

Toward Single Enzyme Molecule Electrochemistry

Allen J. Bard*

Department of Chemistry and Biochemistry and the Center for Electrochemistry, The University of Texas at Austin, Austin, Texas 78712

The article by Lemay and co-workers in this issue focuses on the lithographic fabrication of ultramicroelectrodes (UMEs) with dimensions on the 50 to 100 nm scale and their application to studies of electron transfer reactions of an adsorbed enzyme molecule (a hydrogenase) electrochemically by protein film voltammetry (PFV).¹ The eventual goal of this work is to study single enzyme molecules electrochemically, thus complementing such studies carried out, for example, using fluorescence techniques, as discussed below. The work thus encompasses several fields: nanometer-size electrodes, single-molecule electrochemistry and single enzyme molecule studies.

Nanoelectrodes. Ultramicroelectrodes have had an enormous impact in electrochemistry, for example, in allowing experiments in the microsecond, or, under favorable circumstances, in the nanosecond regime by minimizing troublesome double-layer charging and resistance effects, thus making measurements of fast electron transfer reactions possible.² Ultramicroelectrodes have also increased the spatial resolution of electrochemical studies, as with the scanning electrochemical microscope (SECM).³ These UME virtues are key considerations in the Lemay work.¹ While the earlier UMEs mainly had micrometer dimensions, there have been many studies of nanometer-size electrodes. Recent papers indicate the state of the art of nanoelectrochemistry, where disk-shaped tips with radii as small as 10 nm were reported.^{4,5} Key issues in the use of nanometer-size electrodes are knowledge of their size and geometry, for example, whether the metal electrode protrudes from or is recessed into the insulating portion that surrounds the electrode. For electrodes with radii on the order of 70 nm or more, this can be accomplished with the scanning electron microscope (SEM), as reported in the Lemay work. For electrodes

below 50 nm, SEM often cannot be used because of resolution limitations,⁵ and steady-state voltammetry of the tip can be of use. Because such measurements do not provide information about geometry, approach curves with the SECM, showing how the tip behaves as it approaches an insulating or conducting surface, are needed to prove that the tip is not recessed in a channel. To obtain a useful approach curve, the tip must be moved as close as one to two tip radii from the substrate, which, for a 13 nm tip, is challenging and provides only a short useful approach curve distance range.⁵ Small tips also imply measurements of very small currents, in the picoamp or smaller range.

Single-Molecule Electrochemistry and Spectroelectrochemistry. There have also been studies of single-molecule electrochemistry, although few in number compared to spectroscopic ones and none dealing with enzymes. Because one cannot measure amounts of charge equivalent to only a few electrons in electrochemical cells or the tiny currents that characterize typical electrochemical reactions of only a few molecules, some means of greatly amplifying the current response is needed. In an early approach,^{6–8} this amplification was provided by a close approach (~ 10 nm) of a shrouded tip to a conductor, where a single molecule in solution could be trapped in the gap and shuttled back and forth between the tip, where it was oxidized, and the substrate, where it was reduced. At this distance, given the diffusion coefficient of the solution species, the molecule would make $\sim 10^7$ round trips per second, resulting in a current of about 0.5–1 pA. This same approach was recently described where a tip with radius 5–15 nm could trap a molecule between it and a mercury pool.⁹

In all of these studies, although only a single molecule was trapped, it was

ABSTRACT Single-molecule studies, including those of single enzyme molecules, have led to important new insights about the effects of environment and configuration on the behavior of these molecules. Such information is not available from ensemble studies. Most of these have been based on spectroscopic approaches, but there have been relatively few attempts at single-molecule electrochemistry. In a paper in this issue, progress toward studying a single enzyme molecule by protein film voltammetry is described. This Perspective reviews briefly past work on nanoelectrodes and electrochemical and spectroelectrochemical single-molecule studies, as well as examples of the type of information obtained in past studies of enzymes.

See the accompanying Article by Hoebe et al. on page 2497.

*Address correspondence to ajbard@mail.utexas.edu.

