

Chemical imaging of biological systems with the scanning electrochemical microscope

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Abstract

A brief overview on recent advances in the application of scanning electrochemical microscopy (SECM) to the investigation of biological systems is presented. Special emphasis is given to the mapping of local enzyme activity by SECM, which is exemplified by relevant original systems.

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1. Introduction, an overview

One of the most important and challenging area of analytical chemistry is the in-vivo or in-vitro collection of chemical information from targets of biological origin. The heterogeneity of biological systems requires in many cases a localized, small area measurement of specific analytes that eventually permits a better understanding of the function of the living organs investigated. The obvious spatial prerequisite is often coupled with high temporal resolution and minimal perturbation of the assessed microenvironment by the analytical technique. All these requirements were essential driving forces leading ultimately to the development of miniaturized analytical probes, in particular electrochemical microsensors. The miniaturization of electrodes most likely has its historical roots in the implementation of voltage-sensitive hand-pulled electrolyte-filled glass micropipettes used for the measurement of cell-membrane characteristics [1]. This technology has been used for the preparation of micropipette type ion-selective electrodes and allowed the successful miniaturization of potentiometric glass electrodes [2–4] to tip size as small as 0.1 μm , feasible for intracellular measurements. Later the discovery of ionophores (ion-

selective complexing agents) enlarged the range of ions that could be assessed and in parallel guidelines regarding the preparation and use of the ionophore based potentiometric ion-selective microelectrodes were also addressed [5,6]. Although there are evidences for the early implementation of voltammetric microsensors in biomedical research such as oxygen tension measurements in the brain of cats [7], their application and development experienced in general a significant delay compared to potentiometric microelectrodes. In the early 1970s, Adams recognized that several neurotransmitters can be easily oxidized on carbon electrodes, and thus assessable by voltammetric techniques [8–10]. This discovery associated with the need for spatially localized measurements in biological systems has given a major boost to the development of voltammetric ultramicroelectrodes [11–13]. Therefore research in this area became also very active in the late 1970s inducing a tremendous progress in the theory, application and preparation of voltammetric ultramicroelectrodes (UME) [14,15]. The development of scanning electrochemical microscopy (SECM) by Bard's group [16,17] contributed essentially to the versatility of voltammetry with ultramicroelectrodes, significantly broadening their field of application [18].

Scanning Electrochemical Microscopy is a non-contact scanning probe technique that uses an electrochemical microsensor (electrochemical tip) for imaging targets (analyte, substrate) of different types. The target can be a solid surface

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or a liquid. During controlled spatial movement of the micro-sensor the electrochemical signal, recorded either in voltammetric or potentiometric mode, is rendered to its geometrical coordinates. In voltammetric mode, the diffusion layer and implicitly the steady state current of the tip in the solution of a redox species (mediator) is perturbed by the close proximity of the target and this phenomena is exploited to obtain the topographic image of the target surface [16,19,20]. Non conductive targets simply hinder the diffusion of the mediator to the tip causing the decrease of the current as the tip approaches the surface of the target (*negative feedback*). On the contrary if the target is electrically conducting an amplification of the steady state current, strongly dependent on the tip to target distance, is observed due to the redox recycling of the mediator by the target (*positive feedback*). This effect has been exploited for the electrochemical detection of a single molecule, which is the ultimate detection limit of any analytical technique [21]. The resolution of the topographic imaging was spectacularly improved by reducing the size of the tip and/or using constant distance imaging mode. In this later mode, feedback loops based on shear force (detected by the frequency change of a vibrated tip) [22,23] or atomic force (hyphenated SECM-AFM instruments) [24,25] are employed to preserve a constant distance between the tip and the target. Using thin film of water on mica, formed in high relative humidity atmosphere, allowed the topographic imaging of biological macromolecules such as deoxyribonucleic acids (DNA), keyhole limpet hemocyanin, mouse monoclonal IgG and glucose oxidase with a resolution of ~ 1 nm [26]. Fortunately the scanning electrochemical microscope is more than just a topographic imaging device given that in the area of topographic imaging SECM encounters harsh competition from other scanning probe techniques such as AFM and scanning tunneling microscopy (STM), which are providing much better, in favorable cases even atomic resolution. The main advantage of using SECM is the possibility to map surface reactivity, and thus the assessment of surface reaction kinetics and the chemical imaging of surfaces [17].

