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REVIEW

# Digital biosensors with built-in logic for biomedical applications—biosensors based on a biocomputing concept

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**Abstract** This article reviews biomolecular logic systems for bioanalytical applications, specifically concentrating on the prospects and fundamental and practical challenges of designing digitally operating biosensors logically processing multiple biochemical signals. Such digitally processed information produces a final output in the form of a yes/no response through Boolean logic networks composed of biomolecular systems, and hence leads to a high-fidelity biosensing compared with traditional single (or parallel) sensing devices. It also allows direct coupling of the signal processing with chemical actuators to produce integrated "smart" "sense/act" (biosensor-bioactuator) systems. Unlike common biosensing devices based on a single input (analyte), devices based on biochemical logic systems require a fundamentally new approach for the sensor design and operation and careful attention to the interface of biocomputing systems and electronic transducers. As common in conventional biosensors, the success of the enzyme logic biosensor would depend, in part, on the immobilization of the biocomputing reagent layer. Such surface confinement provides a contact between the biocomputing layer and the transducing surface and combines efficiently the individual logic-gate elements. Particular attention should thus be given to the composition, preparation, and immobilization of the biocomputing surface layer, to the role of the system scalability, and to the efficient transduction of the output signals. By processing complex patterns of multiple physiological markers, such multisignal digital biosensors should have a profound impact upon the rapid diagnosis and treatment of diseases, and particularly upon the timely detection and alert of medical emergencies (along with immediate therapeutic intervention). Other fields ranging from biotechnology to homeland security would benefit from these advances in new biocomputing biosensors and the corresponding closed-loop "add/act" operation.

**Keywords** Biosensor · Biocomputing · Biomolecular computing · Logic gate · Logic network · Enzyme · Biomedical application · Electrode

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## Introduction

Chemical computing [1–4], being a subarea of unconventional computing [5], aims at processing information by chemical means. This emerging research area has developed rapidly from the formulation of single logic gates mimicking Boolean operations such as AND [6, 7], OR [8], XOR [9, 10], NOR [11–14], NAND [15, 16], INHIBIT [17–20], and XNOR [21, 22] to small logic networks [23]. The combination of chemical logic gates in groups or networks resulted in simple computing devices performing basic arithmetic operations [24, 25] such as half-adder/half-subtracter [26–29] and full-adder/full-subtracter [30, 31].

The emerging research field of biocomputing, based on the application of biomolecular systems for processing chemical information, has achieved higher complexity of information processing compared with nonbiological systems owing to the natural specificity and compatibility of

biomolecules allowing their easy assembly in networks [32]. Different biomolecular "tools," including proteins/enzymes [33, 34], DNA [35], RNA [36], and whole cells [37], were used to assemble biocomputing systems processing biochemical information. Various Boolean operations were mimicked by recently pioneered enzyme logic systems [38–41] allowing concerted operation of multienzyme assemblies performing simple arithmetic functions [42]. Although similar logic operations [1–4] and arithmetic functions [24] were also realized using nonbiological chemical systems, the advantage of biomolecular systems has been the relative simplicity of the various assembled logic schemes. Scaling up such systems could result in artificial biocomputing networks with increased complexity, performing various logic functions and mimicking natural biochemical pathways [43–45]. Enzyme-based logic systems were applied to control the states of signal-responsive materials [46–50] and to activate/inactivate switchable bioelectronic devices (silicon chips [51], modified electrodes [52–54], and biofuel cells [55–57]). These switchable systems, controlled by biocomputing logic networks, have laid the foundation for the advanced "smart" systems integrating a "decision"-making part with an operating bioelectronic device.

Most biocomputing systems reported until now represent only the proof of the concept demonstrating the possibility of performing computing/logic operations with the use of biomolecular systems. They are not ready yet for any practical application. To justify these studies, highly speculative statements about their relevance to molecular computer design are formulated in almost every paper. In reality, biochemical systems are not able to compete with electronic computers. Indeed, biocomputing systems are hardly organized in small circuits capable of solving only basic arithmetic operations on the timescale of minutes or even hours [42]. The only exception might be DNA-based computing, which is claimed to be potentially capable of competing with silicon computers owing to the massive parallelism of computing operations. However, even this subarea of biocomputing is not being developed successfully enough in the direction of molecular computers. An expert opinion of Stojanovic [35] can be cited supporting this conclusion: "After ten years of intensive efforts, and large investment, we have to admit that DNA computation is unlikely to make modern silicon computers obsolete, or, indeed, ever to solve any useful computational problem much faster than the average human can." Finally, nonbiochemical computing systems are staying at the same level of development [1–4].

On the other hand, the application of biomolecular logic systems for analytical purposes could yield a novel class of biosensors which are able to accept many input signals and produce binary outputs in the form of "yes"–"no" to identify biomedical problems. This approach has been already successfully applied to analyze protein libraries associated with multiple sclerosis [58]. Logically processed feedback between drug application and physiological conditions can significantly improve drug targeting and efficiency [59]. Recently designed stimuli-responsive hydrogel membranes with pores opening-closing upon commands from enzyme logic systems [46] represent the first step towards "smart" drug-delivery devices controlled by physiological signals and providing personalized medical treatment. The well-developed field of DNA [35] and RNA [36] biocomputing has developed from solving complex combinatorial problems [60] to analyzing biomedical multiparameter physiological conditions [61]. Programmable and autonomous DNA computing systems operating in vitro demonstrated logical multiparameter analysis of disease-related biomolecular markers and could be applied in the future for in situ medical diagnosis and cure [61, 62]. For example, biosensor systems for detecting genetic modifications in avian influenza were developed based on the DNA computing principles [35], when various oligonucleotide signals were logically processed by a DNA logic network [63]. Reversible aggregation of nanoparticles controlled by biochemical signals logically processed by enzyme systems opens up a broad area of hybrid bionanofunctional materials for various biomedical applications [50]. Coupling of enzyme logic systems with controlled self-assembly of nanoparticles allowed logic AND/OR responses to cancer markers (matrix metalloproteinases 2 and 7; MMP2 and MMP7) [64]. The logically controlled aggregation of the superparamagnetic $Fe_3O_4$ nanoparticles was detected by MRI, thus promising easy adaptation of the method to future in vivo medical applications. The results of the logically processed biomolecular signals can be stored in enzyme-controlled set-reset flip-flop memory units [65]. The terminal memory units can be connected to various biocatalytic pathways processing multiple biochemical signals. Programmable memory units based on two different protein kinases as information input signals allowed AND, OR, and NOR logic operations prior to the information storage, thus providing information processing diversity [66].

It should be noted that most of the presently developed biocomputing systems are commonly activated by signals that are not relevant for in vivo biomedical application, and even if the signals have biomedical significance, the biocomputing systems are used at nonphysiological concentrations. This limits the immediate use of the biocomputing systems for biomedical applications. Extensive research, combining efforts in biochemistry, computer science, and medicine, is necessary to develop real biocomputing systems for biomedical applications.

