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Activation of a Biocatalytic Electrode by Removing Glucose Oxidase from the Surface—Application to Signal Triggered Drug Release

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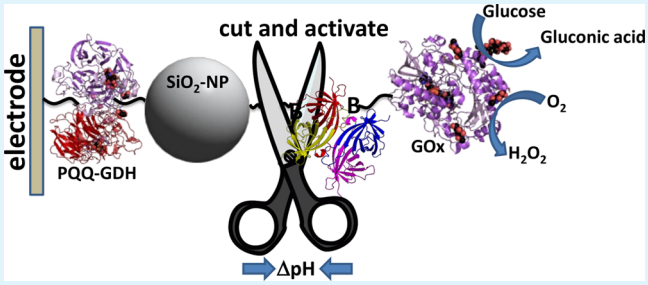
Supporting Information

ABSTRACT: A biocatalytic electrode activated by pH signals was prepared with a multilayered nanostructured interface including PQQ-dependent glucose dehydrogenase (PQQ-GDH) directly associated with the conducting support and glucose oxidase (GOx) located on the external interface. GOx was immobilized through a pH-signal-cleavable linker composed of an iminobiotin/avidin complex. In the presence of GOx, glucose was intercepted at the external interface and biocatalytically oxidized without current generation, thus keeping the electrode in its nonactive state. When the pH value was lowered from pH 7.5 to 4.5 the iminobiotin/avidin complex was cleaved and GOx was removed from the interface allowing glucose penetration to the electrode surface where it was oxidized by PQQ-GDH yielding a bioelectrocatalytic current, thus switching the electrode to its active state. This process was used to trigger a drug-mimicking release process from another connected electrode. Furthermore, the pH-switchable electrode can be activated by biochemical signals logically processed by biocatalytic systems mimicking various Boolean gates. Therefore, the developed switchable electrode can interface biomolecular computing/sensing systems with drug-release processes.



KEYWORDS: *biocatalytic electrode, signal-activated electrode, drug-mimicking release, glucose electrode, alginate electrode*

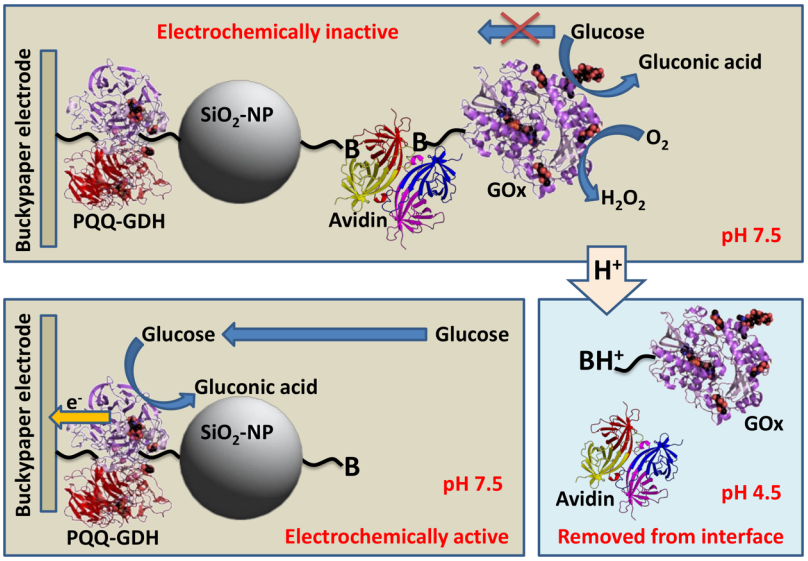
Modified electrodes switchable between electrochemically active and inactive states by various physical and chemical signals have been designed and used for different applications,[1] including unconventional memory units,[2] information processing systems,[3] switchable biosensors,[4,5] and signal-activated biofuel cells.[6−10] Using different signal-responsive materials associated with electrode surfaces, the switchable electrodes were controlled by light signals,[11] magnetic field,[12] temperature changes,[13] applied electrical potentials,[14] and chemical/biochemical inputs.[15,16] Electrode activation by the presence of biomolecular species is particularly important for many biomedical applications.[17] Although some applications, memory operations,[2] for example, require reversible switching between distinct states with different electrode activity, in other applications, like drug release,[18] only the activation of an electrode is sufficient.[19] Reversible transitions between active−inactive states of electrode interfaces are usually controlled by switching signal-responsive materials associated with electrode surfaces.[1] On the other hand, when only electrode activation is needed, it can be achieved by attaching biocatalytic species to the electrode interface through bioaffinity interactions. For example, enzymes can be attached to the electrode surface upon antigen−antibody binding,[20] DNA hybridization.[21] or other biorecognition processes.[22] The electrodes are biocatalytically inactive until the enzymes are attached to the surface. The enzyme attachment and the following electrode activation are driven by the presence of specific biomolecules which result in the formation of enzyme-labeled affinity complexes on the interfaces. The activated electrodes are usually used in various electrochemical biosensors (e.g., immunosensors, DNA sensors, etc.), where the enzymes are used as biocatalytic labels reporting in the case of complex formation.[23] It should be noted that the biorecognition complex formation resulting in electrode activation usually requires human-performed steps: addition of the reacting species, washing the excess of nonreacted molecules from the system, etc. There are very few examples of bioelectrocatalytic interfaces that are already preassembled for operation and only waiting for a triggering signal to start working.[22]

