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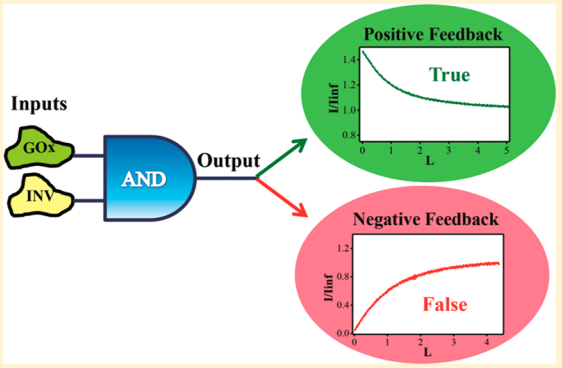
Biomolecular AND Logic Gate Based on Immobilized Enzymes with Precise Spatial Separation Controlled by Scanning Electrochemical Microscopy

Efrat Gdor,[†] Evgeny Katz,[‡] and Daniel Mandler*[,†]

[†]Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

[‡]Department of Chemistry and Biomolecular Science, Clarkson University, Potsdam, New York 13676, United States

ABSTRACT: A surface-localized enzymatic AND gate based on scanning electrochemical microscopy was designed and studied. The gate is composed of an insulating glass surface modified with the enzyme glucose oxidase (GOx) and another surface opposing it made of a microelectrode. The latter was modified with a second enzyme, invertase (INV). The distance separating the modified microelectrode and surface controlled the output of the AND gate produced upon the biocatalytic reaction of the confined enzymes. Specifically, as the GOx-modified glass substrate entered the diffusion layer of the microelectrode, it catalyzed the regeneration of an electron-transfer mediator, ferroceniummethanol, generated electrochemically at the tip by oxidizing glucose, also generated at the tip, by catalytic cleaving of sucrose by INV. To enhance the activity of the GOx, mutarotase was added to convert α- to β-glucose to be further consumed by GOx. Hence, an increase of the current at the microelectrode was obtained by approaching the glass surface only in the presence of all the components. This is the first micrometer-sized biomolecular logic gate, of which we are aware, that is surface-confined and shows the promise held by the localization of biomolecular information-processing species.



1. INTRODUCTION

Recent advances in unconventional computing[1,2] resulted in the rapid development of molecular[3−8] and particularly biomolecular information processing systems (biocomputing systems).[9−14] Boolean logic operations are the most frequently modeled functions performed by molecular and biomolecular systems, and basic binary gates such as AND, OR, XOR, NAND, and NOR have been realized.[9−16] Biocomputing, which partially originates from modeling neural networks,[17] is an attempt to mimic the basic functions of computing using biological components. This was done with proteins/enzymes,[14−16,18] DNA,[13,19,20] RNA,[21] and whole cells.[22−24] This research has been motivated by the promise of novel applications in devising new computation systems,[25] biosensing,[26−28] switchable bioelectronic devices[29] (e.g., biocatalytic electrodes and biofuel cells),[30−33] and signal-controlled "smart" materials.[34]

Analyzing the studies in this field reveals that the grave majority of work in the areas of molecular and biomolecular computing is performed in homogeneous systems,[4−16] unlike conventional computing by solid-state devices that take advantage of confining the processes, mostly as two-dimensional (2D) systems. The latter enables multiple simultaneous and more complex operations. Hence, the development of 2D and 3D compartmentalized biological systems, in which the operational sites are separated physically from each other, could tremendously contribute to generate more complex functions. This requires tools for the designing of biologically based, for example, enzyme-based, localized systems. The latter, namely, local immobilization of **multiple** enzymes that is also termed patterning, is challenging and seldom reported.[35−37]