The present paper provides an overview of the evolution of biomolecular systems (represented primarily by enzyme

logic assemblies) from the formulation of a general concept to multisignal digital biosensors logically processing complex patterns of different physiological markers. It also discusses practical considerations and challenges in the design of such digital biosensors, particularly those based on electrochemical transducers.

## Evolution of enzyme-based logic systems from the demonstration of the concept to biomedical multisignal biosensors

### Motivation for the application of biocomputing systems for biosensing

Common biosensing devices are based on a single input (analyte). Multisignal logic gates rely on multiple target analytes (inputs) to yield yes/no responses. Such digital biosensors are expected to offer high-fidelity biosensing compared with traditional single-input biosensors. By using various biomarkers as inputs for the enzyme gates and automatically processing physiological information, such biochemical logic systems can provide rapid and reliable assessment of the overall physiological condition and would initiate optimal timely therapeutic intervention.

The main challenge in further development of multiparameter biosensing systems with digital logic information processing is scaling up their complexity by assembling individual logic gates into complex logic networks [43–45] aiming at intelligent medical diagnostics via biomolecular logic [67]. The present paper provides an overview of the novel research paradigm of digitally operating biosensors logically processing multiple biochemical signals through Boolean logic networks composed of biomolecular systems, yielding the final output signal as yes/no responses aiming at high-fidelity biosensing compared with common single or parallel sensing devices. Novel biocomputing sensor systems show promise to benefit a wide range of analytical applications ranging from biomedical and environmental monitoring to national defense and food safety. Biocomputing sensor systems of even moderate complexity will allow realizations of closed-loop ("sense/act/treat") systems for biomedical applications, i.e., patient-tailored timely therapy. Such "smart" biosensing-bioactuating systems will benefit other fields ranging from biotechnology to homeland security.

Integrated medical systems based on a sense/delivery feedback loop [68, 69] are expected to revolutionize patient monitoring by enabling personalized medicine in connection with different diseases and conditions. Such fully autonomous systems can be created by coupling sensing and delivery devices for monitoring changes in the level of biomarkers and releasing an appropriate drug in response. Most of the activity towards realizing such feedback-loop systems is currently being devoted to the management of diabetes through integration of an electrochemical glucose sensing element with an insulin-delivery feedback loop for the optimal dose of insulin [69–71]. However, applications of a "sense and act" system extend beyond chronic disease management and could be extremely important in connection with several medical emergencies where a rapid intervention is crucial for the survival of the patient. The successful integration of drug-delivery feedback loops depends on the availability of "smart" interfaces for automated decision making and initiating proper therapeutic interventions. New algorithms and methods are needed in view of the limited experience with real-time control of drug delivery. Such interfacing of the sensing and delivery elements is thus a critical component of integrated medical systems [69, 70].

### Enzyme logic gates—demonstration of the concept

Very interesting and promising results were reported on the formulation of various logic gates based on enzymatic reactions and particularly on the coupling of these logic gates with electronic transducers and signal-responsive materials. The following few examples are given for illustration purposes and are not intended to provide a comprehensive overview of the state of the art in this area.

A system comprised of glucose oxidase and catalase in solution operated as the logic gate "machinery," being activated by two chemical input signals: $H_2O_2$ and glucose (Fig. 1a) [38]. Gluconic acid, which is the product of the biocatalytic oxidation of glucose, was detected by optical means in the presence of special "developing" reagents, and the resulting optical absorbance change, $\Delta A$, was defined as the output signal of the enzyme logic gate (Fig. 1b). The biocatalytic oxidation reaction proceeds in the presence of glucose and $O_2$, where the latter was produced in situ upon $H_2O_2$ reacting with catalase. Therefore, the optical output signal appeared only in the presence of both inputs (combination 1,1), whereas in the absence of either or both input signals (combinations 0,1; 1,0; 0,0, respectively) the system did not produce gluconic acid. To define digital logic values of the output signal, a threshold value separating small-background optical changes defined as 0 output and large absorbance changes defined as 1 output was used (Fig. 1c). The features of this biocatalytic system resemble the Boolean AND logic gate. Similar systems with optical analysis of the output signals were designed to mimic various Boolean logic operations: XOR, INHIBIT, AND, OR, and NOR [38, 39]. Further advances in this direction resulted in the design of universal NAND/NOR logic gates with a modular structure that enables their assembly in networks [41].

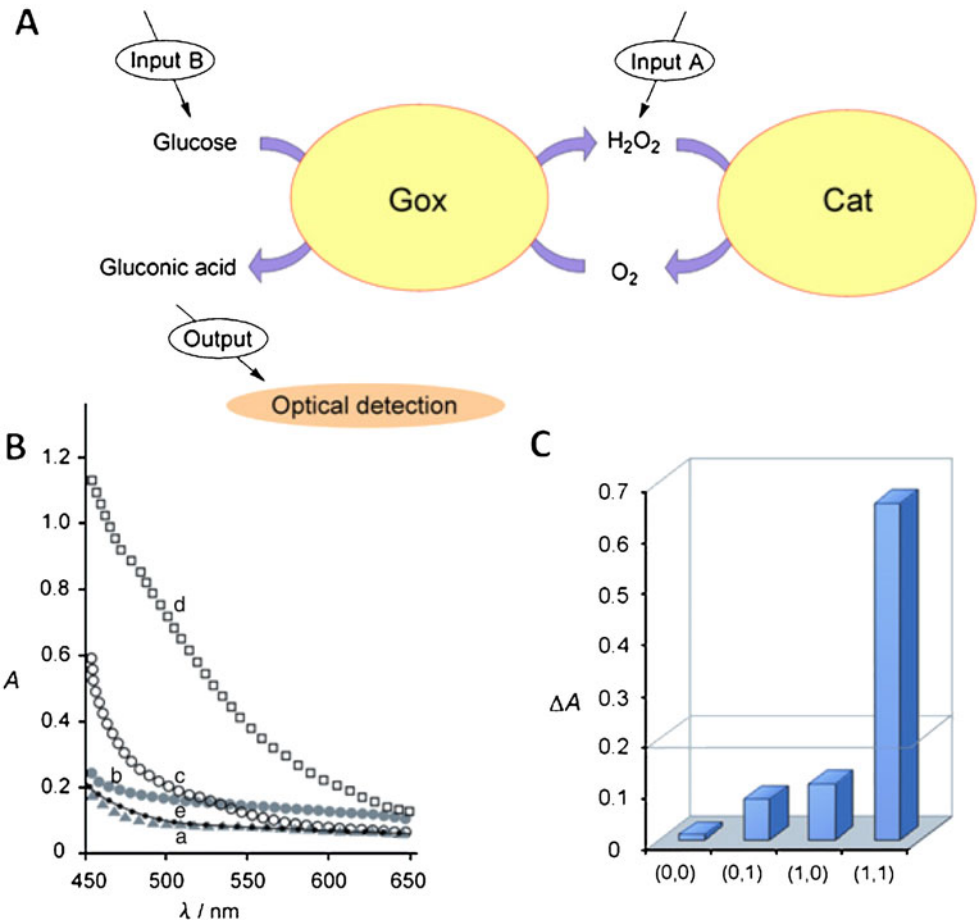


**Fig. 1** **a** Enzyme-based Boolean AND logic gate. **b** Optical changes in the system upon indirect analysis of gluconic acid generated in the presence of different combinations of the input signals (*a* 0,0; *b* 0,1; *c* 1,0; *d* 1,1) and prior to the application of signals (*e*). **c** Bar chart showing the absorbance changes, $\Delta A$, at 500 nm derived from the spectra for various combinations of the input signals, and the threshold value (here 0.2) above which the digital 1 is registered. *GOx* glucose oxidase, *Cat* catalase. (Adapted from [38] with permission. Copyright American Chemical Society, 2006)

