

Multi-enzyme logic network architectures for assessing injuries: digital processing of biomarkers

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A multi-enzyme biocatalytic cascade processing simultaneously five biomarkers characteristic of traumatic brain injury (TBI) and soft tissue injury (STI) was developed. The system operates as a digital biosensor based on concerted function of 8 Boolean **AND** logic gates, resulting in the decision about the physiological conditions based on the logic analysis of complex patterns of the biomarkers. The system represents the first example of a multi-step/multi-enzyme biosensor with the built-in logic for the analysis of complex combinations of biochemical inputs. The approach is based on recent advances in enzyme-based biocomputing systems and the present paper demonstrates the potential applicability of biocomputing for developing novel digital biosensor networks.

Introduction

Molecular¹ and biomolecular² logic gates and their networks processing chemical input signals similarly to computers received high attention and were rapidly developed in the last decade. Being a subarea of unconventional computing,³ they can process chemical information mimicking Boolean logic operations using binary definitions (**1,0**; **YES/NO**) for concentrations of reacting species. Using this approach, chemical reactions could be reformulated as information processing steps with built-in logic operations.⁴ Then, the chemical processes could be programmed similar to computer programming⁵ yielding networks performing several logic operations. Despite the fact that chemical systems, based on organic molecules⁶ or biomolecules,^{7,8} achieved significant success in the formulation of single logic operations and their short sequences mimicking natural biochemical pathways were successfully designed,⁹ there is no clear opinion about their possible applications. The present complexity of the chemical information processing systems is far below that of electronic computers and the time scale of their operation (minutes-to-hours) is too long to be competitive with electronics. There is an optimistic hope that complex combinatorial problems could be solved by biomolecular (*e.g.* DNA-based) systems faster than by regular computers due to massive parallelism of the information processing in chemical systems.¹⁰ However, even this subarea of biochemical computing has not been developed successfully enough in the direction of molecular computers. An expert opinion of Prof. Stojanovic can be cited

for 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”.¹¹ Therefore, on one hand, future development of the chemical systems in the direction of molecular computers being able to compete with electronic computers seems to be very problematic, at least at the present level of the technology. On the other hand, we know that life, in general, and the human brain, in particular, are based on biomolecular systems processing information in a way more efficient than electronic computers. Thus, even if we don't have technology at the moment to mimic life in artificial systems, we still have an example created by Nature which shows at least the perspective for future developments of chemical information processing systems (called for simplicity chemical computing or biocomputing if biomolecular systems are used). An obvious advantage of biocomputing systems over electronic counterparts is their compatibility with biochemical systems and their ability to operate in a biochemical environment.¹² A futuristic view on potential applications of biocomputing systems could even predict intracellular operating biocomputers¹³ as a part of a novel nanomedicine concept.¹⁴

Keeping in mind future potential use of (bio)chemical computing systems for molecular computers¹⁵ based on novel concepts similar to artificial life,¹⁶ we still need to find immediate application for them based on the present level of technology. One of the stimuli for the development of biocomputing systems is their potential application in novel multi-signal responsive biosensors¹⁷ and bioactuators,¹⁸ logically processing complex patterns of biochemical signals (*e.g.* for biomedical applications).^{19,20} Indeed, presently existing biosensors are capable to analyze concentrations of a single analyte (*e.g.* glucose).²¹ In order to perform simultaneous

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analysis of several analytes, complex sensor arrays must be used.²² Signals generated by the multi-sensor arrays require computers to be processed and presented in the final format. Since the data are usually represented by various concentrations shown in an analog form, an expert evaluation should be involved in the decision-making process. Still separate analysis of multiple analytes could be justified if they represent unrelated chemical parameters. However, in most of common applications (particularly in biomedical applications) the measured parameters (analyte concentrations) are related and can be logically processed all together. This novel approach to biosensing can be based on biocomputing systems.²³ They can logically process several related analytes ('inputs') considering their concentrations in the binary format (0,1) and generate the final output signal (0,1; NO/YES) according to the built-in logic program. The program should take into account the relations between analyzed inputs. For a simple example, if according to their nature two signals should appear simultaneously to result in an unambiguous conclusion, logic gate **AND** should be applied for their analysis. More sophisticated logic schemes could be designed for multi-signal analyzing systems. This approach could be particularly powerful for biomedical applications when a single biomarker analysis may not be enough for a conclusion and many biomarkers at different concentration levels should be analyzed to derive logically the corresponding physiological conditions. Based on the recent success in the formulation of logic gates²⁴ and their networks²⁵ operated by enzyme-catalyzed reactions, we designed logic systems composed of individual logic gates for the analysis of pathophysiological conditions originating from various injuries.²⁰ Their multiplexing allowed comprehensive analysis (coding) of different combinations of various injuries.²⁶