The application of SECM to monitor targets of biological origin started in the early 1990s involving topographic imaging of leaf surfaces as well as the determination of local oxygen concentration changes due to photosynthesis [27]. Since then a variety of targets (species) of biological origin including enzymes [28–30], antibodies [31,32], cells [22,33,34], DNA [35–37], etc., have been the subject of SECM studies. The application of SECM in biomedical sciences follows in a certain extent the early line of microelectrode applications, such as metabolism studies and monitoring neurotransmitter secretion of single cells. However, advances in the SECM instrumentation allows now the combined topographical and electrochemical studies of soft biological preparations using a shear-force dependent tip-to-target distance control system [22]. Also the specific SECM feedback theory offers original approaches for cell study such as the ingenious method for

the determination of the redox activity of individual living cells [38]. A growing number of biomedical applications regards the imaging and characterization of immobilized, micropatterned biomolecules with respect of the development of microanalytical systems [30]. The attractiveness of this research direction is not surprising taking into account the popularity and importance of miniaturized analytical chips for DNA analysis [39–45] and immunosensing [46–50]. The role of SECM in this area seems to be primarily the characterization and optimization of sensor structures and detection strategies. Presently routine applications for analysis are limited by the low scanning speed and the usually tedious tip-to-target distance adjustments that have not been completely overcome yet. With a few exceptions [35–37] in SECM the detection of biomolecular interactions is based on enzyme labeling and therefore on the localized measurement of the catalytic activity of enzymes. The assessment of enzymatic activity is also important for the optimization of the immobilization and functional parameters of enzyme sensors [51]. Imaging of local enzyme activities require the availability of electrochemically detectable compounds indicative of the biocatalytic reaction, which is not a difficult task considering the plethora of enzyme electrodes developed in the last 40 years [52, 53]. In many cases the biocatalytic activity, induces changes in the local concentration of molecules or ions readily assessable by electrochemical means, such as oxygen, hydrogen ion or hydrogen peroxide. Moreover, in the case of oxidoreductase enzyme, artificial mediators with ideal electrochemical reversibility that enhances the sensitivity and reproducibility of the measurements, can be used [29]. If there is no electrochemically detectable species involved in the enzymatic reaction, the natural enzyme substrates can be conveniently replaced by synthetic ones that allow the electrochemical monitoring of the progress of the biocatalytic reaction [54–56]. Thus, the mapping of biological samples and in particular targets with biocatalytic activity attracted a lot of interest in the past and this line of research is expected to further grow in importance. In the following sections of this contribution several aspects of imaging biological systems with SECM both in potentiometric and amperometric modes are discussed. Original examples are given to demonstrate the usefulness of SECM in this area.

2. Experimental

2.1. Chemicals and reagents

Acetylcholinesterase enzyme (EC 3.1.1.7, from electric eel, type III as a solution containing 5 mg of ammonium sulfate per mg of protein), glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*), acetylcholine chloride, glucose, bovine serum albumin, cystamine dihydrochloride and glutaraldehyde were purchased from Sigma (St. Louis, MS, USA). The 18-mer amino and thiol modified complemen-

tary oligonucleotids, with analogous sequence to the one located in the 662.–645. position of the *Escherichia coli* *lexA* gene (H_2N -5'-CGTCAGCAGAGCTTCACC-3' and HS-5'-GGTGAAGCTCTGCTGACG-3'), were obtained from the Oligonucleotid Synthesis Group of the Biological Research Center of the Hungarian Academy of Sciences (Szeged, Hungary). All reagents used for the preparation of the solutions were of analytical grade (Fluka, Buchs, Switzerland or Reanal, Budapest, Hungary). The Inorganic Chemistry Department at the Technical University of Budapest generously provided the antimony shuts.

2.2. Preparation of the electrochemical tips

For the preparation of the pH sensitive antimony tips the procedure described in Ref. [57] was used. Briefly antimony shuts were introduced in a thick wall Pyrex® test tube and melted ($t > 630^\circ\text{C}$). The melted antimony was sucked in the lumen of a glass tube with 7 mm external and 1 mm inner diameter. The tube was hand pulled in acetylene-air flame to obtain glass capillaries with outer diameters of about 1 mm. These capillaries were subjected to mechanical pulling with a vertical pipette puller (David Kopf, Model 720, Tujunga, CA, USA) that provided the final diameter of the antimony tips. The resulted micropipette shaped antimony tips were glued in 1 mm inner diameter glass capillaries and the electrical contact between the antimony and a copper wire was established using silver epoxy glue. Platinum tips were prepared by sealing a platinum wire of 5 or 25 μm diameter into a ~ 5 cm long glass capillary followed by polishing the glass to reveal the platinum disk and then conical beveling [57].

