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# Observation of nanoimpact events of catalase on diamond ultramicroelectrodes by direct electron transfer

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**We report electrochemical detection of single-catalase collisions at diamond ultramicroelectrodes and show the operative mechanism involves direct enzyme-mediated charge transfer between electrode and solution. Hydrogen peroxide increases the collision frequency, which fluorescence correlation spectroscopy diffusion measurements suggest stems from an increase in the diffusion rate as the underlying cause.**

The field of particle-electrode collisions (also known as nanoimpact<sup>1</sup>) is a rapidly growing research discipline opening up a broad range of potential applications<sup>2</sup>. This method allows an in-situ direct detection of single nanoparticles in solution<sup>3,4</sup>. In a typical nanoimpact experiment an electrode under a controlled potential is immersed in a solution containing the freely-diffusing analyte. Particles collide stochastically on the electrode where they may directly adsorb, react (reduce or oxidise) or trigger an intermediate redox reaction, all resulting in characteristic current spikes out of which properties of the particle can be extracted, such as concentration, size and shape<sup>2</sup>. This method has been used with inorganic and organic nanoparticles<sup>2</sup>, emulsion droplets<sup>5</sup>, vesicles<sup>6</sup>, DNA<sup>7</sup>, and virus<sup>8</sup> and to measure the motion of micron-sized self-propelled particles<sup>9</sup>.

Single-particle detection of enzymes is of great interest in molecular biology. The measurement of enzymatic activity, function and structure is key to understanding metabolic pathways or determining the origin of genetic diseases<sup>10</sup>. Most tools available today for single-enzyme detection rely on single-molecule fluorescence measurements, which require enzyme labelling. Nano-

impact experiments allow single-enzyme measurements in their native state, without molecular modifications, using cost-effective experimental setups<sup>2</sup>.

Catalase is an important enzyme present in almost all aerobic organisms, and has one of the largest turnover rates known<sup>10</sup>. Recently, single catalase molecules have been detected by an indirect voltammetry method in the presence of H<sub>2</sub>O<sub>2</sub><sup>11,12</sup>; catalase decomposes H<sub>2</sub>O<sub>2</sub> into O<sub>2</sub>, which diffuses to the electrode surface and creates an amplified current signal. In this indirect method, the maximum value of the current from one catalase collision can be predicted by assuming that at excess substrate concentrations the enzyme works at its maximum turnover rate  $k_{cat}$ . However, the measured current exceeded by 3 orders of magnitude the theoretically predicted upper limit values<sup>11</sup>. Therefore the theory for describing individual catalase impacts through the detection of enzyme reaction products at the electrode does not fully hold. An alternative explanation for the occurrence of current spikes could be due to 'direct' electrochemistry, whereby the generated charge transfer between electrode and solution is mediated directly by the enzyme in contact with the surface during a nanoimpact event<sup>11</sup>. To investigate this hypothesis, we designed a nanoimpact experiment held at a potential that would not reduce H<sub>2</sub>O<sub>2</sub> or O<sub>2</sub>, thereby blocking any Faradaic contribution from indirect reactions to the signal, but allowing the direct electron transfer between catalase and the electrode to be detected.

A low background current and minimum noise is essential for nanoimpact experiments. In this study we therefore tested the use of a custom-made boron-doped diamond ultramicroelectrode (BDD-ume). In electrochemistry diamond is known for its chemical inertness, fouling resistance, low background current, low double layer capacitance ( $<10\mu\text{Fcm}^{-2}$ ) and a large electrochemical potential window<sup>13</sup>, and has been reported as an active substrate that can interact with biomolecules, such as DNA<sup>14</sup> and catalase<sup>15</sup>. However, the use of boron-doped diamond ultramicroelectrode for nanoimpact experiments is still limited.