pH-switchable materials immobilized at interfaces of electronic/electrochemical transducers, e.g., silicon chips [51] or conducting electrodes [54], were coupled with enzyme logic gates, producing pH changes in solutions as logic responses to input signals. This allowed electronic transduction of the output signals generated by the enzyme logic gates. Figure 2 shows two biochemical systems with the use of enzymes as input signals: invertase and glucose oxidase to mimic Boolean AND logic operation and esterase and glucose oxidase performing OR logic function [54]. Simultaneous addition of invertase and glucose oxidase (input signals 1,1) to the solution containing sucrose resulted in biocatalytic chain reactions transforming sucrose to glucose and then to gluconic acid, finally resulting in the pH decrease from the initial value of approximately 5.8, reaching pH 3.5 in the nonbuffered solution. If either or both of the enzymes were missing (input signals 0,0; 0,1; 1,0), the reaction did not result in the production of gluconic acid and the pH was not changed from its original value. The system response mimicked the Boolean AND logic function. Another system was composed of a mixture of ethyl butyrate and glucose, reacting upon addition of esterase and glucose oxidase (Fig. 2). Each of the enzymes reacted separately with its own substrate: esterase triggered the hydrolysis of ethyl butyrate, thus releasing butyric acid, whereas glucose oxidase oxidized glucose, producing gluconic acid, both resulting in acidification of the solution. Therefore, these two enzymes, operating together or separately (input signals 1,1; 0,1; 1,0), resulted in the production of acidic solutions. Only if both enzymes were missing (input signals 0,0) the solution pH was unchanged. Thus, this reacting system resembled the Boolean OR logic function. Addition of urease to the solution originally containing urea resulted in the biocatalytic production of ammonia, raising the pH to 8.5–9.0. This reaction was used in both systems to reset the pH values from the values resulting from the operation of the AND/OR logic gates (Fig. 2). The pH changes generated in situ by the biocatalytic reactions were used to switch ON-OFF the interfacial properties of a polymer-modified electrode (Fig. 2). It should be noted that the polymer-modified electrode demonstrated the ON state for soluble anionic redox species when the polymeric chains were protonated and positively charged at pH<4.5; whereas the modified electrode was not active (OFF state) for the redox species when the polymer chains were neutral and hydrophobic at pH>5.5. The ON-OFF states of the electrode interface were characterized by cyclic voltammetry and impedance spectroscopy (Fig. 2).

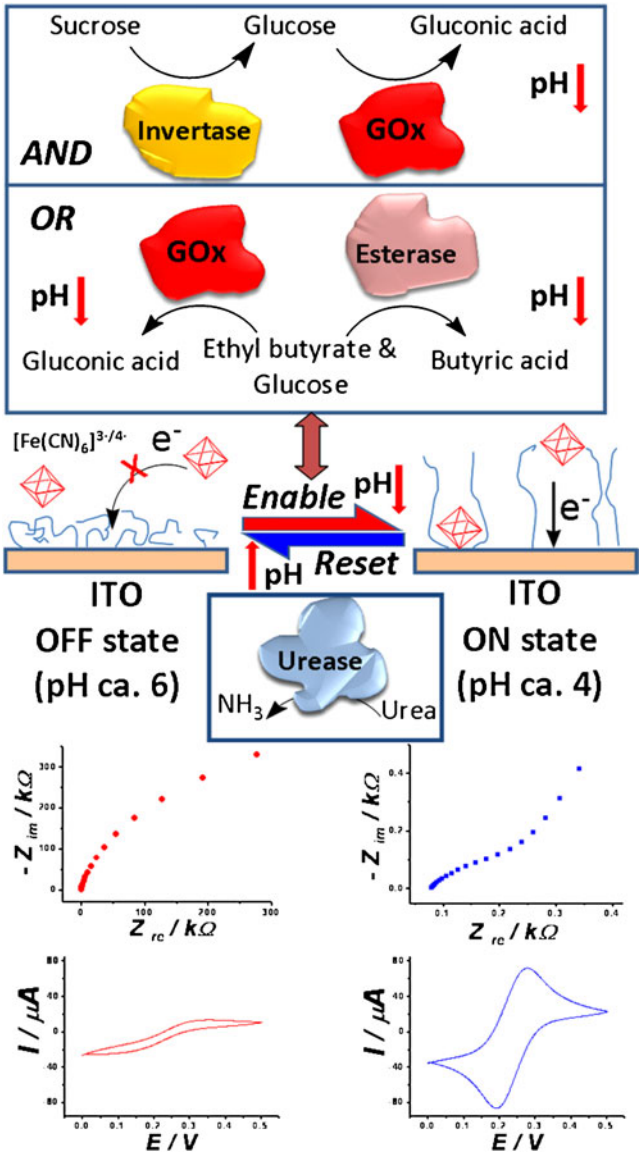


**Fig. 2** Biochemical AND/OR logic gates activated by the enzyme-input signals and their coupling with a pH-switchable polymer-modified electrode. Cyclic voltammograms and impedance spectra show the electronic outputs generated by the ON and OFF states of the electrode. *ITO* indium tin oxide. (From [54] with permission)

Similar enzyme logic systems mimicking Boolean AND/OR logic operations and producing the output signal in the form of solution pH changes were coupled with charging-discharging organic shells around gold nanoparticles associated with a silicon chip surface (Fig. 3a) [51]. This resulted in capacitance changes at the modified interface allowing electronic transduction of the biochemical signals processed by the enzyme logic systems (Fig. 3b,c).

To conclude, these single-gate systems neither solve any real computing problem nor operate as useful biosensors; however, they represent the first important step toward the development of digital biosensors formulated below. It should be noted that very interesting conclusions about the error generation can be derived from the careful analysis of very simple enzyme logic gates [32, 72]. It was shown that upon appropriate optimization of the enzymatic reactions, up to ten logic gates could be concatenated while still allowing low noise in the system [73].

