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When nanoelectrochemistry meets biocatalysis

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Abstract

This mini-review discusses recent advances in nanoelectrochemical techniques for detecting or imaging the bioelectrocatalytic activity of single enzymatic nano-entities. Two current strategies are highlighted, and their performance levels deciphered: nano-impact electrochemistry and nano-SECM imaging. These strategies enable the separation in time or space, respectively, of multiple individual faradaic signals captured from a collection of entities dispersed in volume or on a surface. The exploratory nature of nanoelectrochemistry, applied to bioelectrocatalysis, is generating captivating know-how to study enzyme/electrode processes, and new knowledge to overcome fundamental barriers in heterogenous biocatalysis.

Introduction

Nanoelectrochemistry involves measurement and imaging techniques utilizing specialized nanoscopic tools (nanoelectrodes, nanopipettes, nanogaps, etc.) to address confined electron transfer reactions. A recent focus is on the detection of single entities, where the term *entity* stands for any molecule, particle or soft object involved in an electrochemical event [1,2]. Special expertise, coupled with the development of custom equipment or multimodal platforms, is required to implement the electrochemical study of individual entities that generate low intensity signals [3]. Single molecule electrochemistry was early achieved in a few “nano-gap” configurations, where the very same redox molecule made thousands of round trips per second between closely separated electrodes to be repetitively oxidized and reduced, in order to generate a current intense enough to be recorded [4–7]. This redox cycling scheme is a powerful current amplifying strategy which, combined with redox-labelling, has also been implemented

in electrochemical atomic force microscopy (AFM-SECM) to make imaging of individual biomacromolecules possible [8,9]. Currents were nevertheless low, in the sub-picoamp (pA) range, which required forefront electronics to be used. Indirect methods based on fluorescence emission also offer a noteworthy way to detect single redox molecules, without the need for low-current measurements. They provide the required signal amplification, and thus high sensitivity, by de facto realizing an electron-to-photon conversion [10]. Here, fluorogenic species are individually tracked as they undergo electrochemical conversion between a photoluminescence-emitting redox state to a non-emissive one [11,12].

In the last decade, the predominant approach in single-entity electrochemistry has been the so-called "nano-impact" method [13,14]. This chronoamperometric technique detects individual particles, initially dispersed in a solution, which randomly collide or adsorb onto the surface of an ultramicroelectrode (UME), and so-generate a distinguishable transient faradaic signature (step- or spike-like) that disrupts the electrode current baseline. Many impact detection examples have been reported involving various types of solid nano-objects, including metal or oxide particles as well as nanocarbons. Collisions that lead to the anodic dissolution of the particles yield directly measurable current features in the pA regime, but careful analysis of bandwidth limitations is required in order to resolve the collision dynamics [15–17]. Other types of collision experiments require a current amplification mechanism. For instance, the colliding particle can act as a local electrocatalyst once initiating a rapid redox reaction that generates an electroactive product, which can be detected by the underlying UME [13]. Of note are emerging strategies based on electrochemical nano-impacts of bioconjugated metallic nanoparticles [18]. These techniques aim to develop innovative analytical platforms, as recently exemplified for biomarker detection or protein aggregation characterization [19,20]. Finally, attempts have been made to extend this detection approach to single biocatalytic particles, or even single enzymes as further reported below.

Electrochemists have long been interested in biocatalysis, using redox enzymes (oxidoreductases) as both biorecognition probes and catalysts in electron transfer reactions with an electrode. The field of biocatalysis has multiple practical applications, including utilizing electrochemical biosensors to report the concentration of an analyte or producing bioelectricity in enzymatic fuel cells. Substantial research on bioelectrocatalytic systems is aimed at developing suitable architectures to electrically connect (*i.e.* wire) enzymes to the electrode surface on which they are immobilized. This topic was extensively reviewed by Minteer and colleagues in a very comprehensive article [21]. Dominating approaches involve modifying the electrode surface with nanomaterials, such as metal or carbon particles, or with a porous layer. The goal is to "structure" the transducing interface with high-density contact sites that match the size of enzymes (~5 to 10 nm) in order to enhance enzyme loading, facilitate direct electron transfer (DET) with the electrode, or both [22,23]. Typically, the performance of macroscopic enzyme electrodes is evaluated from the current density (in A/cm²) obtained by ensemble amperometric measurement, *i.e.* at the scale of millions of enzyme molecules hosted on the whole surface of the electrode. Yet it would be more suitable to derive instead the average catalytic current (i_{cat}) generated per enzyme molecule. This would provide a reliable fA/enzyme metric or, equivalently, an apparent enzyme turnover frequency number (in s⁻¹) to be used for comparing the performances of different bioelectrocatalytic systems. In this context of applied research, the averaged i_{cat} per enzyme value can indeed be compared to that calculated from the known catalytic rate (k_{cat}) of the native enzyme in solution, to determine whether the enzymes retain a reasonable catalytic ability once surface-immobilized, or even exhibit a catalytic "enhancement" effect.

To complete the characterization of bioelectrode constructs, numerous surface analysis methods have now developed in order to gain spatial information about the heterogeneity of enzyme-electrode interfaces. In situ microspectroscopies (e-SPR, fluorescence) and scanning

probe microscopies (AFM, EC-STM or SECM) have been used to assess various properties of electrode-immobilized enzymes, including their quantity, density, surface coverage, spatial organization, preferential orientation, conformational distortion, structural or functional integrity, and local chemical micro-environment [24,25]. However, despite these endeavors, determining how the electrocatalytic activity of enzymes is statistically distributed within their heterogeneous ensemble population remains a major experimental challenge. Nanoelectrochemistry applied to biocatalysis offers distinctive methods to explore the interaction between an enzymatic entity and an electrode, as well as to decode how its catalytic activity is converted into an electrochemical current signal. This is made possible through an unparalleled level of analysis that deals with the basic “building” components of a biosystem.

The objective of this concise review is to offer valuable perspectives on the latest advancements in the "nano-bio" electrochemistry field which is centered around the electrochemical examination of individual enzymatic nano-entities, including enzyme-decorated nanoparticles, enzymes clustered on nanosized scaffolds, or even single enzymes. As depicted in the simplified view of Figure 1A, the first approach involves the nano-impact method, which captures distinct *time-separated* faradaic signatures of single enzymatic events at an UME surface. The second approach uses electrochemical microscopy-based methods to image the enzymatic electroactivity of individual biocatalytic particles that are *spatially separated* (Figure 1B).

1. Time-resolved capture of single enzymatic events by nano-impact methods

The collision/blocking mode of nano-impact electrochemistry is a frequently employed technique to identify biological entities at the nanoscale [26]. In this method, an inert bioparticle or biomacromolecule lands on a planar UME surface, and partially obstructs the electrochemical reaction of a soluble redox indicator (such as ferrocene or ferricyanide). As a result, the steady-state current baseline decreases in steps of a few tens of pA intensity at each

successive collision. This approach has been successfully employed for detecting single enzyme collisions, such as glucose oxidase (GOx), horseradish peroxidase (HRP), and catalase, on a nanoelectrode with a 100-nm radius [27]. It is effective and easy to implement for detecting biomacromolecules, but also other larger insulating biological entities (viruses, vesicles or microbial cells). However, since the electrochemical blocking mechanism relies solely on a physical parameter of the entity being detected (its size or "footprint"), it does not provide any functional information about the enzyme activity. Therefore, addressing the lack of specificity in blocking experiments is crucial when implementing new biosensing platforms [28,29]