2.3. Preparation of the targets

For SECM imaging of the enzymatic activity of acetylcholinesterase, the enzyme was immobilized in the tip (20 μm inner diameter) of a micropipette. A mixture of 1 mg bovine serum albumin (BSA), 10 μl of acetylcholinesterase (AChE) (930 IU/ cm^3), 10 μl pH = 8 phosphate buffer and 1 μl of 5% glutaraldehyde was introduced in the tip of the micropipette by capillary action and allowed to react (cross-link) for 15 min.

To create patterns of glucose oxidase (GOx), ultramicroelectrode arrays (UMEA) fabricated by thin film technology (photolithography) have been used. The details of the fabrication process are described in detail elsewhere [58]. The UMEAs consisted of 190 electrically interconnected gold ultramicroelectrodes of $20 \times 20 \mu\text{m}^2$ area displaced in a hexagonal arrangement on a 1.7 mm diameter disk shaped substrate. The spacing between the nearby electrodes was 80 μm . Glucose oxidase was immobilized onto the gold ultramicroelectrodes, by first reacting the gold surface with 50 mM cystamine dihydrochloride for 1 h [59] and then covalently attaching the enzyme to the amino group of the cystamine using glutaraldehyde. The cross-linking was performed in two steps: first the microelectrode array was

placed in 2.5% glutaraldehyde solution for 1 h and after removing the excess of glutaraldehyde an enzyme solution of 70 IU/ml was used to modify the surface. This procedure after thorough rinsing ensured a GOx layer localized on the surface of the microelectrodes.

Single strand oligonucleotides were immobilized onto the same type of UMEAs [58] as used for the GOx immobilization that were prior to the modification electrochemically activated and rinsed with deionized water. Two microliters of thiol modified oligonucleotide solution (0.9 nmol) prepared with 0.02 M triethylammonium-acetate buffer was injected onto the surface of the microelectrode array and allowed to react for 1 h in high relative humidity atmosphere (to avoid the evaporation of the drop). The electrodes were then thoroughly rinsed to remove the excess of oligonucleotide.

2.4. Hybridization and enzyme labeling of the oligonucleotides

Four μl (1.8 nmol) of the complementary oligonucleotide strand solution (prepared with 0.1 M phosphate buffer, pH = 7.4) was dropped onto the oligonucleotide modified surface and then 15 min was allowed for the hybridization. To detect the hybridization, the complementary oligonucleotide strand was covalently labeled with GOx. Therefore after hybridization, the surface of the UMEA was thoroughly rinsed and then contacted for 1 h with 2.5% glutaraldehyde solution to react with the amino group of the complementary oligonucleotide. After removing the excess of glutaraldehyde, 5 μl glucose oxidase solution (1000 IU/ cm^3 prepared with pH = 5.8, 0.1 M phosphate buffer) was injected onto the activated surface. The reaction was performed in water saturated atmosphere for 1 h and then the surface was rinsed again.

2.5. SECM imaging

SECM images were recorded with a laboratory made scanning electrochemical microscope based on three high resolution stepper motor driven translation stages (Newport, Evry, France) and a PGSTAT 10 (Ecochemie, Utrecht, The Netherlands) equipped with a EDC preamplifier for low current measurements. The data acquisition and the positioning system were controlled by self-developed software operating on a Pentium II personal computer. For potentiometric measurements a custom designed high input impedance voltmeter was used ($Z_{\text{in}} = 10^4 \text{ G}\Omega$).

The catalytic activity of immobilized acetylcholinesterase enzyme was monitored in 2 mM acetylcholine solution (1 mM, pH = 8.5 PBS) by potentiometric technique using a 15- μm diameter (pH sensitive) antimony tip. The target (AChE immobilized in the tip of a micropipette) was introduced through the 2 mm diameter hole drilled in the

bottom of a 4-ml volume SECM cell made of Plexiglass. It was fixed in an upright position (perpendicular on the XY plane with the tip at a distance of 1 cm above the base of the cell) then the base of the cell was sealed and the solution was dispensed in the cell. The mapping of local AChE activity was based on monitoring of pH changes in the enzyme's microenvironment induced by the acetic acid generated during the enzymatic hydrolysis of acetylcholine.