## Enzyme logic circuits—scaling up the system complexity

The most challenging aim of future developments in the area of enzyme logic systems and biocomputing in general is scaling up the complexity of the systems by networking the individual parts of a logic circuit. This issue was addressed experimentally when designing networks composed of concatenated enzyme logic gates [43, 44, 74]. The assembled logic networks were analyzed theoretically for optimization and noise reduction [44]. Further development was directed to the coupling of the output signals produced by multienzyme multigate networks with electronic transducers and bioelectronic devices [53, 57].

For example, a logic network composed of three enzymes—alcohol dehydrogenase, glucose dehydrogenase, and glucose oxidase—operating in concert as four concatenated logic gates—was designed to process four different chemical input signals (NADH, acetaldehyde, glucose, and oxygen) (Fig. 4). The cascade of biochemical reactions resulted in pH changes controlled by the pattern of the applied biochemical input signals. The "successful" set of the inputs produced gluconic acid as the final product and yielded an acidic medium, lowering the pH of the solution from its initial value of pH 6–7 to the final value of approximately 4, thus switching ON a polymer-modified interface for the redox process of a diffusional redox probe, $[Fe(CN)_6]^{3-/4-}$. The chemical signals processed by the enzyme logic system and transduced by the sensing interface were read out by electrochemical means using cyclic voltammetry (Fig. 5a). Reversible activation-inactivation of the electrochemical interface was achieved by logic processing of the biochemical input signals and then by the reset function activated in the presence of urease and urea (Fig. 5a, inset). The whole set of input signal combinations included 16 variants, but only 0,0,1,1; 0,1,1,1; 1,0,1,1; 1,1,1,0 and 1,1,1,1 combinations resulted in the ON state of the electrochemical interface (Fig. 5b). This ensemble exemplifies a multigate/multisignal processing enzyme logic system associated with electrochemical transduction readout of the output signal. A similar approach was applied to switch ON-OFF a biofuel cell controlled by an enzyme logic network [57].

It should be noted that previously studied enzyme logic networks did not use any input signals analytically important for biomedical applications and were intended only for the proof of the concept without any real application.

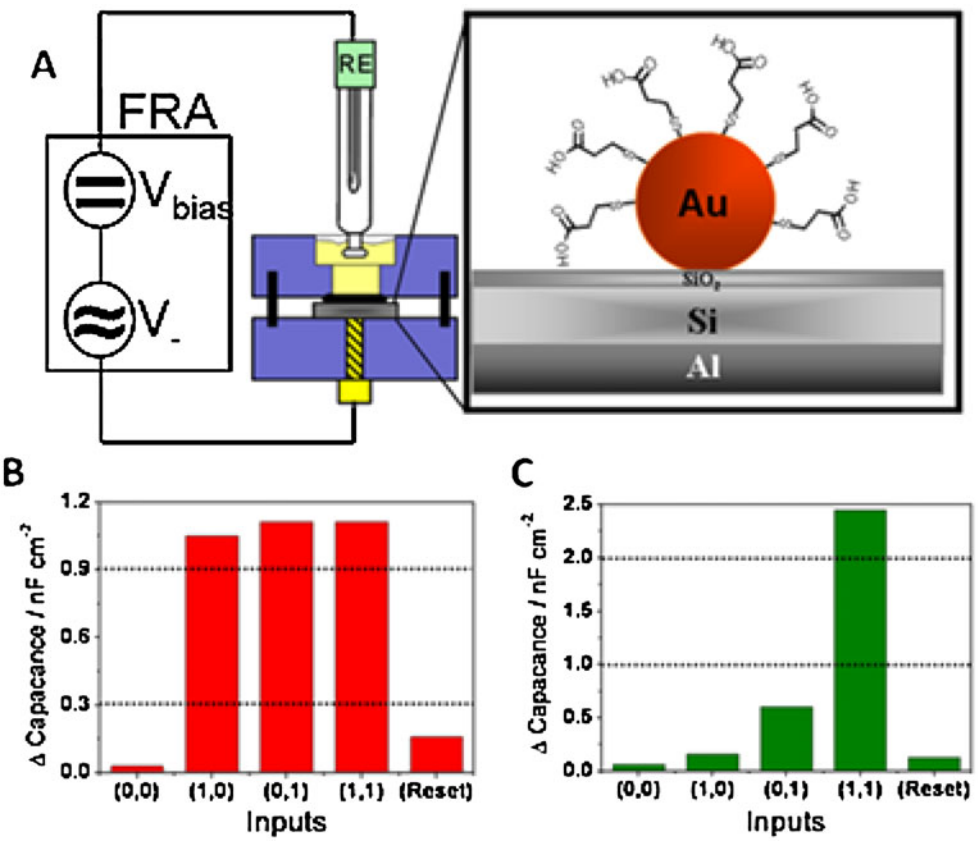


**Fig. 3** **a** Electronic scheme of the signal-transducing device based on a silicon chip modified with gold nanoparticles coated with a pH-sensitive organic shell. **b**, **c** Bar charts showing the output signals generated by OR (**b**)/AND (**c**) enzyme logic gates and transduced by the silicon chip in the form of capacitance changes. The *dashed lines* correspond to the threshold values: the output signals below the first threshold were considered as 0, and the signals above the second threshold were treated as 1. *FRA* frequency-response analyzer, *RE* reference electrode. (From [51] with permission. Copyright American Chemical Society, 2009)

Biomolecular logic gates designed for biomedical analytical applications

Further developments of biomolecular information processing systems (logic gates and their networks) led to the design of biosensoric systems with logically processed signals represented by various biomarkers characteristic of different abnormal physiological conditions [62, 64, 67]. For example, two kinds of complementary nanoparticles functionalized with biotin and avidin, respectively, were protected from the spontaneous aggregation by the attachment of peptide-poly(ethylene glycol) chains [64] (Fig. 6a). Reacting nanoparticles with two proteases (MMP2 and MMP7) resulted in their deprotection upon cutting out the peptide-poly(ethylene glycol) tails and yielded aggregates analyzed by light scattering (Fig. 6b). Since each protease

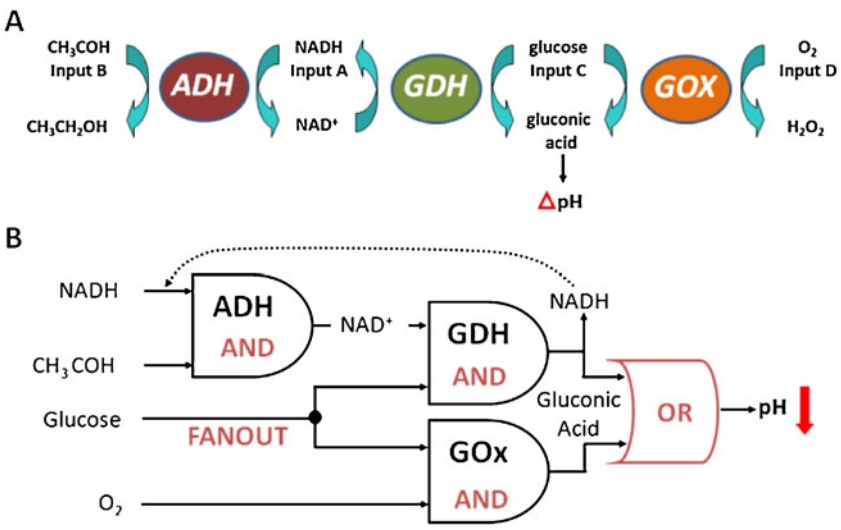


**Fig. 4** **a** Multigate/multisignal processing enzyme logic system producing in situ pH changes as the output signal. **b** The equivalent logic circuitry for the biocatalytic cascade. *ADH* alcohol dehydrogenase, *GDH* glucose dehydrogenase (From [53] with permission. Copyright American Chemical Society, 2009)

