Bericht zum Projekt

Spatially Resolved Activity Measurements of Enzyme Molecules on Surfaces

Im Rahmen eines Max-Buchner Forschungsstipendiums (MBFST-Kennziffer 2740)

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Summary of the project

Enzymes are highly selective biocatalysts and are often also of high catalytic activity. Understanding the mechanism of interfacial charge transfer might be the basis for identifying inorganic catalysts that can serve as electrocatalysts in fuel cells and other electrochemical devices. Therefore, enzymes have been increasingly investigated in the last decades. A present focus of scientific investigation is to study the activity of immobilized enzymes. Those activities on the integral level may not necessarily be identical to individual activities, since e.g. the number of deactivated enzymes after binding to the surface is not known. Research on enzyme behaviour on the level of single to a limited number of enzymes is still in its infancy.

Short Summary of the intermediate report (01.07.2007 – 30.04.2008):

First experiments were performed on horseradish peroxidase (HRP) directly adsorbed on an unmodified Au(111) single crystal surface. These model electrodes were investigated with different electrochemical measurement techniques such as amperometry, electrochemical impedance spectroscopy (EIS) and electrochemical scanning tunneling microscopy (EC-STM) in order to study

the adsorption behavior of HRP on the gold surface and its electrocatalytic activity for the reduction of H_2O_2 to H_2O.

The catalytic activity and the time dependence of the enzyme catalyzed H_2O_2 reduction was mainly studied by amperometric measurements. Therefore, enzymes were pre-adsorbed on the Au(111) surface for 10 minutes. In the presence of 50µM H_2O_2, the Au(111)/HRP system reached a catalytic current density of -3.3µA/cm^2 at 0.8V vs. NHE that remained constant for at least one hour.

Furthermore, in addition to EIS and STM, amperometry was used to study the adsorption of HRP from the solution to a bare Au(111) electrode. Thereby, two different adsorption processes could be identified. An exponentially decreasing adsorption of HRP on active sites takes place until all favourable sites for direct electron transfer are occupied and a monolayer is formed reaching catalytic current densities comparable to those obtained with the Au(111)/HRP system mentioned above. Then, adsorption continues, presumably with the effect of forming multilayer agglomerates that result in a decreased catalytic activity.

However, EC-STM images of the adsorption of HRP and also of preadsorbed molecules on the Au(111) surface could only be obtained with low resolution probably due to weak binding interactions between the gold surface and the enzyme.

Further studies

Extension to other model electrodes

In-situ EC-STM studies on the Au(111)/HRP system showed that HRP preferably binds to low coordinated atoms (step/edges) and to a lesser degree also on flat terraces forming an enzyme monolayer. However, a detailed and high-resolution investigation of HRP directly adsorbed on Au(111) was not possible. Since EC-SPM techniques are per-se ideally suited for measuring the spatially resolved activity of enzyme molecules on surfaces we decided to extend our model electrode systems to HRP on highly oriented pyrolytic graphite (HOPG) and to glucose oxidase (GOD) on HOPG.

Our group already examined the topological and electron transfer properties of the system HOPG/GOD with in-situ EC-STM and cyclic voltammetry earlier [1, 2]. The cyclic voltammograms (CV) showed a well-defined redox peak of the GOD molecules with an equilibrium redox potential at -470 mV vs. SCE. GOD, as possible anode catalyst in a bioelectrochemical fuel cell, catalyzes the oxidation of ß-D-glucose to D-glucono-1,5-lactone and in the presence of oxygen its reduced active site flavin adenine dinucleotide (FAD) $FADH_2$ also reduces oxygen to hydrogen peroxide.