The present work aims at increasing further the complexity of biomarker-analyzing systems by concatenating many logic operations represented by a multi-enzyme system capable to process many variable biomarker signals. Bi-modal way of action is enabled by a built-in **SWITCH** feature activated by the presence or absence of switching inputs in specific gates. Switching between two different sub-systems provides a solution for controlling the biochemical pathways and performance correlation for all multiple **AND** gates in the system. The built-in **SWITCH** feature enables analysis of different physiological conditions within a biochemical logic system operating in a 'single-pot' solution. This conceptually novel approach to biosensors is illustrated in the following sections by a system for the analysis of biomarkers characteristic of the two common injuries: traumatic brain injury (TBI) and soft tissue injury (STI).

Experimental

Chemicals and materials

The enzymes and other biochemicals were obtained from Sigma-Aldrich and used without further purification: creatine kinase from rabbit muscle (CK, E.C. 2.7.3.2), pyruvate kinase from rabbit muscle (PK, E.C. 2.7.1.40), pyruvate dehydrogenase from porcine heart (PDH, E.C. 1.2.4.1), lactate dehydrogenase from porcine heart (LDH, E.C. 1.1.1.27), enolase from bacterial yeast (EN, E.C. 4.2.1.11), pyruvate oxidase from *Aerococcus* sp.

(POx, E.C. 1.2.3.3), peroxidase from horseradish type VI (HRP, E.C.1.11.1.7), glutamate oxidase from *Streptomyces* sp. (GluOx, E.C. 1.4.3.11); creatine anhydrous (Crt), L-cysteine hydrochloride monohydrate (L-Cys), bovine serum albumin (BSA), thiamine pyrophosphate (TPP), adenine 5'-triphosphate sodium salt (ATP), phosphoenolpyruvic acid monopotassium salt (PEP), L(+)-lactic acid (Lac), coenzyme A sodium salt hydrate (CoA), nicotinamide adenine dinucleotide sodium salt (NAD⁺), D(+)-2-phosphoglyceric acid sodium salt (2-PGA), flavin adenine dinucleotide disodium salt hydrate (FAD), adenine 5'-diphosphate (ADP), L-glutamic acid (Glu), 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB). All other standard inorganic salts/reagents were also purchased from Sigma-Aldrich and used as supplied. Ultrapure water (18.2 MΩ cm) from NANOpure Diamond (Barnstead) source was used in all of the experiments.

Instrumentation and measurements

A Shimadzu UV-2450 UV-Vis spectrophotometer with a TCC-240A temperature-controlled cuvette holder and 1 mL poly(methyl methacrylate) (PMMA) cuvettes were used for optical measurements. All optical measurements were performed in temperature-controlled cuvettes/cells at 37 °C mimicking physiological conditions and all reagents were incubated at this temperature prior to experimentation.

A graphical representation of the multi-enzyme biocatalytic system operating in a single-pot solution is outlined in Fig. 1. The input concentrations activating the biocatalytic system are summarized in Table 1. The system operated in two different

