LETTER

Stimuli-Responsive Hydrogel Membranes Coupled with Biocatalytic Processes

Ihor Tokarev, Venkateshwarlu Gopishetty, Jian Zhou, Marcos Pita, Mikhail Motornov, Evgeny Katz,* and Sergiy Minko*

Department of Chemistry and Biomolecular Science and NanoBio Laboratory (NABLAB), Clarkson University, Potsdam, New York 13699-5810

ABSTRACT A nanostructured signal-responsive thin hydrogel membrane was coupled with enzyme-based systems to yield "smart" multisignal-responsive hybrid systems with built-in "logic". The enzyme systems transduce biochemical input signals into structural changes of the membrane, thus resulting in the amplification of the biochemical signals and their transformation into the gated transport of molecules through the membrane. Coupling of the biocatalytic systems with a stimuli-responsive membrane is a promising approach for the development of materials that can regulate transport and release of chemicals/drugs by receiving and processing the biochemical information via biochemical reactions.

KEYWORDS: stimuli-responsive membrane • thin polymer film • biocatalytic process • chemical gating

Signal-responsive materials capable of properties-on-demand changes upon communication with the external environment have recently attracted attention because of their applications in miniaturized devices, "smart" coatings, and drug-delivery systems (1, 2). Typically represented by 2D- or 3D-nanostructured polymer composite systems, signal-responsive materials alter, tune, or turn on and off the shape or dimensions (3), structural alignment or arrangement of components (4), and electronic (5), optical (6), magnetic (7), wetting/adhesion (8), mass-transport (9), or mechanical (10) properties when a physical or chemical signal is received. Bioinspired approaches to signal transduction and property alteration have brought a new dimension to materials science (2, 9, 11). Lessons taken by chemistry and materials science from biological systems have resulted in the development of novel biomolecule-functionalized nanostructured hybrid materials (12). The developed signal-responsive materials are sensitive to a single signal (or sometimes two signals) provided by synthetic or biomolecular receptor groups. For a range of applications, e.g., for analysis, specificity to a single unique chemical signal is considered to be the most important property of the material. However, natural biological systems, in addition to high specificity, demonstrate complex adaptive behavior based on sensitivity to different signals arriving from the environment. Here we demonstrate that a recently formulated chemical approach to information processing (13) can bring novel functions to materials science if integrated with signal-responsive materials. The chemical information processing systems (CIPSs) would be responsible for collecting multiple external signals, processing the received information, and generating a specific signal recognized by the responsive material, which will operate as a chemical actuator, amplifying the signal in the form of macroscopic structural transformations. These transformations will, in turn, be responsible for changes in the material's chemical/physical properties. The resulting hybrid system, composed of an CIPS and a signal-amplifying responsive material, will demonstrate "smart" behavior similar to that of biological systems, where the specificity to received signals is combined with the response to multiple signals. Here we demonstrated the application of this approach to design switchable polymeric membranes with built-in "logic".

CIPSs can be conveniently described using the terminology of binary logic operations if ingredients of the chemical reactions are considered as input signals while the product is considered as an output signal (14). Recent advances in CIPSs have been achieved with biomolecular components such as DNA (15) and proteins/enzymes (16). Application of enzyme-based CIPSs offering specificity in each step of the reactions allowed for the simultaneous operation of several concatenated logic gates without interference in the information processing and processing of multiple chemical input signals to yield a single output signal dependent on a program encoded in the biomolecular system (17). The output signal generated by these enzyme bio-CIPSs could be read out using optical (16, 17) or electrochemical means (18), resulting in optoelectronic or electronic transduction of the biochemically processed information.