A step toward more specific detection of enzymatic entities requires to record nano-impact faradaic events for which the flux of electrons directly arises from the catalytic activity of the targeted enzymes. With this type of collision/catalysis scenario, where an enzyme in its free state randomly diffuses in the sample solution containing its specific substrate, diverse detection schemes have been proposed, leading to a debate on whether nano-impact techniques could achieve single enzyme sensitivity [30–32]. In early studies, single collisions of laccase [33] and HRP [34] enzyme molecules on a gold UME were detected by current spikes of varying durations, depending on how long the adsorbed enzyme remained active. The electrode was biased to an appropriate potential to reduce, respectively, the O₂ and H₂O₂ enzymatic substrate *via* a possible direct electron transfer (DET) mechanism. In essence, these results were discussed in light of the height of the current spikes ("*i_{cat}*"), typically of a few pA, which can only be compatible, at first glance, with a turnover several orders of magnitude greater than the accepted *k_{cat}* value for both of these (slow) enzymes [31]. Later Compton et al. conducted detailed investigations, combining theoretical models and experimental data, to determine if the activity of a single catalase enzyme, a fast oxidoreductase exhibiting a high turnover rate (*k_{cat}* ~10⁵ s⁻¹) to decompose H₂O₂ into O₂, could be individually discerned at a collecting UME. [35,36] In the proposed "collector" model, the electrode potential was negative enough to

reduce O_2 , *i.e.* the catalytic product of the enzyme. Any catalase molecule approaching in the vicinity of the electrode (without physical collision) was then transiently detected via the electrolysis current of the O_2 molecules produced at short diffusion distance. As the magnitude of the experimentally observed faradaic events largely exceeded the value predicted by the computational analysis, the authors didn't attribute the observed current spikes to the local presence of individual catalase, and evoked instead a DET mechanism as observed by others for catalase with a different UME material [37]. Ultimately, they revised this conclusion by completing their simulation framework to consider the inherent fluctuating activity of catalase, accounting for periods of varying activity during enzyme's diffusive walk towards the electrode. This model estimated that higher-than-expected current spikes may exist, and made plausible the experimental observations of single catalase detection [38].

Apart from the attempts to reach single-enzyme resolution, there have been several other papers on characterizing a variety of enzymatic "nano-objects" by the nano-impact technique in the collision/catalysis amplification mode. In these cases, the entity to be detected is a particle, some ~100 nm in size, used as a support or scaffold to host a few hundred copies of a "reporting" enzyme molecule. A priori, a sufficiently large number of enzymes decorating the surface of the particle should make each electrocatalytic collision distinguishable as a current transient in chronoamperograms. A seminal example was the detection of graphene nanosheets, functionalized with ~100 MP11 peroxidase-type enzyme molecules, *via* DET as the collision transduction mechanism [39]. A similar detection scheme was used to measure the electroactive collisions of silica nanoparticles bearing ~400 catalase molecules each [40]. The sensing of individual viral particles of the cytomegalovirus (a large spherical virus of ~100 nm in diameter), immunodecorated by the enzyme GOx and colliding with a UME has also been reported, this time under mediated electron transfer (MET) [41]. This last experiment was conducted in the presence of ferrocene methanol, as a soluble redox mediator, and glucose in

solution. The electrode was biased at an anodic potential so that the ferrocenium cation was generated to activate the GOx catalytic functioning (as depicted Figure 2A). The landing of individual bioelectrocatalytic “virions” on the electrode resulted in bringing to the electrode surface a few thousands of virus-scaffolded GOx molecules, whose catalytic activity was then detected as current steps of a few pA in intensity. In a recently published study, a comparable approach was used to detect single collision of metal-oxide-framework (MOF) particles (~100 nm in diameter) containing a small cluster of GOx enzymes [42]. However, instead of using a ferrocene species, the study used O₂ as a redox mediator. In the latter case, the intensity of current spikes, in the pA-range, seems excessively high when compared to the estimated number of entrapped GOx enzymes per particle, which the authors approximated to ~ 500 molecules. It is thus crucial to note that there is a limitation to the collision technique used to study the enzymatic activity of single bio-hybrid nanoparticles. The challenge is to distinguish between random collision of particularly *active* individual particles from collisions of particularly *large* (aggregated) particles, both resulting in similar large current transients. To alleviate it, Dick. et al. have suggested to meticulously examine simultaneously several electrochemical observables such as the intensity, collision frequency and shape of current transients to validate experimental findings [31].

Obtaining definitive structural details of colliding objects, notably their size, can be better achieved by trying to acquire an independent, physical, information temporally coupled to the nano-impact signals. For instance, the direct observation of collision events by using an optical microscope focused on the collector UME surface, as recently implemented by Renault et al. [43] for imaging graphene particle collisions, or by Lemineur et al. for collision/dissolution schemes [44,45], enables to correlate each current transient to the apparent size of the simultaneously observed individual colliding entity. Nevertheless, this combined approach is limited to particles large enough, and optically contrasted enough, to be resolved by the

microscope, and, obviously it affects the relative simplicity of the nano-impact station by the association of a cumbersome optical platform. To the best of our knowledge, no opto-electrochemical platform has yet been devoted to study electrochemical collision of bio-entities. A second, fast emerging bimodal nano-impact platform relies on the use of “conductive” nanopipettes [46,47]. Nanopipettes and nanopores are used in resistive pulse experiments, where single bio-entities are detected one at a time when they enter through the nano-aperture at the tip of a pulled glass capillary. Each transported entity partially disrupts the ionic current flowing through the aperture (the *non-faradaic* observable of the experiment), resulting in a temporary current “blockade”. Analysis of the magnitude and duration of the blockade events provide details on the size, surface charge, local molecular interactions or conformation fluctuations of biomacromolecules [48,49], including single enzyme dynamics [50,51]. “Conductive” nanopipettes are a kind of pore-based UME, made by deposition of a conductive layer (metallic or carbon-based) onto the cylindrical inner walls of a glass nanopipette. Their increasingly use outlines potential developments in nano-bio-electrochemistry, with the outstanding capability to record time-correlative information, first, a blockade signal certifying the trapping of a single enzymatic entity of interest into the pipette, and second, a faradaic transient at the inner UME transducing the enzymatic activity of this *very same* entity. Some demonstrations toward nano-platforms of this sort have been recently reported. One study has shown the detection of single collisions of HRP enzymes trapped into a conductive nanopipette, *via* DET transduction of the catalytic activity toward H_2O_2 . Intriguingly, bi-directional (anodic and cathodic) current spikes were recorded [52]. An advanced approach has used a “trap-and-detect” operating protocol, enabling in a first stage to “suck-in” a single (or very few of) GOx enzyme molecule(s) into the nanopipette tip, and then to confine the enzymes there, in the presence of glucose, for the enzymatic product H_2O_2 to accumulate (Figure 2B). After a reaction time of a few minutes, the pre-concentrated amount of H_2O_2 was evaluated by full electrolysis

at the internal UME, in order to quantify the rate of the enzymatic reaction which was found to be “accelerated”, as often observed for enzymes confined in small volume [53,54].

Another approach to nanoscale bioelectrochemistry consists in studying the activity of enzymatic entities *confined to a surface*, using electrochemical scanning microscopy (SECM), or rather its derived techniques featuring enhanced spatial resolution. In the subsequent section, we explore some recent findings obtained using nano-SECM methods, which have enabled the detection and imaging of the catalytic activity of enzymatic nano-biostructures. The limitations of most common nano-SECM techniques, either fundamental or practical in nature are also discussed.