Extension to a new EC-SPM technique: Investigation of Enzymes using SECPM

The scanning electrochemical potential microscopy (SECPM) is the latest modification of the electrochemical scanning probe microscopy setup, developed and introduced by Allen J. Bard [3] and Veeco Instruments. The hardware is similar to an EC-STM the only modification consists in replacing the current pre-amplifier by a high-impedance potential difference amplifier. That means, the tip is not used for measuring the tunneling current between tip and sample but as a potential probe. The potential difference between the tip and the applied potential at the working electrode is measured with the potential amplifier and serves as feedback signal in the x-y scanning mode. It offers *in-situ* imaging of samples immersed either in electrolyte solutions or in polar liquids with a resolution of some

nanometers. As the potential changes with the distance across the electrochemical double layer SECPM can also be used to locally map the potential profile of the working electrode. Since the tip scans in the double layer regime SECPM allows contactless surface imaging. Therefore, it can be an important development in SPM techniques especially for non-invasive investigations of biological samples such as DNA, proteins or enzymes deposited on smooth surfaces.

New approach for protein immobilization

SPM techniques have made it possible to characterize or directly observe charge transfer through organic molecules down to the nanoscale and even single molecule levels. For metalloproteins and enzymes however, this approach still remains challenging since difficulties often arise from the stable immobilization of the proteins to the surface, the retention of the biological functions and a sufficient charge transfer between the electrode and the protein. Freshly cleaved HOPG offers only few active sites for protein binding as its surface is hydrophobic. In order to change hydrophobicity into hydrophilicity and to create binding sites for the enzyme molecules (Fig. 1A), the HOPG was electrochemically oxidized in 0.1M $HClO_4$ by performing three potential sweeps from 0.2 to 2.1V vs. NHE. However, even after this pre-treatment step the enzyme coverage on the electrode surface was relatively low (Fig. 1B), therefore, we used carboxyphenyl groups as biocompatible bridge between the protein and the electrode surface in order to achieve coverages of up to a full monolayer of proteins and a fast electron transfer.

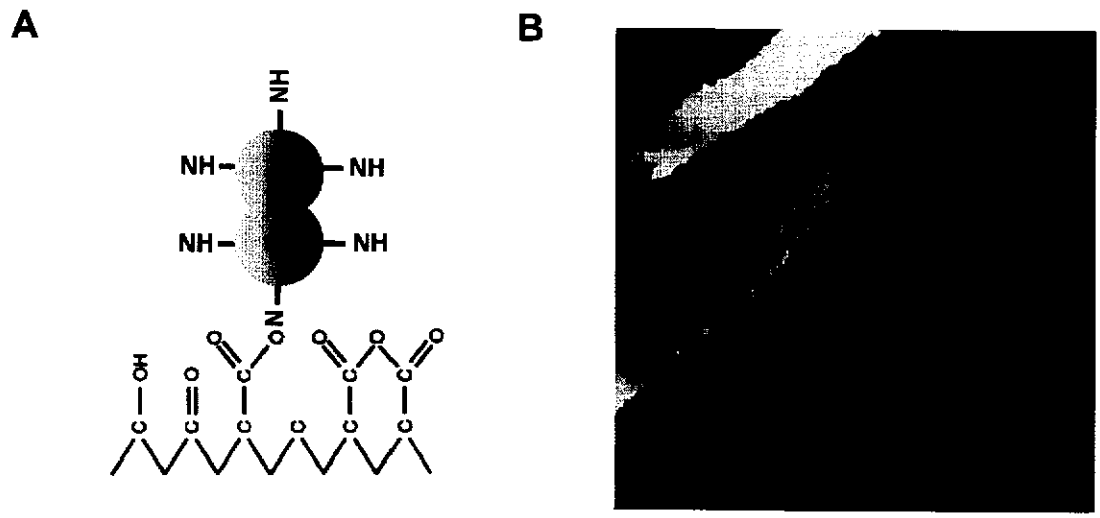


Figure 1: (A) Schematic drawing of the binding mechanism between the oxidized HOPG surface and the amine groups of a protein. (B) EC-STM image of GOD molecules adsorbed on oxidized HOPG.

