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Handling and Sensing of Single Enzyme Molecules: From Fluorescence Detection towards Nanoscale Electrical Measurements

Klaus Mathwig,[b] Qijin Chi,[c] Serge G. Lemay,[d] and Liza Rassaei*[a]

Classical methods to study single enzyme molecules have provided valuable information about the distribution of conformational heterogeneities, reaction mechanisms, and transients in enzymatic reactions when individual molecules instead of an averaging ensemble are studied. Here, we highlight major advances in all-electrical single enzyme studies with a focus on recent micro- and nanofluidic tools, which offer new ways of handling and studying small numbers of molecules or even single enzyme molecules. We particularly emphasize nanofluidic devices, which enable the integration of electrochemical transduction and detection.

1. Introduction

Single-molecule science and technology is an emerging area aiming at studying individual physical, chemical, and biological events (or processes) such as charge transport, enzyme catalysis, and protein expression with potential applications in computation and ultrasensitive analysis. The main motivation for studying single-molecule events is to disclose new fundamental phenomena and properties of target systems, which otherwise remain hidden in an ensemble of molecules. To unravel single-molecule events, sophisticated tools and unique methods are needed, according to the specific nature of the target systems or events.

Enzymes are biomolecular machines that dramatically speed up chemical reactions within organisms. Understanding the underlying mechanisms of enzymatic reactions is essential to gain insight into their reactivity in cellular processes. One way to obtain such insight is by studying the molecular behavior of individual enzymes rather than an ensemble, because individual enzymatic behavior is masked (or hidden) by averaging over an ensemble[1] as well as time-averaging in bulk experiments.[2,3] Variations in the properties of an enzyme distribution mainly arise from individual dynamic enzyme fluctuations[4] or static and dynamic heterogeneities.[4,5] Real-time studies have led to the discovery of transient intermediates, understanding reaction mechanisms at the molecular level, and providing detailed dynamical information.[6]

Today, a large toolkit is available for studying single biomolecules.[7] We have obtained new insights into the functioning of single enzyme molecules over the last 20 years by using highly advanced tools such as optical tweezers,[8] magnetic tweezers,[9] atomic force microscopy (AFM),[10] and scanning tunneling microscopy (STM).[11] Currently, the most common techniques to study single enzyme molecules rely on optical detection:[12] a rise in a fluorescence signal is sensed, owing to either fluorescent labels or markers attached to the molecule of interest or fluorescent tracers that are dissolved within the matrix under study. This leads to great sensitivity, as a single fluorescent molecule can emit thousands of photons.[13]

Current trends in the miniaturization of analytical devices (e.g. micro-/nanofluidic devices) offer new ways of manipulating and sensing individual molecules or very small numbers of molecules. These include fluidic transport of analytes, additional electrical actuation, or confinement of the reaction/detection space down to a volume comparable with that of a cell or an organelle.

As part of these developments, new tools and methods to disclose information at the single-enzyme level are emerging. Here, we first summarize some of the major breakthroughs that have led to significant advances in classical single-molecule enzyme studies. Then, we highlight several recent developments in micro- and nanodevices, which allow the study and handling of few or even single enzyme molecules in a confined reaction space.

[a] *Dr. L. Rassaei*
Laboratory of Organic Materials and Interfaces
Department of Chemical Engineering
Delft University of Technology
Julianalaan 136, 2628 BL Delft (The Netherlands)
E-mail: l.rassaei@tudelft.nl

[b] *Dr. K. Mathwig*
Pharmaceutical Analysis
Groningen Research Institute of Pharmacy
University of Groningen
P.O. Box 196, 9700 AD Groningen (The Netherlands)

[c] *Prof.Dr. Q. Chi*
Department of Chemistry
Technical University of Denmark
2800 Kongens Lyngby (Denmark)

[d] *Prof.Dr. S. G. Lemay*
MESA+ Institute for Nanotechnology
University of Twente
P.O. Box 217, 7500 AE Enschede (The Netherlands)

2. Previous Major Advances in Fluorescence-Based Single-Enzyme Studies

Ground-breaking research in the study of single enzyme molecules[14,15] was pioneered by Rotman, dating back to 1961. His approach was based on the intrinsic amplification of enzymatic reactions by accumulating products from many turnovers of a single enzyme molecule. β-D-Galactosidase molecules were distributed into separate microdroplet containers, in which their products could accumulate. In such an approach, the enzyme molecules were randomly distributed, and the number of molecules in the microdroplets followed a Poisson distribution so that some microdroplets contained only a single enzyme molecule. Then, a fluorogenic substrate, 6-hydroxyfluoran-13-D-galactopyranoside, was introduced to the microdroplets, and the activity of β-D-galactosidase molecules was assayed through the fluorescence of products accumulated in the microdroplets.

