Responsive Interface Switchable by Logically Processed Physiological Signals: Toward "Smart" Actuators for Signal Amplification and Drug Delivery

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Responsive Interface Switchable by Logically Processed Physiological Signals: Toward "Smart" Actuators for Signal Amplification and Drug Delivery

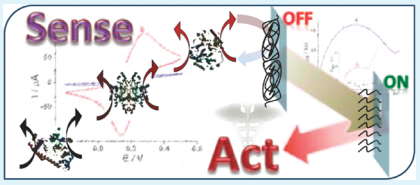
Marina Privman,[‡,†] Tsz Kin Tam,[†] Vera Bocharova,[†] Jan Halámek,[†] Joseph Wang,[*,§] and Evgeny Katz[*,†]

[‡]Empire State College, SUNY, P.O. Box 908, Fort Drum, New York 13602-0908, United States

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

[§]Department of NanoEngineering, University of California—San Diego (UCSD), La Jolla, California 92093, United States

ABSTRACT: Biomarkers characteristic of liver injury, alanine transaminase and lactate dehydrogenase, were processed by an enzyme-based system functioning as a logic **AND** gate. The NAD^+ output signal produced by the system upon its activation in the presence of both biomarkers was then biocatalytically converted to a decrease in pH. The acidic pH value biocatalytically produced by the system as a response to the biomarkers triggered the restructuring of a polymer-modified electrode interface. This allowed a soluble redox species to approach the electrode surface, thus switching the electrochemical reaction ON. The redox transformations activated by the biochemical signals resulted in an amplification of signals. This system represents the first example of an integrated sensing-actuating chemical device with the implemented **AND** Boolean logic for processing natural biomarkers at their physiologically relevant concentrations.



KEYWORDS: signal-responsive interface, logic gate, enzyme, liver injury, biomarker

INTRODUCTION

Application of molecular[1−7] and biomolecular[8−11] information processing systems in biosensors logically processing complex patterns of various chemical input signals[12] has recently received significant attention and shows great promise, particularly for biomedical applications.[13−15] Logic operations performed by enzyme systems[8] were applied for in vitro analysis of biomarkers to identify pathophysiological conditions characteristic of various injuries.[16−19] Systems specifically designed for analysis of biomedical conditions use medically relevant biomarkers as input signals at their physiological concentrations. This poses additional challenge, compared to (bio)molecular logic systems which process external chemical or physical signals without medical relevance and use the physical zero and a conveniently high concentrations of chemical signals to mimic logic **0** and **1** values, respectively. Biomedical logic systems are activated by the digitized chemical inputs being at logic **0** level when the biomarker concentrations are physiologically normal and at logic **1** level upon pathophysiologically elevated concentrations. Sometimes the gap separating the logic **0** and **1** input levels is rather small, imposing additional limitations on the system performance aimed at achieving robust error-free digital logic operation toward high-fidelity diagnostics.

Enzyme logic systems were also applied to control states of signal-responsive materials in the form of a switchable membrane,[20] an emulsion[21] or a nanoparticle assembly,[22] which operated as nanostructured actuators reversibly changing the structure and material properties upon receiving signals from the biomolecular systems. Signal-responsive materials, in the form of polymer brushes, were bound to electrode surfaces allowing reversible switching of electrochemical activities ON/OFF by complex biochemical signals processed through enzyme biocatalytic cascades.[23,24] However, none of the recently developed chemically switchable systems were controlled by biomedically meaningful biomarker-signals, particularly at their physiologically relevant concentrations. This paper presents the first example of a switchable electrode interface logically controlled by the enzyme cascade processing biomarkers characteristic of an injury. A liver injury, using the alanine transaminase (ALT) and lactate dehydrogenase (LDH) as the biomarker inputs for the **AND** logic gate,[17] was selected to illustrate this concept. The biochemically controlled actuation achieved in the present study is the first step toward integrated "smart" 'Sense/Act' (biosensor-bioactuator) systems processing biomedical signals, making programmed decision, and then performing an actuation according to the biomedical needs.

EXPERIMENTAL SECTION

Chemicals and Supplies. Alanine transaminase from porcine heart (ALT, E.C. 2.6.1.2), lactate dehydrogenase from porcine heart (LDH, E.C. 1.1.1.27), glucose dehydrogenase from *Pseudomonas sp.* (GDH, E.C. 1.1.1.47), β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH, 97%), α-ketoglutaric acid sodium salt (≥98%), L-alanine (≥98%), β−D−(+)−glucose (99.5%), *tris*-(hydroxymethyl)aminomethane hydrochloride salt (Tris-buffer), poly-(4-vinyl pyridine) (P4VP, M.W. 160 kDa, ρ = 1.101 g·cm^{-3}) and other