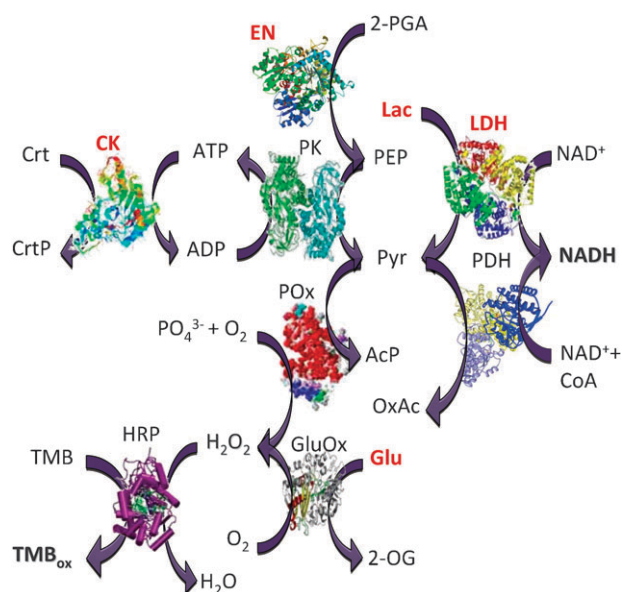


Fig. 1 Multi-enzyme biocatalytic cascade for the analysis of STI and TBI. Biomarker-inputs for STI (CK, Lac, LDH) and for TBI (EN and Glu) are labeled red. Output signals for STI and for TBI are NADH and TMB_{ox}, respectively. Other products of the biocatalytic cascade are the following: acetyl phosphate (AcP), oxaloacetate (OxAc), 2-oxoglutarate (2-OG), creatine-phosphate (CrtP). Note that for simplicity the scheme does not include some reacting cofactors, promoters and byproducts—for the full composition of the system refer to the Experimental section.

Table 1 Concentrations of the inputs activating the biocatalytic cascade for the logic analysis of STI and TBI

Inputs	Logic-0	Logic-1	Ref.
CK ^a	100 U/L ^d	710 U/L ^e	29
LDH ^a	150 U/L ^d	1000 U/L ^e	29
Lac ^a	1.6 mM ^d	6 mM ^e	30
EN ^a	0.42 U/L ^d	1.2 U/L ^e	31
Glu ^a	40 μM ^d	140 μM ^e	32
ATP ^b	0	2 mM	^f
2-PGA ^b	0	8 mM	^f
GluOx ^b	0	0.3 U/L	^f
TMB ^b	0	0.45 mM	^f
PEP ^c	0	2 mM	^f
ADP ^c	0	2 mM	^f
Phosphate ^c	0	31 mM	^f
CoA ^c	0	0.5 mM	^f

^a Biomarker inputs. ^b Auxiliary inputs. ^c Switching inputs. ^d Corresponds to the normal physiological concentration. ^e Corresponds to pathophysiological elevated concentration. ^f Optimized experimentally.

modes: (i) for the analysis of TBI (tested in 31 mM potassium phosphate buffer, pH 7.58, containing 6.7 mM magnesium sulfate) and (ii) for the analysis of STI (tested in 50 mM triethanolamine buffer, pH 7.4, containing 0.2 mM magnesium acetate). The constant part of the system (the “machinery” of the logic network) included the following components: PK (10 units per mL), POx (5 units per mL), PDH (2 units per mL), HRP (5 units per mL), Crt (15 mM), NAD⁺ (10 mM), TPP (450 μM), L-Cys (3.96 mM), FAD (10 μM) and O₂ (in equilibrium with air). The output signal in the TBI mode was measured as the absorbance increase at $\lambda = 655$ nm corresponding to the formation of the oxidized form of the redox mediator, TMB_{ox}.²⁷ The output signal in the STI mode was measured as the absorbance increase at $\lambda = 340$ nm corresponding to the formation of NADH.²⁸ The absorbance measurements were started immediately after mixing the reagents in a cuvette and the final absorbance value was taken at 800 s from the beginning of the reaction. This sampling time was optimal for the effective discrimination of the logic 0 and 1 output signals generated by the system.

