electrochemical detection of single enzymes might take place. On the one hand it is possible that detection may occur via the electrochemical monitoring of the products formed by an enzyme *E* reaction with a substrate *S*:



On the other hand there might be reaction via the so-called direct electron transfer. Either:



or:



where P'' and P''' are not necessarily the same as P , and indeed unless a co-factor is present to provide a source or sink of electrons, it is likely that the initial products P'' and P''' will be different from P .

In our work⁴ on catalase we initially searched for reaction based on detection via product amperometry. Although current spikes were observed, this we reported was unproven and the alternative *possibility* of direct electrochemistry considered, thought possible but again reported as not proved. The paper by Sekretaryova *et al.* and their response make clear that they think their laccase system proceeds via the direct electrochemistry mechanism. Again, the burden of *proof* lies with the authors.

The comments in the Introduction to our paper about the laccase system were in the context of enzyme detection via the enzyme product as is unambiguously specified in our paper and *not* in the context of a direct electron transfer as assumed by Sekretaryova *et al.*. So the latter need not be sensitive to inferred criticism as the data we presented relates to the literature kinetics for enzyme activity in bulk solution which, of course, relates to the product detection mechanism, but not to the direct electrochemistry route. As Sekretaryova *et al.*, rightly in our view, discount the former mechanism as applying to laccase these comments obviously do not impinge on their claimed direct electrochemical single enzyme detection.

Comparative analysis

In the case of detection via product formation, and *not* in the case of ‘direct electrochemistry’, a starting point for the kinetic analysis likely essential and mandatory for proof of detection is the familiar Michaelis-Menten model kinetics which are:

$$\frac{d[P]}{dt} = \frac{k_{cat}[S][E]}{K_m + [S]} \quad (4)$$

where k_2 (s^{-1}) is the maximum turnover rate achieved when:

$$[S] \gg K_m = \frac{k_{-1} + k_2}{k_1} \quad (5)$$

and K_m is the Michaelis constant. We used this model to show that for electrochemical analysis with one-dimensional diffusion detection via simple impacts was unlikely. Equation (4) is an ‘ensemble’ response as we clearly stated, as was the recognition that there are known to be ‘fluctuations in the activity of individual enzymes’. In the case of the system studied by Sekretaryova *et al.* we pointed out that the literature suggests that these fluctuations were likely to be insufficient to allow detection of laccase via product detection, a conclusion which it seems we share with Sekretaryova *et al.*

Modeling

As discussed above, we used Michaelis-Menten kinetics as a starting point for our quantitative analysis of detection via product electrochemistry. Sekretaryova *et al.* comment that ‘conventional averaging ensemble studies overlook the flexibility of the bio-catalyst.’. We agree. Moreover we pointed out this possibility in our paper and discussed, briefly, enzyme fluctuations. The effect on the ‘impact experiment’ is that the frequency of impacts is much reduced and the size of the fewer spikes altered. However in a one-dimensional system, we show that even enzymes featuring a much higher catalytic activity than the mean will not be seen as spikes in the current for the catalase system. In an upcoming publication under different experimental conditions we will show how current spikes *may* be seen in some cases. However

this is of no relevance to the laccase case if direct electrochemistry is the explanation of the observations of Sekretaryova *et al.*.

Experimental evaluation

In their correspondence Sekretaryova *et al.* make four points. In the case of the first and third points we of course agree as we ourselves made exactly the same points in our own paper.

Concerning the second point about materials; these were acquired from commercial sources and, for our work, we accepted the levels of possible contamination provided by the manufacturers.

Last we appreciate that the electrolyte solutions used differ from the enzyme's natural environment and might alter its activity. Our paper did not conclude that the described experimental set-up was suitable for single enzyme detection in its natural environment. Further the levels of H_2O_2 used were the highest concentrations possible consistent with no degradation of the catalase.⁵

Summary

Scientifically, the published work of Sekretaryova *et al.* and ourselves show current spikes related to the presence of enzymes in solution. The cause of the signals is probably not due to electrolysis of the enzyme reaction products but direct electrochemistry *might* explain the observations. However *proof* of the latter requires detailed quantitative analysis against suitable models for impact frequency and size, and current 'spike' shape analysis together with the acquisition of extra kinetic data. At present we believe the claim for single enzyme electrochemistry to be plausible but not proven.