In the present work we thus demonstrate the high suitability

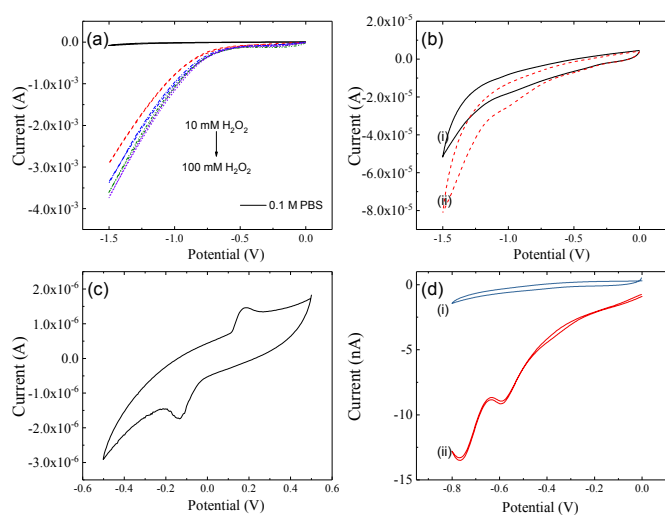
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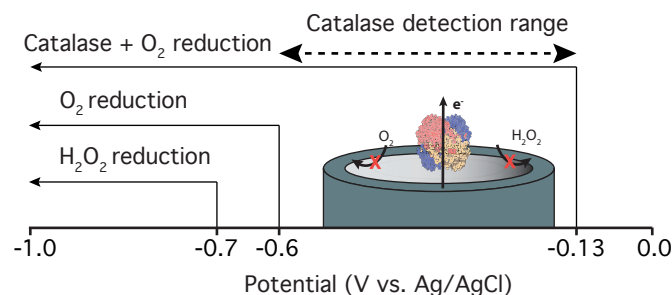


**Fig. 1** Cyclic voltammogram at BDD electrode in 0.1 M PBS with (a) absence of  $\text{H}_2\text{O}_2$  and 10, 25, 50 and 100 mM  $\text{H}_2\text{O}_2$ ; (b) with (i)  $\text{N}_2$  gas and (ii) air bubbling for 10 min; (c) at BDD with drop-coated catalase modification ( $20\ \mu\text{L}$  100 nM mixed with 0.5% nafion solution); (d) at (i) BDD-ume and (ii) Catalase-coated BDD-ume in 0.1 M PBS.

of BDD-ume to observe nanoimpact events of biomolecules. Furthermore we provide clear evidence of the operation of a mechanism of direct electron transfer between catalase and the BDD-ume. Finally, we also report an increase in number of collisions with the electrode, when the enzyme is in the presence of its substrate, suggesting an increase in the diffusion coefficient under these experimental conditions.

The experimental setup comprises a three-electrode system with a working electrode of custom-made BDD-ume of  $10\ \mu\text{m}$  diameter, a reference electrode of Ag/AgCl and a Pt wire counter electrode. The experiments were carried out with a CHI 900B electrochemical detector (CH Instruments, Inc.) equipped with a Faraday cage. The catalase concentration throughout this work was determined by UV spectroscopy (Varian Cary, Agilent) using an extinction coefficient of  $\epsilon_{405} = 324\,000\ \text{M}^{-1}\ \text{cm}^{-1}$ <sup>16</sup>. All the chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

A series of cyclic voltammetric experiments were carried out first on a BDD macroelectrode ( $0.47\ \text{cm}^2$ ), to determine if a potential range existed within which enzyme-mediated direct electron transfer between solution and a diamond electrode could occur in the presence of catalase and dissolved oxygen, but not the reduction of  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  in the absence of catalase. Figure 1a shows voltammograms in the absence and increasing presence of  $[\text{H}_2\text{O}_2]$  in de-aerated 0.1 M phosphate-buffered saline (PBS) at a BDD macroelectrode. The BDD surface is passive for  $\text{H}_2\text{O}_2$  reduction at potentials less than  $-0.7\ \text{V}$  and no distinguishable peaks were observed in the whole range. The same experiment was performed at 0.1 M PBS with  $\text{N}_2$  gas and air bubbling for 10 min (Figure 1b (i) and (ii) respectively). A difference in current between the two CV curves was observed in the potential range from  $-0.6$  to  $-1.5\ \text{V}$ . This difference is due to  $\text{O}_2$  reduction. Figure 1c shows the voltammogram of catalase-modified BDD (drop-coating  $20\ \mu\text{L}$



**Fig. 2** Scheme of the reduction potential of  $\text{H}_2\text{O}_2$ ,  $\text{O}_2$ , catalase and catalase reduced  $\text{O}_2$ , and the identified preferred range for catalase detection while blocking  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  reduction. Catalase illustration adapted from<sup>18</sup>.