For glucose oxidase activity measurements the micro-patterned target was fixed in the bottom of a 4-ml volume cell filled with 130 mM D-glucose solution prepared with 0.1 M phosphate buffer (pH = 5.8). The enzyme activity was followed by detecting the hydrogen peroxide generated during the enzymatic oxidation of glucose at 700 mV vs. Ag/AgCl using either 5 or 25 μm diameter platinum tips.

3. Results and discussions

3.1. Imaging of biological systems by scanning potentiometric microscopy

The use of ion-selective potentiometry in SECM offers a selective detection technique for ions that are difficult to assess by voltammetric means, such as calcium, hydrogen, sodium, ammonium, etc. In contrast with voltammetric tips, potentiometric microelectrodes are passive tips, which mean that the detection does not require the conversion of the detected component at the electrode and feedback effects cannot be generated. These particularities of the potentiometric operation mode can represent advantages or disadvantages depending on the type of application. For instance, the absence of feedback effects impeded their use for tip-target distance determination; however, it also eliminates the contribution of surface conductivity of the target on the recorded signal when measuring enzyme activity. There are

several methods to determine the tip to target distance in potentiometric SECM such as by using an additional amperometric electrode (double barrel electrodes) [60] or solution resistance approach curves [61]. In particular cases such as the pH sensitive antimony electrodes amperometric approach curves based on oxygen reduction in air saturated buffer solution can be used to determine the tip to target distance and then after switching to potentiometric operation mode it allows the mapping of pH profiles in the solution [62]. Since an enormous number of chemical, electrochemical and biochemical processes are associated with pH changes the importance of "pH microscopy" [63] cannot be overestimated.

Many enzyme reactions and metabolic processes of microorganisms induces pH changes [62]. However, when measuring local pH changes the buffer capacity of the sample solution is of essential importance with respect of the magnitude of the signal. Therefore the analytical use of enzyme systems based on detection of pH changes is generally limited to the determination of enzyme inhibitors, where the enzyme assay can be performed in ideal conditions, separated from the incubation in the real sample [64]. These inhibitory systems are important especially in environmental analysis where pesticides and heavy metals, etc., can be assessed based on their inhibitory effect on selected enzyme systems. In a previous paper, the development of a fast response potentiometric acetylcholine biosensor was reported [65]. Since AChE catalyzes the hydrolysis of acetylcholine generating choline and acetic acid, the enzymatic activity could be determined based on pH measurement [51,66] and the effect of the buffer capacity on the response of the enzyme electrode was studied [65]. As an extension of that work the three-dimensional pH profiles generated by the activity of immobilized AChE has been assessed by SECM. Fig. 1 presents the electrode potential of a pH-sensitive antimony tip that maps pH changes in the

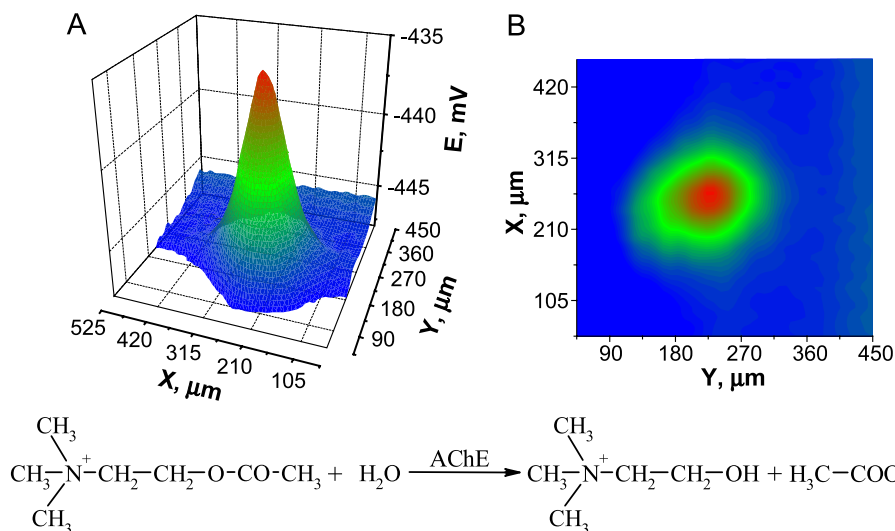


Fig. 1. Mapping the local activity of an immobilized acetylcholinesterase target (localized in the 20 μm diameter tip of a microcapillary) with an antimony tip in a 2 mM acetylcholine solution prepared with 1 mM, pH = 8.5 phosphate buffer: (A) three dimensional image, (B) two dimensional topographic image.

microenvironment of the immobilized acetylcholinesterase target in a poorly buffered solution. The center of the image, corresponding to the location of the immobilized enzyme, shows higher electrode potentials indicating the decrease in pH due to the biocatalytic reaction.