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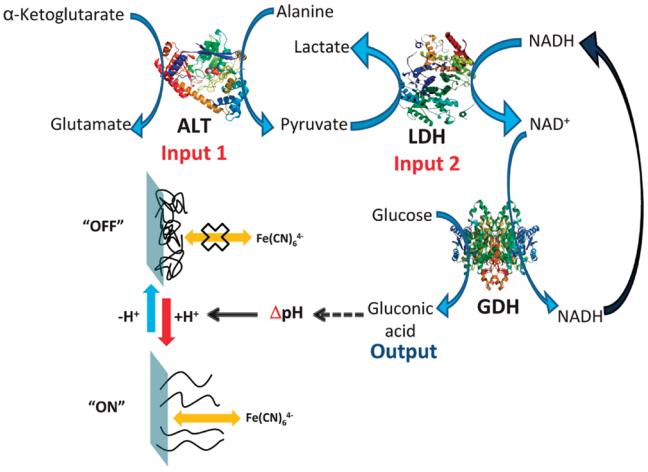
standard chemicals were purchased from Sigma-Aldrich and used as supplied without any further purification. Water used in all of the experiments was ultra pure (18.2 MΩ·cm) from a NANOpure Diamond (Barnstead) source. Indium tin oxide (ITO) single-side coated conducting glass from Aldrich (20 ± 5 Ω/sq surface resistivity) was used for electrochemical measurements.

Composition and Operation of the System. The "machinery" of the signal processing chemical system was composed of 10 U·mL^{-1} GDH, 200 mM alanine, 0.3 mM NADH, 9.7 mM α-ketoglutarate, 20 mM glucose and 10 mM $K_4[Fe(CN)_6]$ dissolved in an aqueous solution containing 100 mM Na_2SO_4 and 1.2 mM Tris-buffer. Logic **0** and **1** levels of ALT (0.02 and 2 U·mL^{-1}) and LDH (0.15 and 1 U·mL^{-1}) input signals were applied to the logic system to realize meaningful circulating levels of these biomarkers under normal and pathophysiological conditions, respectively. The system was activated by the addition of four different combinations of the input signals: **0,0**; **0,1**; **1,0** and **1,1**. The resulting pH changes and electrochemical behavior of the system were monitored for up to three hours. The pH measurements were performed with Mettler Toledo SevenEasy pH-meter. Electrochemical measurements were carried out with an ECO Chemie Autolab PASTAT 10 electrochemical analyzer, using the GPES 4.9 (General Purpose Electrochemical System) software package for cyclic voltammetry and FRA 4.X (Frequency Response Analyzer) for the Faradaic impedance measurements. The working electrode was an ITO electrode (1.2 cm^2 geometrical area) modified with a P4VP polymer brush, following a previously described procedure.[24] A Metrohm Ag|AgCl|KCl, 3M, electrode served as a reference electrode and a Metrohm Pt wire was used as a counter electrode. The state of the electrode interface was studied by recording the Faradaic impedance spectra in the frequency range of 10 Hz to 10 kHz and 0.01 Hz to 10 kHz for the ON and OFF states of the electrode, respectively, while applying a bias potential of 0.17 V. The values of electron transfer resistance, R_{et}, were derived from the recorded impedance spectra using the commercial software ZView, version 2.1b, Scribner Associates, Inc., by fitting the experimental data to the Randles-Ershler equivalent circuitry.[25] Cyclic voltammograms were recorded in the potential range from −0.2 to 0.5 V with a scan rate of 100 mV·s^{-1}, and peak currents were obtained from the second scan. All measurements were performed in a temperature-controlled standard three-electrode cell (ECO Chemie) at 37 ± 0.2 °C mimicking physiological conditions.

RESULTS AND DISCUSSION

The enzymes ALT and LDH were selected as liver injury biomarkers.[26] Their specificity for the injury is rather limited and elevated concentrations of each biomarker separately are not a solid evidence of liver injury. However, the appearance of both biomarkers at their characteristic elevated concentrations provides strong evidence of liver injury. A biocatalytic cascade which includes cooperative action of both biomarkers mimicking the **AND** Boolean logic gate was designed to signal the presence of these biomarkers.[17] Scheme 1 shows the biocatalytic cascade activated by the biomarker inputs, ALT and LDH, primarily resulting in the conversion of NADH to its oxidized form NAD^+. The corresponding optical absorbance changes were used in an earlier work to analyze the performance of the **AND** logic gate upon application of different combinations of the input signals.[17] In the present work, to convert the NAD^+ signal to pH changes affecting the state of the modified electrode, GDH and glucose were added to the system to operate as the last step of the biocatalytic cascade. In situ generation of NAD^+ resulted in the oxidation of glucose biocatalyzed by GDH, thus causing release of two hydrogen ions, while NAD^+ accepts

Scheme 1. Biocatalytic Cascade Used for the Logic Processing of the Chemical Input Signals, Resulting in Situ pH Changes and Activation of the Electrode Interface



only one hydrogen ion upon its reduction, thus resulting in the solution acidification, and producing ΔpH as the final output signal.