100 nM catalase mixed with 0.5% nafion solution onto clean BDD electrode surface and dried for 2 hours) in 0.1 M PBS. A pair of redox peaks appeared at  $+0.18$  and  $-0.13\ \text{V}$ , in agreement with the reported redox potentials for direct oxidation and reduction of catalase species itself<sup>15,17</sup>. The above experiments thus suggest that on the BDD surface, the reduction signal of catalase can be observed in the potential range of  $-0.13$  to  $-0.6\ \text{V}$  where  $\text{H}_2\text{O}_2$  or  $\text{O}_2$  cannot be reduced, as illustrated schematically in Figure 2. Figure 1d shows a cyclic voltammogram at BDD-ume and catalase-modified BDD-ume in 0.1 M PBS in the presence of dissolved oxygen. A larger background current and distinguishable  $\text{O}_2$  reduction peaks are observed at the modified electrode in the potential range from about 0 to  $-0.6\ \text{V}$  which is likely to arise from the reduction of the enzyme and a higher sensitivity toward  $\text{O}_2$  reduction when catalase is present at the surface as noted previously<sup>17</sup>. Since current in this potential range cannot represent the reduction of  $\text{O}_2$  or  $\text{H}_2\text{O}_2$  at the clean diamond electrode (see Figure 1a and b) it can be concluded that significant increase in current represents a catalase collision on the electrode, through a mechanism associated with direct enzyme-mediated charge transfer between the solution and the electrode. This potential range between  $-0.13\ \text{V}$  and  $-0.6\ \text{V}$  therefore provides a suitable range for the detection of catalase via nanoimpacts by a mechanism of direct electron transfer.

Nanoimpact experiments were thus performed at the BDD-ume in 0.1 M PBS containing 100 mM  $\text{H}_2\text{O}_2$  and 10 pM catalase (similar to the experimental conditions in<sup>11</sup>) at a potential of  $-0.2\ \text{V}$ . Figure 3a records the  $i-t$  curve when applying successive potentials of  $+0.2$ ,  $0$  and  $-0.2\ \text{V}$ . The observed spikes at  $-0.2\ \text{V}$  are most likely due to single particle events of catalase monomers or multimers impacting the electrode. This experiment thus confirms the hypothesis of direct electron transfer between the enzyme and the electrode surface. Further, these spikes were also observed in the same solution but without  $\text{H}_2\text{O}_2$  at  $-0.2\ \text{V}$  (figure 3b) but in the presence of dissolved  $\text{O}_2$ . Representative spikes at a magnified scale are shown in Figure 3c and d with a noise current lower than  $2\ \text{pA}$ .

Figure 4 displays representative  $i-t$  curves recorded at  $-0.2\ \text{V}$  in 0.1 M PBS solution with 0, 5, 10 pM catalase and 10 pM catalase combined with 100 mM  $\text{H}_2\text{O}_2$  respectively. No spikes were

