Bio-Electrochemistry

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Imaging Single Enzyme Molecules under In Situ Conditions**

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The electrochemical investigation of redox enzymes has become a broad field of research because the redox properties of the enzymes make them candidates for biosensors and bioelectronic nanodevices.[1] The degree of surface coverage, the surface orientation of the enzymes, that is, the position of the active site, and the activity of a single enzyme are still a question of dispute; this data can not be extracted from integral electrochemical measurements, such as cyclic voltammetry (CV). It has been shown, however, that scanning probe techniques, especially the electrochemical scanning tunneling microscopy (EC-STM),[2,3] can reveal the structure and reactivity of enzymes down to a single-molecule level,[4–6] although some experimental problems remain. To avoid any damage to the protein structure only small tunneling currents I_T can be applied. Therefore, scanning electrochemical potential microscopy (SECPM), a technique which measures the potential at zero current ($I = 0$) may be advantageous. The hardware is similar to an EC-STM, however the tip, is used as a potential sensor. The potential difference between the tip and the applied potential at the working electrode is measured with a high-impedance potential amplifier and serves as a feedback signal in the x–y scanning mode. In addition, SECPM also offers the possibility to map the potential distribution of the interface in the x–z direction, that is, perpendicular to the electrode surface. According to the Gouy–Chapman–Stern theory, at the electrode/electrolyte interface of every electrode there is an electrochemical double layer (EDL), the potential of which decreases with the distance from the electrode surface.[7]

Woo et al.[8] measured the local potential profile of an Au(111) electrode in 1 mM $NaBF_4$ by moving the tip of a home-built instrument based on a modified EC-STM perpendicular to the surface (x–z direction). Hurth et al.[9] performed similar experiments to study the influence of the surface potential and of the electrolyte concentration on the double layer profile of a Pt foil in KCl. Corbella et al.[10] first used the constant potential mode of this technique to investigate the distribution of tungsten in diamond-like carbon films by imaging the surface in the x–y direction. These are the only examples described to date for this technique.

SECPM appears to be especially suited for the investigation of organic and biological molecules adsorbed on electrode surfaces under electrochemical conditions. Herein we report the use of constant-potential-mode SECPM to image single enzymes adsorbed on electrode surfaces. Studying enzymes of different size, chemical composition, and electrochemical properties, we can show that SECPM is able to image biomolecules under in situ conditions with an unprecedented resolution. Comparing SECPM images with EC-STM images indicates that a potentiometric technique, such as SECPM, may be advantageous for imaging enzymes at the solid–liquid interface and has the potential to investigate the dynamic behavior of an enzyme on the surface.

As in all scanning probe techniques the tip preparation has to be tailored to the specific needs of the technique. The tip geometry determines the resolution, because the change in the potential distribution perpendicular and parallel to the electrode surface is different, the tip should be chosen based on the scan mode. For the x–z direction a tip with a short extension Δz along the z-axis and for the x–y mapping a sharp tip (Δx and Δy are small) as in STM are advantageous.

The tip is a metal electrode with an EDL at the solid–liquid interface. Thus when the tip approaches the electrode both EDLs will overlap; again the x–z and the x–y mode have to be distinguished. Moving in the x–z direction towards the surface, the potential measured at the tip is increasingly influenced by the overlapping EDLs. With a model of two overlapping EDLs at identical metals, both following the Gouy–Chapman theory (dilute electrolyte), it is possible to deconvolute the potentials and to calculate the EDL at the electrode surface.[11]

In the x–y mode, the influences of two overlapping EDLs can be considered as almost constant throughout the imaging. In this case it is also possible to deconvolute the potentials. Further discussions of the tip influence are given in the Supporting Information.

The iron-storage protein ferritin and the iron-free conformation apoferritin have a well defined 3D structure. Both proteins consist of 24 subunits forming a spherical protein shell, 12 nm in diameter. Whereas each ferritin can store about 4500 Fe^{III} ions (as $8\,FeO(OH)\cdot FeO(H_2PO_3)$ crystallites) in a core which has a diameter of up to 8 nm,[12] apoferritin only consists of the hollow protein shell.[13] Figure 1 shows two ferritin molecules adsorbed on line defects of an oxidized highly oriented pyrolytic graphite (HOPG) surface imaged in 10 mM PBS (phosphate buffer solution) in constant-current-mode EC-STM ($U_{Bias} = 0.1$ V, $I_T = 0.5$ nA; Figure 1 A1) and constant-potential-mode SECPM ($\Delta U = 5$ mV; Figure 1 B1). Both images show a similar shape of the molecules with a diameter of 7.5 nm (left enzyme) and 6.9 nm (right enzyme)