Patterning is a broad term for various procedures such as microcontact printing,[35,38,39] microspotting,[40−42] droplet-to-droplet array,[43] high-throughput screening,[37,44] and others.[45,46,47] In this paper we will focus mostly on local immobilization, as we decided to approach this subject from a different angle, by immobilizing the enzymes onto opposite-facing solid supports. Among a few reported studies on local immobilization of multiple enzymes, there is a related report on compartmentalized microparticles using a multistep procedure.[36] Another related example was reported by Theilacker et al. who used stamping to produce multicomponent protein arrays.[35] On the other hand, the modification of the entire surface by a single enzyme is commonly used and well-documented.[48−51] Here we propose a different concept whereby a microprobe modified by one enzyme is brought in proximity to a surface that is modified by a second enzyme. This approach enabled generating a Boolean AND gate where the distance between the two operational (enzymatic) sites is controllable. Moreover, as the microprobe is a microelectrode, it also provides the tool for detecting the output of the gate.

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Scanning electrochemical microscopy (SECM), introduced two decades ago,[52] is exactly the tool that is capable of performing such operation. SECM is an electrochemical-based probing technique for studying processes occurring at chemical interfaces.[52,53] SECM has been successfully utilized for mapping various surfaces,[54−57] studying electron and ion transfer reactions,[58] and modifying surfaces locally with high resolution.[40,59,60] Reviewing the literature that deals with enzymes and SECM reveals that the majority of the studies can be divided into a few fields: biosensing,[61−65] surface functionality,[40] imaging (usually coinciding with kinetic studies and enzyme activity),[58,66,67] and even developments and upgrades of SECM by integrating different components.[55,68,69] Immobilization of enzymes onto surfaces is hardly a novelty, nor is the immobilization of an enzyme on a microelectrode (e.g., on a SECM electrode); however, the incorporation of both in the same system, to the best of our knowledge, is not common.[70] The aim of this study is to show the applicability of the SECM technique for constructing and studying a biomolecular enzyme-based logic gate. Specifically, we immobilized glucose oxidase (GOx) onto an insulating solid support, namely, a glass surface, and the enzyme invertase (INV) onto a Pt microelectrode associated with a SECM instrument. The buffered solution contained sucrose as a substrate for GOx and ferrocenemethanol as an electron-transfer mediator. The AND gate was formed by generating a flux of ferrocenium ions at the microelectrode and glucose due to enzymatic cleavage of sucrose by INV. An increase of the ferrocenemethanol steady-state current upon approaching the glucose oxidase-modified surface constitutes the YES output of the AND logic gate. To increase the rate of the enzymatic reaction at the tip, mutarotase was added to the solution. The latter catalyzed the mutarotation of α- to β-glucose consumable by GOx.

2. EXPERIMENTAL SECTION

Instrumentation. A bipotentiostat (CHI-750B, CH Instruments Inc., TX, USA) with a preamplifier was used for controlling the potential in all SECM experiments as well as for conducting chronoamperometric measurements and for cyclic voltammetry (CV). Electrochemical experiments were carried out in a three-electrode cell using a platinum ultramicroelectrode (Pt-UME) as a working electrode, a Ag/AgCl (KCl 3M) electrode as a reference electrode, and a Pt wire as a counter electrode. SECM measurements were performed using a homemade SECM apparatus based on a PX 200 CAP Piezo Translation System (Piezojena, Jena, Germany) with integrated capacitive sensor of 1 nm resolution and x−y motion of 200 μm. In-house software (LabView, National Instrument, TX, USA) was used for positioning the microelectrode. Characterization of the different samples was carried out using high-resolution scanning electron microscopy (MagellanTM XHR SEM, FEI company, Fl, USA), while optical microscopy was performed with an Olympus BX6000 microscope (Tokyo, Japan).