New approach for electrochemical enzyme detection

A new method was developed in order to be able to electrochemically detect also redox-inactive enzymes e.g. papain, a member of the cysteine protease family, using tailor made ferrocene affinity labels (FcAL) [4]. Thereby, the affinity label concept is adopted from optical detection principle where fluorescence affinity labels are used to locate enzyme molecules and to determine their catalytic

activity [5]. For electrochemical detection an electroactive marker, in this case a ferrocene moiety, is tagged to the synthesized affinity label. Ferrocene labeled papain molecules (FcAL@papain) immobilized on HOPG were investigated using cyclic voltammetry. These ferrocene-based affinity labels are ideally suited since they combine the electrochemically active ferrocene moiety which is stable under physiological conditions with the selectivity and activity sensing properties of the affinity label concept.

Results

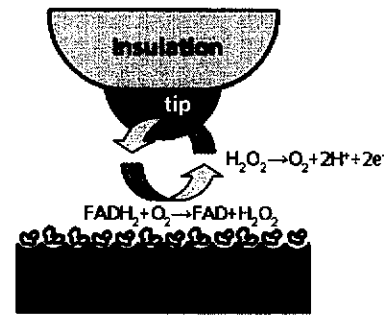
Probing the activity of enzymes using SPM tip as a local current sensor

It has been shown by our group that the EC-STM tip can be used as a local sensor for the investigation of the hydrogen evolution reaction (HER) occurring at a single Pd nanoparticle [6].

This sensing technique can also be applied for similar enzyme catalyzed reactions occurring at the electrode surface, assuming that the number of enzymes and the rate constant are high enough so that the current would be detectable. The reaction of interest in this study will be the reduction of oxygen to hydrogen peroxide by the enzyme GOD. Experiments for local sensing of the catalytic activity of GOD have shown that in the presence of oxygen, the reduced active site of GOD $FADH_2$ reduces oxygen to hydrogen peroxide. Hydrogen peroxide can then be oxidized at the potential controlled STM-tip. In other words the activity of GOD is directly measured by detecting the Faradaic currents that are produced by the reaction product hydrogen peroxide (Scheme 1).

$$GOD(FADH_2) + O_2 \rightarrow GOD(FAD) + H_2O_2$$

$$H_2O_2 \xrightarrow{electrode} O_2 + 2H^+ + 2e^-$$



Scheme 1: Current sensing using the EC STM tip as local probe.

Figure 2A shows the CV of the GOD/HOPG (left axis) and the measured current flowing through the tip which was positioned 10nm above the electrode surface (right axis). While the electrode potential was varied the tip potential was kept constant at 300mV vs. NHE. Figure 2B shows the measured peak current at the tip for different distances between tip and electrode surface. With increasing distance the current decreases significantly.