Published online December 23, 2008.
10.1021/nn800801z CCC: \$40.75

© 2008 American Chemical Society

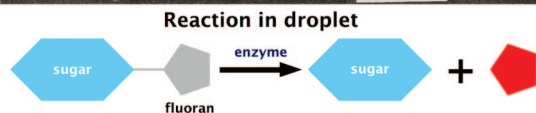
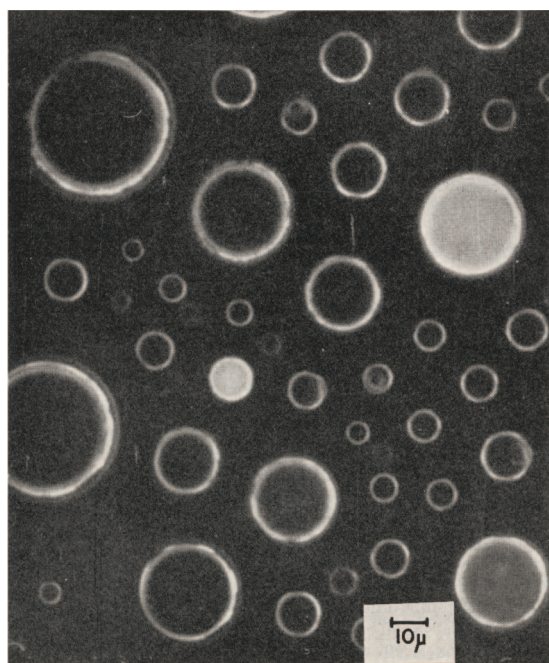


Figure 1. Rotman's experiment with enzymes and substrate contained in aqueous droplets in a silicone oil.¹⁴ Each droplet contains one or more enzyme molecules, the number of which can be determined from the relative fluorescence intensity of each after incubation. The system can be adjusted to a condition where droplets contain either one or no enzyme molecule by choice of the enzyme concentration. The bright droplets show those that are fluorescing. Note that the droplets are spaced apart by electrostatic forces from the charge on each droplet. Reproduced with permission from ref 14. Copyright 1961 by M. Boris Rotman.

sampled many times during the measurement period, so the advantage of probing an isolated single molecule as different from an ensemble was lost; however, this information can be preserved in spectroelectrochemical experiments. Here, a molecule, such as a luminescent polymer, is immobilized on a transparent conductive substrate, *e.g.*, an indium tin oxide (ITO) film on a cover glass.¹⁰ This is used as an electrode in a thin layer electrochemical cell, and the fluorescence from the single molecule is probed as a function of the ITO potential. Oxidation of the molecule causes quenching of the fluorescence. In this case, the fluorescence emission under laser irradiation, whose intensity is a function of the state of oxidation of the molecule, provides the needed amplification. The same approach

was used with single, larger polymer particles¹¹ and also to produce emission electrochemically *via* electrogenerated chemiluminescence (ECL).¹² Here, the emission is produced electrochemically by repeated redox events; while it is usually less intense than laser-induced fluorescence, it also has a much smaller background signal.

A recent alternative for single metal nanoparticle (MNP) electrochemical detection uses an electrocatalytic reaction on the MNP (*e.g.*, proton reduction or hydrazine oxidation) that occurs at a Pt particle, but not on a glassy carbon detection electrode.¹³ Here, when a MNP collides and sticks to the glassy carbon electrode, an electrocatalytic reaction occurs and produces a current that depends on the particle radius and the reactant concentration in solution, which is about 8–10 orders of magnitude larger than that of a single electron transfer to the MNP. In the Lemay work, amplification is obtained by the enzyme molecules, which turn over a high number of substrate molecules present at high concentration (equivalent to the MNP experiment where a single particle turns over a large number of reactant molecules present at high concentration).

Single Enzyme Molecule Studies.

There is a strong motivation to study single enzyme molecules as opposed to the more usual studies of ensembles. In studies of single enzyme molecules, one can observe if there are temporal variations in the activity; does the enzyme turn on and off during its reaction or are there variations in activity that can be accounted for by conformational changes? These kinds of effects would be averaged out in an ensemble of even a relatively small number of molecules.