The present paper reports on a new bioelectrocatalytic system that is preassembled on an electrode surface and mute until one of its components is removed from the interface upon receiving a chemical signal. In the active form the bioelectrocatalytic interface generates anodic current corresponding to glucose oxidation. Surprisingly, this electrode active state is produced by *removing* glucose oxidase (GOx) from the electrode surface. The system is based on two glucose-oxidizing enzymes: PQQ-dependent glucose dehydrogenase (PQQ-GDH; E.C. 1.1.5.2) and glucose oxidase (GOx; E.C.

Received: July 12, 2014
Accepted: August 1, 2014
Published: August 1, 2014

dx.doi.org/10.1021/am504561d | *ACS Appl. Mater. Interfaces* 2014, 6, 13349−13354

Scheme 1. Electrochemically Inactive (top) and Active (bottom) Electrode States and the Transition from the Inactive to Active State by Removing GOx from the Interface upon Cleaving the pH-Sensitive Affinity Bridge in the Presence of a pH Signal



1.1.3.4). Although PQQ-GDH immobilized directly on the electrode surface is capable of direct electron transfer upon biocatalytic glucose oxidation, GOx is located at a distance from the electrode surface and does not communicate with the electrode directly. When glucose is present in the solution, GOx intercepts glucose on its way to the electrode surface and oxidizes it with no current formation. This does not allow PQQ-GDH to produce current because glucose does not reach the PQQ-GDH molecules, being fully consumed by the GOx catalyzed reaction. Removing GOx from the interface allows glucose oxidation by PQQ-GDH and current formation. To remove GOx, it is bound to the surface through a cleavable linker that can be cut by an external signal. When the signal is applied and the linker is cut, GOx is removed from the interface and the electrode is activated. This can be done by light signals when photocleavable spacers are used.[24] Alternatively, chemical signals can cut the linker, e.g., using thiol-cleavable reagents.[25] In the present study, from the broad variety of possible cleavable linkers responding to different signals,[26] we selected a pH-cleavable biomolecular linker. This choice is based on the previously developed enzyme-based logic systems processing multiple biomolecular input signals[27] and producing the final output in the form of pH changes.[16,17] Therefore, the pH-cleavable linker used in this study allows easy integration of the present electrode activated by pH change with the previously designed enzyme logic systems.

