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A biocatalytic cascade with several output signals—towards biosensors with different levels of confidence

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Abstract

The biocatalytic cascade based on enzyme-catalyzed reactions activated by several biomolecular input signals and producing output signal after each reaction step was developed as an example of a logically reversible information processing system. The model system was designed to mimic the operation of concatenated AND logic gates with optically readable output signals generated at each step of the logic operation. Implications include concurrent bioanalyses and data interpretation for medical diagnostics.

Keywords

Bioanalytical methods; Bioassays; Enzymes; Optical sensors; Biocomputing; Logic gates

Biomolecular information processing (biocomputing) [1, 2] is a rapidly developing research subarea of chemical computing [3, 4] and more generally of unconventional computing [5]. Sophisticated processes realized in protein/enzyme [2, 6], DNA/RNA [7, 8] and whole cell [9] systems are considered for information processing, ultimately aiming at biomolecular computers [10] operating faster (or more efficiently in some applications) than electronic

systems due to massive parallelism [7] or novel bioinspired computational approaches [11]. While the computational aim is still far from practical realization, low-scale information processing systems are already practically feasible for various bioanalytical and biosensor applications [12]. Taking an advantage from possible operation in a biological environment [13], biomolecular computing systems have been interfaced with physiological processes for the analysis of complex patterns of biomarkers signaling on various pathophysiological conditions [12], e.g., injuries [14]. Different enzyme-based biocatalytic cascades were designed to mimic operation of various Boolean logic gates (e.g., AND, OR, XOR, NAND, NOR, etc.) and their concatenated networked assemblies [2]. Analytical use of the biocomputing systems allows binary logic output in the form of YES/NO (1,0) with respect to a threshold value separating these values according to biomedical significance [12]. In most of bioanalytical/biomedical applications, the logic 0 input signals are defined as normal physiological concentrations of the corresponding chemicals, while their elevated (or sometimes decreased) pathophysiological concentrations are considered as logic 1 input signals [12]. While the logic 0/1 *input* signals are defined by the application, the logic 0/1 *output* signal values are set by the biochemical processes used in the information processing. “Programs” implemented in the composition of the enzyme cascades allowed the desired processing of biological signals realizing, for example, AND gates where the output signal YES (1) appeared only in the presence of *all* input signals at the logic 1 value, while OR gates produced the output YES in the presence of *any* (at least one) input signal at the logic 1 value. In principle, biocomputing approaches can be designed to provide instant diagnostic interpretations based on biomolecule determinations in concurrent measurements.

Different biomedical scenarios would require different signal processing. Less specific biomarkers should be analyzed as a group utilizing the AND logic to provide higher level of confidence, thus compensating low specificity of each separate signal. Highly specific biomarkers can be applied with the OR logic to broaden the analytical variants. For some specific biomedical applications, the AND/OR logic operations can be combined in complex logic networks, all realized in biomolecular systems [2]. However, in most of presently reported biocomputing systems [1] (particularly based on enzyme cascades) [2] multiple input signals, after stepwise biochemical reactions, resulted in a *single* YES/NO (1,0) output considered as the final interpretive conclusion. Obviously, some information is lost in the information processing. For example, for the signal processing through OR gate, the output YES (logic 1 value) can appear in the following combinations of the input signals: 0,1; 1,0; and 1,1. Signal processing through several concatenated logic gates represented by several biochemical reactions with multiple inputs and with only one output results in the significant loss of information at each step of the process. Despite inevitable loss of information upon Boolean logic operations, this kind of analysis is beneficial for getting fast conclusion providing reliable point-of-care diagnosis and autonomous on-demand therapeutic intervention. On the other hand, the information encoded in the combinations of the input signals can be preserved if the number of output signals is equal to the number of inputs, which is the case in reversible logic gates [15]. (Note that in this context “*reversibility*” has a different meaning than in chemistry; in information processing, “*logical reversibility*” means the possibility to recover initial information from the processed information.) The reversible logic gates may have 2-and-2, 3-and-3, 4-and-4 input–output signals with