Chemicals and Materials. Glucose oxidase (GOx) from *Aspergillus niger* type X−S (E.C. 1.1.3.4), horseradish peroxidase (HRP) type VI (E.C. 1.11.1.7), INV from Baker's Yeast (*S. cerevisiae*) (E.C. 3.2.1.20), bovine serum albumin (BSA), 3-aminopropyltriethoxysilane (APTS, 99%), polydiallyldimethylammonium chloride (PDDA, 20%), ferrocenemethanol (FcMeOH, 97%), disodium hydrogen phosphate (98.5%), glucose (99%), and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, 98%) were purchased from Sigma-Aldrich. Other chemicals were received from various companies: potassium nitrate (≥99.0%), ethanol (99.9%), and hydrogen peroxide (30%) from Merck, hydrochloric acid (32%) and sulfuric acid (96%) from J. T. Baker, mutarotase (MUT) from porcine kidney (E.C. 5.1.3.B1) from Calzyme Laboratories Inc., glutaric dialdehyde (GDA, 25%) from Alfa Aesar, potassium chloride (99%) from Gadot, sucrose (95%) from Frutarom, and sodium dihydrogen phosphate (99%) from Mallinckrodt Chemicals. All chemicals were used as supplied without further purification. Deionized water (18.3 MΩ cm, EasyPure UV, Barnstead, UK) was used in all experiments. Phosphate buffer was prepared from solutions of 50 mM of disodium hydrogen phosphate and sodium dihydrogen phosphate that were mixed to obtain pH 7.00. This buffer was used in all experiments.

Methods. The Pt-UMEs were prepared following conventional procedures.[53] Briefly, borosilicate capillaries (Gerner Glass, Germany, ID/OD 1.60 ± 0.05/2.00 ± 0.05 mm) were heat-pulled using a micropipet puller (P-97, Sutter Instruments Co., CA, USA). Pt wires (25 μm diameter) were inserted into the pulled capillaries and sealed under vacuum with a micropipet puller (PP-830, Narishige group, Tokyo, Japan). The sealed capillaries were polished with 400, 600, and 1200 grit emery paper (Buhler, Lake Bluff, USA) to form a smooth metal disk insulated in a glass sheath. The ratio between the diameter of the insulating casing and the metal disk (RG) was approximately 10. Further polishing of the electrode was accomplished with 9, 3, and 0.3 μm alumina paper (Fibramet discs, Buhler). To obtain electric contact between the platinum and the copper wires, silver epoxy was used. Prior to all experiments, the microelectrodes were polished with 0.05 μm alumina powder. The Pt microelectrodes were electrochemically characterized by inspecting their CV in 2 mM $Ru(NH_3)_6Cl_3$ (0.1 M KCl) solution.

To obtain an INV-modified UME, a modification solution was prepared by dissolving 8 mg of enzyme and 62.5 mg of BSA in 1 mL of phosphate buffer (pH 7, 50 mM) to full dissolution. GDA (14 μL) was added under magnetic stirring. The solution was further stirred for 30 min in ambient conditions. A Pt-UME prepared as described above was activated by CV (0.5 V s^{-1}) in 0.1 M H_2SO_4, sweeping the potential from −0.4 to 1.6 V. After the Pt-UME was rinsed and dried, it was dipped 20 times in the modification solution and dried for 4 h. The modified microelectrode was kept in phosphate buffer (pH 7, 50 mM) until use.

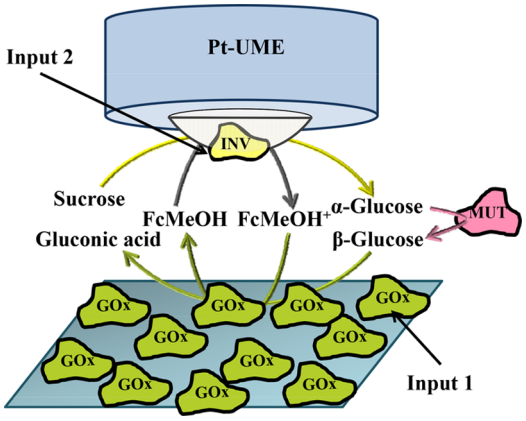
Microscope glass slides were cut into 15 × 8 mm^2 and cleaned as follows: 10 min in piranha solution (1:3 H_2O_2:H_2SO_4) (Warning: piranha solution is extremely energetic and potentially explosive. It must be handled with extreme caution.), then 10 min in ethanol, and finally 10 min in water. The clean slides were dried with nitrogen flow and then heated for 1 h at 100 °C. The glass slides were then derivatized with amino groups by immersing in 2% APTS in ethanol for 10 min, followed by rinsing with ethanol, drying with nitrogen flow, and curing at 110 °C for 2 h. The immobilization of the GOx onto the amino-functionalized glass slides was performed as described by Wittstock et al.[71]