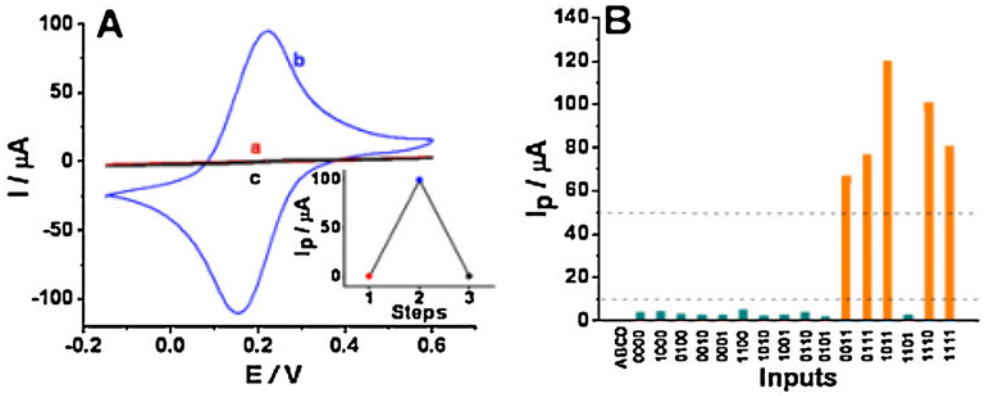


**Fig. 5** Electrochemical transduction of the chemical signals processed by the enzyme logic network shown in Fig. 4. **a** Cyclic voltammograms obtained for an ITO electrode modified with a poly(4-vinylpyridine) polymer brush in *a* the initial OFF state, pH≈6.7, *b* the ON state enabled by the input combinations resulting in acidifying the solution to pH≈4.3, and *c* in situ reset to the OFF state, pH≈8.8. *Inset*: Reversible current changes upon switching the electrode ON-OFF. **b** Anodic peak currents, $I_p$, for the 16 possible input combinations. The *dotted lines* show threshold values separating logic 1, undefined, and logic 0 output signals. (Adapted from [53] with permission. Copyright American Chemical Society, 2009)

was capable of cutting only one kind of the peptide, thus deprotecting only one kind of nanoparticle, the process was completed only in the presence of both proteases in the solution (input signals 1,1), therefore mimicking the AND logic gate. The enzymes used in this study as the input signals represented important cancer biomarkers. MMP2 is overexpressed in many cancers, including breast cancers, and is an indicator of cancer invasiveness, metastasis, and angiogenesis, whereas MMP7, a protease with broader substrate specificity, is thought to facilitate early stages of mammary carcinoma progression [64].

The use of input signals with obvious biomedical meaning was a significant step in the development of enzyme logic systems (compared with the logic gates which used, for example, biomedically meaningless ethyl butyrate as an input). However, the following important issues have not been addressed yet (being partially or completely ignored):

1. The logic 0 values of the input signals were defined as the complete absence of the biomaterial, whereas for practical applications they should be defined as normal physiological concentrations.
2. The logic 1 values of the input signals did not always correspond to the concentrations expected in vivo, but were rather selected as convenient concentrations sometimes significantly exceeding physiological levels.
3. The application of the input signals to different logic schemes was not justified according to their biomedical meaning. For example, proteases MMP2 and MMP7 were applied to activate AND as well as OR logic gates without an explanation of which logic function produces a biomedically relevant conclusion [64].

The next steps in the development of the enzyme logic systems are aimed at addressing these important issues.

## Enzyme logic systems recognizing various injury-related physiological conditions

Recently developed enzyme logic systems were applied for the analysis of different physiological situations associated with several injuries [75, 76]. Different types of injury result in distinct pathophysiological changes reflected by changes in the concentrations of many biochemical substances in a body. Some of these biomolecules undergo major concentration changes during a given injury and could be selected as biological signaling markers useful for biochemical processing, i.e., as input signals for enzyme logic gates/circuits. For example, a biocomputing system composed of a combination of AND and IDENTITY logic gates based on the concert operation of three enzymes—lactate oxidase, horseradish peroxidase, and glucose dehydrogenase—was designed to process biochemical information related to pathophysiological conditions originating from traumatic brain injury and hemorrhagic shock [76] (Fig. 7). Three biochemical substances—glucose, lactate, and norepinephrine—were selected as physiological markers signaling different kinds of injuries, and were applied to demonstrate the concept of biochemical signal processing and autonomous decision making. Specific concentration patterns of these markers can provide sufficient information to identify the type of injury that occurred in the body. An abnormal increase in the concentration of glucose might originate from hemorrhagic shock [77, 78], whereas a higher than normal physiological lactate concentration could be caused by hemor-

