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A Method for the Design and Study of Enzyme Microstructures Formed by Means of a Flow-through Microdispenser

Szilveszter Gáspár,*,† Marcus Mosbach,‡ Lars Wallman,§ Thomas Laurell,§ Elisabeth Csöregi,† and Wolfgang Schuhmann*,‡

Department of Biotechnology, Lund University, P.O. Box 124, SE−22100 Lund, Sweden, Analytische Chemie, Elektroanalytik & Sensorik, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany, and Department of Electrical Measurements, Lund Institute of Technology, P.O. Box 118, SE−22100 Lund, Sweden

Micrometer-sized enzyme grids were fabricated on gold surfaces using a novel method based on a flow-through microdispenser. The method involves dispensing very small droplets of enzyme solution (∼100 pL) during the concomitant relative movement of a gold substrate with respect to the nozzle of a microdispenser, resulting in enzyme patterns with a line width of ∼100 μm. Different immobilization methods have been evaluated, yielding either enzyme monolayers using functionalized self-assembled thiol monolayers for covalent binding of the enzyme or enzyme multilayers by cross-linking or entrapping the enzymes in a polymer film. The latter immobilization techniques allow the formation of coupled multienzyme structures. On the basis of this feature, coupled bienzyme (glucose oxidase and catalase) or three-enzyme (α-glucosidase, mutarotase, and glucose oxidase) microstructures consisting of line patterns of one enzyme intersecting with the patterned lines of the other enzyme(s) were fabricated. By means of scanning electrochemical microscopy (SECM) operated in the generator−collector mode, the enzyme microstructures and their integrity were visualized using the localized detection of enzymatically produced/consumed H_2O_2. A calibration curve for glucose could be obtained by subsequent SECM line scans over a glucose oxidase microstructure for increasing glucose concentrations, demonstrating the possibility of obtaining localized quantitative data from the prepared microstructures. Possible applications of these enzyme microstructures for multianalyte detection and interference elimination and for screening of different biosensor configurations are highlighted.

A major prerequisite for the development of suitable biosensor architectures is the immobilization of the biological recognition element on a transducer surface in order to retain the activity of the biomolecule and, thus, to ensure the operational and long-term stability of the sensor. For miniaturized biosensors, in addition, the spatially resolved immobilization of biological recognition elements is increasingly important. Furthermore, retaining different enzymes at very specific locations of a solid support could be useful in nonseparation-type multianalyte sensing. Thus, microscopic, distinct structures generated on the same support and sensitive to different substrates could be the key to minimizing the sample volume while maintaining a high throughput of samples. Recently, in a review highlighting the importance of "nanobiotechnology" the spatial organization of biomolecules on surfaces has been seen as one way of mimicking the unequaled capabilities of biological systems to convert and transduce energy, synthesize special organic chemicals, create biomass, store information, recognize, sense, signal, move, self-assemble, and reproduce.[1] Therefore, there is a growing interest in biomolecule micro- and nanostructures, and hence, new techniques have to be developed to generate biosensor architectures with improved spatial precision.

The ways to pattern proteins on different substrates can be divided into two classes: *indirect methods* and methods involving *active placement*.[2]

The indirect methods consist of local modification of the reactivity of the surface aiming on the localized immobilization of biomolecules on the functionalized areas. Most of these methods are based on self-assembled monolayers (SAMs). Enzymatically active spots on SAMs were obtained by a combination of localized desorption,which is induced by means of a scanning electrochemical microscope (SECM), followed by chemical derivatization of the obtained gold spot and covalent binding of an enzyme.[3] SECM was also used to locally deposit small gold particles that subsequently were modified with SAMs and used to obtain fluoresceine isothiocyanate or glucose oxidase (GOX) spots on silicon.[4] Stripes (200 μm) of pure ω-hydroxyundecanethiol alternating with ω-hydroxyundecanethiol mixed with thiolated biotin, obtained by microcontact printing, were used to build microstructures of G-protein-coupled receptors in a defined

* Corresponding authors. Szilveszter Gaspar, Phone: +46 46 222 80 98. Fax: +46 46 222 47 13. E-mail: silvester.gaspar@biotek.lu.se. Wolfgang Schuhmann, Phone: +49 234 32 26200. Fax: +49 234 32 14683. E-mail: woschu@anachem.ruhr-uni-bochum.de.

† Lund University.

‡ Ruhr-Universität Bochum.

§ Lund Institute of Technology.

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orientation.[5] An atomic force microscope tip was used to write alkanethiols by "dip-pen" lithography with nanometer line width but without moving toward biomolecule immobilization.[6,7] Self-assembled monolayer patterns were obtained also by microcontact printing.[8] In addition to SAMs, the specific biotin−avidin chemistry was also used to pattern biomolecule structures in an indirect method. Using laser scanning confocal optics and photoactive biotin, complex biotin patterns with line widths of 5−20 μm were produced.[9,10]

In the active placement methods, biomolecules are locally delivered to the surface. The simplest approach uses a glass capillary to generate 20-μm spots of anti-human chorionic gonadropin and anti-human placental lactogen under observation by an optical microscope on a glass substrate.[11] The shooting of small biomolecule-containing droplets by means of an ink jet nozzle has been used to selectively modify ISFET structures,[12] to fabricate DNA microarrays,[13] or to produce spot arrays containing horseradish peroxidase on cellulose paper;[14] however, most of the reviewed approaches are either time-consuming or yield only simple biomolecule microstructures (spots).