2. Electrochemical imaging of single spatially-separated enzymatic particles with nano-SECM techniques.

Scanning probe imaging techniques, specifically AFM and in-situ STM, have been used for a long time to examine enzyme-electrode interfaces in order to determine the spatial distribution and structural uniformity of enzymes [24]. Meanwhile, SECM can be used to map the surface bio(electro)chemical *activity*. The initial requirement is the pre-immobilization of the biocatalytic “material” on a surface, forming incomplete layers with spatially isolated domains or confined spots. During an SECM analysis, an ultramicroelectrode tip is used to scan the surface and produce an electrochemical “tip current” image that can reveal the contours of each electroactive domain by a featured faradaic spot. This type of spatially resolved information has accompanied foundational research on bioelectrodes and bioanalytical developments. Recent reviews have gathered the pioneering works, and their numerous subsequent studies, that have employed SECM to image large *micrometer*-sized enzyme spots [55,56], utilizing either the positive feedback mode (FB), requiring the presence of a soluble redox mediator, or the generation/collection mode (GC) where the product of the local enzymatic reaction is

electrochemically collected at the tip. The main focus of this discussion is on enzymatic "spots" that are limited to the nanometer scale, with the aim of approaching the single enzyme regime. While the ability of SECM to reach this "ultimate" sensitivity has not yet been proven, one can however expect that modern nano-SECM techniques can be used to investigate the electroactivity of somewhat larger biocatalytic entities, like enzymes clusters (or supercomplexes) a few ~10 nm wide. Clustering is defined here as the nano-structured arrangement of a small number of enzymes on a larger particle or scaffold structure, forming a bioconjugate or bio-hybrid entity that in itself becomes a novel catalytic *nano-system* with properties that may be different from those of the constitutive enzyme alone. New nano-analytical methods capable of single bioconjugated particles measurement are of primary interest in fundamental and applied biocatalysis (and not only bioelectrocatalysis), in order to gain a holistic understanding of heterogeneous enzymatic reactions, and to define design rules of efficient biocatalytic nanosystems for sensors and biomedical approaches. A particularly important topic is the elucidation of catalytic "enhancement" mechanisms, observed for a wide range of bio-hybrid systems where enzymes are spatially organized onto metallic nanoparticles or DNA scaffolds [57–59]. Enzymatic enhancement seems to be a ubiquitous phenomenon for both natural (in cell metabolism) and artificial enzymatic complexes, but its physical origin and mechanism are not yet fully understood. SECM with nanoscale resolution is a well-appropriate approach to explore the electrochemical "side" of this field of research, with electrode-attached enzymatic systems.

To date, "conventional" (uncoupled) SECM has been only scarcely employed to image enzymatic activity at the sub-micron scale. A seminal example has reported the imaging of a biological cell, where the surface distribution of a membrane protein receptor has been mapped [60]. For this, the (non-electroactive) proteins were first labelled with Alkaline Phosphatase (AP)-tagged antibody, and a carbon nanoelectrode was scanned over the cell surface. The tip

current, collected from the oxidation of the enzymatic product locally released by the AP enzymes, enabled to reveal the locations of the underlying proteins onto the cell membrane. Interestingly, by switching the tip potential to reduce an added redox mediator, the correlated topographic image could also be obtained (not simultaneously) *via* a constant current hopping operation mode. Only one other example exists showing the use of non-coupled SECM to image enzymatic *nano*-spots containing lactate oxidase molecules, deposited on a gold surface [61]. Improved traditional SECM stations can achieve high-resolution imaging thanks to significant technical advancements. The primary modification involves replacing the ultramicroelectrode tip, which has a size of around 10 μm , with a high aspect-ratio nanoelectrode that has an electroactive area of a few 10 nm, in order to meet the commensurate “nano” resolution. However, using a sharper tip requires coupling the SECM setup with a second physical technique to precisely control the tip-sample “working” distance, which needs to be set at approximately 10 nm. This coupling provides an important advantage to the SECM technique, as it allows obtaining an independently acquired *topographic* image using the feedback control system of the tip-positioning technique, enabling to acquire the *tip current* image in the well-adapted constant distance mode. This eliminates the problem encountered in the most common constant height SECM mode, where the tip current image can be inaccurate in reflecting the local reactivity of the surface, as it is heavily influenced by *non-specific* variations of the tip current caused by the topography (roughness) artifacts.

Popularized by the group of P. Unwin, the most developed coupled SECM techniques made use of a micro-(nano)-pipette-based electrode tip to simultaneously image and functionally interrogate individual entities at the single particle level, or even sub-particles scale [62]. In a first technique, termed SECCM (for Scanning Electrochemical Cell Microscopy), a pulled capillary filled with an electrolyte containing a redox mediator is brought, via its protruding meniscus of liquid, in contact with a conductive surface bearing catalytic particles and biased

as working electrode. A reference and counter electrode are immersed in the back of the pipette. Using this concept, among many other examples, the electrocatalytic activity of gold nanopatterns deposited onto a carbon surface was mapped, with an outstanding (~2 nm) resolution [63]. However, in SECCM, the whole surface is kept dried, the meniscus of solution hanging from the pipette only transiently “wetting” the surface very locally. This obviously makes this technique probably not amenable to image fragile enzymatic biostructures, which do not withstand being dried.

Another nanopipette-based technique, that has been used to probe the electrochemical activity of single particles, is SICM, for Scanning Ion Conductance Microscopy [64]. In this fully *in situ* technique, a flux of ions is forced through the distal nano-aperture of a pipette by applying a voltage between the bulk and the internal solution. Being modulated by the distance between the nano-pipette tip and the surface, this *ionic current* is very sensitive to any change in local topography. Its magnitude can thus be monitored, and used as feedback signal to keep the pipette-surface distance constant, and draw the topographic image. Yet, since the ionic flux is also sensitive to local conductivity changes due to surface reaction generating (or consuming) ions, local reactivity can also be obtained. SICM can thus be used for topographic and electrochemical imaging, but only for conductive surfaces, and with the disadvantage of being indirect, that is to simply measure an ion flux without being able to *trigger* a faradaic process. Nevertheless, the tip positioning capability of SICM can be most useful when coupled to SECM, in a combined SICM-SECM configuration to enable constant distance SECM imaging. This combination, pioneered by the group of T. Matsue, used an electrolyte-filled pulled capillary whose ring-shaped extremity is coated with gold to act as a microelectrode tip. Using a soluble redox mediator, the enzymatic activity of HRP and GOx spots deposited on a glass surface could be imaged, with active domains resolved at the sub-micron scale [65].

A suitable correlative imaging technique for obtaining both topography and tip current images is AFM-SECM, which involves coupling the SECM with an Atomic Force Microscope [66–68]. Of particular interest is the *Mt (mediator tethered)* /AFM-SECM mode, developed by our group, where the SECM redox mediator is tethered to the bio-object to be imaged. This configuration lifts the diffusional constraints of classical SECM and uniquely enables the enzymatic activity of surface-attached enzymes to be probed at the nanometer scale [69,70]. In our studies, an immuno-assembly strategy was designed in order to decorate particles of the *fd* bacteriophage (880 nm long, 6 nm height) with an *integrated* electrocatalytic system, *i.e.* composed of a redox enzyme *and* its “wiring” co-substrate. As depicted in Figure 3A, this involved assembling a layer of IgGs conjugated to the enzyme quinoprotein-dependent glucose dehydrogenase (PQQ-GDH, ~230 units per virus) on the viral surface, followed by a layer of IgGs carrying ferrocenylated (Fc) polyethylene glycol (PEG) chains (~ 4000 Fc units per virus) [71]. The resulting biocatalytic particles demonstrated exceptional electrocatalytic performance toward glucose oxidation, due to efficient *nano-confined* mediated electron transfer between the GDH internal electron co-factor (PQQ) and the particle-tethered Fc mediators, acting as redox co-substrate. Using *Mt*/AFM-SECM nanoscale imaging, with home-designed gold probes adapted to imaging “fragile” objects, we were able to acquire simultaneously the topography and catalytic activity of *individual* viral particles [72]. The statistical distribution of these properties within the viral population, and their correlation, could thus be studied. The distribution of catalytic activity along the particles could also be mapped, revealing a catalytic “hot spot” located in their middle. We showed that this phenomenon was due to the propagation of electrons along the viral scaffold associated with their local enzymatic consumption. This charge transport was modeled as a 1D diffusive process, limited to the length of each particle. This allowed us to theoretically link the intensity of the hot spot to the enzymatic activity, and thus quantify the enhanced kinetic of the enzyme at the scale of individual virions, a world’s premiere [72]. In a further work, by using particles of the Tobacco Mosaic Virus (TMV, 300