Scheme 1 (top) shows the preassembled electrode interface in its electrochemically nonactive state. This interface was produced in several chemical modification steps (see experimental details in the Supporting Information). The material called "buckypaper", made of compressed multiwalled carbon nanotubes (CNTs), was used as the conducting support (see Figure S1 in the Supporting Information).[28] PQQ-GDH molecules were linked to the CNTs using a heterobifunctional cross-linker, 1-pyrenebutanoic acid succinimidyl ester (PBSE), which provides covalent binding with amino groups of protein lysine residues through formation of amide bonds and interacts with CNTs via $\pi-\pi$ stacking of polyaromatic pyrenyl moiety (see Figure S2 in the Supporting Information).[28] Notably, this attachment mode provides efficient direct electrical communication of the enzyme active center, PQQ, with the conducting electrode support, resulting in electrical current upon glucose oxidation.[29] Carboxylic groups of the enzyme molecules were converted to active ester functionalities, which then reacted with SiO_2 particles (200 nm diameter) coated with an aminosilane layer. This step resulted in the coupling of the SiO_2 particles to the enzyme layer on the electrode surface through formation of amide bonds. Then the amino functions of the immobilized SiO_2 particles were reacted with iminobiotin-NHS ester to yield a biotinylated interface. Note that iminobiotin is the analog of biotin which has binding properties to avidin dependent on the pH value. While at basic and neutral pH the free base form of iminobiotin retains the high affinity specific binding to avidin characteristic of biotin, at acidic pH values, the protonated form of the analog interacts poorly with avidin.[30] Using this property, the electrode interface functionalized with iminobiotin was reacted with avidin at pH 7.5 resulting in avidin binding. The iminobiotin/avidin complex was later reacted with GOx which was also functionalized with iminobiotin and with fluorescein isothiocyanate (FITC). This final modification step resulted in bridging GOx and SiO_2 particles through the iminobiotin/avidin/iminobiotin affinity complex. It should be noted that SiO_2 particles were used as a platform for immobilization of GOx in order to provide high surface density and thus high biocatalytic activity of GOx, which is critically important for intercepting glucose and not allowing its penetration deeper into the modified layer on the electrode surface. Therefore, a high surface density of the immobilized SiO_2 particles is important for successful system operation. Figure 1 shows a scanning electron microscopy (SEM) image of the electrode surface with SiO_2 particles demonstrating their high density, ca. 30 particles per μm^2, occupying ca. 62% of the surface. Figure 2A shows a fluorescent image of the surface obtained using confocal microscopy and demonstrating fluorescence (visible in the image as a green color) of the dye-labeled GOx attached to the SiO_2 particles. The GOx surface density of ca. 1020 molecules per μm^2 (ca. 34 molecules per SiO_2 particle) was achieved, assuming the GOx footprint[31] of 58 nm^2 and random disordered packing with 60% coverage. The bioelectrocatalytic

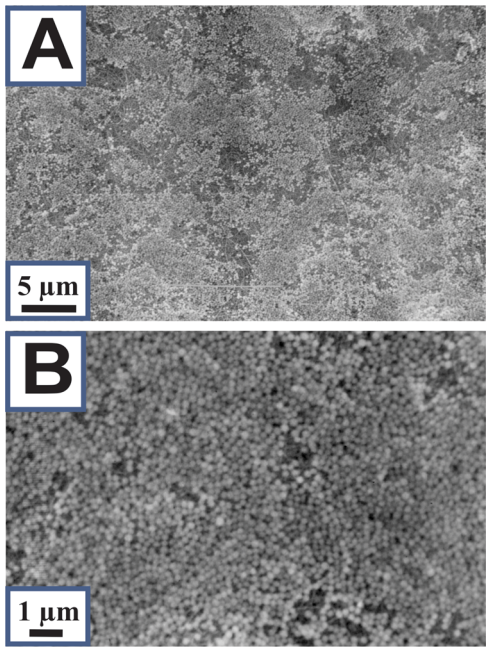


Figure 1. Scanning electron microscopy (SEM) image of the electrode surface with SiO_2 particles attached to the PQQ-GDH layer. A and B represent images with different magnification.

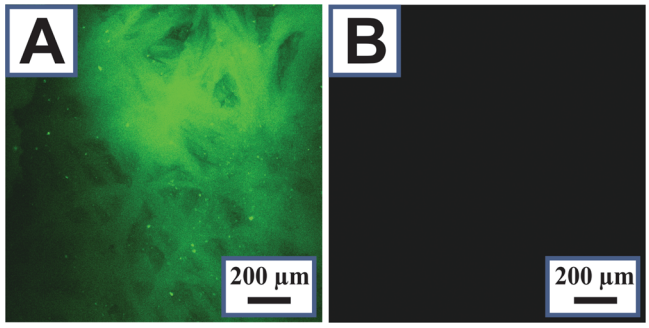


Figure 2. (A) Fluorescent image of the surface obtained with a confocal microscope demonstrating fluorescence (visible in the image as green color) of the dye-labeled GOx attached to the SiO_2 particles. (B) Same electrode surface after the pH-triggered removal of the dye-labeled GOx.