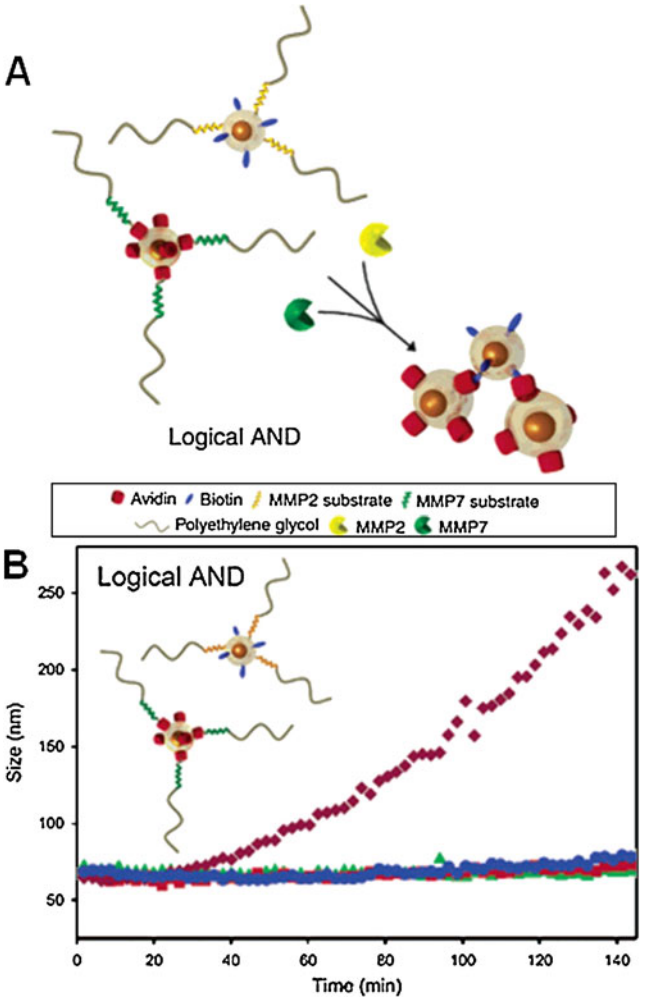


**Fig. 6** **a** Nanoparticles aggregating in the presence of matrix metalloproteinase 2 (*MMP2*) and matrix metalloproteinase 7 (*MMP7*) biomarkers and mimicking the AND logic gate. **b** The hydrodynamic radius in dynamic light scattering is only increased in the presence of both MMP2 and MMP7 input signals; either or none is insufficient to actuate the assembly. (Adapted from [64] with permission. Copyright American Chemical Society, 2007)

rhagic shock or/and traumatic brain injury [78, 79]. A high concentration of norepinephrine can be indicative of any traumatic injury [79, 80]. Thus, glucose, lactate, and norepinephrine were applied as chemical input signals for the enzyme logic circuit. The digitized signals were considered as logic 0 when the inputs were applied at their physiologically normal concentrations: 4 mM glucose, 2 mM lactate, and 2.2 nM norepinephrine. Abnormally high concentrations of glucose (30 mM) characteristic of hemorrhagic shock [77, 78], of lactate (13 mM) observed in the case of both traumatic brain injury and hemorrhagic shock [78, 79], and of norepinephrine (3.5 μM) typical for any traumatic injury [79, 80] were considered as logic 1 values for the input signals. Biochemical processing of different patterns of the biomarkers resulted in the formation of norepiquinone and NADH defined as the output signals (Figs. 8 and 9). Optical and electrochemical means were used to follow the formation of the output signals for eight different combinations of the three input signals. The enzymatically processed biochemical information presented in the form of a truth table enabled the differentiation between normal physiological conditions, pathophysiological conditions corresponding to traumatic brain injury, and hemorrhagic shock to be distinguished, as well as abnormal situations not corresponding to injury. Specifically, the input signals (glucose, lactate, norepinephrine) appearing with the digital pattern 0,1,1 resulted in the output signal combination (norepiquinone, NADH) 1,0 which corresponds to traumatic brain injury symptoms; whereas the input pattern 1,1,1 yielded the output combination 1,1, meaning hemorrhagic shock. It is interesting to note that the characteristic output patterns can be easily represented by decimal numbers corresponding to the binary output code. For example, traumatic brain injury will be represented by 2 (binary 10), whereas hemorrhagic shock will be represented by 3 (binary 11). This way of presenting the analytical results will be particularly convenient when the number of the input/output signals used is large.

The developed system represents a biocomputing logic system applied for the analysis of biomedical conditions related to various injuries. One can anticipate that such biochemical logic gates will facilitate timely automated decision making in connection with an integrated therapeutic feedback-loop system and hence will revolutionize the diagnosis and treatment of injured patients.

The major challenge in developing this kind of digital multisignal biosensor system is obtaining a significant difference between logic 0 and 1 output values. One should remember that the input signals appear at their physiological levels and the input signals with logic value 0 do not have physically zero concentrations. However, there may be a relatively small difference between the physical concentrations of the input signals corresponding to the logic values of 0 and 1. To obtain a significant difference in the output signals, while they are induced by the inputs possessing a small difference, the response function should be sigmoidal rather than linear. In other words, the system should demonstrate nonlinearity with a sharp transition between the states. The first steps in this direction have been already taken experimentally and analyzed theoretically [40]. However, extensive research in this direction to design chemical "filter" systems similar to the electronic counterparts is needed [81]. The thresholds separating logic 0 and 1 values could be personally tailored for a patient by following circulating biomarkers in physiological liquids. This will be an important step in achieving future personalized medicine.

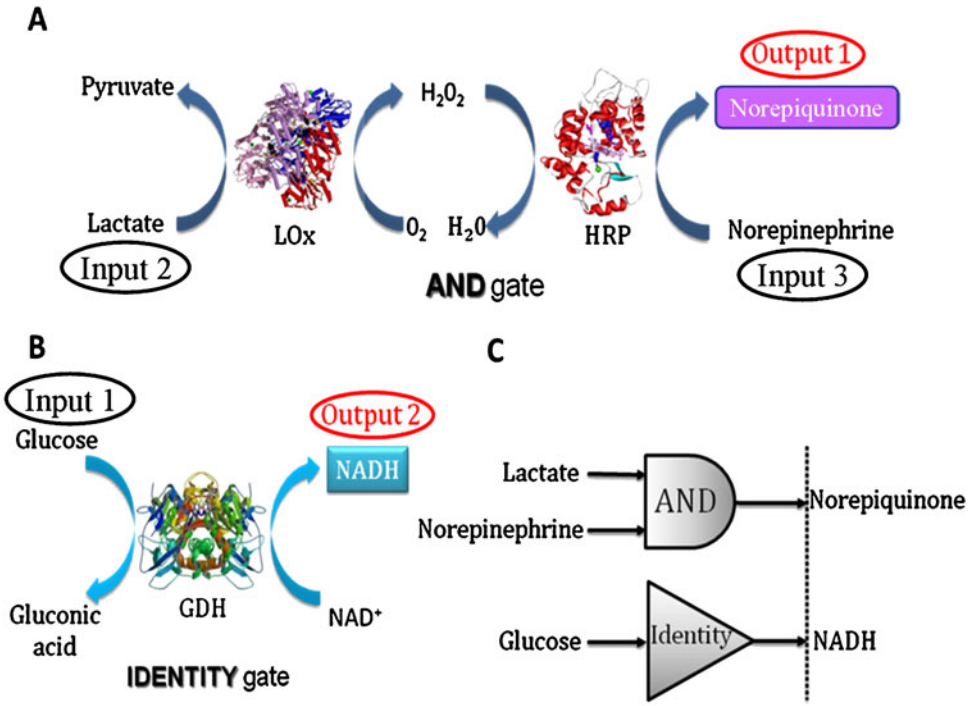


**Fig. 7** Biocomputing system for analysis of trauma brain injury and hemorrhagic shock: biochemical reactions catalyzed by lactate oxidase (*LOx*) and horseradish peroxidase (*HRP*) (**a**) and GDH (**b**) used to perform AND/IDENTITY logic operation and the equivalent logic system used for processing the lactate, norepinephrine, and glucose input signals (**c**). (From [76] with permission)

## Practical considerations in designing digital electrochemical sensors integrated with enzyme logic systems

Unlike present-day biosensing devices using a single input (analyte), devices based on biochemical logic systems require a fundamentally new approach for the sensor design and operation. Such devices will process patterns of multiple physiological markers to yield yes/no responses and hence a high-fidelity biosensing (compared with common single or parallel sensing devices). The design of practical digital biosensors requires careful attention to be paid to the interface of biocomputing systems and the electronic transducer. This involves increasing the system scalability (e.g., using increasing numbers of logic gates) and complexity (i.e., coupling of gates with non-Boolean elements). Particular attention should be paid to the composition, preparation, and immobilization of the biocomputing surface layer, to the role of the system scalability, and to the efficient transduction of the output signals.