Results and discussion

The biocomputing approach to multi-signal processing biosensors with built-in logic was exemplified by a system analyzing complex patterns of biomarkers originating from injury conditions. When an injury occurs due to mechanical or chemical damage to specific organs or tissues, chemical species (proteins and low molecular compounds), normally present only in intracellular compartments, are released into various body fluids. Therefore, the rapid and sensitive detection of these biomarkers is an essential method to obtain a proper injury diagnosis. One disadvantage when detecting enzyme markers with the aim of injury diagnosis is the low-specificity of the biomarkers due to the various pathological conditions underlying their release. However, the specificity of the enzyme-based diagnostic system can be achieved *via* the implementation of logic analysis of complex biomarker patterns. Instead of detecting a single injury biomarker, a biochemical system composed of enzyme logic gates can process two (or eventually several) physiologically relevant

inputs with a corresponding detectable output. The diagnostic capabilities of the biochemical detection system are enhanced when multiple logic gates are concatenated to increase the information processing capacity of the logic system. Hence, such systems can be tuned in order to detect specific injuries while diminishing the possibility of false alarms.

The assessment of two common battlefield injuries, soft tissue injury (STI) and traumatic brain injury (TBI), was performed by a multi-enzyme biocatalytic cascade, Fig. 1, which can be described as a comprehensive concatenated logic system, Fig. 2. The logic network represented in Fig. 2 is actually identical to the biocatalytic cascade depicted in Fig. 1, but it offers another way of its description which is more convenient for the discussion of the system operation. The system architecture includes 8 networked logic gates of the **AND** type. The biocatalytic system operated in two different modes: one for the analysis of TBI and another for the analysis of STI with the possibility to switch between them. The system was designed to analyze 5 different biomarkers appearing in different combinations and to lead to a logic conclusion about the presence or absence of TBI or STI conditions. Three biomarkers creatine kinase (CK), lactate dehydrogenase (LDH) and lactate (Lac) corresponded to the physiological conditions characteristic of STI, while two other biomarkers enolase (EN) and glutamate (Glu) were reporting on the TBI diagnosis. All five biomarkers were applied as biochemical input signals (labeled red in Fig. 2) activating the logic network at two different concentration levels: logic-0 corresponded to the normal physiological concentrations of the biomarkers, while logic-1 was selected at the elevated pathophysiological concentrations corresponding to the respective injuries, Table 1. Simultaneous processing of many biomarkers through the complex biocatalytic cascade required optimization of the biocatalytic reactions by tuning the reaction rates in order to have comparable output signals for various combinations of the biomarker inputs. The optimization was achieved by careful selection of the auxiliary inputs (labeled green in Fig. 2): ATP, 2-PGA, glutamate oxidase (GluOx) and TMB. In order to digitize the logic network operation, the auxiliary inputs were applied at two levels: logic-0 corresponded to the physical zero concentration, while logic-1 was selected experimentally upon optimization of the system, Table 1. The auxiliary inputs did not provide any information about physiological conditions related to the injuries, but they were needed for optimal processing of the biomarker inputs. When they were applied at “0” levels the system was mute and insensitive to the biomarker inputs, while their application at “1” levels provided optimized processing of the biomarker inputs applied in different combinations of “0” and “1” logic levels. Four additional inputs (labeled blue in Fig. 2) PEP, ADP, phosphate and CoA were used to switch the system operation between the TBI and STI modes. These inputs were applied at logic-0 levels corresponding to the physical zero concentrations and logic-1 levels being experimentally optimized, Table 1.

STI operation mode of the logic network

Since none of the used biomarkers is specific enough for the STI diagnosis, only simultaneous appearance of all three