This pioneering work had a significant impact on the field and has since then inspired many fruitful studies. In this regard, one noteworthy example is the discovery of static heterogeneity in the activity of enzyme molecules by Xue and Yeung thirty-five years later in 1995.[16] Their finding was based on enzyme lactate dehydrogenase, which catalyzes the conversion of lactate and pyruvate through simultaneous reduction of the non-fluorescent coenzyme NAD^+ to fluorescent NADH. A similar approach to the Rotman method was used for signal amplification, that is, the fluorescent product (NADH in this case) was accumulated, but instead of confining the enzyme molecules in microdroplets, they were placed in capillaries. Afterwards, the slow diffusion of NADH to create zones around each enzyme molecule was measured. As NADH electrophoresed through the detection volume, peaks were recorded and the area of the peaks was related to the activity of the corresponding enzyme, indicating that different enzyme molecules displayed varying activities.

Three years later, Xie and co-workers gained additional insight into the dynamic heterogeneity of enzymes by developing a method to measure single-enzyme kinetics in real time, which unraveled the static and dynamic disorders of reaction rates.[5] Cholesterol oxidase was selected as a model enzyme and immobilized in an agarose gel. The fluorescence properties of the enzyme cofactor flavin adenine dinucleotide (FAD), which is fluorescent in its oxidized form but non-fluorescent in its reduced form, were monitored. The on/off cycle in FAD fluorescence was followed by observing the differences in fluorescent properties of the oxidized and reduced forms in each reaction cycle. In this way, the time for one complete turnover, as well as the duration of individual states, was determined.

In 2006, in another example of pioneering work, the mechanism of a single enzyme molecule was studied by monitoring catalytic turnovers of β-galactosidase. Here, the photon burst of each product molecule was measured from its fluorogenic substrate, resorufin-β-galactopyranoside.[17] The product of this enzymatic reaction, fluorescent resorufin, was constantly generated and then diffused away from the probed volume. When a similar experiment was carried out for the enzyme ensemble, an excellent agreement between the single molecule data and the ensemble data was found, elucidating that the Michaelis–Menten kinetics model still holds for single-molecule enzymatic reactions. However, it was discovered that the enzymatic rate constant at the single-molecule level broadly fluctuates.

3. Nano-/Microstructuring for Studying and Handling Single Enzymes

In earlier classical single-enzyme studies, individual molecules were either confined in microdroplets or immobilized on a solid surface.[15,18] Although these approaches continue to be crucial in single-biomolecule studies, recent advances in nanotechnology have allowed us to steadily fabricate smaller features toward molecular dimensions. We are now at the edge of using nanofabricated devices[19] to directly study and manipulate individual molecules. Most notable devices are nanopore devices, which have been reviewed extensively.[20–22] In this section, we focus on different micro- and nanodevices offering new ways of fluidic handling as well as electrical and electrochemical handling and sensing for studying enzymatic processes. These tools provide new platforms to study biochemical reactions in geometric confinement. The results can be utilized, for example, to design, functionalize, and program bionanodevices such as nanobiosensors and microbiopower devices.

3.1. Fluidic Handling

Most early classical methods have a common setback, that is, the number of active enzyme molecules is only statistically determined, but not actively controlled. Moreover, an elaborate immobilization process is often required. Although a vast single-molecule toolkit now exists, which allows techniques to be tailored toward specific types of molecules for localizing and imaging,[7] several approaches have been developed to effectively control transport and/or reactions of biomolecules in solutions confined in microfluidic or nanofluidic devices.[23]

Inspired by the Rotman study,[14] Eijkel et al. developed a method based on microdroplets to measure the kinetic activity of individual enzyme molecules. Here, a nano-/microfluidic hybrid device[24] offered the advantage of accurate control over the generation of highly monodispersed aqueous droplets, which are ideally suited as containers for single enzyme molecules, owing to their ultrasmall volume. Single β-glucosidase molecules were trapped in femtoliter droplets generated by the nanofluidic device. The activity of single enzymes was studied in the presence of a fluorescein-β-D-glucopyranoside substrate by monitoring the accumulation of fluorescein (as product) in the droplets by fluorescence microscopy.