Coupling of bio-CIPSs with stimuli-responsive materials is a promising approach for the development of intelligent materials. These kinds of intelligent materials could serve, for example, as drug-delivery systems that regulate the release of drugs based on the "analysis" of chemical signals received in situ (in tissues or blood). The controlled release would be secured by the stimuli-responsive material, while the processing of the chemical information and release of

* E-mail: ekatz@clarkson.edu (E.K.), sminko@clarkson.edu (S.M.).
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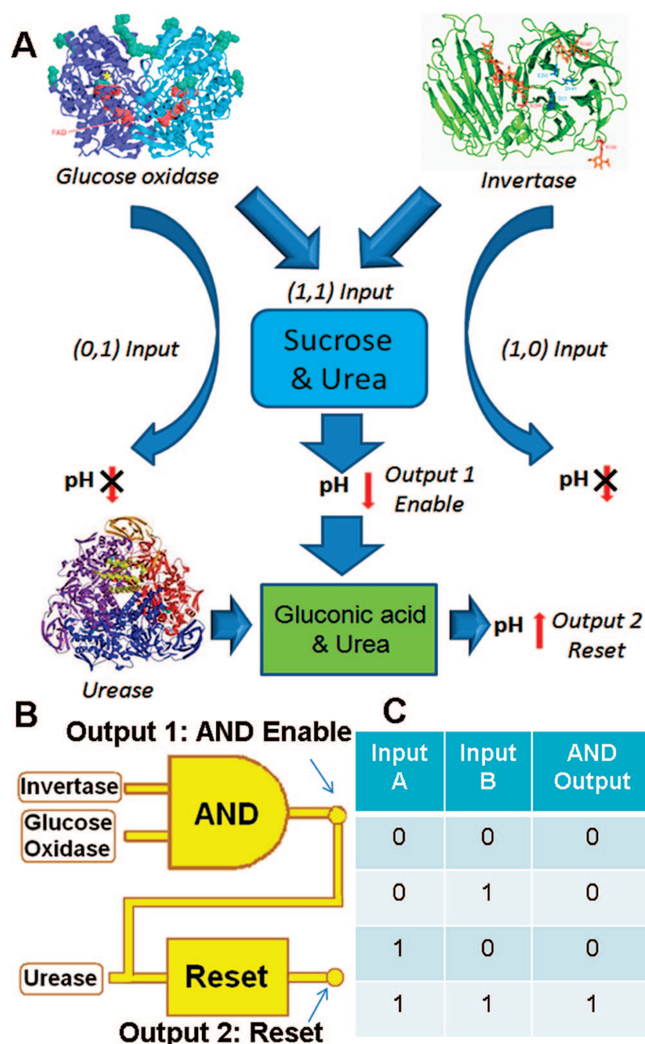
the command signal readable by the responsive material would be performed by the enzymes coupled to the responsive material. In order to couple the enzyme bio-CIPS with the signal-responsive material, the output signal generated by the enzymatic reactions should be in the form of chemical changes acceptable by the material and resulting in the material's structural changes.

Because many enzymatic reactions consume or yield proton ions and many polymer-based signal-responsive systems are sensitive to changes in pH (1, 2), we designed enzyme bio-CIPSs that performed simple **AND/OR** binary logic operations based on biocatalytic reactions and produced pH changes sufficient to induce structural rearrangements in signal-responsive polymeric gel membranes. The prototype bio-CIPS has been described elsewhere 13b, 13c). The **AND** logic gate was composed of an aqueous solution (0.01 M sodium sulfate) containing dissolved sucrose (0.1 M), O_2 (in equilibrium with air), and urea (2 mM), while the enzymes glucose oxidase (GOx, from *Aspergillus niger*, type X-S, E.C. 1.1.3.4) and invertase (Inv, from Bakers yeast, grade VII, E.C. 3.2.1.26), operated as input signals (Figure 1). The absence of each enzyme in the system was considered as the input signal "**0**", while the presence of the enzyme (in a specific optimized concentration: GOx, 5 units mL^{-1}; Inv, 10 units mL^{-1}) was considered as the input signal "**1**". The whole reaction chain included the conversion of sucrose to glucose catalyzed by Inv, followed by the oxidation of glucose catalyzed by GOx and resulting in the formation of gluconic acid, thus yielding acidic pH values. The reaction chain proceeds only in the presence of both enzymes (input signals "**1**,**1**"), while the absence of either or both (input signals "**0**,**0**", "**0**,**1**", and "**1**,**0**") inhibits the formation of the acidic medium (Figure 2). The output signal produced by the biochemical system was considered as "**0**" when the pH changes were small ($\Delta pH < 0.2$) and as "**1**" when $\Delta pH > 1$ (Figure 2, inset). The system demonstrated **AND** logic behavior with a characteristic truth table (Figure 1C). After the reaction was complete, another enzyme input of urease (from jack beans, E.C. 3.5.1.5, 5 units mL^{-1}) was used to catalyze the hydrolysis of urea and to reset the pH value to the original neutral value. The whole **AND-Reset** cycle mimics the performance of the respective electronic circuitry (Figure 1B).