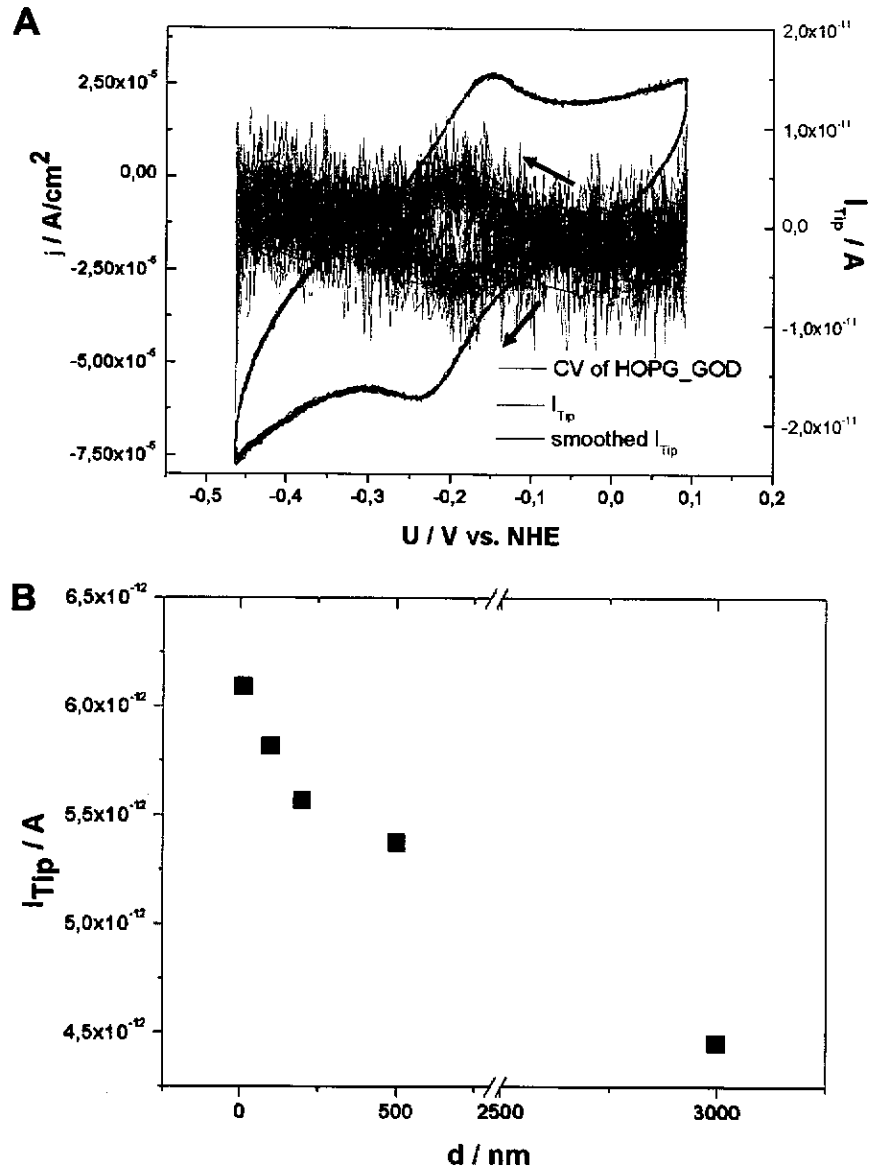


Figure 2: (A) The EC-STM tip was used as local sensor in order to detect faradaic currents due to hydrogen peroxide formation. Black curve: CV of the GOD/HOPG electrode. Green: measured current at the tip. (B) Detected Faradaic current at the tip in dependence of the distance between tip and GOD/HOPG surface.

SECPM on enzymes

We could show that SECPM is especially suited for the investigation of organic and biological molecules adsorbed on electrode surfaces under electrochemical conditions. In a recently published paper we report on the constant potential mode SECPM to image single enzyme molecules adsorbed on HOPG electrode surfaces [7]. Besides the iron storage protein ferritin and its iron free conformation apoferritin the redox enzyme horseradish peroxidase (HRP) was investigated. SECPM results were compared to a STM study.

A, Ferritin and Apoferritin

In the case of ferritin which consists of a protein shell with a diameter of 12nm containing up to 4500 Fe(III) ions in a core [8] and apoferritin which only consists of the hollow protein shell [9] a clear difference in the SECPM images was found. While SECPM was able to visualize the protein structure of the hollow apoferritin with a diameter of 12 nm (Figure 3), the investigation of the apoferritin electrode via EC-STM completely failed. Whereas, EC-STM and SECPM both only resolved the iron core of up to 8nm in diameter for the ferritin molecules (Figure 4). We assume that the potential distribution (SECPM) of the iron atoms inside the cavity of the ferritin molecule dominate in the imaging process and thus superimpose upon the properties of the protein shell.