In the context of the correspondence we reiterate that our paper was not critical of the work of Sekretaryova *et al.*. It was intended as a constructive contribution to the debate. Had we wished to criticise their paper we would have submitted a note to the journal in which it was published and would then doubtless have, amongst others, made the points in the Appendix below. Finally we comment that we trust that the persistent misspelling of the name of the first author of our paper is unintended.

Appendix

Role of the filter

The choice of the potentiostat is generally crucial in nano-impact experiments if physical quantities are to be extracted from the spike shape.^{6;7} The transfer function of the entire signal chain and the employed filters play herein an important role, while particularly the latter may determine the outcome of the measurement.

In a *typical* measurement set-up, such filters are used to remove unwanted elements from the recorded signal, which can for instance be the background noise. In this case, common filters suppress components of the input signal in a chosen frequency regime and at least affect this targeted frequency regime. If there is no overlap between the wanted and the unwanted signal in terms of their spectral components, the unwanted signal may successfully be removed from the measurement.^{N1} The here-addressed work² by Sekretaryova *et al.* discusses data that was recorded 'with noise filtering at 10 Hz' and subsequent removal of 'electrical noise' via a Fourier transform filter equally operated at 10 Hz, as explained in the

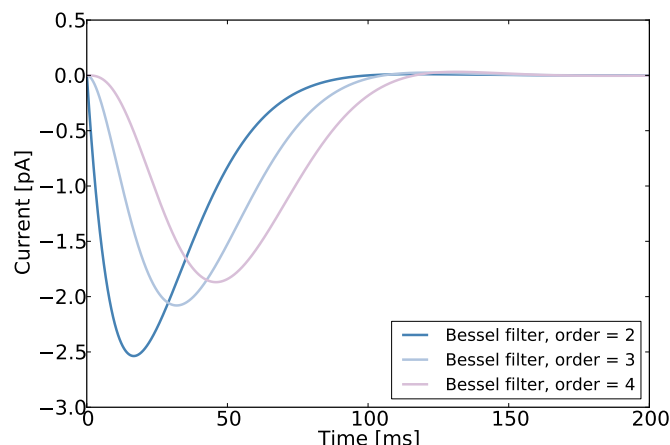


Figure 1: Bessel filter responses to a rectangular pulse featuring a height of -1 nA and a width of 100 μ s calculated at a cut-off frequency of 10 Hz. Note that the vertical scale is pA.

Experimental Details. Though the nature of the filters is not further specified, a closer evaluation of Figure 2c suggests that a low-pass filters were likely used.

We acknowledge that the actual filter types used by Sekretaryova *et al.* are unknown to us and a large number of possibilities exists. To illustrate the effect of standard low-pass filters operated at a cut-off frequency^{N2} of 10 Hz, we herein nonetheless report a model calculation of various filter responses to rectangular input pulse. Figure 1 compares such responses to a pulse featuring a height of -1 nA, which is approximately 250 times greater than the peak height observed in Figure 2c in the work by Sekretaryova *et al.*. A closer investigation of the graph reveals that modelled spike shapes on the one hand are very similar to that spike shape reported in Figure 2c and on the other feature a height that is reduced by two to three orders of magnitude compared to the input signal. If an analysis assumed that the turnover number k_{cat} was proportional to the measured peak height as it is done in the here-addressed publication and the filter had a similar effect on the input signal, data might be equally corrupted.

We trust that Sekretaryova *et al.* have considered this issue, investigated the bandwidth of the enzyme signal and the transfer function of their set-up, and ensured that the measured peak heights are not affected by the filter.