different logic programs converting the input to output signals. Different kinds of reversible logic gates (e.g., Toffoli [16], Fredkin [16, 17], and Feynman [18] gates) have been realized in molecular and biomolecular systems, mostly aiming at future computational applications. While the complexity of the information processing through these gates is needed for computational applications, it might be not necessary for bioanalytical applications, where simple networks of concatenated AND/OR gates might be sufficient for many biomedical scenarios. Keeping in mind bioanalytical rather than computational applications, one can design a biocatalytic cascade mimicking concatenated AND logic gates with multiple inputs and outputs performing reversible information processing specifically for the analysis of biomarker species. The present paper demonstrates for the first time a biocatalytic cascade activated by several enzyme inputs with the optically readable output signals after each biocatalytic step to allow *reversible information processing*, where the original pattern of the input signals can be reconstructed from the obtained output signals. The paper also suggests specific bioanalytical applications and possible extensions of the developed approach.

Scheme 1a shows a biocatalytic cascade designed to implement reversible information processing in a biosensing system. The major backbone of the biocatalytic cascade is highlighted in the scheme with the light orange color. The reaction sequence starts with conversion of 2-phosphoglycerate (PGA) to phosphoenolpyruvate (PEP) biocatalyzed by enolase (phosphopyruvate hydratase) (ENO; E.C. 4.2.1.11). Then in situ produced PEP is converted to pyruvate (Pyr) with the concomitant production of ATP from ADP (ATP = adenosine triphosphate; ADP = adenosine diphosphate) in the process biocatalyzed by pyruvate kinase (PK; E.C. 2.7.1.40). In the next reaction step, Pyr is consumed by lactate dehydrogenase (LDH; E.C. 1.1.1.27) to yield lactate (Lac), also resulting in the oxidation of NADH to NAD⁺. The last reaction step results in conversion of Lac back to Pyr in the process biocatalyzed by lactate oxidase (LOx; E.C. 1.1.3.2), also producing H₂O₂ from abundant O₂. These processes are activated only in the presence of the corresponding enzymes, which can be considered as logic input signals [2].

In the first discussion (for the reason explained later), we will consider only three enzymes, PK, LDH, and LOx, as the logic input signals applied at the variable logic 0 or logic 1 values. This model system is aimed at demonstration of the concept rather than a real biomedical/biosensing application. We will define the logic 0 input value as the absence of the enzymes and logic 1 value as experimentally convenient (optimized) enzyme concentrations (see Supplementary information for the experimental details). ENO, catalyzes the first transformation in the reaction chain, will be considered as a constantly present component of the system with the optimized concentration. The system also includes other components always added to the solution at the same (nonvariable) concentrations prior to the initiation of the biocatalytic reactions; these include two additional enzymes to facilitate detection, horseradish peroxidase (HRP; E.C. 1.11.1.7) and luciferase (Luc), along with enzyme substrates! co factors, PGA, NADH, luciferin (Lucif), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) at experimentally optimized concentrations. Oxygen was always present in the solution in equilibrium with air. One of the output signals generated by the biocatalytic cascade was the absorbance decrease ($\lambda_{\text{max}} = 340 \text{ nm}$) associated with the oxidation of NADH to NAD⁺ biocatalyzed by LDH. Additional

biochemical processes biocatalyzed by luciferase and HRP were used to convert ATP and H_2O_2 produced in the biocatalytic cascade to optically readable signals. The biocatalytic formation of ATP in the presence of the luciferase resulted in bioluminescence (emission at $\lambda_{\text{max}} = 552 \text{ nm}$; note that the wavelength depends on pH) [19] under conditions used for the standard fluorometric assay of ATP [20]. The biocatalytic production of H_2O_2 in the presence of HRP and ABTS resulted in an absorbance increase at $\lambda_{\text{max}} = 420 \text{ nm}$ due to ABTS oxidation [21].