Similarly, the **OR** logic gate was composed of ethyl butyrate (0.01 M), glucose (0.01 M), O_2, and urea (2 mM) dissolved in an aqueous solution (0.01 M sodium sulfate), while two enzymes, GOx (5 units mL^{-1}) and esterase (Est; 5 units mL^{-1}) were used as input signals (Figure 3). Both enzymes activated biocatalytic reactions independently: GOx catalytically oxidized glucose and Est catalytically hydrolyzed ethyl butyrate, both resulting in acidification of the solution (Figure 4). Thus, the system preserved the initial neutral pH ($\Delta pH < 0.2$; the output signal "**0**") only in the absence of both enzymes (input signals "**0**,**0**"), while the reactions (either or both together) yielded the acidic media ($\Delta pH > 1$; the output signal "**1**") upon input signals "**0**,**1**", "**1**,**0**", and "**1**,**1**" (Figure 4, inset), demonstrating behavior



Input A	Input B	AND Output
0	0	0
0	1	0
1	0	0
1	1	1

FIGURE 1. Reaction scheme for the biochemical AND logic gate with the enzymes GOx and Inv used as input signals to activate the gate operation: the absence of the enzyme is considered as "0" input signals and the presence as "1" input signals. The Reset function was catalyzed by urease (A). Equivalent electronic circuit for the biochemical AND-Reset logic operations (B). Truth table of the AND gate showing the output signals in the form of pH changes (ΔpH) generated upon different combinations of the input signals (if $\Delta pH < 0.2$; the output signal "0") (C).

typical for the **OR** gate with the respective truth table (Figure 3C). The logic operation resulting in acidification of the solution was followed by the addition of the reset-enzyme urease, returning the system to the original pH value. The whole reaction set could be expressed in terms of the equivalent electronic system: **OR-Reset** (Figure 3B).

Stimuli-responsive porous polymer membranes in this work are thin films with an array of vertically oriented cylindrical pores (19). The membranes were prepared from a weak polyelectrolyte network whose swelling depends on the pH of the environment. Swelling−shrinking of the network causes switching in the diameter of the membrane's pores between two states: open submicrometer pores and completely closed pores. This behavior was used to regulate a range of properties of the membrane (19) as well as for the transduction of chemical information into an optical signal (20).

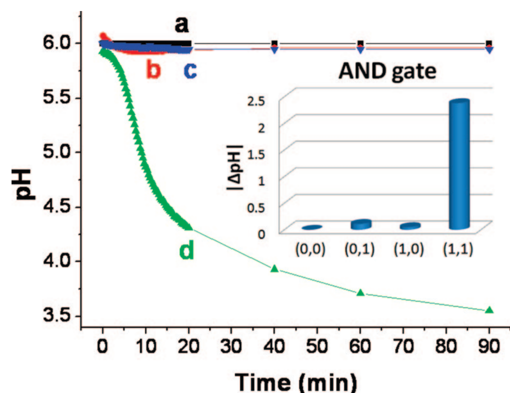


FIGURE 2. Plots for the time-dependent pH changes generated in situ by the AND gate upon different combinations of input signals. The enzymes GOx and Inv were used as input signals to activate the gate operation: (a) reference experiment (no enzymes added, "0,0"); (b) only GOx added, "1,0"; (c) only Inv added, "0,1"; (d) both GOx and Inv added, "1,1". Inset: Bar diagram showing the pH changes as the output signals of the AND gate. The absence of the enzyme is considered as "0" input signals and the presence as "1" input signals.