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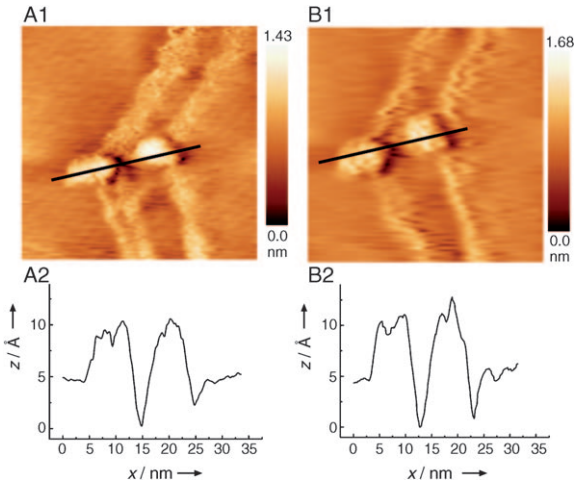

Figure 1. A1) EC-STM image and B1) SECPM image of ferritin on a HOPG electrode (40 nm × 40 nm) with corresponding line scans (A2) and (B2).

and a height of 0.5 nm relative to the HOPG flat surface (line scans in Figure 1 A2, B2). During STM measurements artifacts are sometimes observed, probably arising from tip–electrode interactions, especially when agglomerates of molecules are imaged. The same electrode area can be imaged in SECPM mode without any disturbance.

Apoferritin was also examined using SECPM and STM. SECPM images resolve single molecules (Figure 2 A) that have a diameter of approximately 12 nm (Figure 2 B), whereas the investigation by EC-STM completely failed.

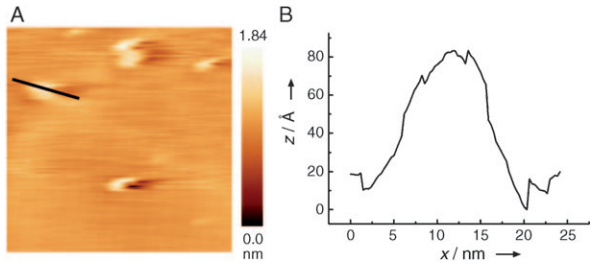

Figure 2. A) SECPM image of apoferritin on a HOPG electrode (90 nm × 90 nm) and B) corresponding line scan.

From these results we assume:

1) With SECPM it is possible to visualize the protein structure of the hollow apoferritin (diameter 12 nm), the low image contrast may result from the rather even potential distribution of the protein;
2) With STM it is not possible to image apoferritin, probably because of the low conductivity of the protein polypeptide;
3) With STM and SECPM it is possible to resolve ferritin molecules with comparable resolution. The measured diameter, however, is much smaller than that of apoferritin. We assume that the conductivity (STM) and the potential distribution (SECPM) of the iron atoms inside the cavity of the ferritin molecule dominate in the imaging process at the expense of the properties of the protein shell.

In contrast to ferritin, the redox enzyme horseradish peroxidase (HRP) contains only one iron atom which is located in the active site, the heme group.[14] Single HRP molecules were imaged in 10 mM PBS by constant-potential-mode SECPM (Figure 3 A; $\Delta U = 5$ mV). All five visible molecules exhibit an open-loop-shaped structure with mean dimensions of $54 \times 52 \times 3.2$ Å^3. The same area was also imaged in STM (Figure 3 B; $U_{Bias} = 0.1$ V, $I_T = 0.5$ nA). The four bright spots represent single molecules with dimensions of $43 \times 34 \times 2.4$ Å^3.

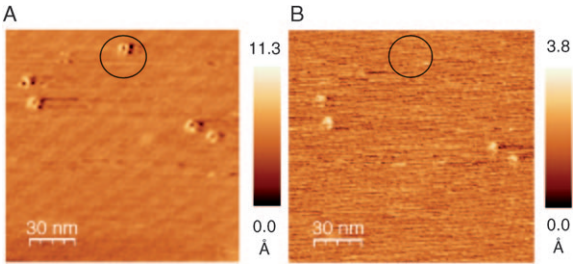

Figure 3. A) SECPM image and B) EC-STM image of HRP on a HOPG electrode.

Comparing STM and SECPM, the molecules could be resolved to a much greater extent in SECPM. Furthermore, in SECPM an additional HRP molecule in the upper part of the image (Figure 3 A, black circle) can be observed which is not visible in STM. A poor electrical contact between enzyme and electrode in STM may inhibit the electron transfer to the tip and thus prevent imaging of the molecules. Since SECPM only maps the charge distribution of the molecule no electron transfer is required.

In addition, the resolution of the STM image is relatively low, especially the size of the molecules is smaller than that reported by Zhang et al.[4] based on ex situ ($62 \times 43 \times 12$ Å^3) and in situ ($68 \times 44 \times 40$ Å^3) STM studies performed on HOPG with $I_T = 0.5$–1.2 nA. A possible explanation for this observation may be again a poor contact of the enzymes with the substrate possibly impairing the STM image. Furthermore, contributions from tip intrusion into the molecule must be taken into account. It has to be considered that in STM, applying a tunneling current of 0.5 nA means that approximately 10^9 $e^- s^{-1}$ flow through the molecule, that is, one electron per nanosecond. This flow may have a negative effect not only on the image quality, but also on the protein structure.