Visualization and characterization of these micrometer-sized biomolecule structures require techniques with high spatial resolution. In SECM, a microelectrode is scanned over the surface of interest using a high-resolution positioning system while topographic and electrochemical information is collected via the current registered by the polarized microelectrode.[15,16] Nowadays, SECM is a well-established technique used to record high resolution images based on monitoring local electrochemical properties of the target surface. SECM has already been used to map enzyme microstructures immobilized on different surfaces. Detecting the enzymatically produced H_2O_2, GOX spots immobilized on SAM modified gold surface were visualized.[3,4] Microspots of 20-μm radius of carcinoembryonic antigen immobilized on a glass substrate and labeled with horseradish peroxidase could be detected using the reduction current arising by the enzymatically oxidized ferrocenylmethanol mediator.[17] The generator−collector mode was mostly used because of the often slow enzyme kinetics.

Recently, we reported on a method for generating microstructures of different enzymes that is aimed at the development of multianalyte-sensing plates. A flow-through liquid picodispenser[18,19] was used in combination with a positioning system to pattern enzyme lines with micrometer spatial resolution for the localized detection of glucose and lactate.[20] Picoliter droplets of the appropriate enzyme solution were dispensed during the concomitant movement of the gold plate relative to the picodispenser to obtain the desired enzyme lines. SECM was used to visualize either the GOX or the lactate oxidase microstructure (or both), depending on the substrate present in the measurement cell.[20]

In this paper, we further extend the versatility of this novel method in constructing multienzyme micropatterns on biosensor interfaces. The picodispenser was used to pattern complex structures (grids) of different enzymes on gold substrates. On the basis of the line structures consisting of enzyme monolayers reported before,[20] multilayer enzyme structures were envisaged using different immobilization methods. Coupled enzyme microstructures of two or more enzymes could be constructed using one enzyme either consuming or producing the product/substrate for the second enzymatic reaction. Such grid structures, consisting of lines of different proteins, present intersection points that may open new ways in both multianalyte detection and interference elimination and as a screening method for identifying optimal interface configurations in biosensors.

EXPERIMENTAL SECTION

Materials. Di(*N*-succinimidyl)-3,3′-dithiodipropionate (Catalog No. 43789) was purchased from Fluka (Buchs, Switzerland). Glucose oxidase, GOX, (Catalog No. G 7141, EC 1.1.3.4., from *Aspergillus niger*, type X-S, 5.7 U mg^{-1}); catalase (Catalog No. C-9322, EC 1.11.1.6., from bovine liver, 2100 U mg^{-1} solid), mutarotase (Catalog No. M-4286, EC 5.1.3.3., from porcine kidney, 4400 U mg^{-1} protein), and ascorbic acid were purchased from Sigma Chemical Co., Deisenhofen, Germany. α-Glucosidase (Catalog No. 22823, EC 3.2.1.20, from yeast, 67 U mg^{-1}) was purchased from Serva (Feinbiochemica GmbH & Co., Heildelberg, Germany). Poly(ethylene glycol) (400) diglycidyl ether, PEGDGE, (Catalog No. 08210) was purchased from Polysciences, (Warrington, PA). A polyvinyl acetate−polyethylene copolymer dispersion, Vinnapas EP 16, was purchased from Wacker, Burghausen, Germany. $K_2HPO_4 \cdot 3H_2O$ (Catalog No. 105099) and KH_2PO_4 (Catalog No. 104873) were from Merck, Darmstadt, Germany. Dimethyl sulfoxide, DMSO, (Catalog No. 34943) was from Riedel de Haen (Seelze, Germany). D-Glucose was purchased from Mallinckrodt Baker B. V. (Deventer, Holland). D-Maltose was obtained from Biomol, Hamburg, Germany. All chemicals were used as received. All solutions were prepared using triple-distilled water from a quartz distillator if not otherwise mentioned.

Generation of Enzyme Microstructures. The procedure generating the enzyme structures was based on a low-volume (∼2.6 μL), silicon microfabricated, flow-through dispenser previously described.[21] This dispenser ejects picoliter-sized droplets on the basis of the impulse movement of a piezoceramic element and a protruding specially designed nozzle. A droplet of enzyme

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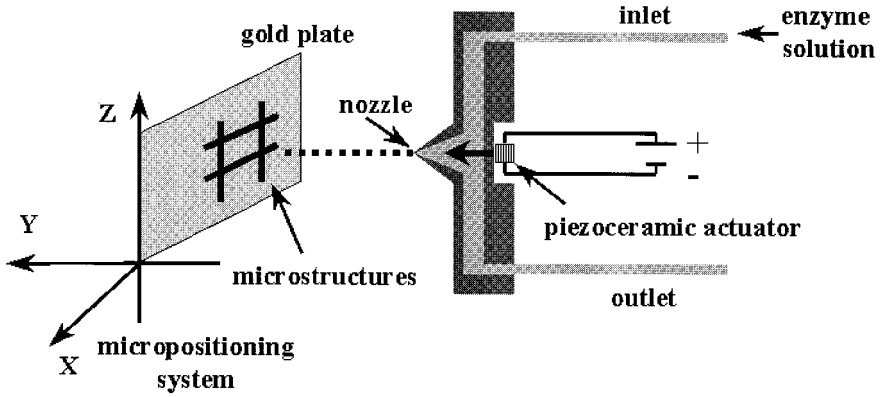

Figure 1. Schematic representation of the flow-through microdispenser, gold plate, and positioning system used to obtain enzyme microstructures.