Noji and co-workers improved the microdroplet method to significantly speed up single-enzyme detection in droplets.[26] They used microfabricated hydrophilic SiO_2 patterns to generate a large ordered array of uniform microdroplets in oil to optically detect single molecules (β-galactosidase). This approach was further scaled up to count single enzymes immobilized on beads in an array of 10^6 microdroplets in parallel.[25] Figure 1 compares various microdroplet-based methods schematically.

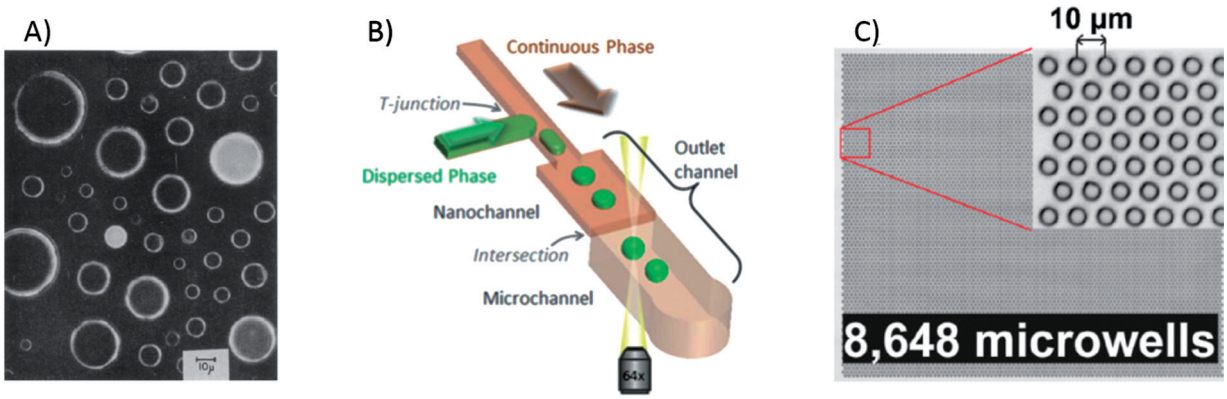

Figure 1. Comparison of three different microdroplet methods used for studying single enzymes. A) Original experiment of dispersed microdroplets with captured enzymes. Reproduced with permission from Ref. [14]. Copyright (1961) National Academy of Sciences. B) Schematic diagram of nanofluidic generation of monodisperse nanodroplets. Reproduced with permission from Ref. [24]. Copyright (2013) Royal Society of Chemistry. D) Microscopy image of a section of a large array of 1 million microdroplets. Reproduced with permission from Ref. [25]. Copyright (2012) Royal Society of Chemistry.

In a different pioneering experiment for nanofluidic control[27] of the reaction of small numbers of enzymes, Kitamori and co-workers employed branched nanochannels and pressure-driven flow to mix the substrate Tokyo-Green–β-galactoside and the enzyme β-galactosidase (see Figure 2 A).[28] Here,

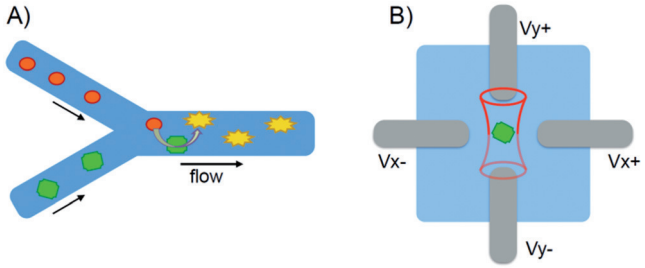

Figure 2. Scheme showing fluidic control of molecular transport. A) Branched nanofluidic channel for enzymatic reactions.[28] The substrate (red) and enzyme (green) react at the intersection of two nanochannels, and the reaction product (yellow) is subsequently detected either optically[28] or electrochemically.[30] B) An ABEL trap[31] for handling single molecules in solution. A feedback loop is used, in which the position of a molecule is determined optically and it is pushed towards the center between electrodes by using electrophoretic forces.

the lateral and vertical confinement to several 100 nm ensured immediate mixing and triggered the reaction directly at the intersection of two nanochannels with well-controlled flow speed. The fluorescence of the product Tokyo-Green was detected downstream in the nanochannel. The kinetic parameters of the reaction were obtained, and the enzyme reaction rates were found to be significantly enhanced in comparison with the same reaction occurred in bulk solutions, most likely arising from higher proton activities contributed by surface interactions. Recently, the same group used pressure-driven flow to study immunochemical reactions between antigens and antibodies immobilized in the nanochannel.[29] The devices have the advantage of achieving a virtually 100% capture rate, owing to the efficient nanoscale confinement.