Actually, single enzyme molecule studies go back to 1961 and

the work of Rotman, who recognized that “a method capable of measuring the activity of single enzyme molecules could initiate studies of a number of biological problems not yet amenable to experimentation.”¹⁴ In this experiment, a solution of β -D-galactosidase and its substrate, D-galactose coupled to 6-hydroxyfluorin, was prepared (Figure 1). The fluorin fluoresces when free, but not when attached to the sugar. This solution was sprayed onto a few drops of silicone oil on a microscope slide to produce aqueous droplets 0.1–40 μ m diameter. From Poisson statistics, given the number of droplets and the number of enzyme molecules, one could determine the number of enzyme molecules per droplet, which varied from 0 to 3, with a number of droplets containing single molecules. For droplets in the diameter range 14–15 μ m, the rate of increase in the fluorescence intensity in a droplet was directly proportional to the number of enzyme molecules per droplet. By observing these over a period of 15 h,

Ultramicroelectrodes

have had an enormous impact in electrochemistry, for example, in allowing experiments in the microsecond or in the nanosecond regime by minimizing troublesome double-layer charging and resistance effects.

Rotman could determine the number of enzyme molecules per drop. These experiments were used to demonstrate that genetically altered β -D-galactosidase was a different enzyme species than the wild type, and not simply a mixture of active and inactive wild-type enzyme. He also showed that heating produced a mixture of active and inactive enzyme molecules rather than partially inactivating all molecules, a finding that could not be made from ensemble measurements.

A number of papers on single enzyme molecule studies have appeared since 1995, which have shown that the activities of single enzyme molecules of the same type can vary and single enzyme molecules can turn on and off.^{15–17} For example, Engelkamp *et al.*¹⁷ employed the setup and chemistry shown in Figure 2 to watch the production of a fluorescent product by an enzyme reaction and demonstrated that there are bursts of reaction for ~ 37 ms while the enzyme is then inactive (“sleeps”) for ~ 97 ms.¹⁷ This suggests that the enzyme moves through many confor-

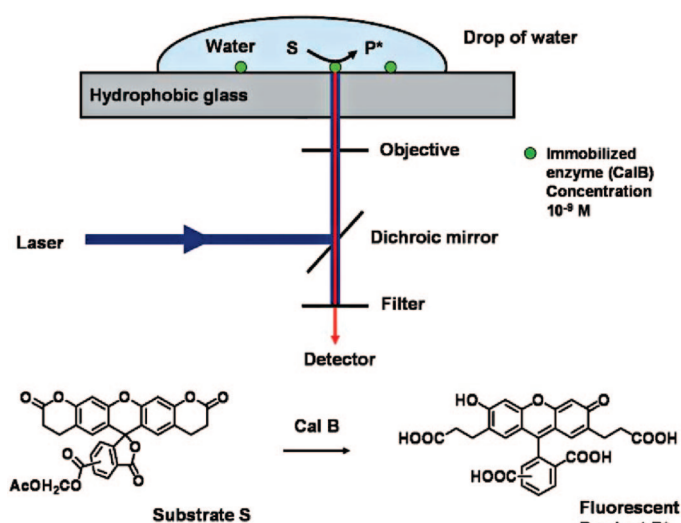


Figure 2. Setup of Engelkamp *et al.*¹⁷ for detecting reaction at a single enzyme molecule immobilized on a glass plate. Reaction of substrate S produces a fluorescent product, as shown. Reproduced with permission from ref 17. Copyright 2006 The Royal Society of Chemistry.

mations with time, with only a few that demonstrate high catalytic activity.

The Outlook for Single Enzyme Molecule Electrochemistry. What are the prospects for single enzyme molecule electrochemistry through PFV? By subtracting cyclic voltammograms at a scan speed of $1.5 \text{ mV} \cdot \text{s}^{-1}$ before and after introduction of the hydrogenase, Lemay and co-workers found a current of 22 fA with about 2 fA rms noise with filtering and a bandwidth of 3 Hz with an estimated 8 to 46 enzyme molecules on a $100 \times 100 \text{ nm}^2$ electrode. Assuming a $k_{\text{max}} = 10^4 \text{ s}^{-1}$, the response from a single enzyme would be 1.6 fA. From the previous spectroscopic studies, it appears that a time resolution of 10–50 ms is desirable. This suggests that considerable advances are needed for direct PFV observations of single enzyme molecules. The spectroscopic approaches in single-molecule studies often determine the rate of the reaction by following the product of the substrate reaction. Perhaps an alternative approach to PFV would be a scheme to amplify the current from the electrochemistry of the substrate product, as by a rapid catalytic reaction, to yield a larger current proportional to the enzyme turnover rate.