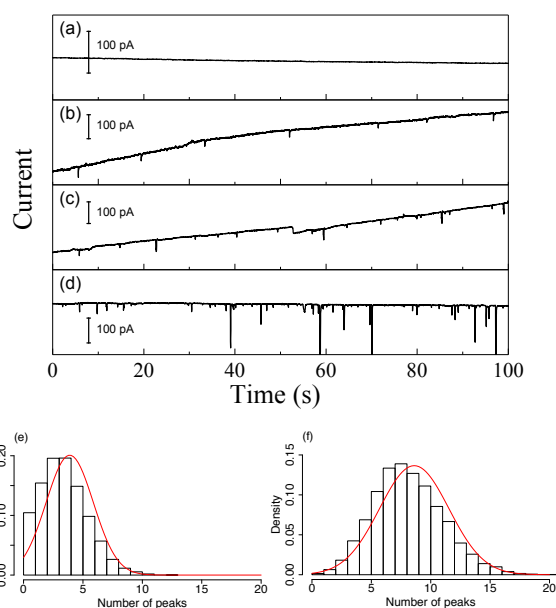
detected in the absence of catalase (Figure 4a), while spikes appeared when adding 5 pM (Figure 4b). With higher concentration (10 pM, Figure 4c), more spikes appeared, and a further increase in spike frequency was observed in the presence of 100 mM H<sub>2</sub>O<sub>2</sub> (figure 4d).

We identified current spikes using a peakfinder algorithm (findpeaks, MATLAB, Mathworks), applying a current threshold of 10 pA to a smoothened raw signal and then counting zero-point crossings in the first derivative of the signal. Figure 4e-f show the average number of spikes per 10s, corresponding to a sample size of 1000 observations and 10000 replicates, randomly sampled over the duration of the experiment (2000 s). We measured an average spike count of 3.8 (standard error of the mean 0.02) for 10 pM catalase and 8.6 (standard error of the mean 0.02) for 10 pM with H<sub>2</sub>O<sub>2</sub>. This corresponds to a change in the mean inter-spike time interval from 2.4s to 1.1s, in 10 pM catalase without and with H<sub>2</sub>O<sub>2</sub> respectively (histogram and fitted exponential distribution shown in ESI 1). A twofold increase in concentration of catalase also was found to result in a higher number of spikes (Figure 4 b and c). This is in agreement with a theoretical expression of the steady-state diffusion-controlled flux of particles to an ultramicroelectrode surface  $J$  given by<sup>3</sup>:

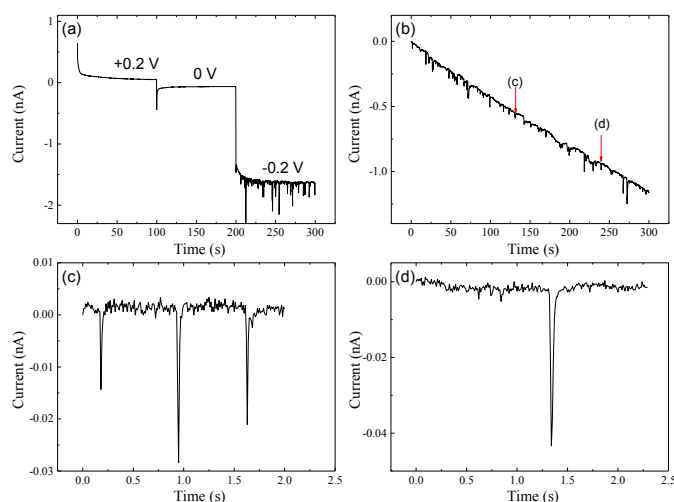
$$J = \frac{4Dc}{\pi r} \quad (1)$$

where  $D$  is the particle diffusion coefficient,  $C$  is the particle concentration and  $r$  is the radius of the electrode.

To observe the effect of adding hydrogen peroxide in the mobility of catalase, we measured its diffusion by fluorescence correlation spectroscopy (FCS). Catalase (from bovine serum 1mg/mL) was labelled with a fluorescent dye (NHS-Rhodamine, Thermo-