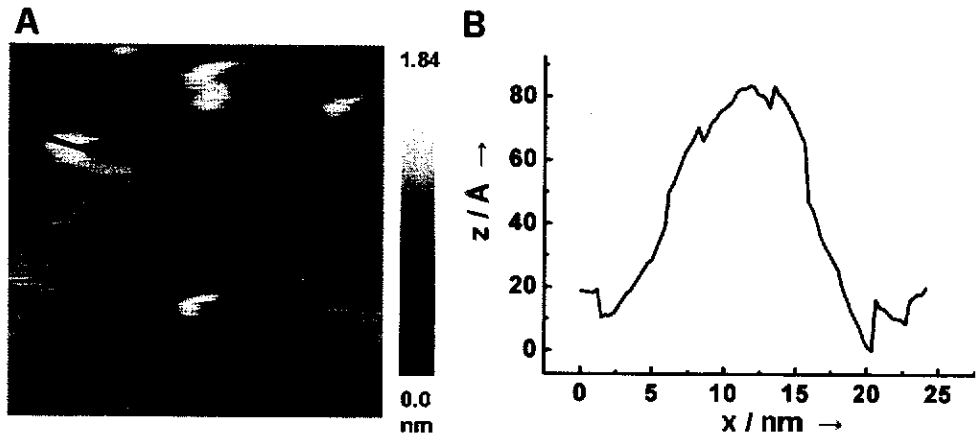


Figure 3: (A) SECPM image of apoferritin on HOPG (90 x 90 nm^2) and (B) corresponding line scan.

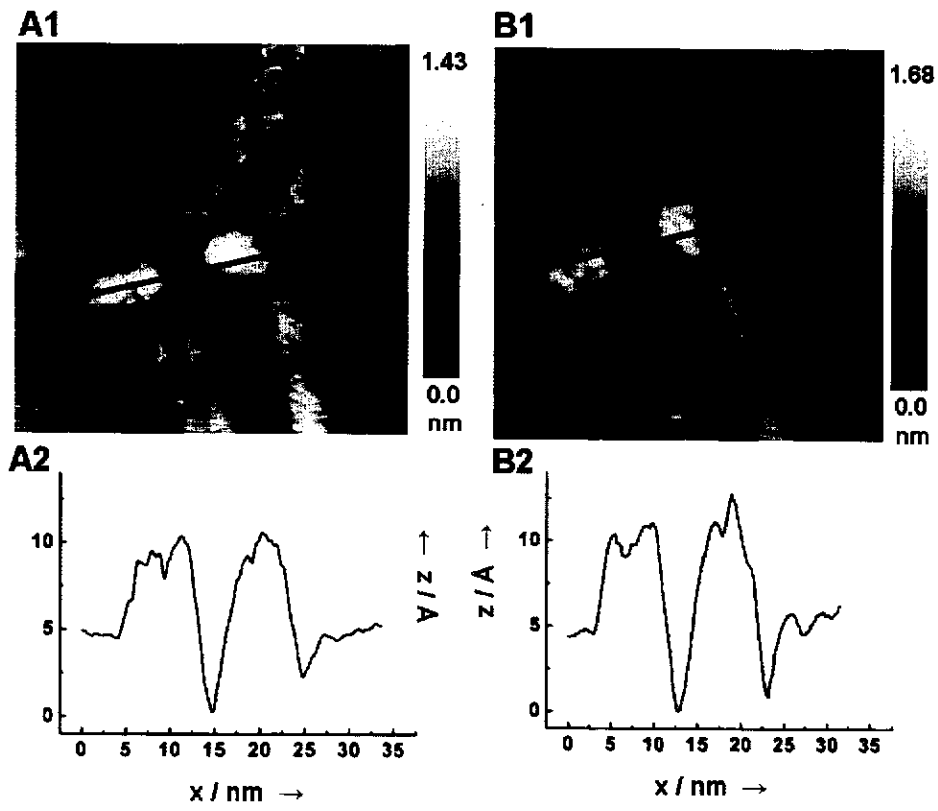


Figure 4 (A1) EC-STM image and (B1) SECPM image of ferritin on a HOPG electrode (40nm x 40nm) with corresponding line scans (A2) and (B2).

B, Horseradish peroxidase (HRP)

In contrast to ferritin, HRP contains only one iron atom located in the active site, the heme group [10]. Figure 5 A shows a SECPM image of five HRP molecules adsorbed on HOPG. In SECPM mode all five visible molecules exhibit an open-loop-shaped structure with mean dimensions of 54 x 52 x 3.2 $Å^3$.