3. RESULTS AND DISCUSSION

This work deals with the design of localized biological Boolean operators based on enzymatic cooperative reactions between enzymes immobilized on opposing surfaces. The approach

taken to fulfill this task included modification of both a glass surface and a microelectrode with two different enzymes, GOx and INV, serving as inputs 1 and 2, respectively. The AND gate as designed by us is portrayed in Scheme 1. The conditions for

Scheme 1. Schematic Representation of the AND Gate with the Enzymes Localized on a Pt-UME and Glass Solid Support, Where the Distance between the Reacting Enzymes Was Controlled by Scanning Electrochemical Microscopy



a successful AND gate, studied thoroughly, included the enzyme compatibility, substrate choice, and the effect of the concentrations of each component (the substrates, the buffer, etc.). The enzymes should be chosen so that their catalytic activities neither coincide nor interfere. In addition, the reactants and products of the enzymes should be configured to avoid any spontaneous reaction between them. In other words, all the reactions in the system should be feasible in the presence of the enzymes only. Finally, the enzymes or their products and reactants should not inhibit any desired reaction.

3.1. Homogenous Biocatalytic Reaction for Constructing the Enzyme AND Gate. Keeping in mind the AND gate shown in Scheme 1 as the ultimate aim, we studied first the biocatalytic cascade operated in a solution. This is similar to most of the previous studies in which enzymes as well as other components of logic gates were dissolved in solution, and detection was accomplished by spectroscopic or electrochemical means.[14,72] Therefore, we initially dissolved GOx and INV in an aerated buffer solution and studied their reaction upon increasing concentration of sucrose. The sucrose was cleaved to glucose and fructose by INV. The generated glucose was expected to be oxidized by oxygen in the process biocatalyzed by GOx, yielding gluconic acid and H_2O_2. Sucrose was added to the reacting solution at intervals of ca. 60 s, and the in situ-generated H_2O_2 was analyzed by chronoamperometry, whereby a constant potential of 0.6 V (vs Ag/AgCl) was applied to a Pt-UME. Figure 1 shows the chronoamperometric responses of the system corresponding to the electrochemical oxidation of H_2O_2. The result shown in Figure 1, curve a, demonstrates formation of H_2O_2 delayed by ca. 3 min. This cannot be attributed to the slow electrochemical kinetics or slow diffusion (note that addition of H_2O_2 to the solution results in the immediate electrochemical response). Therefore the observed lag period originates from the enzyme biocatalytic reactions; this effect was observed and discussed in other similar systems. Apparently, INV-catalyzed cleavage of sucrose results in the formation of α-glucose, which is a much less-effective substrate for GOx, with the activity of 0.64% as

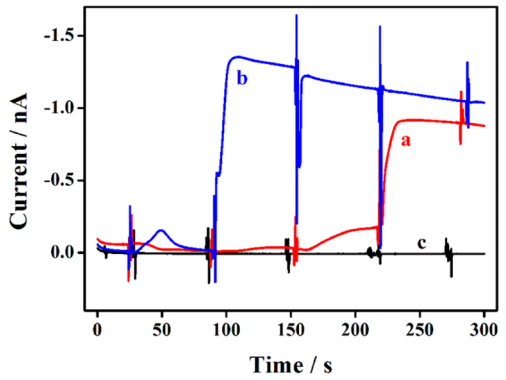


Figure 1. Chronoamperometry recorded with a Pt-UME in a solution containing (a) INV (332.8 U/mL) and GOx (129.7 U/mL), (b) GOx (129.7 U/mL), INV (332.8 U/mL), and MUT (506 U/mL), and (c) no enzymes in a control experiment. Sucrose (20 μL, 0.5 M) was added to the solutions in intervals of ca. 60 s. The experiments were performed in a phosphate buffer solution (50 mM, pH 7) upon application of 0.6 V to the Pt-UME.