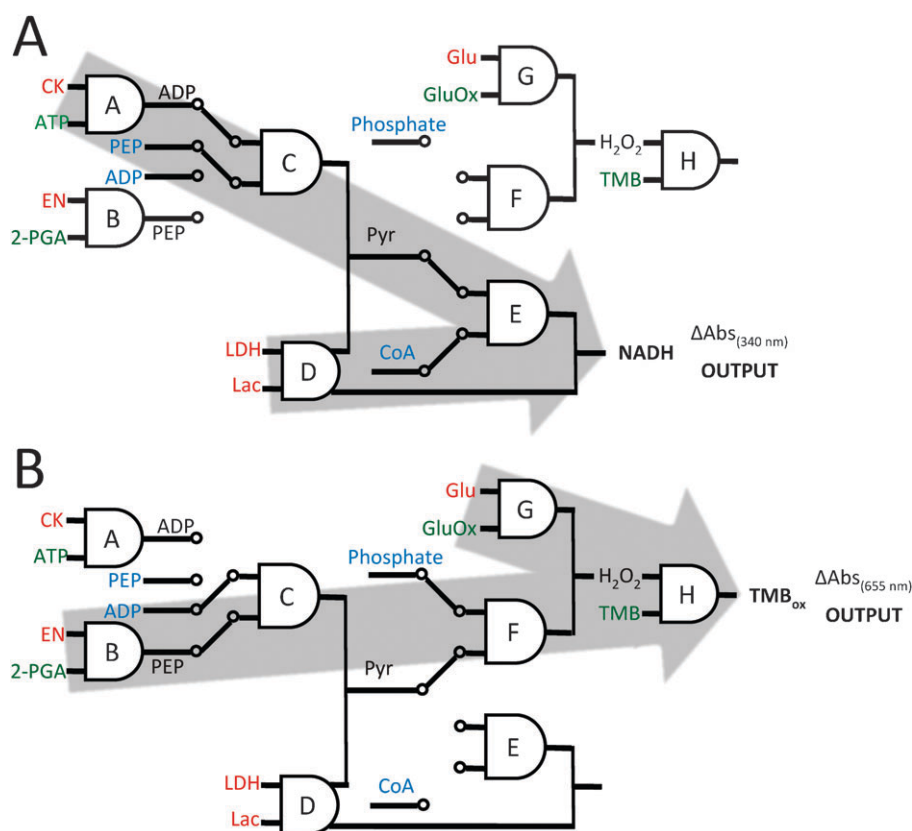


Fig. 2 Equivalent logic schemes for the concatenated logic gates analyzing STI and TBI corresponding to the biocatalytic cascade shown in Fig. 1. (A) The system switched to the STI analysis mode. (B) The system switched to the TBI analysis mode. Biomarker inputs are red labeled, auxiliary inputs are green labeled, and switching inputs are blue labeled. The switches-regulated pathways for the STI and TBI operational modes are indicated by arrows.

STI-related biomarkers (CK, LDH and Lac) at logic-1 values would provide the reliable conclusion about the STI conditions. The system was operating in the following way (see Fig. 1 for the biochemical representation and Fig. 2(A) for the logic equivalent circuitry—the STI pathway is highlighted): the biocatalytic reaction of CK (STI biomarker) and ATP (gate A) resulted in the production of ADP. Further reaction of ADP with PEP biocatalyzed by PK (gate C) resulted in the formation of pyruvate (Pyr), which then reacted in the presence of CoA and PDH (gate E) to yield the reduced NADH considered as the output signal. Note that the pathway composed of A–C–E gates was activated only when the biomarker input CK, auxiliary input ATP and switching inputs PEP and CoA appeared at logic-1 values. Simultaneous application of the LDH and Lac inputs (STI biomarkers) at logic-1 (gate D) resulted in the reduction of NAD^+ and further increase of the NADH output. It should be noted that this pathway resulted in the concomitant production of Pyr which was passing through gate E producing one more equivalent of NADH, thus further amplifying the output signal. Fig. 3(A) shows the optical changes in the system measured at $\lambda = 340 \text{ nm}$ (NADH absorbance) for different combinations of the biomarker inputs. Only simultaneous application of all three biomarker inputs at logic-1 values (input combination 1,1,1) resulted in the high optical absorbance changes allowing an unambiguous conclusion

about the STI condition. The experimentally derived threshold level of 0.5 OD allowed perfect separation of the logic-0 and 1 levels for the output signal being different at least by 2-fold, Fig. 3(B). It should be noted that in all measurements shown in Fig. 3 the auxiliary (ATP) and switching (PEP and CoA) inputs were applied at logic-1 values to allow optimal performance of the analytical pathway. At the same time the switching inputs ADP and phosphate were applied at logic-0 levels to inhibit the alternative TBI pathway.