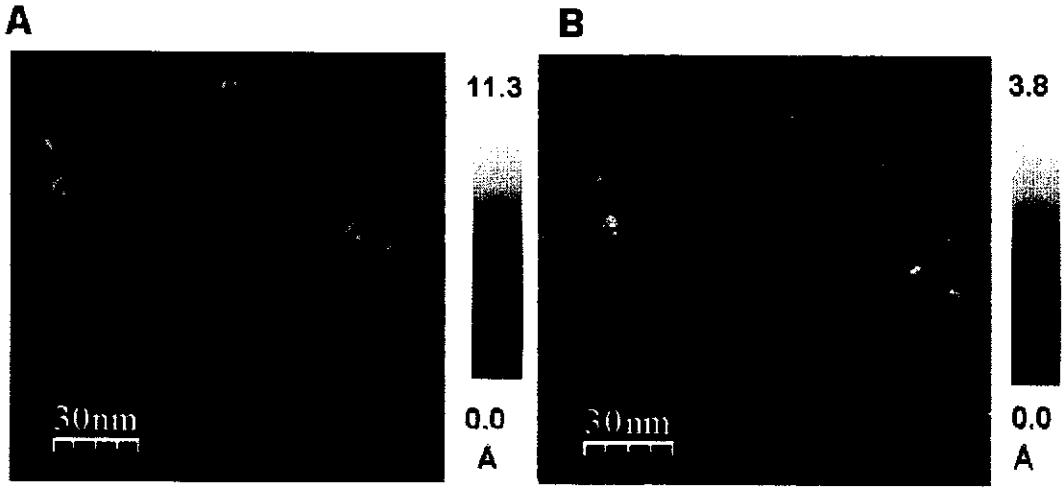


Figure 5: (A) SECPM image and (B) EC-STM image of HRP/HOPG electrode.

The same area was also imaged in STM (Figure 5B U_{Bias} = 0.1 V, I_T = 0.5 nA). The four homogeneous bright spots represent single molecules with dimensions of 43 x 34 x 2.4 $Å^3$. 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 5A black circle) can be observed which is not visible in STM. A poor electrical contact between enzyme and electrode 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. Furthermore, contributions due to tip intrusion of the molecule moiety must be taken into account. One has to consider that applying a tunneling current of 0.5 nA in STM means that approximately 10^9 e^-/s flow through the molecule or one e^- passes every ns. This 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 6A) of two HRP molecules the open-loop structure is clearly recognizable. Figure 6B shows the corresponding contour plot of Figure 6A. The lines represent constant height slices with a constant height difference of 0.27 Å. From X-ray crystallographic data [10] it is concluded that the heme is located in a pocket between the distal and the proximal domain of the molecule (Figure 6C). Comparing the 3D image and the X-ray structure one could suggest that the open-loop seen in SECPM corresponds to the pocket in the protein shell where the active center of the enzyme is located. In order 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 using the software package YASARA [11] (Figure 6D). 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 6D black circle) as well as in the SECPM image (Figure 6A and Figure 6B).

Our results show that SECPM is able to image biomolecules under in-situ conditions with an unprecedented resolution. SECPM seems to be a promising electrochemical SPM technique for mapping the charge distribution of adsorbed molecules.

Comparing SECPM images with EC-STM images indicates that a potentiometric technique such as SECPM may clearly be advantageous for imaging enzymes at the solid-liquid interface and has the potential to investigate their dynamic behavior on the surface.