compared with that of β-glucose.[73] The attempts to increase the phosphate buffer concentration to 0.65 M to facilitate mutarotation of α- to β-glucose did not result in faster H_2O_2 generation, in contrast to a previous report.[74] Therefore, an additional enzyme, mutarotase (MUT), was introduced to the solution for biocatalytic transformation of α- to β-glucose. Figure 1, curve b, shows chronoamperometric responses of the system to the additions of sucrose in the presence of three enzymes, GOx, INV, and MUT, in the solution. It is evident that already, after the first addition of sucrose, a noticeable increase of the current is obtained, and at the second addition a plateau is reached, indicating the improved efficiency of the GOx-biocatalyzed reaction. Hence, the three-enzyme system complies with the AND gate requirements, where the inputs can be either the electron donor and acceptor, for example sucrose and oxygen, respectively, or alternatively the enzymes INV and GOx (note that the use of enzymes as logic inputs was already reported in several studies).[75−77] The output is in both cases the current registered on the Pt-UME.

3.2. Homogeneous−Heterogeneous Reaction for Constructing the Enzyme AND Gate. The next step was confining one element of the biocatalytic system so that a semiheterogeneous system is achieved. This required immobilization of one enzyme onto a solid support. In view of the final system (Scheme 1), we decided to attach INV to a Pt-UME. Immobilizing GOx onto the microelectrode would have required attaching INV to the large surface to complete the heterogeneous system. This would have led to the continuous generation of glucose on the large surface and the formation of a thick diffusion layer of glucose. As a result it would have been impossible to fine-tune the overlap between the diffusion layers of the microelectrode and that of the surface by controlling the tip−surface distance.

The immobilization of an enzyme onto a microelectrode is not trivial, and modified microelectrodes are not commercially available. Various approaches for the modification of microelectrodes[78−80] were studied and attempted by us. We needed a method ensuring the immobilization of the enzyme but also preserving electron transfer to and from the electrode surface. We are not aware of methods for the immobilization of enzymes directly onto the microelectrode surface; rather, a binding matrix is necessary. A few approaches for enzyme

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embedding into matrices formed on microelectrodes have been reported and include (electro)polymerization using sol–gel or polypyrrole-derived matrices and covalent immobilization using, for example, GDA or poly(ethylene glycol) diglycidyl ether.[81] These matrices must be permeable to the enzyme substrates as well as to electroactive species, which are oxidized or reduced on the electrode surface.

Finally, we adopted the method by Ciobanu et al.,[78] which is based on the formation of a protein (BSA) matrix cross-linked by GDA in which the enzyme INV was incorporated. Figure 2A,B shows optical micrographs of the modified 25 μm