This system is rather complex since the pH change, indicative of the enzymatic activity, should be interpreted by taking into account also the buffer system (type, capacity, pH) and the geometry of the setup. Numerical methods have been extensively used for the quantitative treatment of different complex processes investigated by SECM [67]. Since we were interested not only in the visualization but also in a quantitative assessment of the enzymatic activity of surface confined enzymes we proposed to use numerical methods for the interpretation of the data. Therefore we have developed a digital simulation software based on the method of finite differences applicable even for complex three dimensional geometries. Performing the simulation under the same conditions as the experiments were performed and correlating the experimentally (Fig. 1) and simulated local pH changes resulted from the biochemical activity of AChE immobilized in the tip of a microcapillary (Fig. 2), provided an average enzymatic activity of 300 U/cm³ at 25 °C. The simulated pH change along the symmetry axis of the microcapillary can be used to optimize the reaction layer thickness of a microsensor to give maximal pH change (sensitivity) [68].

Considering the importance of electrolyte concentration in biological systems, presently the application of potentiometric tips in SECM is under its real potential. Forward pointing SECM applications such as studies of osteoclastic bone resorption (Ca²⁺) [69], secretion studies of epithelial cell monolayers (Cl[−]) [70], potential dependent switch off-switch on of urease activity (NH₄⁺) [71] have been proposed. There are several aspects, which beside the development of SECM instrumentation can increase the weight of potentiometric tips in SECM studies. Thus, advances in the field of ion-selective potentiometry makes feasible the construction of more robust solid internal contact electrodes, that could overcome handling difficulties experienced with the fragile micropipettes [72–74]. Moreover, recently spectacular improvements of the detection limit of ion selective electrodes (down to pM) have been reported [75–78]. The normally observed detection limits (μM) were determined by the leaching of primary ions from the ion-selective membrane into the sample solution that increased the surface concentration of the detected ion. The improvements are based on the elimination of this undesired leaching, the existence of which was eventually proved also by SECM studies [79].

3.2. Amperometric mapping of enzyme activity

A large number of bioanalytical methods such as immunoassays are based on the detection of biorecognition events by means of enzyme labels [80]. The major advantage of using enzyme labels is the amplification effect provided by