nm long) modified to display a short terminal domain (~40 nm long), immunologically distinct from the rest of the protein shell, it was possible to selectively decorate the end of the viral particles with the integrated PQQ-GDH/PEG-Fc system (Figure 3B). *Mt*/AFM-SECM imaging then allowed us to visualize and quantify the functional activity of the ten or so enzyme molecules occupying the terminal domain of the virus, which sets a record in terms of the lowest number of enzyme molecules interrogated by an electrochemical imaging technique [73].

Conclusion

By leveraging the advanced investigative capabilities of nanoelectrochemistry tools, researchers have been able to explore bioelectrocatalysis processes across a wide range of scales, from individual enzymes (1-10 nm) to larger enzyme nanostructures (10-100 nm). Nano-impact techniques have ventured into the daring task of detecting the electrocatalytic current of a single enzyme. With most enzymes whose k_{cat} commonly ranges from 1 to 10^4 s^{-1} , A. J. Bard predicted as early as 2008 that the electrochemical detection of a single enzyme would require a current sensitivity below the femtoamp [30]. Considering that the sub-femtoamp range is anyway difficult to achieve with purely amperometric measurements [74], it seems doubtful that the nano-impact method has actually met single enzyme sensitivity. So, the question remains: can an enzyme, at the whim of its conformational fluctuations, transiently function at a turnover rate "extraordinarily" higher than its average rate defined by its k_{cat} ? To address this problem, new approaches are emerging that use nanoconfinement of single enzymes in the very small volume of a conductive nanopipette. This, like other "amplification" schemes, allows the entrapped enzyme enough time to produce a redox analyte in a significant concentration that can be detected electrochemically. This strategy is certainly suitable for measuring individual enzyme turnover, although it loses the high-throughput advantage of the impact technique. At present, nano-collision methodologies start to be turned towards the development of future "digital" bio-analytical platforms, where enzymes anchored on nanoparticles may have a role

to play in future detection schemes [29]. For a viable analytical development, rigor is required to properly analyze the collisional current/time traces and to decipher whether the faradaic signatures come from a single particle or an agglomerate.

The limits in current sensitivity mentioned above for nano-impacts are also applicable for nano-SECM. This second technique has rather evolved by a top-down approach, following the continuous decrease in size of scanning tip from micro- to nanoscale to detect, and image, an increasingly small number of enzymes immobilized on surface patterns. SICM-SECM technique could be further explored, coupled with enzyme labelling of membrane, for living cells imaging[64]. The AFM-SECM combination has been developed into an advanced imaging mode capable of functionally addressing soft bio-functional objects in the 10-100 nm range, with a particular focus on integrated enzyme systems built on viral scaffolds. A small cluster of less than ~10 glucose dehydrogenase enzymes (turning at an enhanced $k_{cat} = 8500 \text{ s}^{-1}$) has been imaged, with the *tour de force* of collecting bioelectrocatalytic current in the sub -10 fA range. New innovative biomaterials as DNA origamis could be now used as scaffold to construct more complex, and spatially ordered, enzymatic nanosystems to further explore the catalytic enhancement of DNA-attached enzymes [75,76]. This is a new route toward the electrochemical investigation of artificial *multi-enzymes* structures, starting from the co-immobilization of two class of enzymes co-operating in a catalytic *cascade* on the same scaffold. AFM-SECM could allow to decipher the cascade mechanisms and to validate the mere existence of "substrate channeling" that seems to operate in cascades [77], being one of the rare techniques that directly offer a level of observation at the scale of single nanosystems.

Conflict of interest statement

Nothing declared

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References

Papers of particular interest have been highlighted as:

* of special interest

** of outstanding interest

- [1] Y. Wang, X. Shan, N. Tao, Emerging tools for studying single entity electrochemistry, *Faraday Discuss.* 193 (2016) 9–39. <https://doi.org/10.1039/c6fd00180g>
- [2] L.A. Baker, Perspective and Prospectus on Single-Entity Electrochemistry, *J. Am. Chem. Soc.* 140 (2018) 15549–15559. <https://doi.org/10.1021/JACS.8B09747>
- [3] P. Unwin, Concluding remarks: next generation nanoelectrochemistry-next generation nanoelectrochemists, *Faraday Discuss.* 233 (2022) 374–391. <https://doi.org/10.1039/d2fd00020b>
- * **This article interweaves the history of science with the progress of electrochemistry, leading up to the present-day nanoelectrochemistry, while also looking towards its future advancements.**
- [4] F.-R.F. Fan, A.J. Bard, Electrochemical Detection of Single Molecules, *Science* 267 (1995) 871–874. <https://doi.org/10.1126/science.267.5199.871>
- [5] P. Sun, M. V Mirkin, Electrochemistry of Individual Molecules in Zeptoliter Volumes, *J. Am. Chem. Soc.* 130 (2008) 8241–8250. <https://doi.org/10.1021/ja711088j>
- [6] S. Kang, A.F. Nieuwenhuis, K. Mathwig, D. Mampallil, S.G. Lemay, Electrochemical single-molecule detection in aqueous solution using self-aligned nanogap transducers, *ACS Nano* 7 (2013) 10931–10937. <https://doi.org/10.1021/nn404440v>
- [7] J.C. Byers, B. Paulose Nadappuram, D. Perry, K. McKelvey, A.W. Colburn, P.R. Unwin, Single Molecule Electrochemical Detection in Aqueous Solutions and Ionic Liquids, *Anal. Chem.* 87 (2015) 10450–10456. <https://doi.org/10.1021/acs.analchem.5b02569>
- [8] L. Nault, C. Taofifenua, A. Anne, A. Chovin, C. Demaille, N. Besong-Ndika, Jane Cardinale, Daniela Carette, T. Michon, J. Walter, J. Besong-Ndika, D. Cardinale, N. Carette, T. Michon, J. Walter, Electrochemical Atomic Force Microscopy Imaging of Redox-Immuno-marked Proteins on Native Potyviruses: From Subparticle to Single-Protein Resolution, *ACS Nano* 9 (2015) 4911–4924. <https://doi.org/10.1021/acsnano.5b00952>
- [9] K. Chennit, Y. Coffinier, S. Li, N. Clément, A. Anne, A. Chovin, C. Demaille, High-density single antibody electrochemical nanoarrays, *Nano Res.* (2022). <https://doi.org/10.1007/s12274-022-5137-1>
- [10] K. Mathwig, T.J. Aartsma, G.W. Canters, S.G. Lemay, Nanoscale methods for single-molecule electrochemistry, *Ann. Rev. Anal. Chem.* 7 (2014) 383–404. <https://doi.org/10.1146/annurev-anchem-062012-092557>
- [11] L. Jeuken, M. Orrit, G. Canters, Single-molecule fluorescence in redox chemistry, *Curr. Opin. Electrochem.* 37 (2023) 101196. <https://doi.org/10.1016/J.COEELEC.2022.101196>