The signal-responsive 100-nm-thick polyelectrolyte gel membrane (Figure 5) in this work was prepared by salt-induced phase separation of sodium alginate (from brown algae, medium viscosity, ≥2000 cP for a 2% solution at 25 °C) and gelatin (from porcine skin, type A) and cross-linked by $CaCl_2$ (Figure 5d) (21). The average pore diameter was 380 ± 116 nm, as estimated for this film at the half-depth of the pores. The membrane operates by gel swelling in response to changes in the pH; this leads to shrinkage of the pores and consequently to a change in the permeability. The membrane was deposited onto an indium−tin oxide (ITO) glass electrode (20 ± 5 Ω sq^{-1}, Aldrich) for electrochemical characterization or onto a porous substrate (track-etched polyester membrane, pore size of 200 nm, Sterlitech Corp.) for permeability measurements. The scanning probe microscopy (SPM) topography images obtained in situ in a liquid cell (Multimode Microscope, Veeco Instruments, Plainview, NY; Figure 6), electrochemical impedance spectroscopy (ECO Chemie Autolab; Figure 7a), and probe molecules (fluorescent dye Rhodamine B) diffusivity through the polyelectrolyte membrane (Figure 7b) were explored to monitor the behavior of the membrane coupled with the enzyme-based logic gates. The experiments proved a strong dependence of the swelling of the polyelectrolyte membrane on the pH: the pores were open at pH < 4 and completely closed at pH > 5. To activate the **AND** biochemical logic gate (Figure 1), we added one or both enzymes (GOx and Inv) as input signals to the solution with dissolved sucrose, O_2, and urea, which wet the membrane at pH 6. In the absence of each enzyme in the system (input signals "**0**,**0**"), the membrane pores were closed (Figures 5a and 6a); the impedance measurements on the membrane deposited on the electrode showed an electron-transfer resistance, R_{et}, of ca. 2.5 kΩ for a diffusional redox probe, $[Fe(CN)_6]^{3-/4-}$, 10 mM (Figure 7a), and no diffusion of the dye was detected through a membrane deposited on the porous substrate (Figure 7b). Obviously, the same behavior of the membrane was documented in the case when only one of the enzymes (input signals