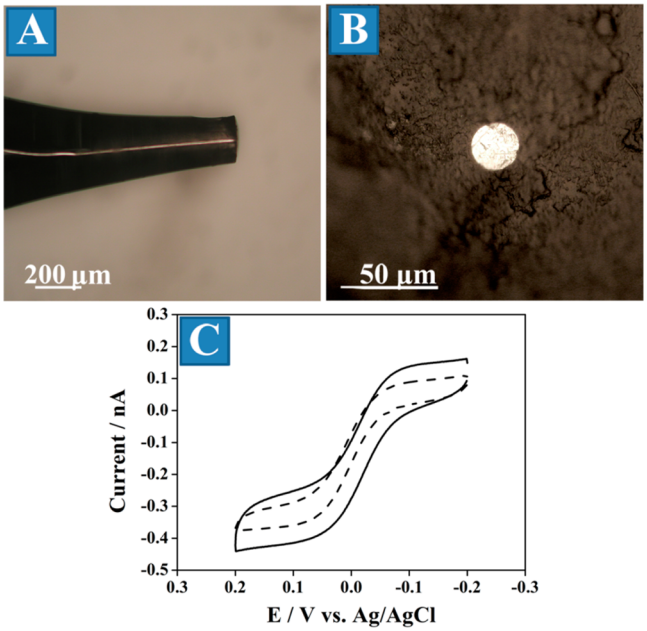

Figure 2. Optical micrographs of the INV-modified Pt-UME. (A) Side view. (B) Top view. (C) Cyclic voltammograms of a Pt-UME (continuous line–bare; dashed line–modified by invertase) recorded in a solution of 0.2 mM FcMeOH in 0.05 M phosphate buffer, pH 7.0, and scan rate of 25 mV·s^{-1}.

diameter Pt-UME. The matrix can be clearly seen on the glass sheath; however, it is unclear whether the protein is also adsorbed onto the metallic part of the Pt-UME. Regardless, this method allowed retaining the electrochemical activity of the Pt-UME toward species in the solution, as desired, shown in Figure 2C. The latter shows CVs obtained on a bare and INV-modified Pt-UME in a solution containing 0.2 mM FcMeOH used as a redox probe. As can be seen, the diffusion-controlled steady state current that was obtained is almost the same for the modified and bare microelectrodes. This suggests that the immobilization of the enzyme did not inhibit the interfacial electron transfer process. Moreover, the permeability of FcMeOH through the binding matrix must be very facile. It is worth mentioning that this modification procedure gave satisfactory results, in contrast with other immobilization methods tested such as electropolymerizing 2-aminophenol[78] or casting.[80] Evidence for the INV activity after immobilization will be discussed later.

Figure 3 depicts the current recorded with the system composed of a Pt-UME modified with INV in a buffered solution containing GOx and FcMeOH. The potential applied to the microelectrode was 0.15 V, which caused the oxidation of FcMeOH to its radical cation that, in turn, was reduced by

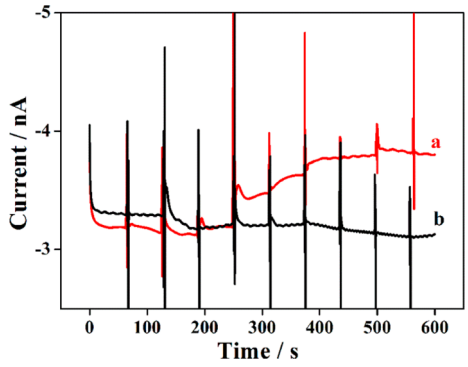

Figure 3. Chronoamperometry recorded with the INV-modified Pt-UME in a buffer solution (50 mM, pH 7) consisting of FcMeOH (1 mM) and GOx (129.7 U/mL). Additions every ca. 60 s of (a) sucrose 20 μL, 0.5 M (in the same buffer solution), and (b) buffer solution in a control experiment.

glucose, catalyzed by GOx. To this solution, a fixed amount of sucrose was added at discerned times. As a blank, a buffer solution was added instead of the sucrose. As can be seen (Figure 3, curve a), an increase of the current is detected only after a few minutes of adding sucrose. In the blank experiment (Figure 3, curve b), where buffer with no sucrose was added, the current did not change considerably, clearly suggesting the crucial role played by the sugar. The delayed increase in current (Figure 3, curve a) is consistent with our previous findings regarding the lower efficiency of α-glucose as a substrate for GOx.