solution having a volume of ~100 pL was dispensed following each current pulse applied on the piezoceramic element using a function generator (Hewlett-Packard, Waldbronn, Germany, model 33120 A) and a laboratory power supply (Conrad Electronics, Hirschau, Germany). The dispensed droplet produces on the target plate a spot with a typical diameter of ~100 μm. With one *xyz* stage (Owis, Staufen, Germany), the microdispenser was properly positioned, while with a second micropositioning system, the target substrate was moved relative to the microdispenser nozzle in order to obtain the needed enzyme pattern from overlapping enzyme spots (see Figure 1). Silicon wafers (Wacker Chemie, Stuttgart, Germany) coated with Au (1000 Å) in a high-vacuum evaporation apparatus (Univex 300, Leybold, Köln, Germany) and cut in pieces of 0.5 × 0.5 cm were used as substrate. Three different immobilization chemistries were used to immobilize the enzymes on the gold substrate:

I. SAMs of an activated ester (di(*N*-succinimidyl)-3,3′-dithiodipropionate) were made on Au substrates by dipping the cleaned gold surfaces in 10 mM activated ester solution in DMSO for 90 min. Droplets of enzyme solution (~1 mg mL^{-1} enzyme in distilled water) were dispensed on these modified Au plates while moving the plates to obtain the needed pattern. The coupling chemistry based on the activated ester headgroup of the SAM and the amino groups on the protein shell was allowed to cure for 2 h.

II. Droplets of enzyme solution (~1 mg mL^{-1} enzyme in distilled water) containing also a cross-linker, PEGDGE (~2.5 mg mL^{-1}) were dispensed on clean Au plates. The coupling chemistry between the cross-linker and enzymes was allowed to cure for at least 2 h.

III. Droplets of enzyme solution (~1 mg mL^{-1} enzyme in distilled water) containing ~5 mg mL^{-1} Vinnapas EP 16 polymer dispersion were dispensed on clean Au plates. The polymer was retaining the enzyme on the surface after curing at room temperature for 12 h, as described earlier.[22]

SECM Measurement System. Pt-disk microelectrodes (50-μm diameter) for SECM measurements were fabricated by sealing the Pt wire in glass tubes using a laser puller.[23] The electrodes were polished using 0.3-μm alumina paste (LECO, Kirchhein, Germany) and were ultrasonicated in distilled water prior to use. A Ag/AgCl wire and a Pt wire as pseudoreference electrode and counterelectrode, respectively, completed the three-electrode measuring system in an electrochemical cell having a volume of 1 mL. The potential was applied to the microelectrode using a Petit Ampere LC-3C potentiostat (Bioanalytical Systems, West Lafayette, IN). The SECM setup used in this study was described previously.[24] The microelectrode was positioned using the negative feedback effect imposed on the O_2 reduction reaction. To visualize the enzyme microstructures, the microelectrode was polarized to 600 mV vs Ag/AgCl, a potential at which the oxidation of H_2O_2 occurs in a diffusion-controlled manner. In the generator−collector mode of the SECM, the observed oxidation current was either increasing or decreasing, depending on the nature of the scanned enzyme structure (either producing or consuming H_2O_2). The measurement solution was either an 1 M glucose solution in 0.1 M phosphate buffer at pH 7.0 or a 0.083 M maltose solution in the same buffer when the microstructure involved α-glucosidase and mutarotase.

RESULTS AND DISCUSSION

As mentioned earlier, SECM is a suitable technique for mapping the electrochemical properties of various surfaces. Figure 2A schematically shows the principle of the visualization of the enzymatic activity in locally confined enzyme microstructures by the SECM setup used in the present communication.

GOX oxidizes glucose by reducing its natural cofactor, O_2, producing H_2O_2 exclusively at the site of the immobilized enzyme activity. The microelectrode is first positioned in close proximity to the sample surface and adjacent to the enzyme microstructure using the feedback current overlaying the diffusion-controlled O_2 reduction at the microelectrode (poised to −350 mV vs Ag/AgCl). To invoke the generator−collector mode, the microelectrode is then polarized at a potential sufficiently high to convert the enzymatically generated reaction product. While scanning the microelectrode over the surface in the *x* and *y* directions, the current increases significantly only over the immobilized GOX structures as a result of the high local concentration of enzymatically produced H_2O_2. Taking into account the dimensions of the enzyme structure and the overall electrolyte volume of the measuring cell, no significant H_2O_2 accumulation in the bulk will be obtained, leading to a stable background current during the course of the measurement. Plotting the current recorded in