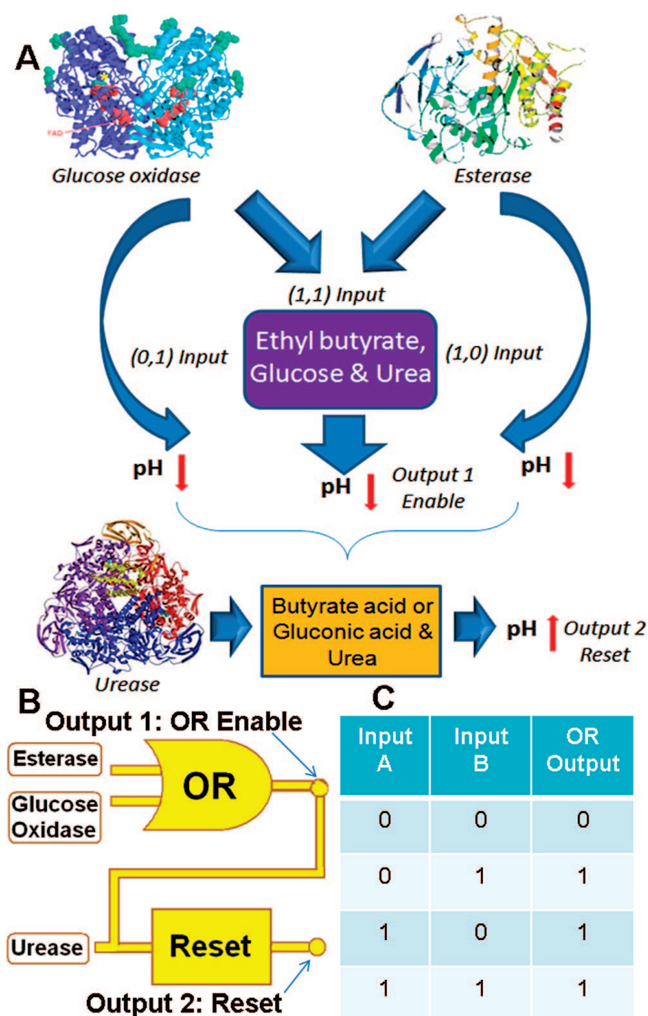


FIGURE 3. Reaction scheme for the biochemical OR logic gate with the enzymes GOx and Est used as input signals to activate the gate operation: the absence of the enzyme is considered as "0" input signals and the presence as "1" input signals. The Reset function was catalyzed by urease (A). Equivalent electronic circuit for the biochemical OR-Reset logic operations (B). Truth table of the OR gate showing the output signals in the form of pH changes (ΔpH) generated upon different combinations of the input signals (if ΔpH < 0.2; the output signal "0") (C).

"**0**,**1**" and "**1**,**0**") was added to the system. However, if both enzymes were added (input signals "**1**,**1**"), the enzymatic reactions resulted in a pH decrease from 6 to 4 and an opening of the pores of the membrane (Figures 5b and 6b); R_{et} dropped down to ca. 0.5 kΩ (Figure 7a), and the dye fluorescent spectra were detected in the filtration chamber from the inverse side of the membrane, indicating diffusion of the dye through the open pores (apparent diffusion coefficient of ~1.1 × 10^{-11} m^2 s^{-1}; Figure 7b). The state of the system was reset by adding urease. Hydrolysis of urea resulted in the elevation of the pH to the original value of pH 6. In the experiment, we observed closed pores, a reversible increase of R_{et} to ca. 2.5 kΩ, and an interruption of transport of the dye through the membrane.

A similar experiment was conducted with the enzyme-based **OR** gate shown in Figure 3. In this case, all input signals "**0**,**1**", "**1**,**0**", and "**1**,**1**" resulted in open pores of the membrane, while reset of the system returned the mem-

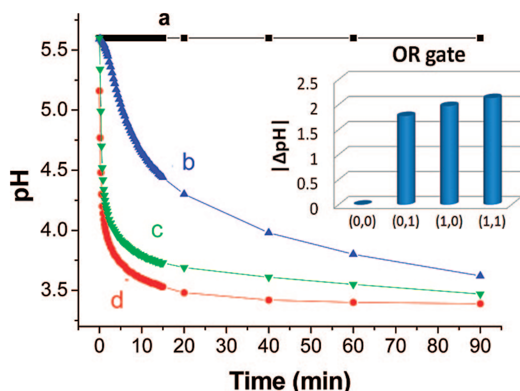


FIGURE 4. Plots for the time-dependent pH changes generated in situ by the OR gate upon different combinations of the input signals. The enzymes GOx and Est were used as input signals to activate the gate operation: (a) reference experiment (no enzymes added, "0,0"); (b) only Est added, "1,0"; (c) only GOx added, "0,1"; (d) both GOx and Est added, "1,1". Inset: Bar diagram showing the pH changes as the output signals of the OR gate. The absence of the enzyme is considered as "0" input signals and the presence as "1" input signals.

brane to the state of closed pores, resulting in the respective changes in R_{et} in the impedance measurements (Figure 7a) and the membrane permeability for the dye (Figure 7b).

Finally, we have demonstrated that not only enzymes in solutions can be used as the input signals. An alternative

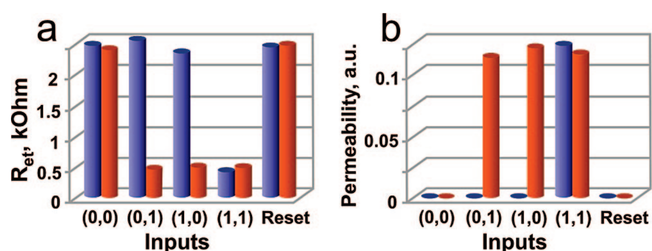


FIGURE 7. Electron-transfer resistance, R_{et}, of the pH-responsive polyelectrolyte membrane deposited on the electrode surface derived from the impedance spectroscopy measurements obtained upon different combinations of input signals (a). Permeability (ratio of the membrane permeability deposited on the supporting filter to the permeability of the filter with no membrane) for Rhodamine B obtained upon different combinations of the input signals (b). Blue and red bars correspond to the AND and OR gates, respectively.