### Surface immobilization of the biocomputing machinery

As common in conventional electrochemical biosensors, the success of the enzyme logic biosensor depends, in part, on the immobilization of the biocomputing reagent layer. Unlike early studies in our laboratories [75, 76] where the multiple gate constituents and inputs were dissolved in a solution, practical stand-alone biochemical logic sensors require optimal surface confinement of the biocomputing layer. A careful engineering of the enzyme microenvironment (on the transducer surface) is essential for the optimal performance, considering the increased complexity of logic-gate biosensors (compared with common simpler enzyme electrodes). The goal is not only to provide a contact between the biocomputing layer and the transducing surface, but also to combine efficiently the individual logic-gate elements (to ensure efficient coupling of the enzyme cascade). Such efficient coupling should be accomplished while maintaining high enzymatic stability and retaining the individual reagents. Avoiding leakage of cosubstrates is particularly crucial for on-body sensing applications. Particular attention should be paid to the composition of the "reagent layer," especially to the relative level of the multiple enzyme and cosubstrate components of the logic network machinery, as well as to the corresponding buffer salts and enzyme stabilizers. The selection of the enzymes should also ensure the absence of cross-reactions among the individual biocatalytic gates. The surface confinement actually allows an ordered (optimal) placement of the individual gates (through a layer-by-layer configuration), hence ensuring a more efficient and rational coupling of the enzyme cascade and avoiding cross talk (compared with solution experiments where the entire biochemical machinery is homogeneously mixed). Depending on the specific sensing goal and the biomedical scenario, the level of the surface-confined reagents (enzymes, cosubstrates, mediators, etc.) should be tailored to account for significantly different input concentrations or for substantially different enzyme activities. The permeability of the coating should also be optimized for tuning the transport of the

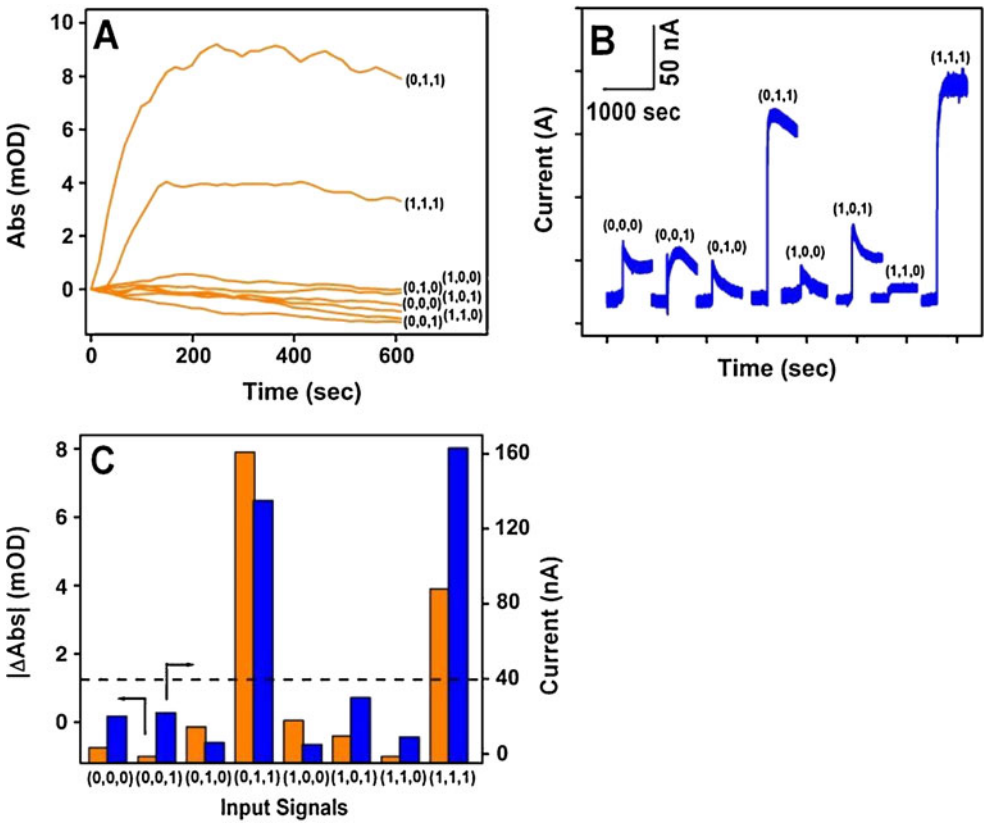


**Fig. 8** Time-dependent signals corresponding to the formation of norepiquinone generated by the combined AND-IDENTITY logic system upon application of various combinations of the input signals (glucose, lactate, and norepinephrine) measured by optical means (**a**) and amperometrically (**b**). **c** Bar chart featuring the combined AND-IDENTITY logic operation of the optical and electrochemical systems. Absorbance measurements were performed at 465 nm. Electrochemical measurements were performed at-0.25 V. The *dashed line* shows the threshold values separating digital 0 and 1 output signals produced by both systems. (From [76] with permission)

individual inputs (and hence their internal levels), while excluding potential interferences and protecting the surface. Different coatings (e.g., Nafion, cellulose acetate) can be used to obtain such controlled transport and protection. Additional protective layers may be required, particularly in connection with continuous biomedical applications. The major issue of biocompatibility (as well as of power) should also be addressed in connection with on-body sensing. Additional physical and chemical immobilization schemes can be considered, including mixed self-assembled monolayers in connection with immune-based logic sensor systems or coimmobilization within carbon-composite electrodes for the enzyme logic gates. The former will be particularly useful for minimizing nonspecific adsorption events while ensuring high reactivity/accessibility of the target antigens. The effect of the surface confinement of the biocomputing layer upon the behavior and performance of the system, compared with the behavior observed in homogeneous solution, should be examined. Such evaluation will involve biochemical gates of increased complexity and scalability. Changes in the enzyme kinetics and response times need to be compared (vs. analogous solution experiments). The different enzyme constituents may be affected differently by their surface confinement, and these differences may also influence the performance of digital biosensors. A wide variety of factors limiting the overall behavior of biocomputing biosensors thus need to be elucidated and identified.