REFERENCES AND NOTES

1. Hoeben, F. J. M.; Meijer, F. S.; Dekker, C.; Albracht, S. P. J.; Heering, H. A.; Lemay, S. G. Toward Single-Enzyme Molecule Electrochemistry: [NiFe]-Hydrogenase Protein Film Voltammetry at Nanoelectrodes. *ACS Nano* **2008**, *2*, 2497–2504.
2. Ultramicroelectrodes. In *Handbook of Electrochemistry*; Zoski, C. G., Ed.; Elsevier: Amsterdam, 2007; pp 155–260.
3. *Scanning Electrochemical Microscopy*; Bard, A. J.; Mirkin, M. V., Eds.; Marcel Dekker: New York, 2001.
4. Zhang, B.; Galusha, J.; Shiozawa, P. G.; Wang, G.; Berggren, A. J.; Jones, R. M.; White, R. J.; Ervin, E. N.; Cauley, C. C.; White, H. S. Bench-Top Method for Fabricating Glass-Sealed Nanodisk Electrodes, Glass Nanopore Electrodes, and Glass Nanopore Membranes of Controlled Size. *Anal. Chem.* **2007**, *79*, 4778–4787.
5. Sun, P.; Mirkin, M. V. Kinetics of Electron-Transfer Reactions at Nanoelectrodes. *Anal. Chem.* **2006**, *78*, 6526–6534.
6. Fan, F.-R. F.; Bard, A. J. Electrochemical Detection of Single Molecules. *Science* **1995**, *267*, 871–874.
7. Fan, F.-R. F.; Kwak, J.; Bard, A. J. Single Molecule Electrochemistry. *J. Am. Chem. Soc.* **1996**, *118*, 9669–9675.
8. Fan, F.-R. F.; Bard, A. J. Electrochemical Detection of Single Molecules. *Acc. Chem. Res.* **1996**, *29*, 572–578.
9. Sun, P.; Mirkin, M. V. Electrochemistry of Individual Molecules in Zeptoliter Volumes. *J. Am. Chem. Soc.* **2008**, *130*, 8241–8250.

In studies of single enzyme molecules, one can observe if there are temporal variations in the activity; does the enzyme turn on and off during its reaction or are there variations in activity that can be accounted for by conformational changes?

10. Palacios, R. E.; Fan, F.-R. F.; Bard, A. J.; Barbara, P. F. Single-Molecule Spectroelectrochemistry (SMS-EC). *J. Am. Chem. Soc.* **2006**, *128*, 9028–9029.
11. Palacios, R. E.; Fan, F.-R. F.; Grey, K.; Suk, J.; Bard, A. J.; Barbara, P. F. Charging and Discharging of Single Conjugated-Polymer Nanoparticles. *Nat. Mater.* **2007**, *6*, 680–685.
12. Chang, Y.-L.; Palacios, R. E.; Fan, F.-R. F.; Bard, A. J.; Barbara, P. F. Electrogenerated Chemiluminescence of Single Conjugated Polymer Nanoparticles. *J. Am. Chem. Soc.* **2008**, *130*, 8906–8907.
13. Xiao, X. Y.; Bard, A. J. Observing Single Nanoparticle Collisions at an Ultramicroelectrode by Electrocatalytic Amplification. *J. Am. Chem. Soc.* **2007**, *129*, 9610–9612.
14. Rotman, B. Measurement of Activity of Single Molecules of β -D-Galactosidase. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *47*, 1981–1991.
15. Tan, W. H.; Yeung, E. S. Monitoring the Reactions of Single Enzyme Molecules and Single Metal Ions. *Anal. Chem.* **1997**, *69*, 4242–4248.
16. Min, W.; English, B. P.; Luo, G.; Cherayil, B. J.; Kou, S. C.; Xie, X. S. Fluctuating Enzymes: Lessons from Single-Molecule Studies. *Acc. Chem. Res.* **2005**, *38*, 923–931.
17. Engelkamp, H.; Hatzakis, N. S.; Hofkens, J.; De Schryver, F. C.; Nolte, R. J. M.; Rowan, A. E. Do Enzymes Sleep and Work? *Chem. Commun.* **2006**, 935–940.