In a 3D representation of the SECPM image (Figure 4 A) of two molecules the open-loop structure is clearly recognizable. Figure 4 B shows the corresponding contour plot of Figure 4 A. The lines represent constant height slices with a constant height difference of 0.27 Å. From X-ray crystallographic data it is concluded that the heme is located in a pocket between the distal and the proximal domain of the molecule (Figure 4 C).[14] Comparing the 3D image and the X-ray structure it could be suggested that the open-loop seen in

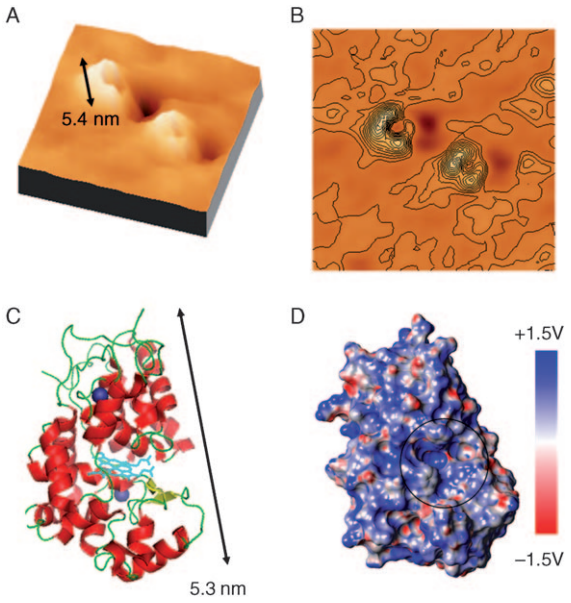

Figure 4. A) 3D SECPM image of HRP adsorbed on HOPG (expansion of Figure 3A). B) Contour Plot of (A), 45 nm × 45 nm, 10 contours with 0.27 Å nm^{-1}. C) 3D plot of the X-ray crystal structure of HRP. The heme is turquoise, the calcium atoms are blue, α-helices are red and β-sheets are yellow (plotted with PyMOL,[16] accession code 1w4w from PDB.[17]). D) Surface potential of an HRP molecule in 10 mM PBS (pH 7) simulated by YASARA.[15]

SECPM corresponds to the pocket in the protein shell where the active center of the enzyme is located. To compare the SECPM images with the charge distribution, the electrostatic surface potential of an HRP molecule immersed in 10 mM PBS (pH 7, $T = 298$ K) was simulated by YASARA (Figure 4D).[15] The potential map allows for a qualitative comparison with the SECPM data. The position of the active site in the pocket of the protein shell can also be identified in the potential map (Figure 4D, black circle) as well as in the SECPM image (Figure 4A and B).

Our results show that SECPM is a promising electrochemical scanning probe technique for mapping the charge distribution of adsorbed molecules. Single HRP molecules can be imaged with a high resolution. Since the local potential is measured in the EDL region and no current flows between tip and sample, we believe that SECPM has a great potential for the investigation of single molecules in their native environment and also under reaction conditions. The ability to perform local reactivity measurements depending on the surface potential may then open up new perspectives for studying not only electrocatalysis at redox enzymes, but also a variety of electrochemical surface science problems under in situ conditions.

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[1] A. Alessandrini, M. Salerno, S. Frabboni, P. Facci, *Appl. Phys. Lett.* **2005**, *86*, 133902.
[2] R. Sonnenfeld, P. K. Hansma, *Science* **1986**, *232*, 211.
[3] K. Itaya, E. Tomita, *Surf. Sci.* **1988**, *201*, L507.
[4] J. D. Zhang, Q. J. Chi, S. J. Dong, E. K. Wang, *Bioelectrochem. Bioenerg.* **1996**, *39*, 267.
[5] M. Wang, S. Bugarski, U. Stimming, *J. Phys. Chem. C* **2008**, *112*, 5165.
[6] M. Wang, S. Bugarski, U. Stimming, *Small* **2008**, *4*, 1110.
[7] A. J. Bard, W. R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, Wiley-VCH, Weinheim, **2001**.
[8] D. H. Woo, J. S. Yoo, S. M. Park, I. C. Jeon, H. Kang, *Bull. Korean Chem. Soc.* **2004**, *25*, 577.
[9] C. Hurth, C. Z. Li, A. J. Bard, *J. Phys. Chem. C* **2007**, *111*, 4620.
[10] C. Corbella, E. Pascual, G. Oncins, C. Canal, J. L. Andujar, E. Bertran, *Thin Solid Films* **2005**, *482*, 293.
[11] C. Baier, W. Schmickler, U. Stimming, unpublished results.
[12] R. R. Crichton, *Angew. Chem.* **1973**, *85*, 53–62; *Angew. Chem. Int. Ed. Engl.* **1973**, *12*, 57–65.
[13] R. R. Crichton, *FEBS Lett.* **1973**, *34*, 125.
[14] N. C. Veitch, *Phytochemistry* **2004**, *65*, 249.
[15] E. Krieger, T. Darden, S. Nabuurs, A. Finkelstein, G. Vriend, *Proteins Struct. Funct. Genet.* **2004**, *57*, 678.
[16] W. L. DeLano, De Lano Scientific, Palo Alto, CA, USA **2002**.
[17] "Protein Data Bank": H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235.