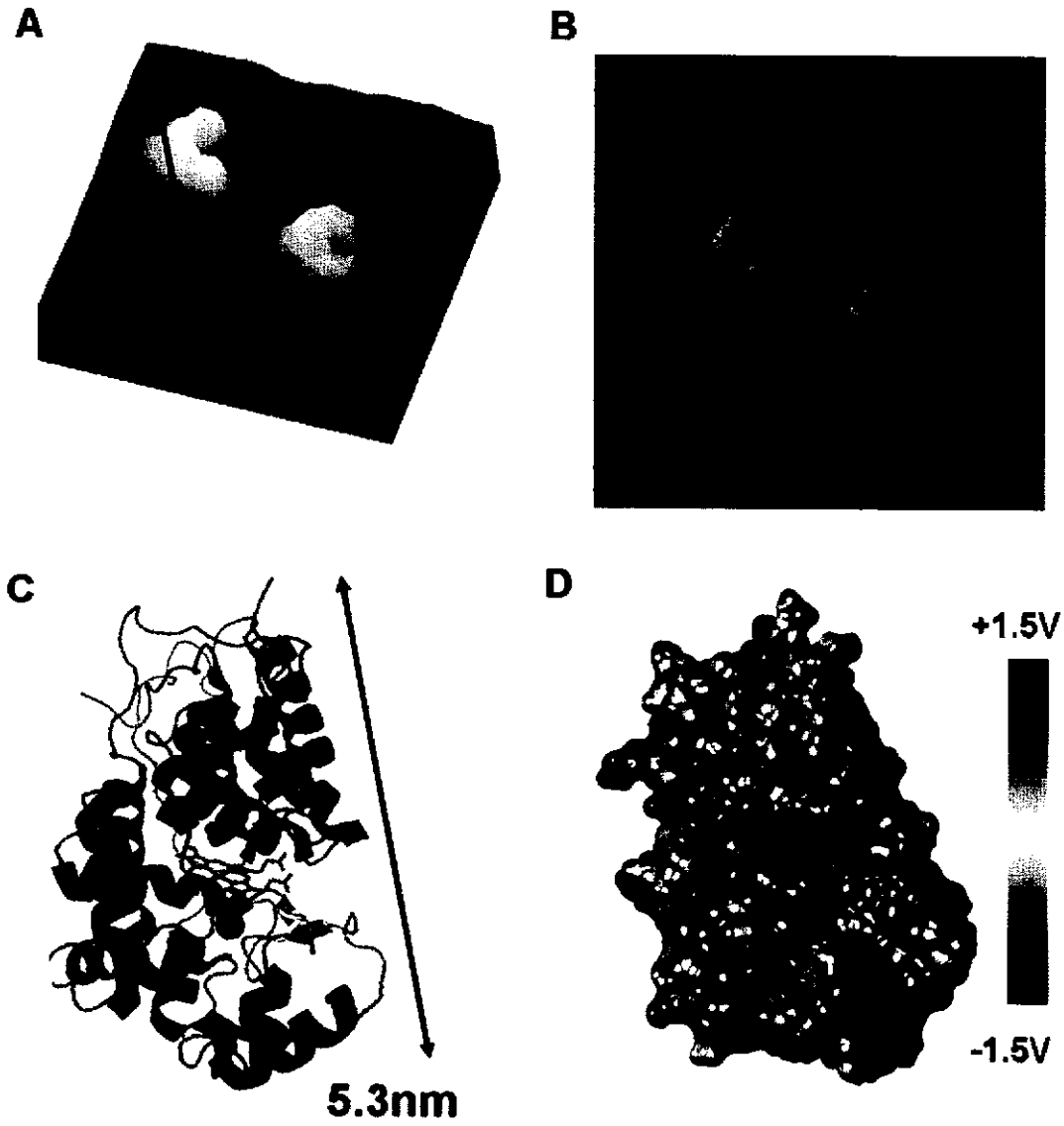
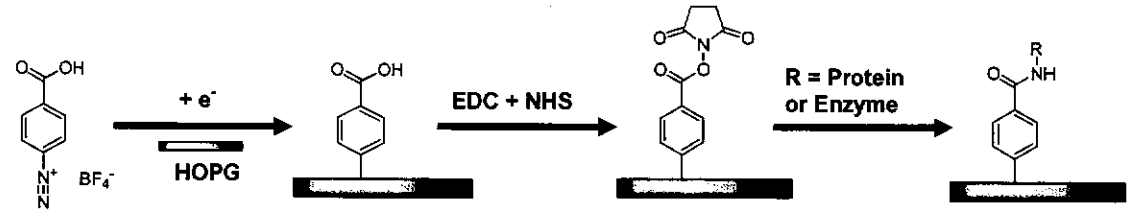