- [12] Y. Fan, R. Hao, C. Han, B. Zhang, Counting Single Redox Molecules in a Nanoscale Electrochemical Cell, *Anal. Chem.* 90 (2018) 13837–13841.
<https://doi.org/10.1021/acs.analchem.8b04659>
- [13] S. Goines, J. E. Dick, Review—Electrochemistry’s Potential to Reach the Ultimate Sensitivity in Measurement Science, *J Electrochem Soc.* 167 (2020) 037505.
<https://doi.org/10.1149/2.0052003jes>
- * **A comprehensive and well-discussed view of the nano-impact technique for electrochemical detection of single entities.**
- [14] S. V. Sokolov, S. Eloul, E. Kätelhön, C. Batchelor-McAuley, R.G. Compton, Electrode–particle impacts: a users guide, *Phys. Chem. Chem. Phys.* 19 (2017) 28–43.
<https://doi.org/10.1039/c6cp07788a>
- [15] P.A. Defnet, T.J. Anderson, B. Zhang, Stochastic collision electrochemistry of single silver nanoparticles, *Curr. Opin. Electrochem.* 22 (2020) 129–135.
<https://doi.org/10.1016/j.coelec.2020.06.004>
- [16] D.A. Robinson, M.A. Edwards, H. Ren, H.S. White, Effects of Instrumental Filters on Electrochemical Measurement of Single-Nanoparticle Collision Dynamics, *ChemElectroChem.* 5 (2018) 3059–3067.
<https://doi.org/https://doi.org/10.1002/celec.201800696>
- [17] J. Ustarroz, M. Kang, E. Bullions, P.R. Unwin, Impact and oxidation of single silver nanoparticles at electrode surfaces: one shot versus multiple events, *Chem. Sci.* 8 (2017) 1841–1853. <https://doi.org/10.1039/C6SC04483B>
- [18] A.D. Castañeda, D.A. Robinson, K.J. Stevenson, R.M. Crooks, Electrocatalytic amplification of DNA-modified nanoparticle collisions via enzymatic digestion, *Chem. Sci.* 7 (2016) 6450–6457. <https://doi.org/10.1039/c6sc02165d>
- [19] K.A. Kirk, A. Vasilescu, D. Andreescu, D. Senarathna, S. Mondal, S. Andreescu, Collision-Based Electrochemical Detection of Lysozyme Aggregation, *Anal. Chem.* 93 (2021) 2026–2037. <https://doi.org/10.1021/acs.analchem.0c03578>
- [20] J. Li, X. Liang, R. Zhong, M. Liu, X. Liu, H.-L. Yan, Y.-G. Zhou, Clinically Applicable Homogeneous Assay for Serological Diagnosis of Alpha-Fetoprotein by Impact Electrochemistry, *ACS Sens.* 7 (2022) 3216–3222.
<https://doi.org/10.1021/acssensors.2c01887>
- [21] H. Chen, O. Simoska, K. Lim, M. Grattieri, M. Yuan, F. Dong, Y.S. Lee, K. Beaver, S. Weliwatte, E.M. Gaffney, S.D. Minter, Fundamentals, Applications, and Future Directions of Bioelectrocatalysis, *Chem. Rev.* 120 (2020) 12903–12993.
<https://doi.org/10.1021/acs.chemrev.0c00472>
- ** **The most comprehensive review of recent years on enzymatic and microbial bioelectrocatalysis, and its applications to biosensors, biofuel cells and bioreactors**
- [22] I. Mazurenko, V.P. Hitaishi, E. Lojou, Recent advances in surface chemistry of electrodes to promote direct enzymatic bioelectrocatalysis, *Curr. Opin. Electrochem.* 19 (2020) 113–121. <https://doi.org/https://doi.org/10.1016/j.coelec.2019.11.004>

- [23] R.D. Milton, T. Wang, K.L. Knoche, S.D. Minter, Tailoring Biointerfaces for Electrocatalysis, *Langmuir* 32 (2016) 2291–2301.
<https://doi.org/10.1021/acs.langmuir.5b04742>
- [24] D. Zigah, E. Lojou, A. de Poulpiquet, Micro- and Nanoscopic Imaging of Enzymatic Electrodes: A Review, *ChemElectroChem* 6 (2019) 5524–5546.
<https://doi.org/https://doi.org/10.1002/celec.201901065>
- * **A recent article that includes an very interesting overview of near-field techniques used to study immobilized enzyme surfaces**
- [25] G.C. Sedenho, A. Hassan, J.C.P. de Souza, F.N. Crespilho, In situ and operando electrochemistry of redox enzymes, *Curr. Opin. Electrochem.* 34 (2022) 101015.
<https://doi.org/10.1016/J.COEELEC.2022.101015>
- [26] Z. Deng, C. Renault, Detection of individual insulating entities by electrochemical blocking, *Curr. Opin. Electrochem.* 25 (2021) 100619.
<https://doi.org/10.1016/J.COEELEC.2020.08.001>
- [27] J.E. Dick, C. Renault, A.J. Bard, Observation of Single-Protein and DNA Macromolecule Collisions on Ultramicroelectrodes, *J. Am. Chem. Soc.* 137 (2015) 8376–8379. <https://doi.org/10.1021/JACS.5B04545>
- [28] J.H. Zhang, Y.G. Zhou, Nano-impact electrochemistry: Analysis of single bioentities, *TrAC-Trends Anal. Chem.* 123 (2020) 115768.
<https://doi.org/10.1016/J.TRAC.2019.115768>
- [29] T.H.T. Nguyen, J. Lee, H.Y. Kim, K.M. Nam, B.K. Kim, Current research on single-entity electrochemistry for soft nanoparticle detection: Introduction to detection methods and applications, *Biosens Bioelectron.* 151 (2020) 111999.
<https://doi.org/10.1016/J.BIOS.2019.111999>
- [30] A.J. Bard, Toward Single Enzyme Molecule Electrochemistry, *ACS Nano* 2 (2008) 2437–2440. <https://doi.org/10.1021/nn800801z>
- [31] K.J. Vannoy, A. Ryabykh, A.I. Chapoval, J.E. Dick, Single enzyme electroanalysis, *Analyst.* 146 (2021) 3413–3421. <https://doi.org/10.1039/D1AN00230A>
- [32] C. Davis, S.X. Wang, L. Sepunaru, What can electrochemistry tell us about individual enzymes? *Curr. Opin. Electrochem.* 25 (2021), 100643
<https://doi.org/10.1016/j.coelec.2020.100643>
- [33] A.N. Sekretaryova, M.Y. Vagin, A.P.F. Turner, M. Eriksson, Electrocatalytic Currents from Single Enzyme Molecules, *J. Am. Chem. Soc.* 138 (2016) 2504–2507.
<https://doi.org/10.1021/JACS.5B13149>
- [34] L. Han, W. Wang, J. Nsabimana, J.W. Yan, B. Ren, D. Zhan, Single molecular catalysis of a redox enzyme on nanoelectrodes, *Faraday Discuss.* 193 (2016) 133–139.
<https://doi.org/10.1039/C6FD00061D>
- [35] E. Kätelhön, L. Sepunaru, A.A. Karyakin, R.G. Compton, Can Nanoimpacts Detect Single-Enzyme Activity? Theoretical Considerations and an Experimental Study of

Catalase Impacts, ACS Catal. 6 (2016) 8313–8320.