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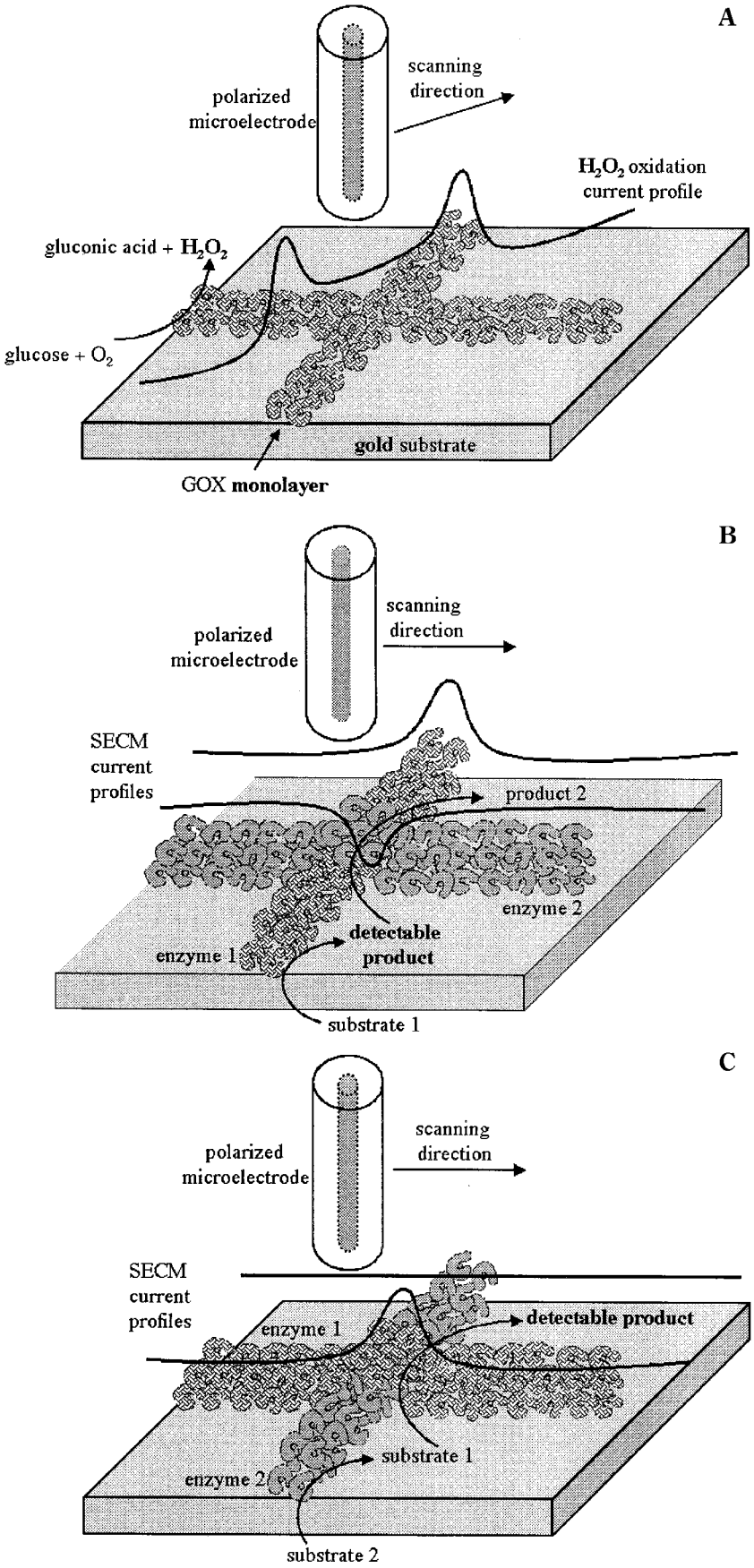

Figure 2. Schematic representation of the working principle for the visualization of enzyme microstructures by SECM (A). Coupled enzymes with the second enzyme consuming the (electrochemically detectable) reaction product of the first enzyme. The solid line shows the expected SECM current profile (B). Coupled enzymes with the second enzyme generating the substrate for the first enzyme. The solid line shows the expected SECM current (C).

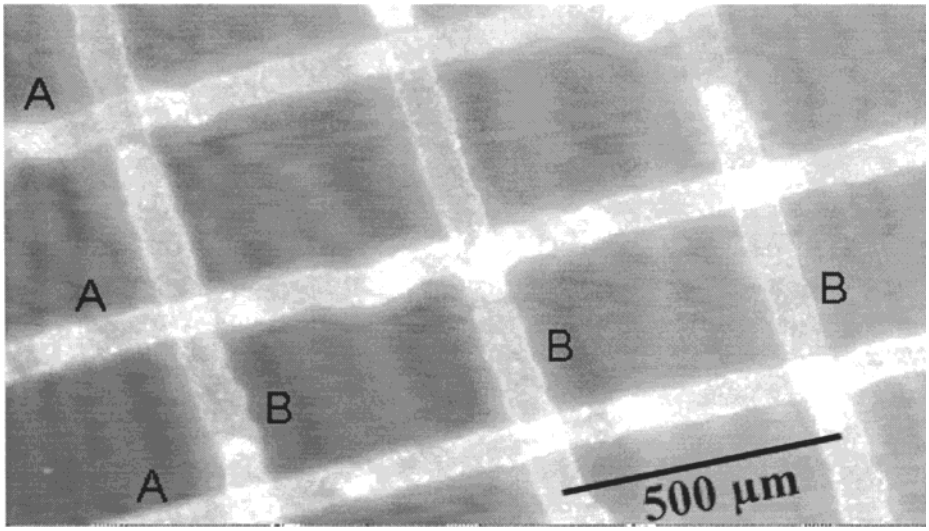

Figure 3. Image of an enzyme grid structure using immobilization method III based on Vinnapas EP16 polymer suspension that was obtained using a digital camera. Each line has a width of ~100 μm, and the distance between two lines is 500 μm.