Distinguishing enzyme signals form background noise

In the analysis of spikes in nano-impact experiments is often limited by the background noise. Though the potentiostat may offer a suitable sensitivity and bandwidth, other sources of noise may dominate the measurement and obstruct a further analysis. To this end, it is generally crucial to develop an exact definition of current features that are considered to be a spike. A suitable definition would herein for instance include all spikes that exhibit a peak height exceeding the noise level by a factor of five or ten.

In Figure 2d, Sekretaryova *et al.* provide a histogram of the number of detected spikes versus the turnover number k_{cat} , where each bar depicts a range of $\Delta k_{cat} = 1.0 \cdot 10^5 \text{ s}^{-1}$. The lowest value of k_{cat} considered is $1.0 \cdot 10^5 \text{ s}^{-1}$ and it is indicated that 15 spikes were found to correspond to k_{cat} values between $0.5 \cdot 10^5 \text{ s}^{-1}$ and $1.5 \cdot 10^5 \text{ s}^{-1}$. The respective range of measured peak currents I_p for this one bar can be calculated via Equation (3) in the paper's Supporting Information:

$$\begin{aligned} I_p^{min} &= 4 \cdot e^- \cdot k_{cat}^{min} = 4 \cdot e^- \cdot 0.5 \cdot 10^5 \text{ s}^{-1} = 32 \text{ fA} \\ I_p^{max} &= 4 \cdot e^- \cdot k_{cat}^{min} = 4 \cdot e^- \cdot 1.5 \cdot 10^5 \text{ s}^{-1} = 96 \text{ fA} \end{aligned} \quad (6)$$

and yields:

$$I_p \in (32 \text{ fA}, 96 \text{ fA}) \quad (7)$$

The Supporting Figure S1 however reveals that the level of background noise, which as indicated by Sekretaryova *et al.* varies significantly between different measurements, exhibits a noise amplitude of up to about 75 fA to 100 fA, which corresponds to a difference of 150 fA to 200 fA between noise minima and maxima.^{N3}

The above observation gives rise to the question whether some of the experimental data shown in Figure S1, which features a noise amplitude that is at least of the magnitude of the considered spike heights, was omitted in the analysis or different spike definitions (for instance thresholds) were used to analyse different measurements. In either case no conclusions could be drawn from Figure 4d.

Plausibility of the observed spike shapes

In the Figures 2a and 2d of the article², Sekretaryova *et al.* show spikes featuring durations ranging from about 150 ms to 9 s, while the trends of the latter are clearly not affected by the filter set at 10 Hz. If the tail represented the progressing denaturation of the enzyme or structural changes as stated in the text, the reader may wonder why this happens via a continuous and continuously-differentiable decay in enzymatic activity. Accepting that different structural conformations may lead to different enzymatic activities, it is not immediately plausible why on a time scale of 9 s it is thermodynamically-favourable that the enzyme deactivation proceeds along a phase-space trajectory that in terms of enzyme activity places the visited states in such order. It is further not directly clear why intermediate states, i.e. states featuring almost the entire continuous range of activities from 100 to 0 percent, exist at all and the deactivation does not progress in a binary or step-wise fashion.

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[N1] On a more general note, we emphasise that a large number of filtering approaches exists and that in some cases more specialised filters may even distinguish between signals of overlapping spectral components if additional information on the input signal is available. Various filters may have an *entirely different* effect on the recorded signal.

[N2] We point out that different software packages and textbooks may define the cut-off frequencies of higher order filters differently. The here-depicted data was calculated via the *SciPy* package and uses the corresponding definition.

[N3] Sekretaryova *et al.* published a correction⁸ to this figure stating that some points were omitted in the original publication and are now considered. We therefore assume that all spikes evaluated in the original publication remain part of the amended analysis in the correction though spikes featuring k_{cat} values of less than $2.0 \cdot 10^5 \text{ s}^{-1}$, which translates to a peak current of 128 fA, are now omitted in the corrected histogram without mentioning.