activity of the assembled electrode interface was characterized by cyclic voltammetry in the presence of glucose at pH 7.5. As expected, the cyclic voltammograms in the absence and presence (20 mM) of glucose were almost the same, Figure 3A (curves *a* and *b*, respectively), thus confirming that the modified electrode was not generating any current corresponding to glucose oxidation. However, the absence of the bioelectrocatalytic activity may originate from various reasons, not necessarily due to the expected glucose interception by GOx. To prove the proposed mechanism of the electrode operation, we removed dissolved O_2 from the solution and the cyclic voltammetry experiment was repeated. At this time, the electrode clearly demonstrated electrocatalytic current corresponding to glucose oxidation (see Figure S3 in the Supporting Information). This can be explained by the halted biocatalytic reaction of GOx in the absence of O_2, which is the natural electron acceptor for GOx, whereas the bioelectrocatalytic activity of PQQ-GDH does not require O_2. In an additional experiment H_2O_2 formation in the solution was demonstrated

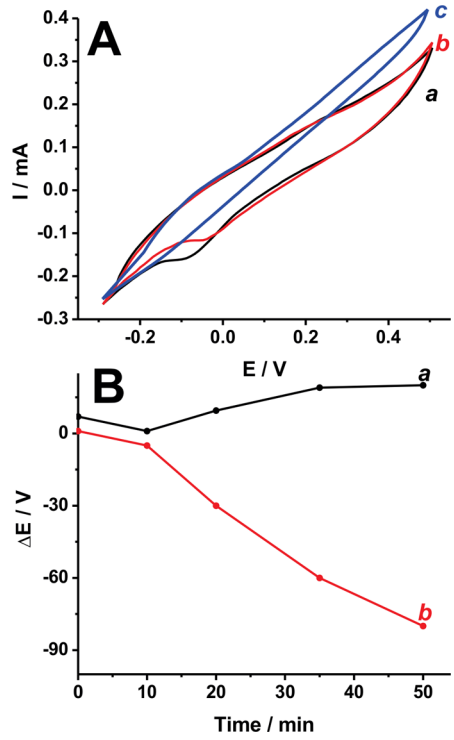
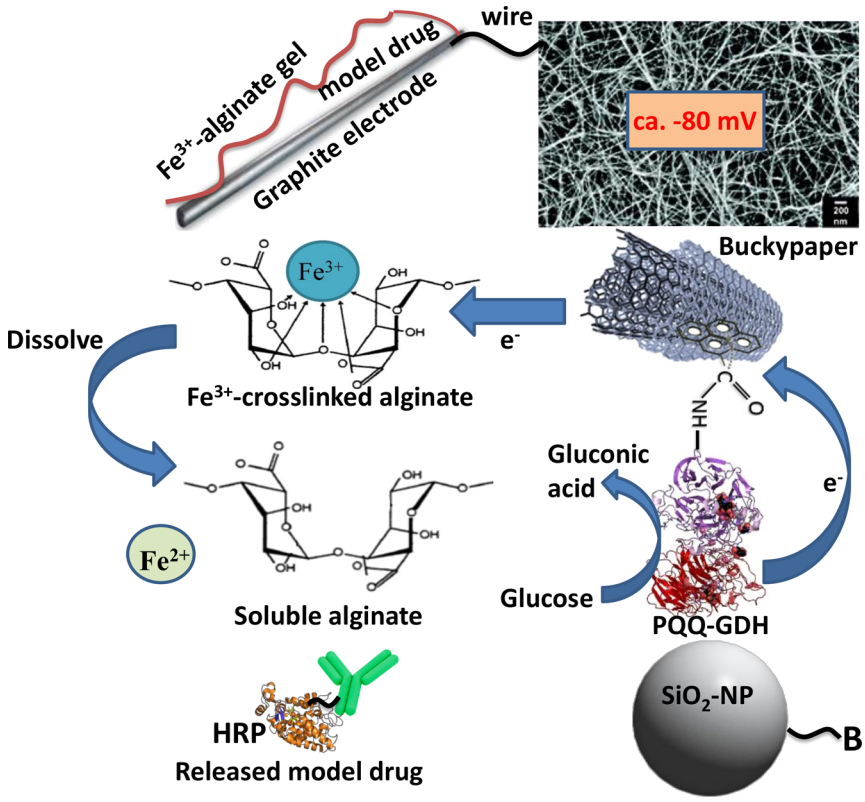