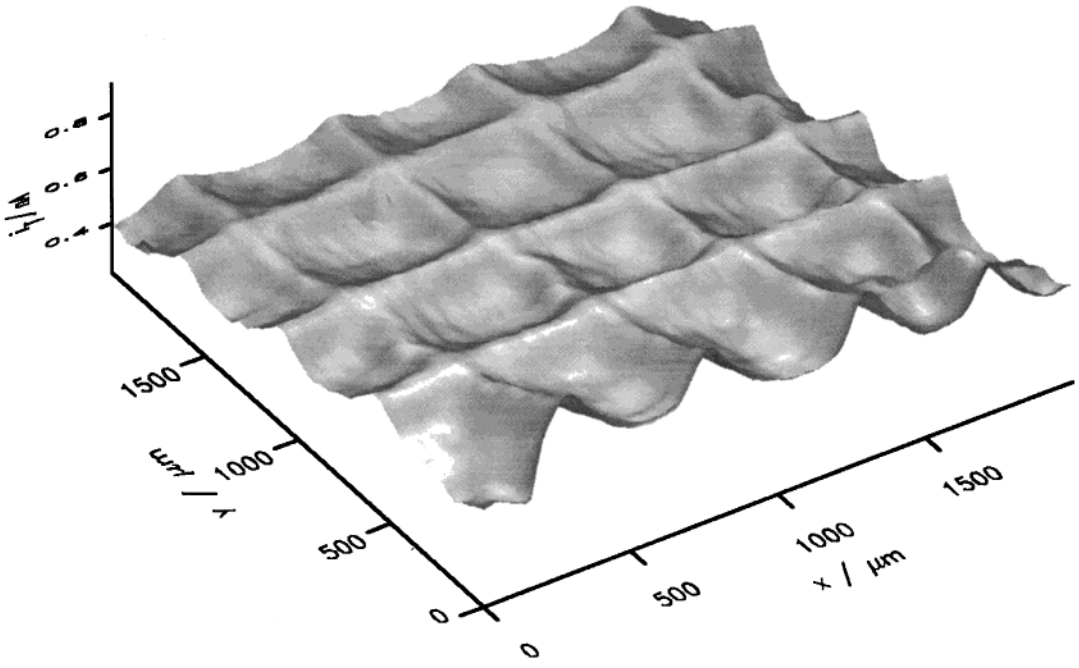

Figure 4. SECM image of a GOX grid structure. Each line has a width of ~100 μm, and the distance between two lines is 500 μm. Solution dispensed to obtain the structure: 8.8 mg mL^{-1} Vinnapas EP 16 and 1.2 mg mL^{-1} GOX.

multiple scans vs the microelectrode position results in an image of the immobilized enzyme activity.

When the enzyme microstructure was built on top of a functionalized thiol-based SAM having an activated ester headgroup (method I), the resulting enzyme structure consisted of only an enzyme monolayer (see schematic representation in Figure 2A). Although this method does not involve any chemical activation of functionalities at the protein, the monolayer architecture has some limitations, especially when more complex structures (like crossing lines) are envisaged. The deposition of different enzymes on top of each other or multilayer structures of the same enzyme is impossible using this method. Moreover, very clean Au surfaces are an indispensable prerequisite for the formation of highly ordered SAMs, which requires tedious cleaning procedures and also represents a source of irreproducibility. Therefore, we have evaluated two other possible immobilization methods, consisting of either cross-linking the enzyme by simultaneous dispensing of an enzyme/cross-linker mixture (method II) or entrapping the enzyme within a polymer film by dispensing a polymer/enzyme solution with properly adapted viscosity (method III). Because of the formed multilayer network or polymer film, these immobilization methods ensure a higher enzyme loading than method I, which is of importance, especially for electrode configurations using enzymes with low activity. The immobilized enzyme activity can, in addition, be tailored by a sequential deposition of multiple droplets on top of each other. Moreover, these immobilization methods are applicable to nonconducting surfaces as well.

The major advantage of these methods is, however, the possibility of developing complex multienzyme structures by sequential dispensing of preparations containing different enzymes, together with an appropriate cross-linker or polymer