<https://doi.org/10.1021/acscatal.6b02633>

- [36] C. Lin, E. Kätelhön, L. Sepunaru, R.G. Compton, Understanding single enzyme activity via the nano-impact technique, Chem. Sci. 8 (2017) 6423–6432. <https://doi.org/10.1039/C7SC02084H>
- [37] L. Jiang, I. Santiago, J. Foord, Observation of nanoimpact events of catalase on diamond ultramicroelectrodes by direct electron transfer, Chem. Comm. 53 (2017) 8332–8335. <https://doi.org/10.1039/C7CC04085G>
- [38] C. Lin, L. Sepunaru, E. Kätelhön, R.G. Compton, Electrochemistry of Single Enzymes: Fluctuations of Catalase Activities, J. Phys. Chem. Lett. 9 (2018) 2814–2817. <https://doi.org/10.1021/ACS.JPCLETT.8B01199>
- [39] D. Li, J. Liu, C.J. Barrow, W. Yang, Protein electrochemistry using graphene-based nano-assembly: an ultrasensitive electrochemical detection of protein molecules via nanoparticle-electrode collisions, Chem. Commun. 50 (2014) 8197–8200. <https://doi.org/10.1039/c4cc03384a>
- [40] C. Chan, L. Sepunaru, S. V Sokolov, E. Kätelhön, N.P. Young, R.G. Compton, Catalytic activity of catalase–silica nanoparticle hybrids: from ensemble to individual entity activity, Chem ; Sci. 8 (2017) 2303–2308. <https://doi.org/10.1039/C6SC04921D>
- [41] J.E. Dick, A.T. Hilterbrand, L.M. Strawsine, J.W. Upton, A.J. Bard, Enzymatically enhanced collisions on ultramicroelectrodes for specific and rapid detection of individual viruses, Proc. Natl. Acad. Sci. U.S.A. 113 (2016) 6403–6408. <https://doi.org/10.1073/PNAS.1605002113>
- ** A convincing example of electrochemical collision measurements of nanometer-sized bioparticles, here large virus particles carrying a significant number of redox enzymes (GOx) to make possible the detection of current steps of a few pA, via a soluble redox mediator-based transduction. New bioanalytical approach based on a “digital” detection was also proposed.**
- [42] H. Tang, H. Wang, J. Du, D. Zhao, M. Cao, Y. Li, Intrinsic Catalytic Activities from Single Enzyme@Metal–Organic Frameworks by Using a Stochastic Collision Electrochemical Technique, J. Phys. Chem. Lett. 12 (2021) 5443–5447. <https://doi.org/10.1021/acs.jpcllett.1c01389>
- [43] A.D. Pendergast, C. Renault, J.E. Dick, Correlated Optical-Electrochemical Measurements Reveal Bidirectional Current Steps for Graphene Nanoplatelet Collisions at Ultramicroelectrodes, Anal. Chem. 93 (2021) 2898–2906. <https://doi.org/10.1021/acs.analchem.0c04409>
- [44] J.F. Lemineur, J.M. Noël, C. Combella, F. Kanoufi, Revealing the sub-50 ms electrochemical conversion of silver halide nanocolloids by stochastic electrochemistry and optical microscopy, Nanoscale 12 (2020) 15128–15136. <https://doi.org/10.1039/d0nr03799k>
- [45] J.F. Lemineur, P. Ciocci, J.M. Noël, H. Ge, C. Combella, F. Kanoufi, Imaging and Quantifying the Formation of Single Nanobubbles at Single Platinum Nanoparticles

during the Hydrogen Evolution Reaction, ACS Nano 15 (2021) 2643–2653.
<https://doi.org/10.1021/acsnano.0c07674>

- [46] R. Jia, M. V. Mirkin, The double life of conductive nanopipette: A nanopore and an electrochemical nanosensor, Chem. Sci. 11 (2020) 9056–9066.
<https://doi.org/10.1039/d0sc02807j>
- [47] R.-J. Yu, K.-L. Chen, Y.-L. Ying, Y.-T. Long, Nanopore electrochemical measurement for single molecular interactions and beyond, Curr. Opin. Electrochem. 35 (2022) 101063. <https://doi.org/https://doi.org/10.1016/j.coelec.2022.101063>
- [48] H. Chen, Y. Lin, Y.-T. Long, S.D. Minter, Y.-L. Ying, Nanopore-based measurement of the interaction of P450cam monooxygenase and putidaredoxin at the single-molecule level, Faraday Discuss. 233 (2022) 295–302. <https://doi.org/10.1039/D1FD00042J>
- [49] H. Tang, H. Wang, D. Zhao, M. Cao, Y. Zhu, Y. Li, Nanopore-Based Single-Entity Electrochemistry for the Label-Free Monitoring of Single-Molecule Glycoprotein–Boronate Affinity Interaction and Its Sensing Application, Anal. Chem. 94 (2022) 5715–5722. <https://doi.org/10.1021/acs.analchem.2c00860>
- [50] R.-J. Yu, Q. Li, S.-C. Liu, H. Ma, Y.-L. Ying, Y.-T. Long, Simultaneous observation of the spatial and temporal dynamics of single enzymatic catalysis using a solid-state nanopore, Nanoscale 15 (2023) 7261–7266. <https://doi.org/10.1039/D2NR06361A>
- [51] H. Ma, R.J. Yu, Y.L. Ying, Y.T. Long, Electrochemically confined effects on single enzyme detection with nanopipettes, J. Electroanal. Chem. 908 (2022) 116086. <https://doi.org/10.1016/J.JELECHEM.2022.116086>
- [52] X. Shen, R. Liu, D. Wang, Nanoconfined Electrochemical Collision and Catalysis of Single Enzyme inside Carbon Nanopipettes, Anal. Chem. 94 (2022) 8110–8114. <https://doi.org/10.1021/acs.analchem.2c01554>
- [53] Y. Wang, R. Pan, D. Jiang, D. Jiang, H.-Y. Chen, Nanopipettes for the Electrochemical Study of Enhanced Enzymatic Activity in a Femtoliter Space, Anal. Chem. 93 (2021) 14521–14526. <https://doi.org/10.1021/acs.analchem.1c03341>
- [54] R. Pan, D. Wang, K. Liu, H.-Y. Chen, D. Jiang, Electrochemical Molecule Trap-Based Sensing of Low-Abundance Enzymes in One Living Cell, J. Am. Chem. Soc. 144 (2022) 17558–17566. <https://doi.org/10.1021/jacs.2c06962>
- ** Recent demonstration of the measurement of the catalytic activity of a small numbers of GOx enzymes entrapped in the confined volume of a conductive nanopipette. By using larger particles like liposomes (containing GOx), this innovating setup allows to couple a resistive-pulse readout, for counting how many individual liposomes enter the nanopipette volume, and subsequently the faradaic measurement of the activity of so-confined enzymes by the electrolysis current (or charge) of the enzymes-produced H₂O₂.**
- [55] F. Conzuelo, A. Schulte, W. Schuhmann, Biological imaging with scanning electrochemical microscopy, Proc. Math. Phys. Eng. Sci. 474 (2018) 20180409. <https://doi.org/10.1098/rspa.2018.0409>