The biocatalytic cascade was initiated by adding the enzyme input signals, PK, LDH, and LOx, in all possible combinations of 0 and 1 logic inputs. Notably, the system was activated by three logic inputs and the results were read by three output signals, thus resulting in a 3-and-3 input–output reversible logic system. Scheme 1b shows the logic equivalent circuit corresponding to the biocatalytic cascade shown in Scheme 1a. The circuit includes the Identity (ID) logic gate concatenated with two following AND logic gates. The output signals are read after each logic step. Logic output B corresponding to the formation of ATP is produced at the logic values 1 or 0 when input B (PK) is applied at logic values 1 or 0, respectively (representing the ID gate). Logic output C corresponding to the formation of NAD^+ (detected optically as the decreasing absorbance of NADH) is produced at the logic value 1 only when inputs B and C are both applied at logic values 1 (representing the AND gate). Logic output D corresponding to the formation of H_2O_2 is produced at the logic value 1 only when inputs B, C, and D are all applied at logic values 1 (representing the second concatenated AND gate). The initial combination of all three input signals can be recovered by analyzing the generated output signals.

The biocatalytic cascade can be modified by adding additional reaction steps or by considering already existing components as variable inputs. The latter does not affect the biocatalytic cascade, but increases complexity of the logic scheme. Indeed, if the first enzyme in the biocatalytic cascade (ENO) is also considered as a variable input A, then the ID gate is transformed to the AND gate dependent on the logic values of two inputs A and B (Scheme 1c). In this case, the number of input signals becomes larger than the number of outputs, and the system loses the property of logic reversibility, which means that the logic values of inputs A and B cannot be recovered from the output B. This is the reason why in the beginning of the system discussion, we limited the logic input signals to three enzymes only. The logic network with four inputs and three outputs appearing at different reaction steps (Scheme 1c) still has important implications in bioanalytical systems as will be discussed below.

Meanwhile, let us consider the experimental results analyzing the 4-and-3 input–output system. Figure 1 shows the experimental spectra and corresponding bar charts for the optically detected output signals appearing for different combinations of the input signals. Despite its simplicity, this figure is based on large amounts of experimental information. Figure 1A, B shows the results of the fluorometric assay of ATP, which is output B of the first AND gate (Scheme 1c). The high intensity of the bioluminescence corresponding to output B (logic 1) was obtained only when input signals A and B were applied both at logic values 1 (corresponding to the presence of enzymes ENO and PK), while the low bioluminescence (logic 0) was obtained for other combinations of inputs A and B (0,0; 0, 1;

and 1,0) as expected for the AND logic gate. Figure 1 C, D shows the NADH absorbance changes ($\lambda_{\text{max}} = 340 \text{ nm}$) corresponding to output C. The large absorbance change, logic 1 (note that the NADH absorbance is decreasing due to the NADH consumption in the reaction catalyzed by LDH; Scheme 1a), was obtained only in the 1,1, 1 combination of input signals A, B, and C. When any of these first three inputs was applied at logic 0 level (corresponding to the absence of the enzyme), the absorbance change was very small (logic 0). Finally, Fig. 1E, F shows the absorbance changes, output D, corresponding to the assay of H_2O_2 (through the HRP-ABTS reaction). The large absorbance change (logic 1) was obtained only in the 1, 1,1,1 combination of input signals A, B, C, and D. When any of four inputs was applied at logic 0 level (corresponding to the absence of the enzyme), the absorbance change was very small (logic 0). Each analyzed output signal was dependent on the preceding inputs and independent of the following inputs. This seems to be a trivial conclusion which in reality is not so trivial. Indeed, simultaneous presence of many enzymes and substrates/cofactors could result in some unexpected additional pathways, cross-talking between enzymes, which are not reflected in the simplified reaction pathway (Scheme 1a). Thus, the experimental proof of the logic system operation was important to support the expectations from the reaction scheme.