scenario involves immobilized enzyme molecules built into materials, while the substrate and another enzyme (e.g., sucrose and Inv) can be used as input signals. For example, we immobilized GOx (using a carbodiimide procedure, enzyme activity of 0.014 units cm^{-2}) on the surface of an alginate membrane imbedded in a solution of urea (2 mM) and O_2 at pH 6. In this case sucrose (0.1 M) and Inv (10 units mL^{-1}) were used as input signals, resulting in the **AND** logic operation. The absence of both Inv and sucrose or either was considered as "**0**,**0**", "**0**,**1**", and "**1**,**0**" input signals. For these input signals, no changes were observed in the membrane

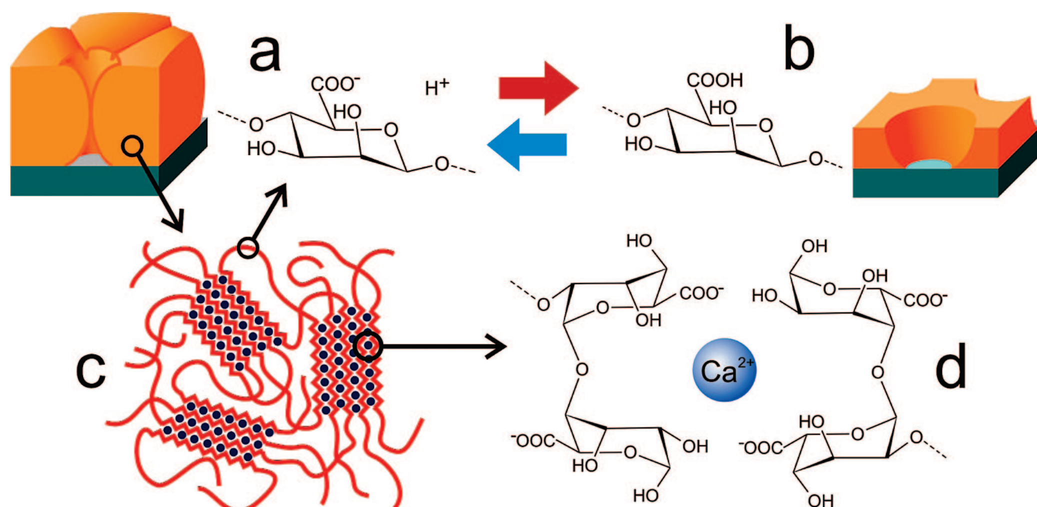


FIGURE 5. Schematic representations of a single pore of the polyelectrolyte membrane switched between the closed (a) and open (b) states. The structure of the alginate hydrogel comprised of D-mannuronic acid and L-guluronic acid residues cross-linked with divalent ions (Ca^{2+}) in part d to give an egg-box-like conformation (c). The swelling and shrinking of the hydrogel is attributed to the ionization (a) and protonation (b) of the unbound carboxyl groups at pH > 5 and pH < 4, respectively.

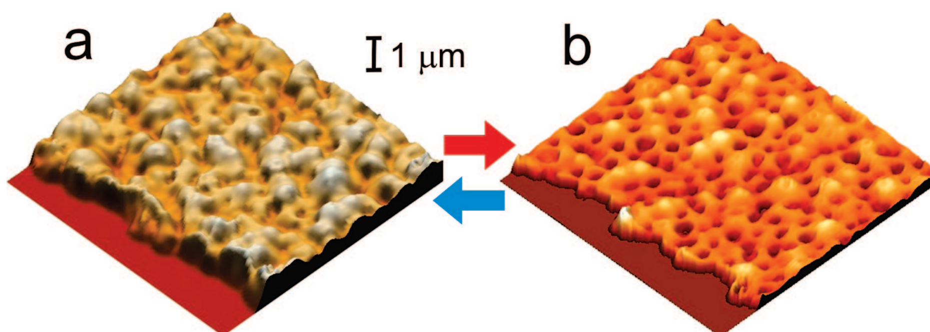


FIGURE 6. SPM topography images (10 × 10 μm^2) of the swollen (a) and shrunken (b) pH-responsive polyelectrolyte membrane.