3.3. Heterogeneous Reaction for Constructing the Enzyme AND Gate. In contrast to the difficulties encountered in the course of immobilizing the enzyme onto the Pt-UME, the enzyme attachment onto the glass surface was relatively straightforward. A few methods were studied to optimize the enzyme activity. Eventually, a procedure developed by Wittstock et al. was chosen.[71] This method is based on layer-by-layer (LbL) deposition, whereby the amino-derivatized glass was dipped alternatively in two modification solutions, one which contained the enzyme, acting as a negatively charged layer (GOx is negatively charged when above pH ~5), while the other contained the positively charged PDDA. Images of the glass modified by GOx are shown in Figure 4. The property that is most dramatically altered is the surface roughness, along with its morphology. The surface (Figure 4A) is substantially rougher as a result of the modification. Increasing the

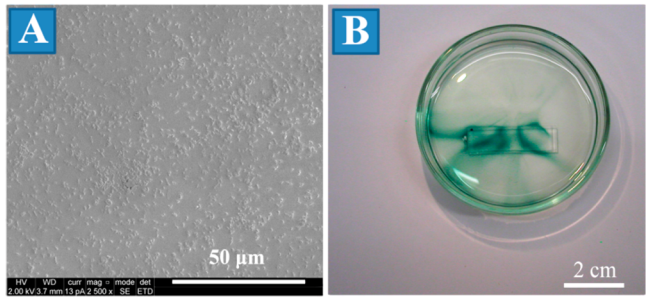

Figure 4. (A) SEM micrograph of the GOx-modified glass surface. (B) Diffusion of the oxidized ABTS from the glass slide on which GOx was immobilized. The experiment was performed in the presence of HRP (125 U/mL), ABTS (1.9 mM), and glucose (27.8 mM) in solution composed of phosphate buffer solution (50 mM, pH 7).

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roughness of the surface as a result of enzyme attachment was observed also for the microelectrode. This affects to some extent the minimal distance that the microelectrode could be brought to the surface without touching it, although it had no adverse effect on the current detection of the microelectrode, as can be seen in Figure 2C, vide supra. The activity of the glass-immobilized GOx was verified by adding glucose to an aerated solution containing HRP and an electron donor, such as ABTS. The development of the blue oxidized ABTS, which is a standard GOx assay, (Figure 4B) was clearly seen by a naked eye and confirmed the oxidation of glucose by oxygen, catalyzed by GOx, and the formation of hydrogen peroxide that is further reduced to water by ABTS and catalyzed by HRP.

Once the different enzymes were immobilized and the substrates chosen, their cooperative activity could be studied using SECM. As detailed above, the INV immobilized on the Pt-UME cleaves the sugar, while the resulting glucose unit diffuses to the surface and is oxidized by the GOx immobilized on the glass support. Since the enzymes' kinetics are limited only by the availability of their corresponding substrates, our hypothesis was that control over the entire process depended on the distance between the Pt-UME and the glass support, given a high concentration of the substrates.

Figure 5 shows the steady state feedback current measured at the Pt-UME while approaching the glass surface. The current is

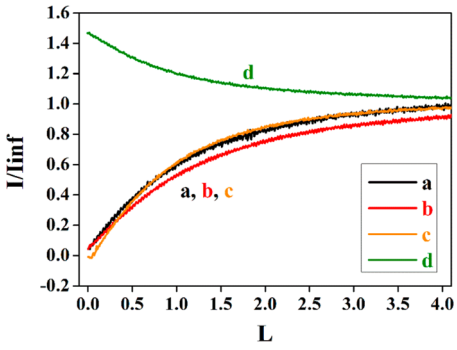

Figure 5. Feedback approach curves, obtained by applying 0.05 V to the Pt-UME. (a) Bare Pt-UME approaching a bared glass surface. (b) Bare Pt-UME approaching a GOx-modified surface. (c) INV-modified Pt-UME approaching a bare glass surface. (d) INV-modified Pt-UME approaching a GOx-modified surface. The solution included MUT (506 U/mL), FcMeOH (0.2 mM), and sucrose (100 mM) in a phosphate buffer solution (50 mM, pH 7).