At this point, let us consider the bioanalytical meaning of the designed system and its potential future applications. The enzymes operating as the input signals in the system described above can be used as labels for biorecognition/bioanalytical processes (e.g., in immunoassays similar to ELISA or in DNA analyses). The enzyme labels can be caused to bind to a biosensor/assay surface only when the corresponding analyte species appear. Thus, intelligent responses with different levels of confidence can be obtained from the system. For example, suppose we want to measure the possible presence of four analytes at levels above predetermined thresholds. When the first two analytes A and B are present (Scheme 2), the enzyme labels ENO and PK will generate the first analytical response with the minimum level of confidence based on the simultaneous presence of two analytes above predetermined thresholds. Additional presence of other analytes, C and then D, will result in the appearance of LDH and then LOx, thus resulting in additional output signals, each one increasing confidence of the diagnostic response by reflecting the presence of two additional analytes. Using this approach, the first two analytes should represent the most important biomarkers in a diagnostic assay, while additional analytes should be less important biomarkers which can increase the confidence of the biomedical conclusion, but their appearance in the absence of the first biomarkers is not meaningful for the diagnostic conclusion. In other words, the analytes at the beginning of the biocatalytic cascade should have the highest importance for the enzyme labels with those with the decreasing importance at the end of the cascade. Depending on the number of the analyzed species and the number of output signals. the analytical system can be considered as a reversible logic system (e.g., 3-and-3 input–output system as described above) or concatenated logic network without logic reversibility (e.g., 4-and-3 input–output system, also exemplified above). Both kinds of systems can find analytical applications and can be extended to other enzyme-labels and different biocatalytic cascades. The present example system demonstrates the concept of the multilevel output bioanalytical logic. We are currently applying this approach to the detection of diagnostic panels of oral cancer biomarker

proteins where the enzymes will be used as biocatalytic labels on the biomarker-specific antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Optical output signals generated by the biocatalytic cascade upon application of the input signals in different logic combinations. **(A)** Typical fluorescence spectra corresponding to output B in logic 1 (**a**) and 0 (**b**) values (Note that the spectra and bars shown in B were normalized to 1.). **(B)** Fluorescence output (emission at $\lambda_{\text{max}}=552$ nm) corresponding to the formation of ATP (output signal B): (**a**) input signals A and B applied at 1,1 combination; (**b**) input signals A and B applied at 0,0; 0,1 and 1,0 combinations. **(C)** Typical absorbance spectra corresponding to the output C in logic 1 (**a**) and 0 (**b**) values. **(D)** Optical absorbance changes ($\lambda_{\text{max}}=340$ nm) corresponding to the decreasing concentration of NADH (output signal C): (**a**) input signals A, B, and C applied at 1,1,1 combination; (**b**) input signals A, B, and C applied at all other combinations where at least one signal was applied at 0 level. **(E)** Typical absorbance spectra corresponding to the output D in logic 1 (**a**) and 0 (**b**) values. **(F)** Optical absorbance changes ($\lambda_{\text{max}}=420$ nm) corresponding to the formation of H_2O_2 (output signal D) detected through the HRP-ABTS assay system: (**a**) input signals A, B, C and D applied at 1,1,1,1 combination; (**b**) input signals A, B, C and D applied at all other

combinations where at least one signal was applied at 0 level. The *dash lines* show the threshold levels separating the output 0 and 1 logic values

Scheme 1.

a Schematics of the biocatalytic cascade and input/output signals used in the information processing system. **b** Equivalent logic system corresponding to the 3-and-3 input–output reversible logic system. **c** Equivalent logic system corresponding to the 4-and-3 input–output logic system composed of three concatenated AND gates

Scheme 2.

Schematic of the potential application of the studied biocatalytic cascade in the bioanalytical application where the enzymes are used as labels for the detection of biomarker analyte species. The biocatalytic cascade provides the multilevel output responses with the increasing confidence level upon appearance of additional input signals