with closed pores. Adding both Inv and sucrose ("**1**,**1**" input signals) resulted in a decrease of the pH down to 4 and opening of the pores of the membrane. The **Reset** function was performed using urease (5 units mL^{-1}), resulting in elevation of the pH value and return of the membrane to the closed state.

It is obvious that many possible combinations can be explored to integrate responsive membranes and biomolecular logic gates by introducing enzymes into the system either through binding them directly to the material of the membrane or injecting them into the membrane's environment. Further scaling up of the enzyme-based logic systems to bio-CIPS networks (17) composed of several concatenated logic gates operating together could allow complex logical processing of biochemical information and its transduction into macroscopic changes of signal-responsive membranes. In this case, the changes in the materials properties would be controlled by many different biochemical signals collected and processed by the enzyme system, providing an efficient means for the fabrication of "smart" multisignal responsive drug-delivery systems, sensors, miniaturized switchers, microfluidic devices, etc., that can operate without communication to an external computer and without an external power source.

EXPERIMENTAL SECTION

Membrane Preparation. Solutions of sodium alginate and gelatin were prepared by dissolving 0.1 g of each polymer separately in 5 mL of Millipore water (18 $\mu\Omega$ cm) at 60 °C. A solution for film deposition was prepared in two steps: (1) mixing the alginate and gelatin solutions in a 1:1 (v/v) ratio and stirring the mixture for 1 h at 60 °C and (2) adding sodium chloride (0.5 wt %) to the resulting solution and stirring for 30 min at the same temperature. Because of aging, the deposition solution must be used fresh to guarantee the reproducibility of the results. Films were spin-cast onto silicon wafers or ITO electrodes pretreated with (3-glycidoxypropyl)trimethoxysilane (GPS) at 3000 rpm for 2 min. Then, they were immersed in a 0.3 M calcium chloride solution for 15 min, rinsed with Millipore water, and dried in a nitrogen flow. The $CaCl_2$ treatment led to ionic cross-linking of the alginate phase, to dissolution of the gelatin phase, and, consequently, to the formation of insoluble films with a macroporous structure (membranes).

The GPS treatment was needed to chemically bind membranes to a substrate surface and thus to eliminate the membranes' lift-off during swelling. The substrates were modified with GPS monolayers according to the following procedure. The wafers were cleaned in an ultrasonic bath with dichloromethane, then treated with a cleaning solution containing NH_4OH and H_2O_2 for 1 h at 60 °C, and carefully rinsed with Millipore water. The cleaned substrates were kept in a 1 wt % solution of GPS in dry toluene overnight. The GPS-modified substrates were then rinsed twice with toluene and once with ethanol to remove the unreacted GPS. Prior to film deposition, the GPS-modified substrates were annealed at 120 °C for 30 min (because we found that the films were more stable on the annealed substrates). The presence of a residual amount of gelatin in the alginate gel membranes was essential for the chemical attachment of the films to the GPS-modified substrates due to reaction of the epoxy rings of GPS with the amino groups of gelatin (21).

Immobilization of GOx on the Membrane's Surface. Enzyme immobilization on the membrane surface was carried out in two steps. First, carboxyl groups of the alginate gel were preactivated for 1 h at 4 °C in a 0.1 M acetate buffer containing 0.5 wt % 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (22). Then the sample was transferred into a 0.1 M acetate buffer (pH 4.1) containing 0.4 wt % GOx. The immobilization was allowed to proceed at 4 °C overnight. After that, the sample was rinsed with an acetate buffer to remove the unbound GOx.

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