TBI operation mode of the logic network

Opposite to STI, the TBI biomarkers (EN and Glu) are rather specific and can report on the injury presence even appearing alone. (It should be noted that a generic EN enzyme was used instead of neuron specific enolase released from damaged brain.) The system was operating in the following way (see Fig. 1 for the traditional biochemical representation and Fig. 2 for the logic equivalent circuitry—the TBI pathway is highlighted): the biocatalytic reaction of EN (TBI biomarker) and 2-PGA (gate B) resulted in the formation of PEP. The next step included the reaction of PEP with ADP (switching input) biocatalyzed by PK (gate C) and resulted in the production of Pyr. Further reaction of Pyr with phosphate (switching input) biocatalyzed by POx (gate F) yielded H_2O_2 which reacted with TMB in the presence of HRP (gate H).

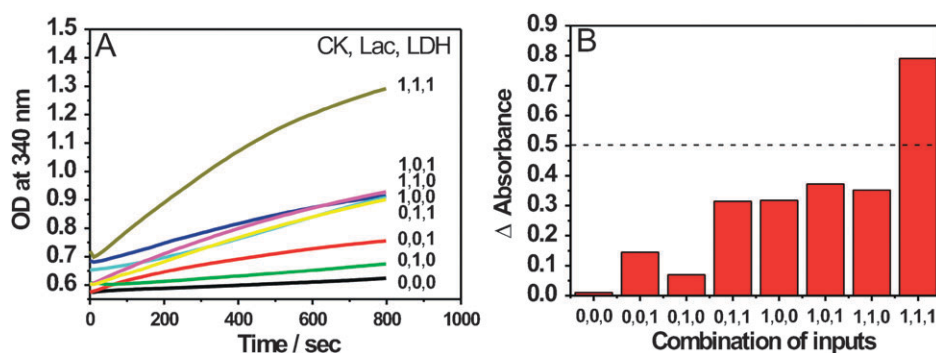


Fig. 3 (A) Optical detection of the output signal (NADH) generated by the logic system operating for the STI analysis obtained upon different combinations of the injury biomarker input signals (CK, Lac, LDH). (B) Bar chart for the output signals generated by the enzyme logic system for the analysis of STI at 800 seconds. The STI diagnosis corresponds to the output signal above the decision threshold (dashed line). The logic system composition is given in the Experimental section and the biomarker input concentrations corresponding to the logic-0 and 1 values are specified in Table 1. The auxiliary (ATP) and switching (PEP and CoA) inputs were at logic-1 for all combinations of the biomarker inputs.

This reaction resulted in the oxidation of TMB and produced the absorbance increase at $\lambda = 655$ nm characteristic of TMB_{ox} being considered as the final output signal from the pathway composed of B–C–F–H gates. The same signal was generated through another pathway composed of G and H gates: the biocatalytic reaction of Glu (TBI biomarker) and GluOx resulted in O_2 reduction and yielded H_2O_2 (G gate). Then H_2O_2 reacted with TMB in the presence of HRP to yield TMB_{ox} with the respective absorbance changes (H gate). Careful optimization of the system (by tuning the concentrations of the auxiliary inputs) allowed comparable output signals produced in both the pathways. Fig. 4(A) shows the optical changes in the system measured at $\lambda = 655$ nm (TMB_{ox} absorbance) for different combinations of the biomarker inputs. Any or both biomarkers appearing at logic-1 level resulted in high absorbance changes signaling about TBI conditions. The experimentally derived threshold level of 0.02 OD allowed perfect separation of the logic-0 and 1 levels for the output signal being significantly different for the input combination 0,0 and all other combinations (0,1; 1,0; 1,1), Fig. 4(B). It should be noted that in all measurements shown in Fig. 4 the auxiliary (2-PGA, TMB and GluOx) and switching (ADP and phosphate) inputs were applied at logic-1 values to allow optimized performance of the analytical pathway. At the

same time the switching inputs PEP and CoA were applied at logic-0 levels to inhibit the alternative STI pathway.