3.2. Electrical Handling/Actuation

Although the generation and handling of micro-/nanofluidic droplets enable improved studies of molecules trapped within them, a different and more direct way of handling single molecules, in addition to the more established toolkit, is to confine them in a space, not by the walls of a nanochannel or droplet, but by overcoming their random diffusion through an anti-Brownian electrokinetic (ABEL) trap.[31] In such a device, the position of a single molecule can be monitored precisely in a microfluidic channel by using fluorescence microscopy, and electrokinetic forces actuated by electrodes are used to push molecules back into the center of the device. Thus, feedback actuation effectively overcomes Brownian motion (see Figure 2 B). Although it is a very demanding experimental technique,[32] an ABEL trap enables the confinement of a molecule in solution to a volume much smaller than typical nanochannel dimensions. The technique was also used to study individual redox events of a single enzyme in solution.[33] Here, the catalytic reduction of nitrite by a trapped blue copper nitrite reductase (a multicenter metalloenzyme) was optically monitored by changes in the fluorescence energy transfer in a labeled enzyme molecule.

Microelectrodes located in nanofluidic devices can also be used, not just for electrokinetic transport, but also to control the redox state of enzyme molecules. The Bohn group employed zero-mode waveguides to study single FAD coenzyme molecules immobilized on gold electrodes in an array of nanodevices through pyrroloquinoline quinone linker.[34] Here the gold electrodes play two roles: they confine the optical field in the zero-mode waveguides and they function as the electrochemical working electrode. In this way, it was possible to control the single-molecule blinking dynamics of FAD by controlling the bias potential at a gold electrode and, thus, the oxidation state of FAD.

The same group recently applied the same detection method of electrochemical actuation and fluorescence readout to study molecules in solution diffusing in and out of nanopores.[35]

3.3. All-Electrical/Electrochemical Sensing

Next to optical detection, other novel attractive methods have been emerging to monitor and manipulate single enzyme molecules. One promising approach relies on electrical detection. Although these techniques have not reached the level of optical sensing in terms of versatility and sensitivity, electrical detection often offers additional advantages of further miniaturization and integration.

Over the past decade, scanning probe microscopies (SPM) have offered unique means to study single molecules electronically. Specifically, the combination of scanning tunneling microscopy (STM) or atomic force microscopy (AFM) with electrochemical measurements has allowed the study of single-molecule events of biological or related macromolecules in situ in chemical or/and electrochemical environments.[36–42] Figure 3

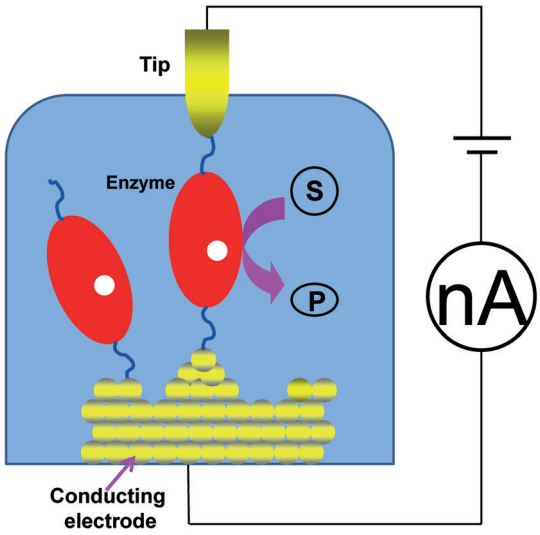

Figure 3. Schematic illustration of the STM-based technique, measuring the electronic properties of protein electron-transfer and/or enzyme (electro)catalysis reactions at the single-molecule level (not drawn to scale; S: substrate, P: product). For simplicity, we omitted a counter electrode and a reference electrode.

conceptually illustrates an STM-based measurement of electronic change in an enzymatic (electro)catalytic reaction. Here, individual enzyme molecules are confined in a nanogap between the working electrode and the STM tip. This configuration enables an enhanced measurement resolution down to the single-molecule level. Direct molecular imaging, scanning tunneling spectroscopy, and break-junction techniques can all be exploited for the in situ monitoring of single-molecule electronic actions. The approach has been attempted for a wide range of proteins or/and enzymes from heme proteins[43] and blue copper proteins[44] to iron–sulfur proteins.[45] In addition, electrochemical AFM has offered the possibility to quantify the physical or conformational changes of enzyme molecules during their enzymatic actions.[37]

In other electrical detection schemes, the activity or the kinetics of enzymatic reactions, which yield redox-active products, can be monitored by electron exchange (oxidation or reduction) at an appropriate electrode bias voltage.