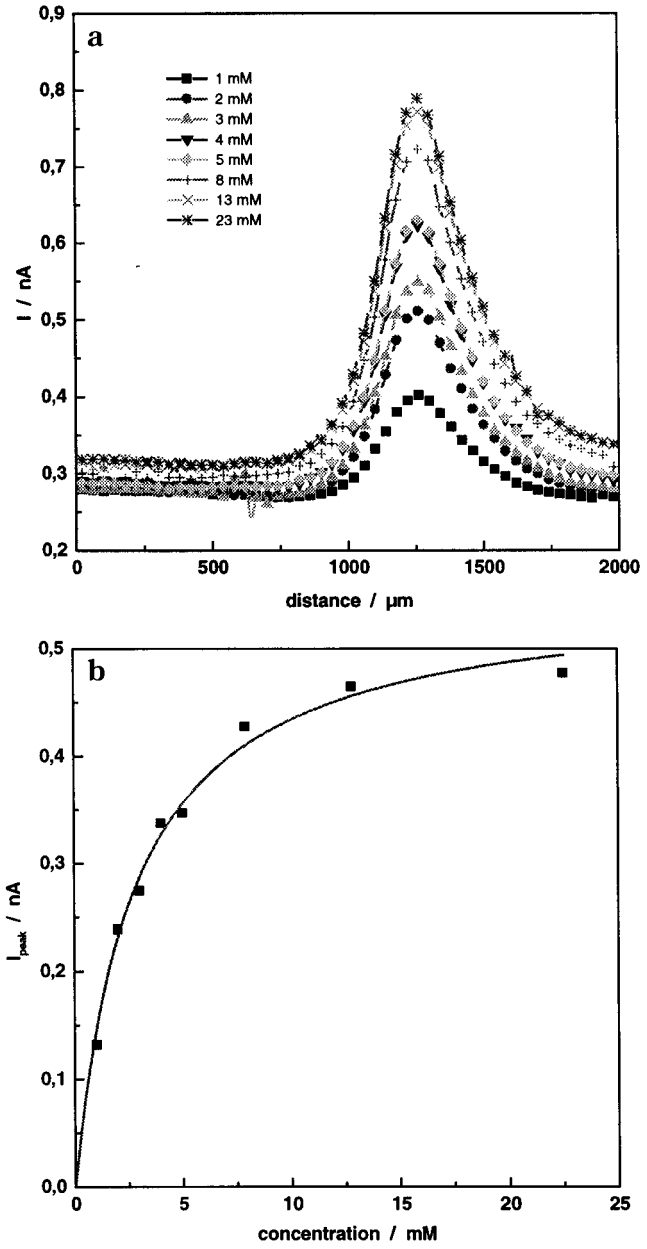

Figure 5. H_2O_2 oxidation currents (A) and calibration curve (B) obtained by single-line scans of the SECM over a GOX line (width of ~ 100 μm) for increasing glucose concentrations. Solution dispensed to obtain the structure: 1.9 mg mL^{-1} PEGDGE and 1.1 mg mL^{-1} GOX.

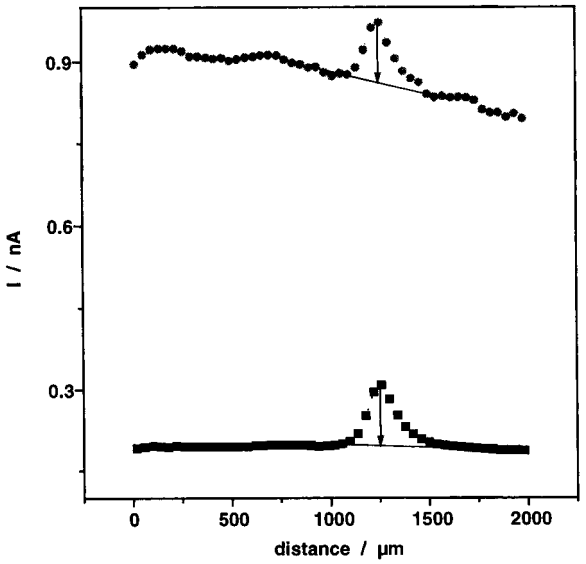

Figure 6. H_2O_2 oxidation currents obtained by single-line scans of the SECM over a GOX line (width of ~ 100 μm) for 4 mM glucose in the presence (●) and in absence (■) of 0.1 mM ascorbic acid. Solution dispensed to obtain the structure: 1.9 mg mL^{-1} PEGDGE and 1.1 mg mL^{-1} GOX.

dispersion. By doing so, microstructures with two or more interacting enzymes can be obtained. Considering, for example, a certain enzyme, (enzyme 1), which converts substrate 1 into an electroactive product, there are many possibilities of interaction of this product with a second (or third) enzyme. Figure 2B,C considers only two of the possible cases. In Figure 2B, the electrochemically detectable product generated by enzyme 1 is consumed by enzyme 2. In a corresponding SECM experiment, imaging a cross of a line containing enzyme 1 with a second line containing enzyme 2 would reveal the enzyme 1 line by a high current and that of enzyme 2 by a low current (see current profiles in Figure 2B).

In Figure 2C, another possible situation is schematically shown in which the substrate of enzyme 1 is the product of the reaction catalyzed by enzyme 2. In this case, a SECM tip visualizing concentration profiles of the reaction product of enzyme 1 will exclusively see the intersection points of the two enzyme lines where the close proximity of both immobilized enzymes allows the formation of the final electroactive product via the locally generated substrate of enzyme 1 by enzyme 2. Many other coupled enzyme systems can be imagined, and there are in principle no limitations imposed by the microdispensing of various enzymes; however, the availability and properties of the enzymes and the chosen way to monitor the reaction sequences via a suitable electroactive product with a microelectrode integrated into the SECM may limit the approach. In the following sections, several examples of enzyme microstructures obtained by using the immobilization method II or III and the detection of H_2O_2 using the generator−collector mode of the SECM, are investigated to demonstrate the feasibility of the proposed novel method.

Monoenzyme Microstructures. Figure 3 presents an image of an enzyme microgrid, obtained by entrapping the enzyme in a Vinnapas EP16 polymer film following the immobilization method III. Similar structures could be generated using the other described immobilization methods. Theoretically, any kind of immobilization method, which consists of handling liquid phases, could be applied, and only a careful selection of the substrate with the proper wetting properties is needed to generate precisely patterned complex microstructures.

A SECM image of a GOX microgrid, fabricated by using immobilization method III detecting the enzymatically generated H_2O_2 in the generator−collector mode (Figure 4) has been obtained. The well-defined activity image demonstrates clearly the good spatial resolution of the developed patterning method. Possible applications of such microstructures for multianalyte detection or high-redundancy monoanalyte detection can be envisaged on the basis of the calibration curve recorded for glucose on a particular section of such a structure (see Figure 5A,B).