normalized by dividing the measured current of the microelectrode by the current at infinity, while the distance is normalized by dividing it by the radius of the microelectrode. It can be seen that a negative feedback current, that is, a decrease in the steady-state current, is obtained while approaching an unmodified glass with an unmodified microelectrode in the presence of FcMeOH and sucrose (curve a). Furthermore, negative feedback currents are also obtained upon adding only one enzyme, that is, by either approaching a GOx-modified glass by an unmodified Pt-UME (curve b), or upon approaching an unmodified glass using an INV-modified Pt-UME (curve c). The YES signal, that is, a positive feedback current, was obtained only by approaching the GOx-modified surface with the INV-modified Pt-UME. These results construct the fundamentals of the AND gate as specified in the truth table (Table 1). In the presence of INV and GOx, the

Table 1. Truth Table of the Presented System Mimicking Boolean AND Gate[a]

GOx	INV	current
0	**0**	**0**
0	**1**	**0**
1	**0**	**0**
1	**1**	**1**

[a] "**1**" and "**0**" as the Boolean representations of YES and NO inputs and responses. Note that the presence and absence of the enzymes GOx and INV were defined as logic inputs **1** and **0**, respectively, while the increasing and decreasing feedback currents measured on the Pt-UME were defined as the output signals **1** and **0**, respectively.

current increases. As a result of the INV activity a gradient of glucose was obtained, whereas the applied potential to the microelectrode formed a gradient of ferrocenium ions. When the microelectrode was in close proximity to the surface, the ferrocenemethanol was regenerated by the GOx, which increased the current, resulting in a YES signal.

The approach curves were obtained in a solution containing 200 mM sucrose, 0.2 mM ferrocenemethanol, and 0.5 mg of MUT in a phosphate buffer solution. From the CVs carried out far from the surface (Figure 2C), a potential of 0.05 V was chosen for the approach (Figure 5). This low potential, which is clearly not in the plateau region of the CVs (as can be deduced from Figure 2C), was chosen to obtain a positive feedback current. Generating higher fluxes of the oxidized $FcMeOH^+$ (by applying more positive potentials to the Pt-UME) could not be managed by the enzyme GOx attached to the surface.[71] Under these conditions the entire process is diffusion-controlled and not limited by the enzyme activity. As can be seen in Figure 5, the only positive feedback current that is obtained is the one with the presence of glucose in the system, resulting from the cleavage of the sucrose. As in a typical nonconductive surface, every arrangement combining fewer components than the entire system results in a negative feedback current. It is important to note that the positive feedback current of the complete system shown in Figure 5 is the optimal current obtained; however, in many cases we obtained a less pronounced positive feedback current.

4. CONCLUSIONS

We presented a localized enzymatic logic gate utilizing SECM. The concept is based on controlling the distance between two enzymes, one of which is attached to a nonconducting surface, while the other is attached to a microelectrode. Each enzyme catalyzes a reaction that generates products that react together. Therefore, a YES output was obtained in the form of a positive feedback current upon approaching one surface to the other. Hence, the microelectrode served both to generate the input as well to record the output. This work shows the promise that the localization of logic gates holds, which is demonstrated here in a functioning AND gate. The use of surface-confined biocatalytic species with the controlled distances between them is very important for the optimization of the performance of these gates and for scaling up the complexity of the biomolecular information processing systems.[82] The immobilization of the enzyme, in addition to its confinement to two dimensions, provides many other advantages. It increases the enzyme stability by forcing a specific configuration (rather than in solution), reduces inhibition, and minimizes or even eliminates protein contamination of the product.[83] More

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sophisticated gates based on patterning multienzymes on surface are currently under examination.

AUTHOR INFORMATION

Corresponding Author

*E-mail: daniel.mandler@mail.huji.ac.il. Tel.: 972 2 6585831. Fax: 972 2 6585319.

Notes

The authors declare no competing financial interest.

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