By using a mixing principle similar to the nanochannel junction shown in Figure 1A, Xia and co-workers developed an electrochemical amperometric detection method for enzymatic reactions in branched nanochannels.[30,46] Glucose and glucose oxidase were mixed in a nanochannel Y-junction. Electrochemical oxidation of the by-product H_2O_2 in enzymatic reactions was detected by using an ultramicroelectrode positioned at the nanochannel outlet. Here, a relatively large number of molecules reacted in the nanochannel, and a single-molecule detection level has not yet been achieved. Nonetheless, target molecules were handled and their catalytic reactions were triggered in a confined nanospace. We believe that the number of enzyme and substrate molecules can be drastically reduced in the future by further reducing the nanochannel cross-section size. In a further step, the same group enriched glucose oxidase in an integrated micro-/nanofluidic pre-concentration device and studied its reaction kinetics by amperometric sensing.[47] Homogeneous enzymatic reactions occurred when glucose was electrokinetically transported to interact with enzyme molecules, and the by-product (H_2O_2) was detected by using an ultramicroelectrode located at the nanochannel outlet.

In general, electrical detection might not be as sensitive as optical sensing, because the inherent noise of electrical amplifiers used in signal transduction does not allow the direct recording of only a few or a single electron(s) transferred at an electrode (following, for example, enzymatic activity). By using state-of-the-art electrical amplifiers at room temperature, the smallest detectable current needs to be significantly higher than 1 fA,[48] which corresponds to (in excess of) 10000 electrons transferred per second. As a consequence, it appears that we could not foresee direct electrical monitoring of a single enzymatic reaction in the near future without a dramatic improvement in intrinsic amplification. However, Hoeben et al.[49] have recently offered a pathway toward electrical detection of single-molecule enzyme activity. In their approach, [NiFe]–hydrogenase molecules were immobilized on the surface of a gold nanoelectrode. The enzyme molecules exhibit a very high catalytic turnover rate reaching 1500– 9000 s^{-1},[50] enabling a $2e^-$ electrooxidation of each dihydrogen molecule into two protons. Thus, each enzyme can generate an impressively high electrochemical current, and less than 50 active enzyme molecules were needed. This study has shed light on the feasibility of electrical detection in enzymatic reactions of single molecules.

More recently, we have implemented a different approach of intrinsically amplified detection in a nanofluidic device through the integration of elements for enzymatic recognition, detection, and electrochemical signal transduction within a 6 fL volume.[51] Here, we locally immobilized tyrosinase through a thiol bond into a microfabricated electrochemical nanogap transducer and used redox cycling to amplify the electrochemical signal of redox-active product molecules (see Figure 4). Tyrosinase turned over electrochemically inactive monophenol into the electroactive couple catechol/quinone with a rate of approximately 14 s^{-1}.[52] By biasing one electrode at an oxida-

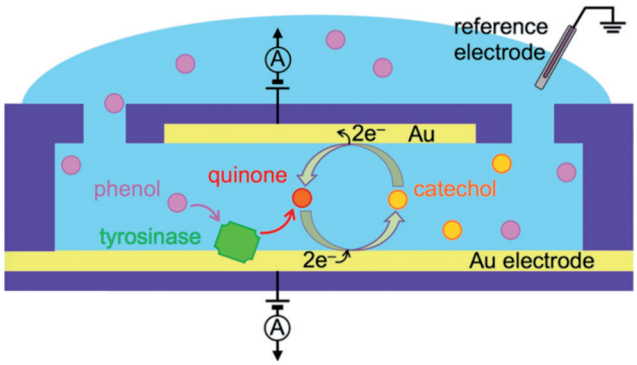

Figure 4. Schematic diagram of enzymatic biodetection through direct electron transfer. Tyrosinase enzyme molecules immobilized in a nanochannel with a height of about 200 nm generate electrochemically active redox products (quinone). They catalyze inactive phenols at a rate of approximately 14 s^{-1}. The generated quinone/catechol couple subsequently undergoes redox cycling (ca. 50 μs shuttling time) between the Au electrodes embedded in the floor and the ceiling of the channel, thus leading to a highly amplified current per enzyme molecule. Reprinted with permission from Ref. [51]. Copyright (2014) American Chemical Society.