Figure 3. (A) Cyclic voltammograms obtained on the modified electrode after its complete assembling including PQQ-GDH and GOx in the absence (*a*) and presence (*b*) of 20 mM glucose demonstrating the bioelectrocatalytically inactive state. The pH-induced removal of GOx results in the electrochemically active state demonstrating the bioelectrocatalytic current (*c*). The cyclic voltammograms were obtained in the background electrolyte solution containing MOPS-buffer (50 mM), Na_2SO_4 (100 mM) and $CaCl_2$ (1 mM) and dissolved O_2, pH 7.5, with the potential scan rate 1 mV s^{-1}. (B) Electrode potential changes upon addition of glucose (20 mM) to the solution: (*a*) in the presence of GOx on the modified electrode surface, pH 7.5, (*b*) after pH-change-induced removal of GOx from the electrode interface (the potential dynamics was measured when the pH was returned to the initial value of 7.5). The potential was measured vs Ag/AgCl reference electrode in a background solution containing MOPS-buffer (50 mM), Na_2SO_4 (100 mM) and $CaCl_2$ (1 mM) and dissolved O_2.

in the presence of O_2 as a result of the GOx biocatalyzed reaction while H_2O_2 was not detected when oxygen was removed from the solution (see experimental details in the Supporting Information). In an additional set of experiments the bioelectrocatalytic activity of the modified electrode was analyzed by cyclic voltammetry after each modification step (all experiments were performed at pH 7.5). The cyclic voltammograms obtained after immobilization of PQQ-GDH, after SiO_2 attachment, after biotinylation and after avidin binding were very similar and all of them clearly demonstrated the glucose bioelectrocatalytic oxidation (see Figure S4, sections A–D, in the Supporting Information; these cyclic voltammograms are also similar to curve *c* in Figure 3). Only the last step of the GOx attachment to the avidin complex on the surface resulted in the complete inhibition of the bioelectrocatalytic current (see Figure S4, section E, in the Supporting Information and Figure 3A, curve *b*). It should be remembered that the presence of GOx on the electrode surface did not inhibit the glucose oxidation (note the H_2O_2 formation due to the GOx biocatalytic activity). However, the GOx biocatalyzed process did not result in current formation on the electrode surface,

dx.doi.org/10.1021/am504561d | *ACS Appl. Mater. Interfaces* 2014, 6, 13349–13354

Scheme 2. Formation of Reductive Potential on the Biocatalytic Electrode after Removing GOx from the Interface Converting the Electrode to Its Biocatalytically Active State and Concomitant Electrochemical Dissolution of Fe^{3+}-Cross-Linked Alginate Film on the Connected Electrode Resulting in the Release of Drug-Modelling Species



whereas the PQQ-GDH which is capable of direct electron communication with the electrode was not provided with glucose intercepted by GOx at the external interface of the layered film on the electrode surface.

To activate the modified electrode, it was reacted with citrate buffer (100 mM, pH 4.5). This resulted in protonation of iminobiotin decreasing its affinity to avidin, thus resulting in dissociation of the iminobiotin/avidin/iminobiotin complex and removing GOx from the interface, Scheme 1 (bottom). Figure 2B shows no fluorescence from the interface after removing the fluorescein-labeled GOx from the surface (compare with Figure 2A before removing GOx). Note that the SiO_2 particles were not removed from the surface, thus the SEM image of the surface was similar to that shown in Figure 1. The cyclic voltammogram obtained after this step (recorded for consistency at pH 7.5 to keep the PQQ-GDH activity unaltered) showed the bioelectrocatalytic current similar to that observed before attaching GOx to the interface, Figure 3A, curve *c*, (compare this curve with curves *b* in Figure S4, sections A–D in the Supporting Information) thus confirming the electrode activation upon removing GOx from the interface. Figure 3B, curve *b*, shows the formation of a negative potential of ca. −80 mV (measured vs Ag/AgCl reference electrode) upon dissociation of the affinity complex and removing GOx from the interface caused by the pH change. Notably, the potential was not lowered in the presence of glucose if GOx was not removed from the interface by the pH signal, Figure 3B, curve *a*.