The biocatalytic cascade producing pH changes can be completed only in the presence of both the input signals, thus mimicking the **AND** logic operation. It should be noted, however, that the logic **0** levels of the input signals correspond to the normal physiological concentrations of ALT and LDH, rather than to their complete absence. Thus, the input combinations **0,0**; **0,1**; and **1,0** also result in the reactions shown in Scheme 1, but yield substantially smaller pH changes than the combination **1,1**, which corresponds to the pathophysiological elevated concentrations of the two biomarkers for liver injury. The challenge in the present work has been achieving the electrode activation only with the signal combination **1,1**, while all other combinations preserve the OFF state of the electrode. To realize this specific switchable behavior of the P4VP-modified electrode, its pH-controlled transition from the OFF to ON state[24] must be coordinated with the pH output signals produced by the biocatalytic system.

Interfacial properties of the P4VP-modified electrode are dependent on the protonation state of the polymer brush which is swollen and permeable for anionic redox species in its positively charged protonated state. The shrunken neutral state of the polymer brush is nonpermeable for redox species, where the electrode is inactive. The reversible transition of the polymer brush between the charged and neutral state, corresponding to the ON and OFF states of the electrode, respectively, can be achieved by changing the pH value of the external solution.[24] However, it might be inhibited by adsorption of proteins; the switchable behavior of the electrode interface requires verification for each specific composition of the solution. Figure 1 shows the titration curve corresponding to the electrode activity derived by cyclic voltammetry at variable pH values in the solution containing all chemicals included in the sensing system except the input signals. The electrode activity is switched OFF at solution pH values higher than 5, while there is a sharp transition to the ON state of the electrode below this pH value. The pK_a = 4.7 of the polymer brush was derived from the experimental titration curve.

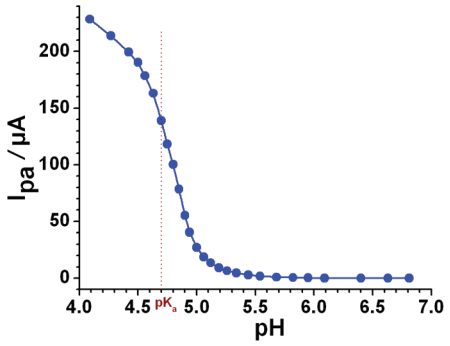


Figure 1. Variation of the anodic peak current measured by cyclic voltammetry upon titration of the sensing system to different pH values. Cyclic voltammograms were recorded after allowing 10 min for the working electrode to equilibrate at each pH. The system composition is specified in the Experimental Section.

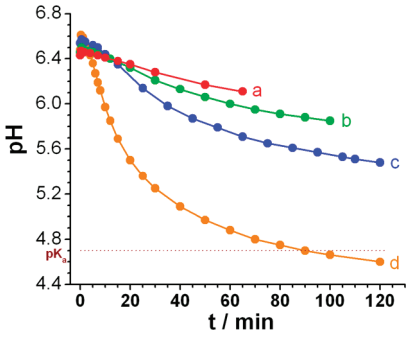


Figure 2. pH changes generated in situ by the biocatalytic cascade activated with various combinations of the two chemical input signals, ALT, LDH: (a) **0,0**; (b) **0,1**; (c) **1,0**; (d) **1,1**. The dotted line corresponds to the pK_a value of the P4VP-brush obtained from the titration curve in Figure 1. The system composition is specified in the Experimental Section.

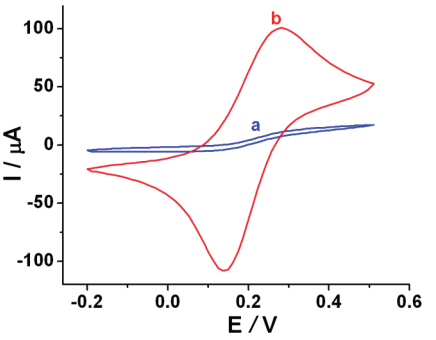


Figure 3. Cyclic voltammograms obtained for the ITO electrode modified with the P4VP-polymer brush in (a) the initial OFF state at t = 1 min after the initiation of the biocatalytic cascade, pH 6.3, and (b) the ON state enabled by the ALT, LDH input combination **1,1**, at t = 80 min, pH 4.75. The system composition is specified in the Experimental Section.