tion potential and the other electrode at a reduction potential, each product molecule repeatedly exchanged electrons by redox cycling, as it cycled by diffusion between the electrodes. The diffusive travelling time across the 200 nm gap between electrodes is significantly shorter than 1 ms, leading to a highly amplified current signal. In this example, the analyte was confined to a 6 fL volume. With a relatively large electrode area of 10×3 μm^2, about 5000 enzyme molecules in the nanodevice were needed for electrocatalytic detection. In the nanospace confinement, the enzymatic reaction still followed the classic Michaelis–Menten kinetics model. We could, thus, hope that further optimization and improvement of this approach would enable electrochemical studies of single enzyme molecules in the near future.

We have also demonstrated that nanogap devices are sensitive enough to detect a single redox-active molecule.[53] However, their extension to study single enzyme molecules requires that the number of active enzymes in the device is drastically reduced. This reduction could be achieved either by reducing the enzyme concentration or decreasing the enzyme incubation time in the device, or even by the combination of both approaches. However, reaching a single-enzyme-molecule level in the device still remains a challenge. This goal could be realized either by using appropriate inhibitors, which irreversibly deactivate the enzyme over time, or by applying a highly negative electrochemical potential that reduces the number of thiol–gold bonds through reduction desorption and, consequently, releasing most enzyme molecules from the electrode surfaces. Either of these approaches can be electrically monitored by recording the current drop per enzyme molecule that is deactivated or leaves the device. For tyrosinase, with a turnover rate of approximately 14 s^{-1}, the production of 14 electroactive molecules per second leads to a comparatively large current signal drop per enzyme (a current in excess of about 100 fA if the products undergo redox cycling for 1 s before diffusion out of the device). Therefore, in such a configuration, the activity of a single enzyme molecule can be monitored by detecting a larger number of redox-active product molecules. Effectively reducing the number of enzyme molecules in a nanochannel requires not only a dramatic dilution of enzyme concentration in the nanochannel, but also a substantial minimization of the nanochannel volume and the electrode area (e.g. by employing electron-beam lithography with sub-10 nm resolution for the definition of these structures).

Electrically detecting a single biocatalytic event will be far more challenging (even if several enzyme molecules are active) than detecting a single active enzyme.

4. Conclusions and Outlook

New technologies are emerging, which offer novel pathways for studying single-molecule enzymatic reactions, largely owing to advances in nanotechnology and microfabrication. Nanoscale devices, such as nanofluidic channels, enable the confinement and handling of few enzyme molecules in an extremely small volume. When these devices are coupled with electrochemical transduction, we can devise many new ways to handle and manipulate biomolecules. Although the ambition towards all-electrical studies of enzymes at the single-molecule level are not yet fulfilled by current nanodevices, increasingly promising steps have taken place. Future progress should be directed not just at pushing the limits of sensitivity (or the smallest number of detected analytes), but also toward more robust immobilization methods as well as fluidic and electrical handling of (single) enzyme molecules. In the future, advanced molecular handling and microstructured devices in which biochemical processes are monitored electrochemically will provide unprecedented insights into bioreactions at the single-molecule level in confined volumes. At the ultimate level of sensitivity, they might lead to a nanobiosensor that ideally combines the reliable sensing of single molecules or single chemical reactions (enzymatic or immunological reactions or DNA sensing) with all-electrical signal transduction elements integrated into one device.

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[1] A. M. van Oijen, P. C. Blainey, D. J. Crampton, C. C. Richardson, T. Ellenberger, X. S. Xie, *Science* **2003**, *301*, 1235 – 1238.
[2] V. I. Claessen, H. Engelkamp, P. C. Christianen, J. C. Maan, R. J. Nolte, K. Blank, A. E. Rowan, *Annual Review of Anal. Chem.* **2010**, *3*, 319 – 340.
[3] R. D. Smiley, G. G. Hammes, *Chem. Rev.* **2006**, *106*, 3080 – 3094.
[4] X. S. Xie, *J. Chem. Phys.* **2002**, *117*, 11024 – 11032.
[5] H. P. Lu, L. Xun, X. S. Xie, *Science* **1998**, *282*, 1877 – 1882.
[6] D. B. Craig, E. Arriaga, J. C. Wong, H. Lu, N. J. Dovichi, *Anal. Chem.* **1998**, *70*, 39A – 43A.