- [56] I. Morkvenaite-Vilkonciene, A. Ramanaviciene, A. Kisieliute, V. Bucinskas, A. Ramanavicius, Scanning electrochemical microscopy in the development of enzymatic sensors and immunosensors, *Biosens. Bioelectron.* 141 (2019) 111411. <https://doi.org/https://doi.org/10.1016/j.bios.2019.111411>
- [57] G.A. Ellis, S.A. Díaz, I.L. Medintz, Enhancing enzymatic performance with nanoparticle immobilization: improved analytical and control capability for synthetic biochemistry, *Curr. Opin. Biotechnol.* 71 (2021) 77–90. <https://doi.org/https://doi.org/10.1016/j.copbio.2021.06.021>
- [58] J.N. Vranish, M.G. Ancona, S.A. Walper, I.L. Medintz, Pursuing the Promise of Enzymatic Enhancement with Nanoparticle Assemblies, *Langmuir* 34 (2018) 2901–2925. <https://doi.org/10.1021/acs.langmuir.7b02588>
- [59] Y. Gao, C.C. Roberts, A. Toop, C.A. Chang, I. Wheeldon, Mechanisms of Enhanced Catalysis in Enzyme–DNA Nanostructures Revealed through Molecular Simulations and Experimental Analysis, *ChemBioChem* 17 (2016) 1430–1436. <https://doi.org/https://doi.org/10.1002/cbic.201600224>
- [60] Y. Takahashi, A.I. Shevchuk, P. Novak, B. Babakinejad, J. Macpherson, P.R. Unwin, H. Shiku, J. Gorelik, D. Klenerman, Y.E. Korchev, T. Matsue, Topographical and electrochemical nanoscale imaging of living cells using voltage-switching mode scanning electrochemical microscopy, *Proc. Natl. Acad. Sci. U.S.A.* 109 (2012) 11540–11545. <https://doi.org/10.1073/pnas.1203570109>
- * **Pioneering experiment using a nano-resolved SECM approach to map the surface of living cells, via enzymatic labeling, with an associated topographic acquisition.**
- [61] J.M. Abad, A.Y. Tesio, E. Martínez-Periñán, F. Pariente, E. Lorenzo, Imaging resolution of biocatalytic activity using nanoscale scanning electrochemical microscopy, *Nano Res.* 11 (2018) 4232–4244. <https://doi.org/10.1007/s12274-018-2011-2>
- [62] C.L. Bentley, J. Edmondson, G.N. Meloni, D. Perry, V. Shkirskiy, P.R. Unwin, Nanoscale Electrochemical Mapping, *Anal. Chem.* 91 (2019) 84–108. <https://doi.org/10.1021/acs.analchem.8b05235>
- [63] C.L. Bentley, M. Kang, P.R. Unwin, Nanoscale Structure Dynamics within Electrocatalytic Materials, *J. Am. Chem. Soc.* 139 (2017) 16813–16821. <https://doi.org/10.1021/jacs.7b09355>
- [64] X. Xu, D. Valavanis, P. Ciocci, S. Confederat, F. Marcuccio, J.-F. Lemineur, P. Actis, F. Kanoufi, P.R. Unwin, The New Era of High-Throughput Nanoelectrochemistry, *Anal. Chem.* 95 (2023) 319–356. <https://doi.org/10.1021/acs.analchem.2c05105>
- [65] Y. Takahashi, A.I. Shevchuk, P. Novak, Y. Murakami, H. Shiku, Y.E. Korchev, T. Matsue, Simultaneous Noncontact Topography and Electrochemical Imaging by SECM/SICM Featuring Ion Current Feedback Regulation, *J. Am. Chem. Soc.* 132 (2010) 10118–10126. <https://doi.org/10.1021/ja1029478>
- [66] J. V Macpherson, P.R. Unwin, Combined Scanning Electrochemical–Atomic Force Microscopy, *Anal. Chem.* 72 (2000) 276–285. <https://doi.org/10.1021/ac990921w>

- [67] C. Kranz, G. Friedbacher, B. Mizaikoff, A. Lugstein, J. Smoliner, E. Bertagnolli, Integrating an Ultramicroelectrode in an AFM Cantilever: Combined Technology for Enhanced Information, *Anal. Chem.* 73 (2001) 2491–2500. <https://doi.org/10.1021/ac001099v>
- [68] J. Abbou, C. Demaille, M. Druet, J. Moiroux, Fabrication of Submicrometer-Sized Gold Electrodes of Controlled Geometry for Scanning Electrochemical-Atomic Force Microscopy, *Anal. Chem.* 74 (2002) 6355–6363. <https://doi.org/10.1021/ac020385z>
- [69] A. Anne, E. Cambril, A. Chovin, C. Demaille, C. Goyer, Electrochemical Atomic Force Microscopy Using a Tip-Attached Redox Mediator for Topographic and Functional Imaging of Nanosystems, *ACS Nano* 3 (2009) 2927–2940 <https://doi.org/10.1021/nn9009054>
- [70] A. Anne, C. Demaille, C. Goyer, Electrochemical Atomic-Force Microscopy Using a Tip-Attached Redox Mediator. Proof-of-Concept and Perspectives for Functional Probing of Nanosystems, *ACS Nano* 3 (2009) 819–827. <https://doi.org/10.1021/nn8007788>
- [71] K. Torbensen, A.N. Patel, A. Chovin, A. Anne, C. Demaille, L. Bataille, T. Michon, E. Grelet, Immuno-Based Molecular Scaffolding of Glucose Dehydrogenase and Ferrocene Mediator on fd Viral Particles Yields Enhanced Bioelectrocatalysis, *ACS Catal.* 9 (2019) 5783–5796. <https://doi.org/10.1021/acscatal.9b01263>
- [72] T.O. Paiva, K. Torbensen, A.N. Patel, A. Anne, A. Chovin, C. Demaille, L. Bataille, T. Michon, Probing the Enzymatic Activity of Individual Biocatalytic fd-Viral Particles by Electrochemical-Atomic Force Microscopy, *ACS Catal.* 10 (2020) 7843–7856. <https://doi.org/10.1021/acscatal.0c01920>.
- ** Very first example where a nano-SECM technique succeeds in measuring the enzymatic catalysis of a hundred redox enzymes carried by a bioparticle nano-support (a virus capsid). The observed catalytic enhancement is described in its physical mechanism, directly at the nanoscale.**
- [73] T.O. Paiva, A. Schneider, L. Bataille, A. Chovin, A. Anne, T. Michon, C. Wege, C. Demaille, Enzymatic activity of individual bioelectrocatalytic viral nanoparticles: dependence of catalysis on the viral scaffold and its length, *Nanoscale* (2022) <https://doi.org/10.1039/D1NR07445H>
- * Record detection of the electrocatalytic current of less than 10 enzymes immobilized on a nano-bioscaffold, obtained by AFM-SECM in the “mediator-tethered” mode**
- [74] R. Gao, M.A. Edwards, J.M. Harris, H.S. White, Shot noise sets the limit of quantification in electrochemical measurements, *Curr. Opin. Electrochem.* 22 (2020) 170–177. <https://doi.org/10.1016/j.coelec.2020.05.010>
- [75] S. Ranallo, A. Porchetta, F. Ricci, DNA-Based Scaffolds for Sensing Applications, *Anal. Chem.* 91 (2019) 44–59. <https://doi.org/10.1021/acs.analchem.8b05009>
- [76] N.C. Seeman, H.F. Sleiman, DNA nanotechnology, *Nat Rev Mater.* 3 (2017) 17068. <https://doi.org/10.1038/natrevmats.2017.68>

- [77] I. Wheeldon, S.D. Minter, S. Banta, S.C. Barton, P. Atanassov, M. Sigman, Substrate channelling as an approach to cascade reactions, *Nat. Chem.* 8 (2016) 299–309.
<https://doi.org/10.1038/nchem.2459>

Figure 1.

Illustration of two nanoelectrochemical methods for detecting the electrocatalytic current of single enzymatic nano-entities: by random nano-impact of free-diffusing particles on an ultramicroelectrode surface (on the left), or by using a scanning nanoelectrode (nano-SECM) interrogating surface-immobilized single entities (on the right).