The results demonstrated above suggest immediate possible applications for the pH-activated electrode. The negative potential and the corresponding anodic current produced on the electrode upon its activation by removing GOx from the interface can be used for electrochemically stimulated drug release (eventually for the release of any species, but drug release is the most obvious application). This is illustrated by Scheme 2, which shows coupling of the biocatalytic electrode with an alginate-modified electrode releasing a drug-mimicking species upon electrochemically stimulated alginate dissolution. Alginate molecules cross-linked with Fe^{3+} cations form a gel thin-film on the electrode surface.[32] Electrochemical reduction of Fe^{3+} to Fe^{2+} results in gel dissolution since Fe^{2+} cations are not able to cross-link alginate[32] (see Figure S5 in the Supporting Information for illustration of different interaction of Fe^{3+} and Fe^{2+} with alginate). The alginate gel dissolution results in the release of entrapped species, which might be different nanospecies mimicking drugs or real drug molecules (e.g., lysozyme), as was demonstrated in the recent series of publications.[33−36] As an example of a drug-mimicking species, horseradish peroxidase-labeled antigoat IgG-antibody (HRP-Ab) was loaded in the alginate film and then released upon electrochemically stimulated film dissolution. The HRP-Ab species released at different time intervals were collected on an ELISA plate functionalized with the complementary polyclonal antirabbit IgG-antibody from goat to make an immune-complex with the released HRP-Ab, which was analyzed through the standard immune-assay procedure using 3,3′,5,5′-tetramethyl-benzidine (TMB) as the color producing substance, Figure 4 (right, scheme). In the presence of the HRP-Ab conjugate and upon addition of H_2O_2, TMB was biocatalytically converted to the oxidized form TMB_{ox} to yield absorbance measured at λ = 655 nm. (Experimental details on the alginate thin-film preparation with the entrapped HRP-Ab species, later alginate

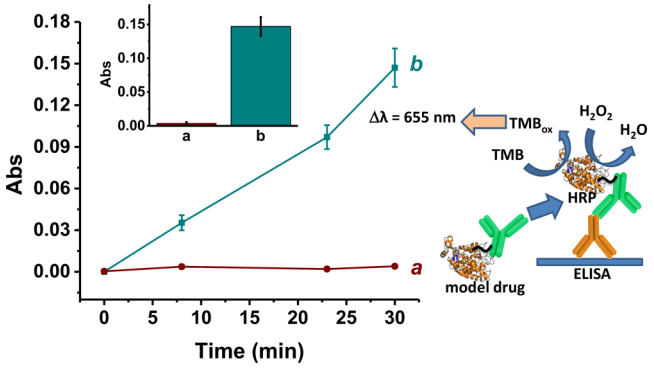


Figure 4. Time-dependent release of the drug-mimicking HRP-Ab species from the alginate film when the biocatalytic electrode was activated by removing GOx from the interface (*b*) and the noncontrolled leakage of the HRP-Ab from the alginate film when the connected electrode is not active in the presence of GOx on its interface (*a*). The optical absorbance characterizing the released species corresponds to the oxidized TMB in the ELISA assay of the released HRP-Ab conjugate (schematics shown at the right). Inset: The bar chart comparing the signal-induced release of the entrapped HRP-Ab species (*b*) and their noncontrolled leakage (*a*) after 30 min following the connection of the alginate electrode and the switchable electrode in its active (*b*) and inactive state (*a*).

dissolution resulting in the HRP-Ab release, and then HRP-Ab enzymatic assay are collected in the Supporting Information; additional information is available in refs 32−36). Figure 4 curve *b*, shows the kinetics of the HRP-Ab release upon the alginate film dissolution stimulated by the reductive potential/current generated by the glucose oxidizing electrode after removal of GOx as described above. Figure 4, curve *a*, shows, for comparison, a very small leakage of HRP-Ab from the alginate film which is preserved intact if GOx is not removed from the modified interface.

Although the electrical output signal generated by the biocatalytic electrode upon its activation was used for triggering drug-mimicking release, the input signal in the form of pH change, removing GOx and activating the electrode, can be produced by various biocatalytic systems mimicking Boolean logic operations and processing multiple biochemical signals.[27] Several examples of the enzyme-based biocatalytic systems mimicking Boolean logic gates and their concatenated networks producing pH changes as the final output signal were reported recently[27] and used for the activation of electrodes coated with pH-sensitive signal-responsive polymers,[1,3] including those integrated in biofuel cells.[6] The biomolecular input signals processed by the logic systems included biomarkers specific to various biomedical dysfunctions (e.g., related to liver injury).[17] Overall, the present report fills the gap between systems logically processing biomolecular signals and producing the binary output YES/NO in the form of pH changes and other systems mimicking drug release upon receiving the pH signal. At the present stage of research, we are not yet attempting practical biomedical applications in vivo, but we feel that the continuation of this research will certainly lead to possible medical applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the NSF award # CBET-1066397 (Clarkson University). Financial support from the Ministerio de Economía e Innovación, grant CTQ2012-34238 (Complutense University of Madrid) is gratefully acknowledged.

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