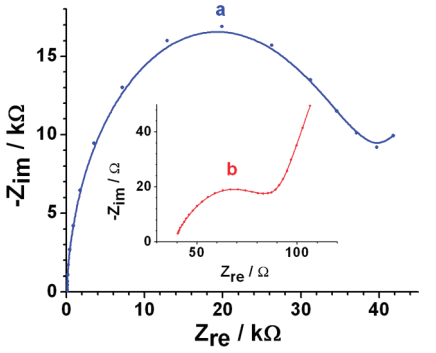


Figure 4. Sample impedance spectra in the form of Nyquist plots for (a) the OFF state of the electrode, with the initial pH 6.6, electron transfer resistance, R_{et}, ~40 kΩ, and (b) the ON state enabled by the input combination **1,1**, with the pH 4.76 generated in situ, R_{et}, ~60.3 Ω. (Note different scales for curves a and b). The system composition is specified in the Experimental Section.

In the next experiment we applied the two input signals, ALT and LDH, in four different combinations: **0,0**; **0,1**; **1,0** and **1,1**, to generate the pH changes in situ upon activation of the biocatalytic cascade shown in Scheme 1. Figure 2 shows the time-dependent pH changes for the four input combinations. The important feature of the system is a large separation of the pH changes generated in the presence of **1,1** input signals from all other combinations. The pH changes produced in situ in the presence of **1,1** input signals reach the pK_a value of the polymer brush at a reaction time of 90 min, while other signal combinations do not produce this pH value at any reaction time (measured up to 3 h). Such distinct pH-time profiles allowed selective electrode activation in the presence of the **1,1** input combination characteristic of liver injury, Scheme 1.

Figure 3 shows cyclic voltammograms obtained with the P4VP-modified electrode being in the original OFF state at pH 6.3 (curve a) and after the electrode was activated by **1,1** input signals reaching the pH 4.75 generated in situ in 80 min (curve b). While the electrode being in the initial OFF state shows only very small activity for the soluble redox probe, the same electrode after its activation with **1,1** input signals shows a reversible cyclic voltammogram characteristic of the diffusional electrochemical process. It should be noted that all the other input combinations did not change the electrode state, even for much longer reaction times demonstrating the cyclic voltammograms similar to curve a in Figure 3. Consistent results were obtained upon measuring the Faradaic impedance spectra with the switchable electrode. Figure 4 (curve a) shows the impedance spectrum at the initial OFF state of the electrode with the electron transfer resistance, R_{et}, ~40 kΩ. Application of the **1,1** input combination resulted in the opening of the electrode interface and the decrease of the R_{et} to 60.3 Ω (curve b). It should be noted that all other combinations of the input signals did not change the R_{et} value, demonstrating impedance spectra similar to the one shown in Figure 4 (curve a).

It should be noted that we also tested input values corresponding to intermediate concentrations of the biomarkers corresponding to mild liver injury conditions. However, the P4VP membrane was not open upon these inputs since the pH value produced by the biocatalytic system did not reach the value low enough for the polymer protonation needed for its restructuring. This means that only severe injury conditions can provide enough changes in the system in order to trigger the polymer opening and electrode activation.

The primary redox species changing the concentrations upon the operation of the **AND** logic gate, NADH/NAD^+, appear at concentrations of 0.3 mM, while the redox species reacting at the switchable electrode, $[Fe(CN)_6]^{4-}$, has a much higher

concentration of 10 mM. The primary biochemical species, processed through the biocatalytic cascade, trigger the opening of the electrode surface for the electrochemical reaction with a high concentration of the reacting species, thus resulting in the amplification of the output signal.

CONCLUSION

We demonstrated the possibility of chemical actuation triggered by appropriate combinations of medically relevant biomarkers applied under pathophysiologically elevated concentrations. A switchable polymer-modified electrode was selectively activated by the **1**,**1** input signals combination, which was processed by the enzyme logic system operating as the **AND** gate and transduced to pH changes controlling the interfacial properties of the electrode.

Small concentration changes of the NADH/NAD^+ system were converted into a large current corresponding to the electrochemical process of the $K_4[Fe(CN)_6]$ redox probe, thus amplifying the output signal generated by the enzyme logic system. A switchable electrode controlled by biomarker inputs can be integrated into a biofuel cell[27] being activated upon pathophysiological conditions characteristic of a specific injury. The system could be also redesigned in the future to open a channel in a microfluidic device for a drug release triggered upon receiving the output signal from the enzyme logic, thus opening the opportunities for the 'Sense/Act' (biosensor-bioactuator) integrated systems. Future research will be directed at a system operating in the human serum solutions mimicking real biological samples.[16] Another challenge will be the integration of the signal-processing system logic with the switchable polymer interface to generate local interfacial pH changes[28] rather than a bulk pH change in the solution.

AUTHOR INFORMATION

Corresponding Author

*Tel: +1 (315) 268 4421 (E.K.); +1 (858) 246 0128 (J.W.). Fax: +1 (315) 268 6610 (E.K.); +1 (858) 534 9553 (J.W.). E-mail: ekatz@clarkson.edu (E.K.); josephwang@ucsd.edu (J.W.).

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