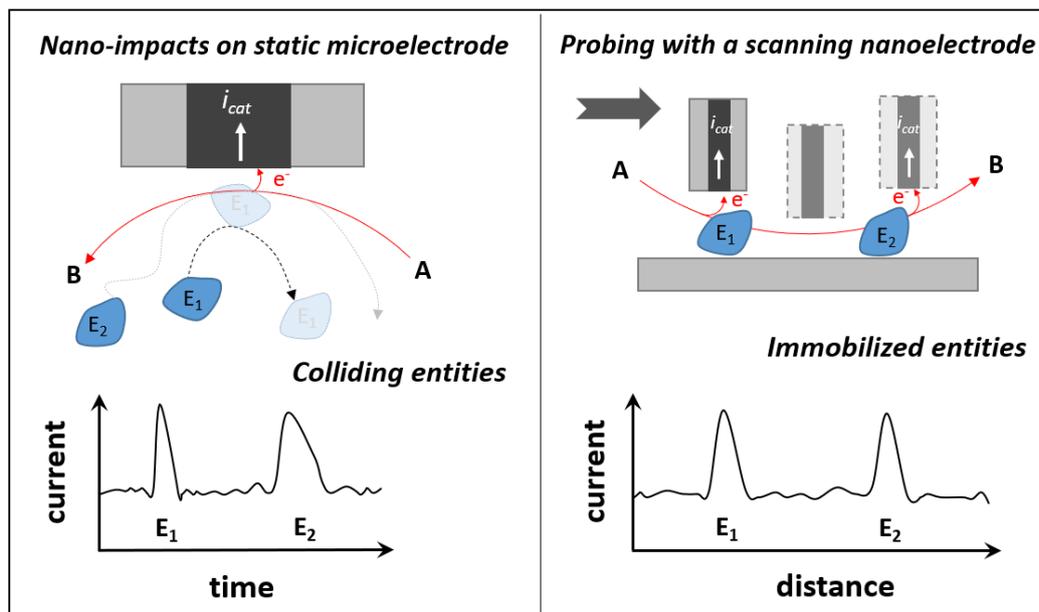


Figure 2:

a) Nano-impact detection, *via* mediated electron transfer and enzymatic labelling, of particles of the cytomegalovirus CMV immunodecorated by Glucose Oxidase (GOx). Top: the collecting ultramicroelectrode (1 μm in size) was biased to generate ferrocenium ions (FcMeOH^+), acting as redox co-mediator for the GOx-catalyzed glucose oxidation. Bottom: each individual collision of GOx-decorated virus particle was detected by a current step of a few pA in intensity, thanks to the enzymatic amplification scheme. Reproduced from [41], Copyright 2016 National Academy of Sciences. b) Electrocatalysis detection of a small number of enzyme nano-confined in a small volume. Top: schematic of innovating setup to capture a few GOx enzymes (less than 10 molecules) in the inner tip volume of a conductive nanopipette, using a three-step method (sort/trap/detect) with controlled electroosmotic flow (EOF). In presence of glucose, H_2O_2 is produced by the trapped enzymes and oxidized at the internal platinum microelectrode. Bottom: (a) The enzymatic activity was tracked by the increase (vs. a background) of the coulombic charge in pC over time. (b) Significant increase can be observed with an applied appropriate EOF to trap the enzyme vs “without trapping”. (c) Charge increases values measured for 13 independent acquisitions, and (d) correlation between the charge increases and the number of GOx estimated to be present inside the trapped volume. Adapted from [54], Copyright 2022 American Chemical Society.

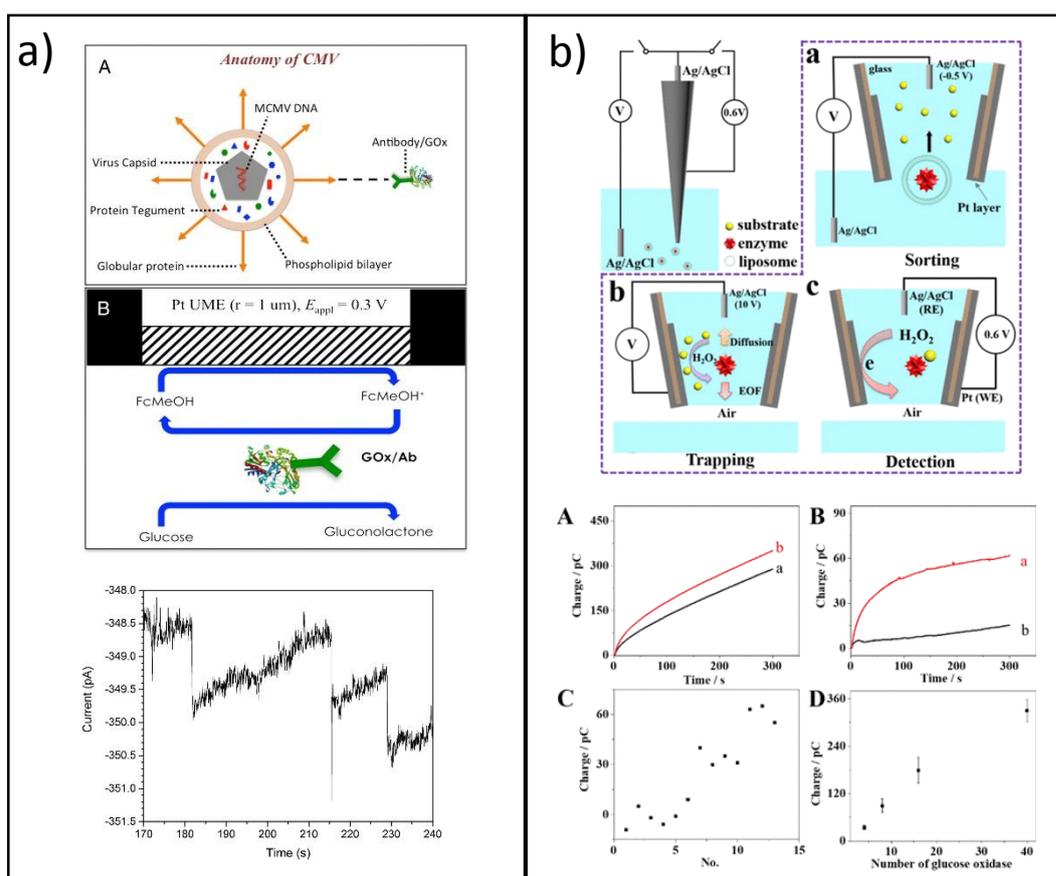


Figure 3:

a) Components list for the immunoassembly of an *integrated* enzymatic nanosystem on the fd bacteriophage virus particle, containing the PQQ-dependent Glucose Dehydrogenase (PQQ-GDH) enzyme and its “attached” Fc redox co-substrate. Below, the *mediator-tethered* AFM-SECM-based principle of the nanoelectrochemical addressing of such a single bioparticle adsorbed on an isolated surface. The current recorded at the tip comes from the glucose substrate catalytically oxidized across the Fc-mediated electroenzymatic reaction. On the right, representative AFM-SECM images in absence/presence of glucose in solution, showing the independently acquired topography and electrocatalytic current images of individual “virions”. Reproduced with permission from [72], Copyright 2020 American Chemical Society. B) Mt/AFM-SECM imaging of the GDH/PEG-Fc integrated system reduced to less than 10 enzyme molecules, anchored at the ~40 nm long dissimilar domain of a TMV-like viral particles. Adapted with permission from [73], Copyright 2022 Royal Society of Chemistry.