Switching between the STI and TBI modes

Operation of the logic system in the STI mode results in production of ADP as a product of the biocatalytic reaction at gate A. Further reaction of ADP in gate C requires the presence of PEP which is not produced by the system when it operates in the STI mode (note that the gate B is mute because of the absence of EN input). Therefore PEP should be added artificially in order to activate gate C. Opposite to this, when the system operates in the TBI mode, PEP is produced *in situ* in gate B, while ADP which is also needed for the operation of gate C is missing (note that gate A is mute because of the absence of CK). Therefore, in this case ADP should be added artificially in order to activate gate C. Finally, for activation of the network in the STI mode the switching inputs PEP and ADP should be applied at the logic-1 and 0 values, respectively, while for the TBI mode they should be at the opposite 0 and 1 values. This switch allowed the use of gates C and F in two different modes of operation. Similarly, in order to switch between two operational modes, gates F and E should be selectively activated by the correct concentrations of phosphate and CoA. Specifically, phosphate and CoA were

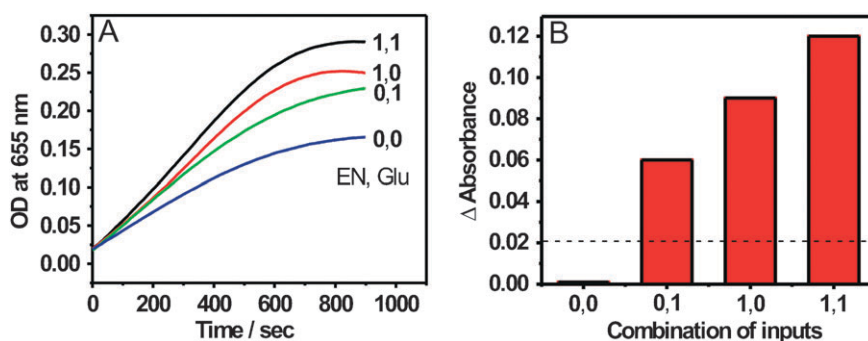


Fig. 4 (A) Optical detection of the output signal (TMB_{ox}) generated by the logic system operating for the TBI analysis obtained upon different combinations of the biomarker input signals (EN, Glu). (B) Bar chart for the output signals generated by the enzyme logic system for the analysis of TBI at 800 seconds. The TBI diagnosis corresponds to the output signals above the decision threshold (dashed line). The logic system composition is given in the Experimental section and the input concentrations corresponding to the logic-0 and 1 values are specified in Table 1. The auxiliary (2-PGA, TMB and GluOx) and switching (ADP and phosphate) inputs were at logic-1 for all combinations of the biomarker inputs.

applied at logic-0 and 1 levels in the STI mode and at logic-1 and 0 levels in the TBI mode.

It should be noted that we were not able yet to provide the optimized operation of both the modes under exactly the same solutions—the TBI was operating in 31 mM potassium phosphate buffer, pH 7.58, containing 6.7 mM magnesium sulfate, while the STI mode was realized in 50 mM triethanolamine buffer, pH 7.4, containing 0.2 mM magnesium acetate. Further work will be needed to bring the system (or eventually to design another system) being able to operate in the same environment but in different switchable modes.

Conclusions

The present study demonstrated that even a very sophisticated multi-enzyme/multi-step biocatalytic cascade can provide reliable diagnostics of physiological conditions upon logic analysis of complex patterns of various biomarkers. The obtained results experimentally confirm our recent theoretical prediction that enzyme logic networks with up to 10 concatenated logic gates should be able to process biochemical information within a reasonable noise level.³³ The designed system exemplify the novel approach to multi-signal processing biosensors mimicking natural biochemical pathways and operating according to the biocomputing concept.² Further studies will be needed to transfer this approach from a conceptual demonstration to real-life biosensor applications. The future biosensor devices will be based on electrochemical methods rather than optical analysis used in the present study. This will require a lot of scientific and engineering work to integrate multi-enzyme systems in a rational design with mini-invasive electrodes before a real practically applicable biosensor becomes possible. It should be noted that in addition to the biocomputing and biosensor challenges, additional biomedical studies will be needed to formulate analyzed biomarkers and their normal and pathophysiological concentrations reflecting specific medical problems. The broadening of the possible applications of this concept will result in the design of various bioelectronic devices and bioactuators controlled by complex patterns of multiple inputs. Microrobotics and bioimplantable computing systems are among the most likely applications to benefit from advances in biomolecular computing. Future progress in these areas will depend on the development of novel computing concepts and design of new signal-responsive and information processing materials contributing to molecular information technology.³⁴

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