Correspondence on "Can Nanoimpacts Detect Single-Enzyme Activity? Theoretical Considerations and an Experimental Study of Catalase Impacts"

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Correspondence on "Can Nanoimpacts Detect Single-Enzyme Activity? Theoretical Considerations and an Experimental Study of Catalase Impacts" by E. Kätelhön, L. Sepunaru, A.A. Karyakin, R.G. Compton (ACS Catalysis, 2016, 6(12), 8313-8320**; DOI:** 10.1021/acscatal.6b02633).

Two recent papers[1-2] have shown that telectrochemical measurements performed on individual catalytic entities differ significantly from conventional *in vitro* studies performed using large quantities of particles, where with the response is averaged over the whole population of catalytic objects. Single-molecule measurements allow the resolution of information on catalyst functionality at the lowest level of structural organization and tailored to *in vivo* conditions in a living cell, where only a few biocatalyst molecules are present. We believe that this recently emerged approach of collision electrochemistry[3-4] can be applied to redox enzyme molecules [5-6] introducing a new technique, which is complementary to the existing single molecule enzymology toolkit[7-8], for precise characterization of enzymatic activity.

However, in a recent article published in this journal[9], Kättelhön et *al*. were critical of results obtained using collision electrochemistry of single enzyme molecules, e.g. claiming that our results contradict the literature. Although these authors make some excellent points, we stand by our original findings[6] and would like to point out some critical issues that they may not have considered.

Comparative analysis. The authors repeatedly refer to turnover numbers obtained from standard enzymatic assays and compare these with results from single enzyme molecule electrocatalysis. There are several reasons why the catalytic constant obtained from single molecule experiments cannot be compared with data obtained from studies that average data from a population of molecules. Due to conformational fluctuations, the effects of which are masked in ensemble studies, a single enzyme molecule exhibits a variety of catalytic rates. Therefore, it does not possess a single rate constant, but exhibits a distribution of rates that under normal circumstances are recorded as an average rate.[10] Because of this molecular flexibility, the turnover number obtained from Michaelis-Menten kinetics have different microscopic interpretations.[11-12] We believe that comparison of our results with the turnover number of 560 s^{-1} previously obtained for *Rhus vernicifera* laccase[13] is irrelevant for the following reasons. *Firstly*, the active site of plant laccase from *Rhus vernicifera* differs noticeably from that of the fungal laccase *Trametes versicolor* that we studied. The potential of the primary acceptor site, T1, of *Rhus vernicifera* laccase is about 400 mV (*vs* NHE), whereas the potential of T1

in *Trametes versicolor* is about 780 mV (*vs* NHE). The T1 ligation is also different for these two enzymes.[14] These factors influence the kinetics and catalytic mechanism of the two enzymes.[15] *Secondly*, the value of 560 s^{-1} for the turnover number quoted by Kättelhön *et al.*[9] was obtained from ensemble studies, where the reaction between the reducing substrate and the enzyme is the rate determining step under close-to-equilibrium conditions. In our case, a significant overpotential is applied to the electrode as a replacement for the reducing substrate, thus making the experimental conditions far from equilibrium. This dramatically influences the biocatalytic kinetics! In particular, either intramolecular electron transfer or reaction with oxygen become the rate-determining step at such high overpotentials. The reported rate constants for intramolecular electron transfer (>25000 s^{-1}) and for reaction with oxygen ($k > 5\times10^7$ M^{-1} s^{-1})[16] are several orders of magnitude higher than that for the reaction of the reducing substrate, and moreover, were still obtained from ensemble measurements averaging the whole population of enzyme molecules. Our measurements quantify only catalytic collisions with very high turnover numbers, yielding current spikes which are clearly visible over the measurement noise. This suggests that the average values of the rate constant obtained in the absence of kinetic control (i.e. electron transfer between the electrode and laccase is not a rate-determining step) are realistic.

Kättelhön *et al.*[9] suggest that the laccase bioelectrocatalysis observed by us on a 0.1 s time domain on polycrystalline gold, is in some way contradictory to the inactivation observed over a much longer time domain[17], while it is actually in agreement with the previously reported phenomenon. The spike-shaped amperometric responses are likely due to a brief period of productive electron transfer followed by a loss of activity due to the biocatalyst flattening onto the polycrystalline gold surface[17]. Moreover, laccase does actually show direct anisotropic catalysis towards oxygen reduction on gold (111) [18-19], chemically-modified polycrystalline gold [20-21] and porous gold [22-26].

Modeling. We also believe that the applicability of the theoretical analysis presented by the Kättelhön *et al.*[9] for single enzyme collisions is rather limited, e.g. exemplified when they try to explain their own experimental results,which should have been understood already from their “back of the envelope” estimation. In the theoretical model presented, the authors assume that "the enzyme kinetics follow the Michaelis-Menten model and steadily transform substrate into product independent of the enzyme position." This assumption immediately implies two important limitations. *Firstly*, conventional averaging ensemble studies overlook the flexibility of the biocatalyst, which crucially affects the kinetics at the molecular level.[14] Omitting this contribution could significantly oversimplify the model. *Secondly*, the model considers single enzyme quantification via product electrolysis, which

as noted by the authors, is not the case for the direct electron transfer reported in the papers they criticise [6, 27].

Experimental evaluation. The experimental model utilized for evaluation of single molecule bioelectrocatalysis is rather weak due to uncertainties in the experimental conditions. *Firstly*, as was anticipated by the authors, catalase activity in solution with a high concentration of hydrogen peroxide (100 mM) leads to production of small bubbles (nanobubbles) of oxygen available for avalanche-type reduction at -1 V (vs SCE). The visible appearance of bubbles for "catalase-positive" microorganisms [28-29] is well known in the field of Microbiology and used for microbial identification.. However, this is not the case for laccases. *Secondly*, the presence of contaminants in the commercial heme-protein (e.g. iron-containing substances) might significantly contribute to the observed amperometric phenomena via electrocatalysis of hydrogen peroxide reduction at -1 V (vs SCE). We confirm that this issue might also affect the measurements reported by us. Therefore, a clear demonstration ofbiocatalytic activity must be accompanied by inhibition studies to unequivocally establish their validity. *Thirdly*, as was also mentioned by the authors, catalase undergoes direct electron transfer at a variety of carbon materials [30-40] to different electron acceptors such as hydrogen peroxide, which contributes to the complexity of electrode phenomena making quantification via oxygen electrolysis uncertain. *Lastly*, being an oxidative biocide [41-42], the hydrogen peroxide at high concentrations could cause uncontrollable enzyme denaturation, which might lead to changes in kinetic characteristics.

Overall, this discussion illustrates the long path ahead of us to fully grasp all the phenomena happening when a single biocatalyst molecule interacts at an electrode interface. These intriguing studies to elaborate the functional performance of single molecules may face many obstacles.However, we believe that based on experimental data on enzyme collisions from various groups [5-6], observed current spikes can be assigned to catalytic currents via direct communication between an enzyme and an electrode, although quantitative interpretation of the experimentally observed values at this stage remains challenging.

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