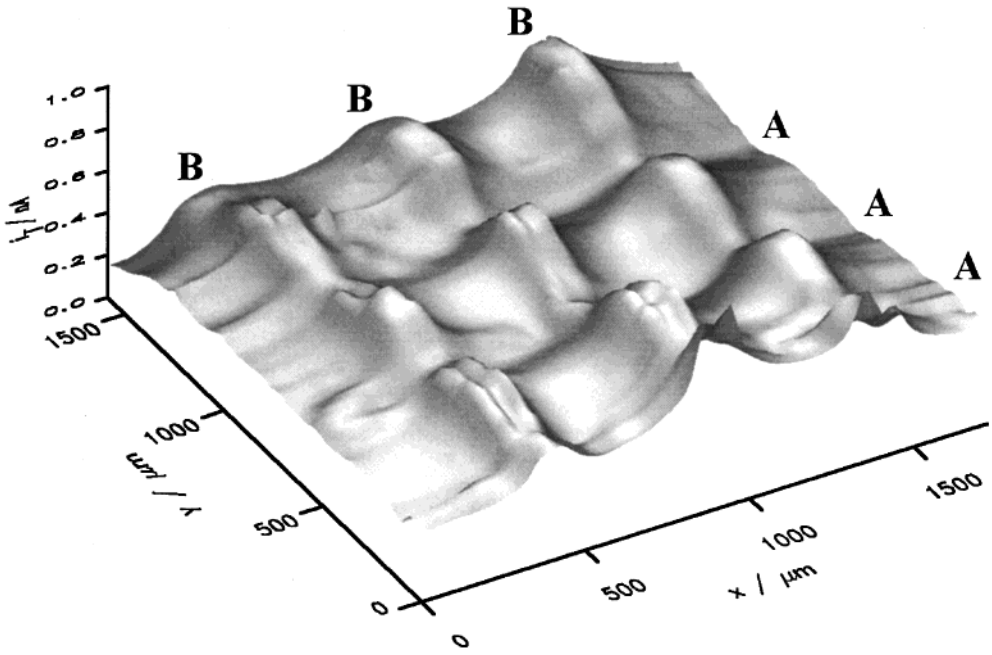

Figure 7. SECM image of a GOX−catalase bienzyme grid structure. Each line has a width of ~100 μm, and the distance between two lines is 500 μm. Solutions dispensed to obtain the structure: 2.5 mg mL^{-1} PEGDGE and 1.0 mg mL^{-1} GOX (for lines B), and 2.5 mg mL^{-1} PEGDGE and 1.0 mg mL^{-1} catalase (for lines A).

When the microelectrode is scanned several times in a constant *z* position across the same section of a 100-μm GOX line, the addition of increasing amounts of glucose to the electrolyte after each line scan resulted in current profiles shown in Figure 5A, which are clearly representing the increased formation of H_2O_2 due to increased glucose concentration. On the basis of this possibility to detect local changes of H_2O_2, nonseparation-type monitoring of different substrates can be achieved using enzyme microstructures with different oxidases patterned on the same substrate.

In addition, the grid structure allows the elimination of any influence from interfering compounds. Figure 6 represents a sequence of single-line scans over a 100-μm GOX structure. In the presence of 4 mM glucose, the position of the GOX line on the Au plate is revealed by a glucose concentration-dependent current peak. After the addition of 0.1 mM ascorbic acid (one of the main interfering agents in biological samples), an increased background current was recorded independent of the position of the microelectrode. On the top of this increased background current attributed to the direct oxidation of ascorbic acid at the microelectrode, the glucose proportional peak current remained unchanged. Moreover, the close proximity of the microelectrode to the sample surface at least partly blocks the diffusion of ascorbic acid to the electrode and, hence, even reduces the observed background current. This small background current facilitates the detection of glucose in the presence of interfering compounds, because only the height of the current peak over the enzyme microstructure has to be measured on top of the baseline, which is representing the influence of the interfering compounds.

Multienzyme Microstructures. A more detailed investigation of the current image over the GOX grid structure (see Figure 4) clearly shows a distinct current increase over the grid sections where two lines are intersecting each other. Obviously, at these grid points, an increased enzyme activity is attained, which clearly leads toward the generation of multienzyme microgrids where at the intersection points, two (or more) enzymes are overlaid, thus allowing their productive or destructive interaction. Coupled enzyme systems are currently used in different biosensor designs either in a multilayer or a monolayer bienzyme approach. Imagining an enzyme structure similar to the one shown in Figure 3, but with the lines A containing enzyme 1 and the lines B enzyme 2, nine bienzyme intersection points with possible variations in the enzyme 1-to-enzyme 2 ratio (adjusted by the number of dispensed droplets) would be obtained, corresponding to nine different biosensor configurations with individual properties concerning linear detection range, detection limit, and sensitivity against interfering compounds. Thus, it should be possible by using SECM line scans over the intersecting bienzyme microstructure to evaluate in a single experiment the sensor properties of nine sensor configurations.

To support this working hypothesis, a system schematically shown in Figure 2B (enzyme 2 is consuming the product generated by the catalytic action of enzyme 1) has been chosen. A suitable model system representing this scheme is the combination of GOX with catalase. Catalase, disintegrating H_2O_2 produced via the glucose oxidation reaction, has frequently been applied in combination with oxidases to remove interferences from H_2O_2 in amperometric biosensors. By coupling GOX with catalase, the following reaction sequences occur:

on the GOX structure: β-D-glucose + $O_2 \rightarrow$ gluconic acid + H_2O_2
on the catalase structure: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

A microgrid structure has been generated with catalase lines in the horizontal and GOX lines in the vertical direction. Figure 7 shows the SECM image obtained by detecting the enzymatically produced H_2O_2 (at the GOX line). The catalase lines can be clearly visualized by the significant current decrease due to the enzymatic H_2O_2 consumption.