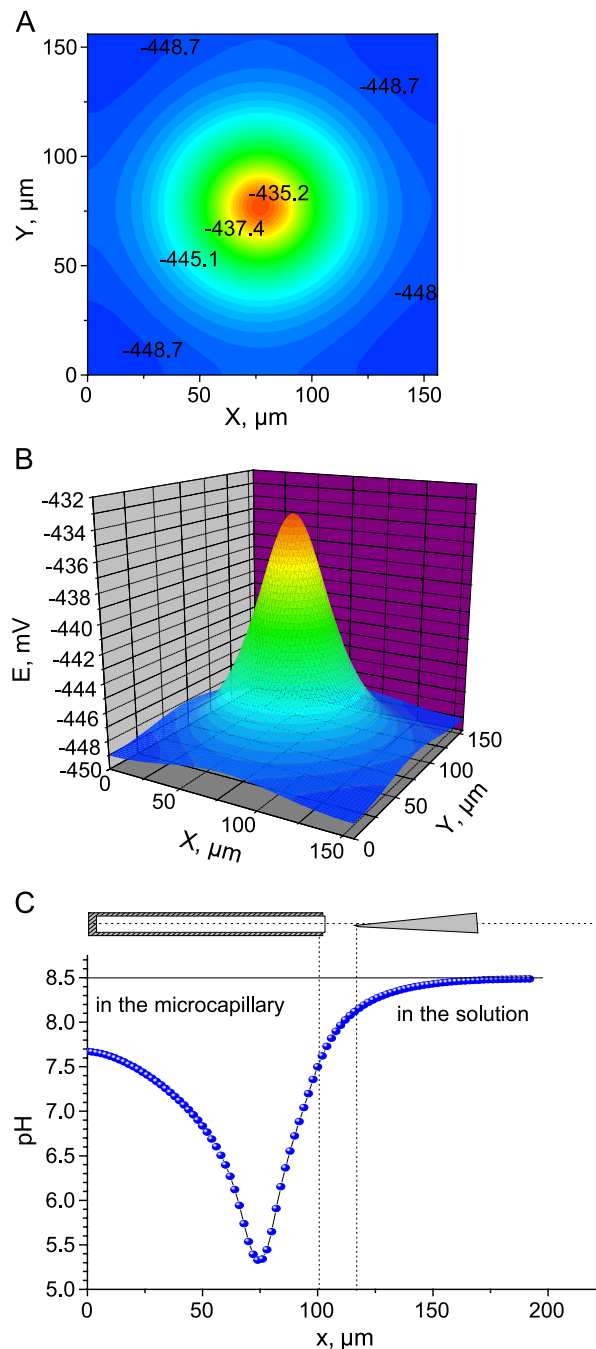


Fig. 2. Three-dimensional simulation based on the finite differences method (2 μm spatial and 7×10^{-5} s temporal resolution) of the system presented in Fig. 1 (A) topographic (B) three-dimensional and (C) pH profile along the symmetry axis of the enzyme containing microcapillary.

their biocatalytic activity. At substrate saturation this amplification effect, that depends on the turnover rate of the enzyme (for glucose oxidase ~20,200 mol/min per mol of enzyme) [81] and the amount of immobilized enzyme, results in the generation (or consumption) of a large number of detectable molecules. Therefore methods based on enzyme labels such as enzyme-linked immunosorbent assay (ELISA) are widely used to detect trace amounts of substances with clinical or environmental importance. The

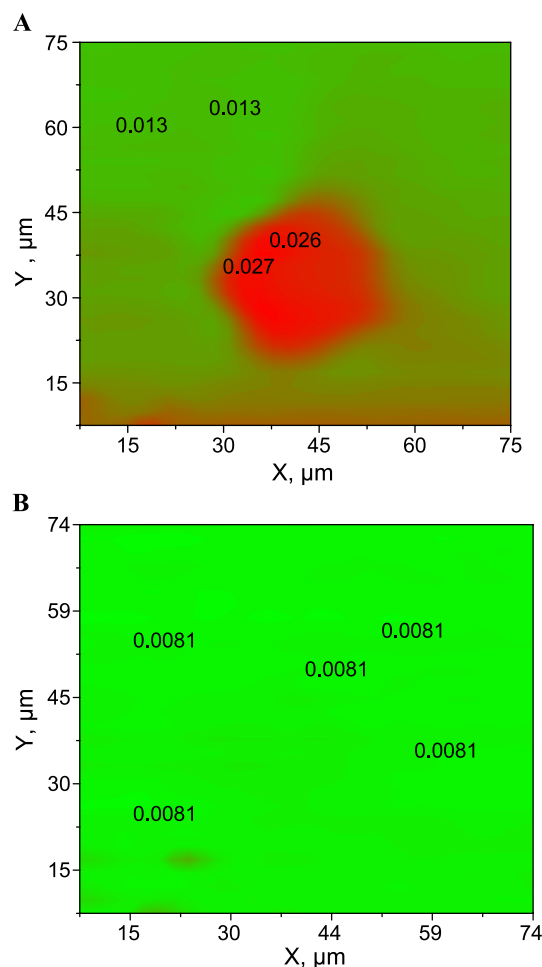


Fig. 3. Topographic image of the local activity of a $20 \times 20 \mu\text{m}^2$ area glucose oxidase layer recorded with a $5\text{-}\mu\text{m}$ diameter platinum tip: before (A) and after inhibition by mercury (II) ions (B). The labels on the image represent the tip current (nA) due to the oxidation of hydrogen peroxide generated during the enzymatic reaction.

sensitivity of the method can be improved by increasing the concentration of the detectable product achieved either by increasing the time allowed for the enzymatic reaction (that, however, lengthens the analysis time) or decreasing the reaction volume. Since by definition SECM is well suitable to detect molecules in extremely small volumes [21] it has provided very sensitive means for determining the local activity of immobilized enzymes [30,31,59,82–84]. A major advantage of the SECM detection is that does not require incubation time because the products of the enzymatic reactions are detected directly in the close proximity of the target [82]. Also the biorecognition reactions can be localized in very small areas by micropatterning techniques [84–87], which not only decreases the reagent consumption but also provides the basis for multianalyte assays. Eventually, the miniaturization of the reactive spots is an essential requirement to obtain steady state concentration profiles of the products over the enzyme modified surface [88].