[7] N. G. Walter, C.-Y. Huang, A. J. Manzo, M. A. Sobhy, *Nat. Methods* **2008**, *5*, 475 – 489.
[8] S. K. Vashist, A. Venkatesh, K. Mitsakakis, G. Czilwik, G. Roth, F. von Stetten, R. Zengerle, *BioNanoScience* **2012**, *2*, 115 – 126.
[9] Y. Rondelez, G. Tresset, T. Nakashima, Y. Kato-Yamada, H. Fujita, S. Takeuchi, H. Noji, *Nature* **2005**, *433*, 773 – 777.
[10] P. Hinterdorfer, Y. F. Dufrêne, *Nat. Methods* **2006**, *3*, 347 – 355.
[11] J. Zhang, A. M. Kuznetsov, I. G. Medvedev, Q. Chi, T. Albrecht, P. S. Jensen, J. Ulstrup, *Chem. Rev.* **2008**, *108*, 2737 – 2791.
[12] C. Joo, H. Balci, Y. Ishitsuka, C. Buranachai, T. Ha, *Annu. Rev. Biochem.* **2008**, *77*, 51 – 76.
[13] K. P. Janssen, G. De Cremer, R. K. Neely, A. V. Kubarev, J. Van Loon, J. A. Martens, D. E. De Vos, M. B. Roeffaers, J. Hofkens, *Chem. Soc. Rev.* **2014**, *43*, 990 – 1006.
[14] B. Rotman, *Proc. Natl. Acad. Sci. USA* **1961**, *47*, 1981.
[15] A. B. Theberge, F. Courtois, Y. Schaerli, M. Fischlechner, C. Abell, F. Hollfelder, W. T. Huck, *Angew. Chem. Int. Ed.* **2010**, *49*, 5846 – 5868; *Angew. Chem.* **2010**, *122*, 5982 – 6005.
[16] Q. Xue, E. S. Yeung, *Nature* **1995**, *373*, 681 – 683.
[17] B. P. English, W. Min, A. M. van Oijen, K. T. Lee, G. Luo, H. Sun, B. J. Cherayil, S. Kou, X. S. Xie, *Nat. Chem. Biol.* **2006**, *2*, 87 – 94.
[18] R. A. Sheldon, *Advanced Synthesis & Catalysis* **2007**, *349*, 1289 – 1307.
[19] L. Rassaei, P. S. Singh, S. G. Lemay, *Anal. Chem.* **2011**, *83*, 3974 – 3980.
[20] B. N. Miles, A. P. Ivanov, K. A. Wilson, F. Doğan, D. Japrung, J. B. Edel, *Chem. Soc. Rev.* **2013**, *42*, 15 – 28.
[21] K. Mathwig, T. Albrecht, E. D. Goluch, L. Rassaei, *Anal. Chem.* **2015**, *87*, 5470 – 5475.
[22] S. Howorka, Z. Siwy, *Chem. Soc. Rev.* **2009**, *38*, 2360 – 2384.
[23] A. M. Streets, Y. Huang, *Current opinion in biotechnology* **2014**, *25*, 69 – 77.
[24] R. Arayanarakool, L. Shui, S. W. Kengen, A. van den Berg, J. C. Eijkel, *Lab on a chip* **2013**, *13*, 1955 – 1962.
[25] S. H. Kim, S. Iwai, S. Araki, S. Sakakihara, R. Iino, H. Noji, *Lab on a Chip* **2012**, *12*, 4986 – 4991.
[26] S. Sakakihara, S. Araki, R. Iino, H. Noji, *Lab on a Chip* **2010**, *10*, 3355 – 3362.
[27] T. Tsukahara, K. Mawatari, T. Kitamori, *Chem. Soc. Rev.* **2010**, *39*, 1000 – 1013.
[28] T. Tsukahara, K. Mawatari, A. Hibara, T. Kitamori, *Anal. Bioanal. Chem.* **2008**, *391*, 2745 – 2752.
[29] K. Shirai, K. Mawatari, T. Kitamori, *Small* **2014**, *10*, 1514 – 1522.
[30] C. Wang, D.-K. Ye, Y.-Y. Wang, T. Lu, X.-H. Xia, *Lab on a Chip* **2013**, *13*, 1546 – 1553.
[31] Q. Wang, R. H. Goldsmith, Y. Jiang, S. D. Bockenhauer, W. Moerner, *Acc. Chem. Res.* **2012**, *45*, 1955 – 1964.