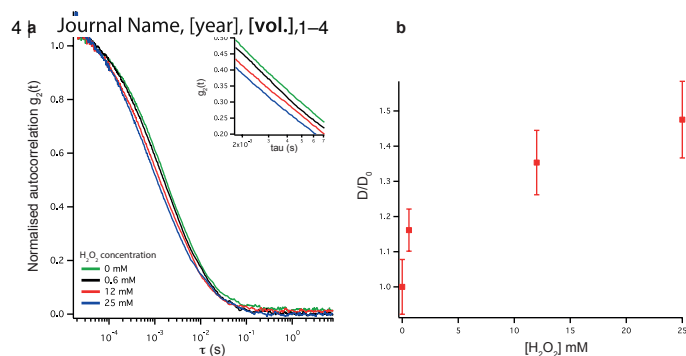
**Fig. 4** (a)-(d) Typical  $i$ - $t$  curve at BDD-ume at -0.2 V in a solution containing (a) 0.1 M PBS and (b) 5 pM catalase; (c) 10 pM catalase; and (d) 10 pM catalase mixed with 100 mM H<sub>2</sub>O<sub>2</sub>; (e)-(f) Histogram of detected number of spikes shows the average number of spikes per 10s, corresponding to a sample size of 1000 observations and 10000 replicates, randomly sampled over the duration of the experiment (2000 s). A fitted normal distribution yields an average spike count of 3.8 (standard error of the mean 0.02) for 10 pM catalase in (e) and 8.6 (standard error of the mean 0.02) for 10 pM with H<sub>2</sub>O<sub>2</sub> in (f).



**Fig. 3** (a)  $i$ - $t$  curve at BDD-ume in the solution containing 0.1 M PBS, 100 mM hydrogen peroxide and 10 pM catalase at successive applied potentials of +0.2, 0 and -0.2 V versus Ag/AgCl; (b)  $i$ - $t$  curve at BDD-ume in the solution containing 0.1 M PBS and 10 pM catalase at applied potentials of -0.2 V versus Ag/AgCl; (c) and (d) representative spikes obtained from (b) as indicated. The time and current axes in (c) and (d) have been offset for clarity.

scientific) in a 10-fold molar excess, incubated overnight and purified by size exclusion gel filtration spin columns (BioSpin 6, Bio-Rad). 1 nM concentration of purified enzyme was mixed with H<sub>2</sub>O<sub>2</sub> at indicated concentrations and the fluorescence intensity was recorded immediately afterwards for 30s. We used a custom-made confocal microscopy setup (setup details in ESI 2). The obtained fluorescence intensity signal was processed in a multiple-tau digital correlator to generate intensity autocorrelation functions  $g_2(t)$  (Figure 5a). Obtained autocorrelation curves were fitted to a model of single-species diffusion using a least-square regression algorithm to yield relative diffusion values reported in Figure 5b. We observed an increase in diffusion of catalase higher than 30% in agreement with results reported by others for a wide range of enzymes<sup>19,20</sup>. The heat released in the catalytic reaction has been suggested to play a role in the observed enhanced diffusion phenomenon<sup>19,21</sup>. However, this hypothesis has been challenged by other models that attribute it to a hydrodynamic effect<sup>22</sup>.

We observed an increase in number of spikes when hydrogen peroxide was added to a catalase solution. This increase could be due to several factors such as, a longer residence time of the enzyme on the electrode, a stronger enzyme-electrode interaction, an increase in concentration or faster diffusion of the enzyme<sup>2,23</sup>. Provided that most of the experimental conditions remain the same upon addition of H<sub>2</sub>O<sub>2</sub> and that the spike fre-



**Fig. 5** Fluorescence correlation spectroscopy (FCS) diffusion measurements of fluorescently-labelled catalase. (a) Normalised autocorrelation  $g_2(\tau)$  curves of catalase in the presence of different concentrations of  $\text{H}_2\text{O}_2$ . Zoom-in shown in inset. (b) Relative diffusion coefficients extracted by fitting  $g_2(\tau)$  to a single-species diffusion model.

quency and diffusion coefficient are linearly related, we favour the hypothesis in the light of the results above that an increase in diffusion coefficient of the enzyme might be the main contribution to the observed increased collision events. Several theories have been put forward that speculate about the origin of the propulsion mechanism of enzymes, which remains subject to debate, such as, collective heating<sup>21</sup>, chemoacoustic effect<sup>19</sup> and hydrodynamic coupling<sup>22</sup>. However, none of the experimental observations report a direct single-particle detection of enzymatic nanomotors without resorting to molecular labelling<sup>19,20</sup>. We believe that nanoimpact voltammetry paves the way towards studying the self-propulsion of enzymatic nanomotors like catalase in their native state.

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