However, one of the main reasons for using coupled enzyme systems on electrochemical biosensor interfaces is the detection

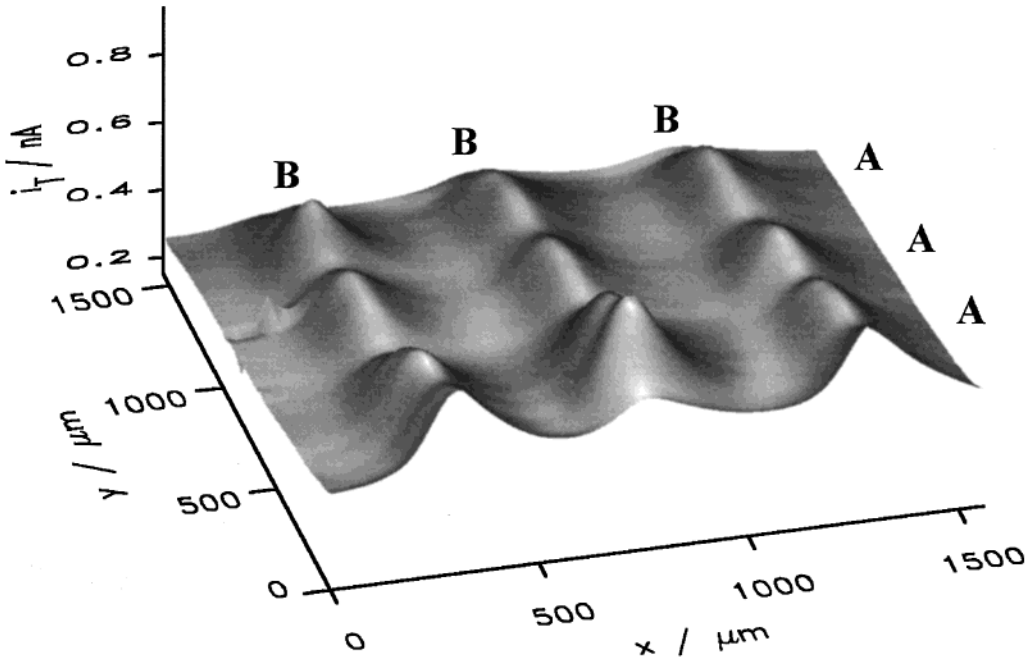

Figure 8. SECM image of an α-glucosidase−mutarotase−GOX multi-enzyme microstructure. Each line has a width of ∼100 μm, and the distance between two lines is 500 μm. Solutions dispensed to obtain the structure: 5.8 mg mL^{-1} Vinnapas EP 16 and 1.4 mg mL^{-1} GOX (for lines B); and 5.8 mg mL^{-1} Vinnapas EP 16, 1.3 mg mL^{-1} α-glucosidase, and 0.9 mg mL^{-1} mutarotase (for lines A).

of substrates that are converted primarily by an enzyme yielding a nonelectroactive product (or at least nonelectroactive in an interference-free potential window). Such a system is schematically presented in Figure 2C where enzyme 2 is generating the substrate for enzyme 1, and enzyme 1 is turning it into an electrochemically detectable product. As an example, lines A consist of co-immobilized α-glucosidase and mutarotase and lines B consist of GOX.

The following reaction sequences are taking place in order to finally permit the detection of maltose via the H_2O_2 generated at the GOX lines:

on the α-glucosidase structure: maltose + H_2O → 2α-D-glucose

on the mutarotase structure: α-D-glucose → β-D-glucose

on the GOX structure: β-D-glucose + O_2 → gluconic acid + H_2O_2

Figure 8 shows a clearly increased current at the intersections between lines A and B, indicating the conversion of maltose by α-glucosidase and the subsequent oxidation of the glucose at the intersecting GOX, leading finally to a proportional H_2O_2-oxidation current. This clearly demonstrates the possibility of generating complex enzyme microstructures in which different enzymes are active and are working together in either a destructive or a constructive manner. Simultaneous determination of saccharides has great importance for dairy products and often involves expensive chromatographic separation techniques.[25−27] Here, the proposed enzyme microstructures in combination with SECM imaging could be applicable to simultaneously monitor a variety of different mono-, di-, oligo-, and polysaccharides by combining GOX lines crossing with lines of different immobilized hydrolases.

CONCLUSIONS

The generation of complex enzyme microstructures using a flow-through microdispenser with a typical droplet volume of 100 pL has been demonstrated using three different immobilization techniques, namely, covalent binding at functionalized thiol monolayers, cross-linking using a bifunctional reagent, and entrapment within a polymer hydrogel. The obtained microstructures have typical line widths of ∼100 μm. Immobilization on SAMs results in structures with an enzyme monolayer, and entrapping in polymer films or cross-linking allows the formation of multilayer enzyme structures. Thus, the last two immobilization methods are suitable for generation of multienzyme microstructures. GOX−catalase- and α-glucosidase−mutarotase−GOX-coupled enzyme patterns have been generated to demonstrate the feasibility of the proposed enzyme structures. The multienzyme grids designed by microdispensing in combination with SECM imaging offer the possibility of using the intersections (where two or more enzymes are deposited on the top of each other) for optimizing multilayer biosensor designs to develop nonseparation-type multianalyte detection systems and offer an effective way to interpret the contribution of electrochemically active interferences. Moreover, a SECM line scan across the obtained enzyme microstructures could be proved to allow a quantitative determination of enzyme substrates.

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