Figure 6: (A) 3D SECPM image of HRP adsorbed on HOPG (Zoom in of Fig. 3A). (B) Contour plot of (A) (45 x 45nm^2), 10 intermediate contours with 0.27 Å/nm. (C) 3D plot of the X-ray crystal structure of HRP. The heme is colored in turquoise, the calcium atoms are shown in blue, α-helical and β-sheet regions are shown in red and yellow (plotted with PyMOL [12], accession code 1w4w from PDB [13]). (D) Surface potential of an HRP molecule in 10 mM PBS (pH 7) calculated with the software YASARA [11].

Improvement of the Immobilization of Proteins and Enzymes on HOPG



Scheme 2: Covalent immobilization of proteins and enzyme on carboxyphenyl terminated HOPG surfaces using EDC and NHS.

A novel carboxyphenyl covalent immobilization technique for proteins on carbon supports which was recently introduced for heme proteins bound to glassy carbon [14], has been successfully adopted to the HOPG electrode surface. Thereby, the surface is functionalized with aromatic 4-carboxyphenyl groups by the electrochemical reduction of diazonium cations. Proteins and enzymes can then be attached to the functionalized HOPG through the covalently binding between $-NH_2$ terminal groups of the proteins and –COOH terminal groups of the 4-carboxyphenyl (Scheme 2). Preliminary results with the metalloprotein azurin showed that this technique leads to an increase in the protein coverage on the electrode surface up to a complete monolayer and furthermore, to a significant enhancement (by two orders of magnitude) in the direct electron transfer between electrode and protein.

Electrochemical Detection of Papain using FcAL

Pristine and ferrocene-labeled papain molecules (FcAL@papain) are adsorbed on highly oriented pyrolytic graphite (HOPG) and are electrochemically investigated using cyclic voltammetry in 10mM PBS (pH 7) with a scan rate of 100mV/s. CVs obtained for both electrodes are shown in Figure 8. As expected, the native papain molecules showed no redox behavior (black line), whereas a significant redox behaviour of the FcAL@papain molecules with distinct oxidation and reduction peaks (red line) could be observed. Preliminary results (not shown here) indicate that it is also possible to measure the catalytic activity of papain using the FcAL.

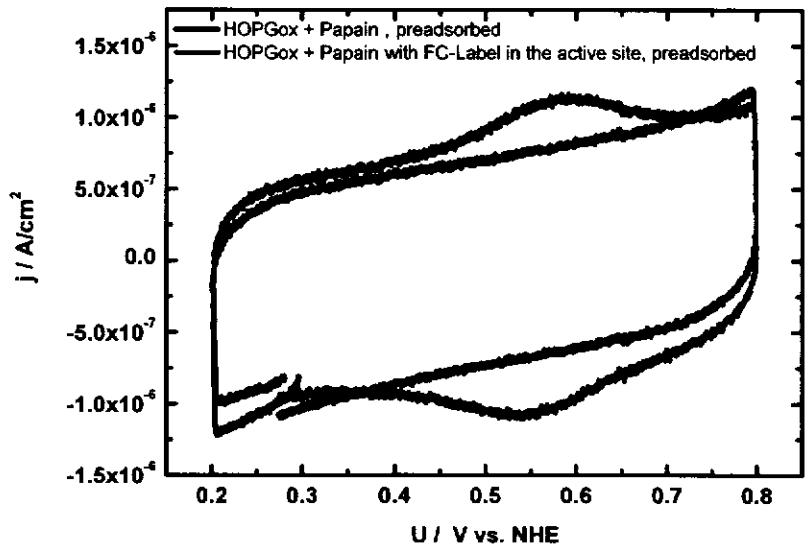


Fig. 8: Cyclic voltammograms of crude (black line) and ferrocene-labeled (red curve) papain adsorbed on HOPG.

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