[32] A. P. Fields, A. E. Cohen, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 8937 – 8942.
[33] R. H. Goldsmith, L. C. Tabares, D. Kostrz, C. Dennison, T. J. Aartsma, G. W. Canters, W. Moerner, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17269 – 17274.
[34] J. Zhao, L. P. Zaino III, P. W. Bohn, *Faraday Discuss.* **2013**, *164*, 57 – 69.
[35] L. P. Zaino, D. A. Grismer, D. Han, G. M. Crouch, P. W. Bohn, *Faraday Discuss.* **2015**, DOI: 10.1039/C5FD00072F.
[36] Q. Chi, O. Farver, J. Ulstrup, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 16203 – 16208.
[37] E. A. D. Pia, Q. Chi, D. D. Jones, J. E. Macdonald, J. Ulstrup, M. Elliott, *Nano Lett.* **2011**, *11*, 176 – 182.
[38] E. A. Della Pia, Q. Chi, J. E. Macdonald, J. Ulstrup, D. D. Jones, M. Elliott, *Nanoscale* **2012**, *4*, 7106 – 7113.
[39] Q. Chi, J. Zhang, P. S. Jensen, H. E. Christensen, J. Ulstrup, *Faraday Discuss.* **2006**, *131*, 181 – 195.
[40] V. Climent, J. Zhang, E. P. Friis, L. H. Østergaard, J. Ulstrup, *J. Phys. Chem. C* **2012**, *116*, 1232 – 1243.
[41] X. Hao, J. Zhang, H. E. Christensen, H. Wang, J. Ulstrup, *ChemPhysChem* **2012**, *13*, 2919 – 2924.
[42] X. Hao, N. Zhu, T. Gschneidtner, E. Ö. Jonsson, J. Zhang, K. Moth-Poulsen, H. Wang, K. S. Thygesen, K. W. Jacobsen, J. Ulstrup, Q. Chi, *Nat. Commun.* **2013**, *4*, 2121.
[43] T. Albrecht, W.-W. Li, W. Haehnel, P. Hildebrandt, J. Ulstrup, *Bioelectrochemistry* **2006**, *69*, 193 – 200.
[44] E. P. Friis, J. E. Andersen, Y. I. Kharkats, A. Kuznetsov, R. J. Nichols, J.-D. Zhang, J. Ulstrup, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1379 – 1384.
[45] J. Zhang, J. Ulstrup, *J. Electroanal. Chem.* **2007**, *599*, 213 – 220.
[46] C. Wang, Z. H. Sheng, J. Ouyang, J. J. Xu, H. Y. Chen, X. H. Xia, *ChemPhysChem* **2012**, *13*, 762 – 768.
[47] C. Wang, S.-J. Li, Z.-Q. Wu, J.-J. Xu, H.-Y. Chen, X.-H. Xia, *Lab on a Chip* **2010**, *10*, 639 – 646.
[48] K. Mathwig, T. J. Aartsma, G. W. Canters, S. G. Lemay, *Annual Review of Anal. Chem.* **2014**, *7*, 383 – 404.
[49] F. J. Hoeben, F. S. Meijer, C. Dekker, S. P. Albracht, H. A. Heering, S. G. Lemay, *ACS Nano* **2008**, *2*, 2497 – 2504.
[50] F. J. Hoeben, I. Heller, S. P. Albracht, C. Dekker, S. G. Lemay, H. A. Heering, *Langmuir* **2008**, *24*, 5925 – 5931.
[51] L. Rassaei, K. Mathwig, S. Kang, H. A. Heering, S. G. Lemay, *ACS Nano* **2014**, *8*, 8278 – 8284.
[52] L. Rassaei, J. Cui, E. D. Goluch, S. G. Lemay, *Anal. Bioanal. Chem.* **2012**, *403*, 1577 – 1584.
[53] S. Kang, A. F. Nieuwenhuis, K. Mathwig, D. Mampallil, S. G. Lemay, *ACS Nano* **2013**, *7*, 10931 – 10937.

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