Two general amperometric techniques are used to detect local enzyme activity by SECM: the feedback mode and

substrate generation/tip collection mode (GC). The first technique used exclusively for imaging oxidoreductase enzymes requires the presence of a redox mediator that acts as artificial reversible electron mediator for the enzyme. In analogous mode with the positive feedback at conducting surfaces, the mediator is recycled at the enzymatic spots which results in increased current signals [29,30]. The second amperometric technique, the generation-collection mode, can be applied whenever electrochemically detectable species are involved in the enzymatic reaction. There is a quantitative requirement in GC mode for the detection of the activity of surface confined enzymes, which is very useful when designing microanalytical systems: $k_{\text{cat}}\Gamma_{\text{enz}} \geq \frac{c'D}{r_s}$, where k_{cat} is the catalytic reaction rate at substrate saturation, Γ_{enz} is the enzyme surface concentration, r_s is the radius of the enzyme modified spot, c' and D are the detection limit and the diffusion coefficient of the detected component, respectively [30]. The criteria basically compares the rate of product generation in the enzymatic reaction with its diffusion away from the probed surface taking in consideration the detection limit of the amperometric technique.

Fig. 3 shows that the activity of a glucose oxidase monolayer confined to an area of $20 \times 20 \mu\text{m}^2$ area can be readily imaged based on the detection of the generated hydrogen peroxide. The featureless image obtained when repeating the scan in the same conditions but after irreversible inhibiting the enzyme with mercury (II) ions demonstrates that there are no feedback interferences in GC mode.

Despite of extensive ELISA studies according to our knowledge SECM has been never applied to detect DNA

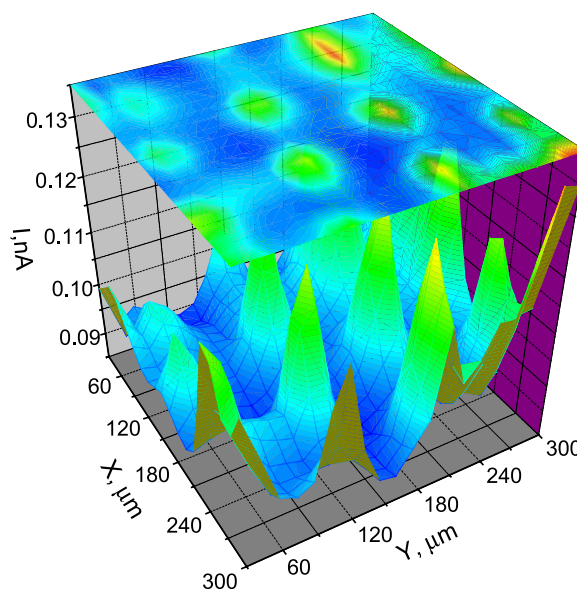


Fig. 4. Mapping of DNA hybridization events with SECM. The captured 18-mer oligonucleotide was labeled with glucose oxidase, therefore the enzyme activity determined by detecting the hydrogen peroxide generated during the enzymatic reaction is indicative of the hybridization process. The image was recorded in a 130 mM D-glucose solution prepared with 0.1 M phosphate buffer (pH = 5.8) using a $12.5 \mu\text{m}$ radius platinum tip.

hybridization using enzyme labeling. Therefore we have performed preliminary experiments that proved the feasibility of the SECM technique for this task. Patterns of an eighteen-mer oligonucleotids were prepared on microelectrode arrays and after hybridization with the complementary strand the later was labeled with glucose oxidase. The hybridization event could be followed by monitoring the activity of the enzyme labels as shown in Fig. 4.

4. Conclusion

The overview on the application of SECM to biological systems and the selected original examples clearly demonstrates the potential of scanning electrochemical microscopy in the field of biology and biomedical science. The technique proved to be lately very useful for controlling the preparation and the characterization of biosensors and sensor structures, as well as the optimization of their performance.

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