## Optimal transduction of biocomputing signal processes

Electrochemical transduction of the system outputs also requires careful attention in view of the "digital" character of the system and the possible multiple output signals. Special attention must be paid to the electrochemical detection mode, to the selection of the electrode materials, and to the attainment of optimal signal-to-noise characteristics. Simultaneous measurements of multiple output signals requires different transduction strategies (compared with the common fixed-potential measurements in common single-output sensors). Desirably, these could be accomplished using a single working electrode, hence simplifying the design of enzyme logic biosensors (compared with multielectrode systems). This may involve a potential scan for the simultaneous measurement of the multiple outputs, as illustrated in Fig. 10 for three common outputs; the scan rate and potential window also need to be optimized. Alternatively, multipotential steps (pulses) to different values could be used for the detection of the corresponding outputs.

Optimal electrochemical detection of multiple outputs (with minimal interference from coexisting sample constituents) may require evaluation of various electrocatalysts (e.g., Prussian blue for hydrogen peroxide or methylene green for NADH). A single (more universal) catalytic layer, offering the simultaneous detection of the outputs without signal overlap, is highly desired. The minimization of noise associated with

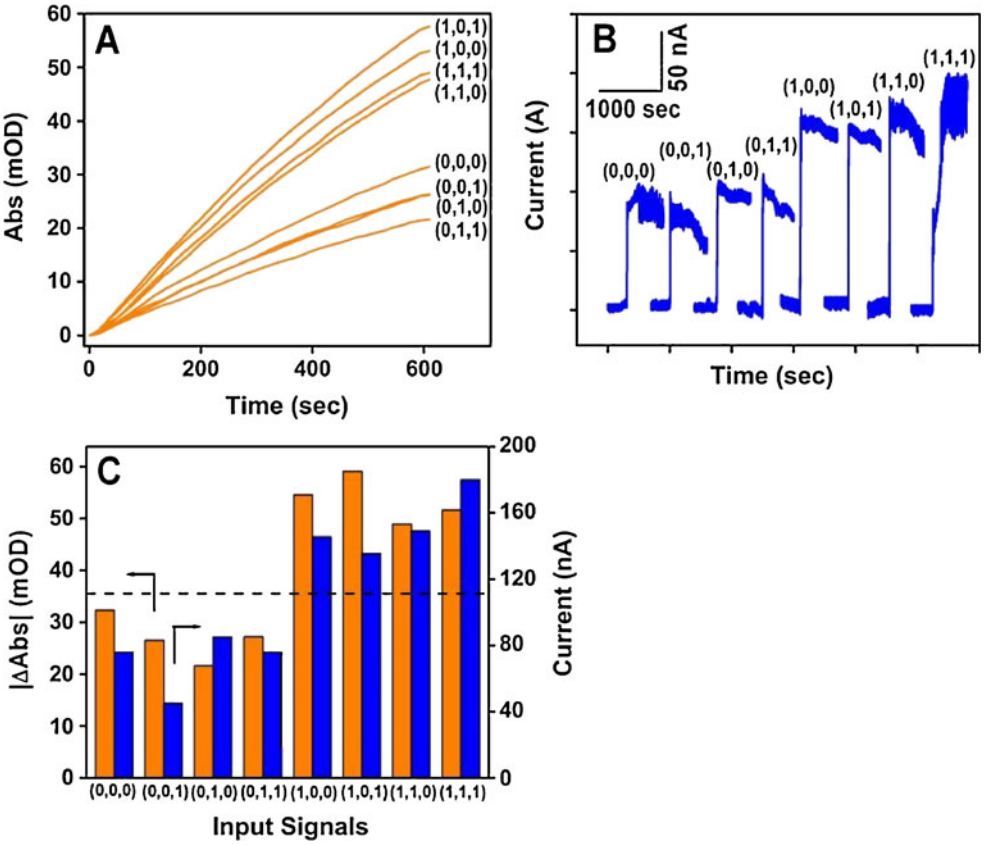


**Fig. 9** Time-dependent signals corresponding to the formation of NADH generated by the combined AND-IDENTITY logic system upon application of various combinations of the input signals (glucose, lactate, and norepinephrine) measured by optical means (**a**) and amperometrically (**b**). **c** Bar chart featuring the combined AND-IDENTITY logic operation of the optical and electrochemical systems. Absorbance measurements were performed at 340 nm. Electrochemical measurements were performed at +0.75 V. The *dashed line* shows the threshold values separating digital 0 and 1 output signals produced by both systems. (From [76] with permission)

increased system scalability often relies on the presence of secondary cosubstrates or enzymes to be identified as suitable. Attention must be also paid to establishing the digital "background" (0) values corresponding to "normal" (physiological) levels of the target analytes. Signal amplification schemes, involving nanoscale materials [82] or product accumulation and recycling [83], can be evaluated for enhancement of the sensitivity in connection with ultralow levels of certain inputs (e.g., biomarkers or bioagents). Such amplification schemes will also benefit biocomputing sensors involving a narrow 0–1 digital range.

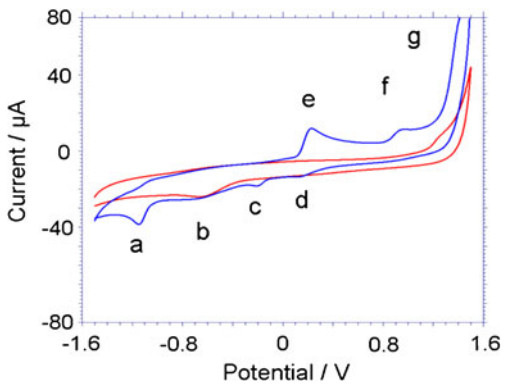


**Fig. 10** Cyclic voltammograms (50 mV $s^{-1}$) for the background in phosphate-buffered saline (*red*) and for a solution containing $NAD^+$ (**a**), $O_2$ (**b**), norepinephrine-quinone (**c**, **d**), norepinephrine (**e**, **f**), and NADH (**g**) (*blue*)

## Conclusions and perspectives

Biochemical computing and logic-gate systems based on biomolecules have the potential to revolutionize the field of biosensors. Interfacing of biocomputing elements with sensing processes would allow multisignal analysis followed by biochemical processing of the data, giving a final digital ("yes" or "no") analytical answer. Such "yes/no" information also allows for the direct coupling of the signal processing with signal-responsive materials and chemical actuators to offer a closed-loop "sense/act" operation. Biochemical networks can offer robust error-free operation upon appropriate optimization of their components and interconnections. The chemical stability of the biomolecular components will be improved upon their immobilization in signal-responsive materials or functional interfaces. Further development of this research area requires the cooperative work of engineers, biochemists, and computer specialists. The ultimate goal of this work will be the design of a microfluidic lab-on-a-chip performing multienzyme-catalyzed cascades and operating similarly to an electronic chip by being able to integrate large networks for processing biochemical signals.

Sensing devices based on biochemical logic systems require a fundamentally new approach for the sensor design and operation and hence careful attention to be paid to the interface of biocomputing systems and electronic transducers. The resulting digital biosensors would benefit diverse and important fields that require immediate intervention or corrective action on the basis of reliable analytical data, ranging from environmental monitoring to homeland security.

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