Enzyme logic gates for the digital analysis of physiological level upon injury

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Abstract

A biocomputing system composed of a combination of AND/IDENTITY logic gates based on the concerted operation of three enzymes: lactate oxidase, horseradish peroxidase and glucose dehydrogenase was designed to process biochemical information related to pathophysiological conditions originating from various injuries. Three biochemical markers: lactate, norepinephrine and glucose were applied as input signals to activate the enzyme logic system. Physiologically normal concentrations of the markers were selected as logic 0 values of the input signals, while their abnormally increased concentrations, indicative of various injury conditions were defined as logic 1 input. Biochemical processing of different patterns of the biomarkers resulted in the formation of norepi-quinone and NADH defined as the output signals. Optical and electrochemical means were used to follow the formation of the output signals for eight different combinations of three input signals. The enzymatically processed biochemical information presented in the form of a logic truth table allowed distinguishing the difference between normal physiological conditions, pathophysiological conditions corresponding to traumatic brain injury and hemorrhagic shock, and abnormal situations (not corresponding to injury). The developed system represents a biocomputing logic system applied for the analysis of biomedical conditions related to various injuries. We anticipate that such biochemical logic gates will facilitate decision-making in connection to an integrated therapeutic feedback-loop system and hence will revolutionize the monitoring and treatment of injured civilians and soldiers.

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1. Introduction

The majority of battlefield, criminal or traffic accident deaths occur within the first 30 min after injury. Accordingly, rapid evaluation of physiological conditions and immediate treatment of an injured person are extremely important for enhancing the survival rate (Baker et al., 1992). Medical intervention without obtaining reliable information about the exact nature of the injury might be meaningless or even dangerous since various injury scenarios (brain injury, trauma, shock, fatigue, etc.) require different therapeutic treatments (Zasler et al., 2006; Feliciano et al., 2007; Altura et al., 1983). However, in many cases (particularly for injured soldiers in a battlefield) rapid evacuation to a hospital is impossible and the medical diagnosis is too late. Thus, high fidelity real-time diagnostics is of critical importance for reliable decision-making and optimal treatment immediately after the injury has occurred. Eventually, such reliable and rapid analysis of the type of injury could be coupled to an automatic drug-delivering feedback-loop, leading to a timely therapeutic intervention and hence to decreased mortality and severity of the post-traumatic conditions.

Application of standard medical analyses to an injured person requires hospital conditions and cannot be performed without sophisticated equipment and highly trained personnel. Evaluation of the injury conditions directly on an “on-the-spot” basis should involve novel approaches based on modern advances in the area of biosensors (Curreli et al., 2008; Mascini and Tombelli, 2008; Wang, 2006). However, a common biosensor device, selective to one specific target analyte, cannot provide sufficient information to make a medical diagnostics regarding the kind of injury and the required treatment. This conclusion should be based on the simultaneous analysis and comparison of several physiological markers that are known to vary for different types of injury. Following the traditional biosensor approach, several sensing devices should be applied simultaneously, all of them will be equipped with proper electrical and computer interfaces and energy sources, which will process the obtained information and make the final decision. The area of biotechnology involving the sensing and transduction of signals, for instance, for drug delivery in implantable devices, has been rapidly evolving. In this approach, each step increases in complexity of the “decision-making” algorithms involved, and the output...
of this system must interface with the external electronic devices required for further information/signal processing. Some engineering progress has been achieved in this direction through the use of biosensor arrays in restricted and safety-critical environments (Astaras et al., 2008). However, this approach is not practical in remote locations (e.g. for soldiers in a battlefield) and it has little advantages over regular diagnostics in a hospital. Thus, the formulated aim requires a novel unconventional approach to the decision-making (sense-and-treat) biosensor.

The recently pioneered concept of biochemical information processing (biocomputing) allows for the analysis of various chemical signals, their comparison and processing according to a special chemical built-in “program” using only chemical means without the involvement of electronic computers (Shao et al., 2002). Biochemical processing of information with biomolecules and biological components by realizing and networking logic “gates” that mimic Boolean digital logic offers great promise for clinical diagnostics. Indeed, biomolecules are selective and usable in complex biochemical environments. Such systems are also expected to be appropriate for interfacing of the biocomputing “devices” with processes in living organisms, and hence can be useful for future practical biomedical applications. Integration of biocomputing elements with sensing processes would allow multi-signal analysis followed by chemical processing of the data, giving the final answer regarding the physiological conditions of an injured person in a digital (“YES” or “NO”) form. In the next development step, the biocomputing system – analyzing the biochemical information and making decision – could be connected to a chemical actuator releasing an appropriate drug to treat an injured person. Biocomputing elements of even moderate complexity will allow an effective interface between complex physiological systems and nano-structured signal-responsive materials and/or electronic systems. Thus, integration of the biocomputing concepts into sensing technology will contribute to a new emerging technological paradigm: networking of information processing stages that involve only biochemical processes, aiming at eliminating the “wires” and “batteries” and reducing the overall need for electrical power supplies at the stages of information processing, carried out on-site in implantable devices.

Biocomputing (Shao et al., 2002) being a sub-area of unconventional chemical computing (De Silva and Uchiyama, 2007; Credi, 2007) ranges from application of biomolecules (proteins, Tomizaki and Mihara, 2007; Unger and Moulit, 2006; enzymes, Sivan et al., 2003; Strack et al., 2008a; Baron et al., 2006; DNA, Stojanovic et al., 2005) to the use of whole biological cells (Simpson et al., 2001) for processing biochemical signals in a digital form according to Boolean logic operations. Recent extensive research in the area of the enzyme-based logic systems allowed the formulation of different Boolean logic gates (Strack et al., 2008a; Baron et al., 2006). Further scaling up the complexity of the enzyme logic systems allowed for their concatenation in sophisticated logic networks processing various patterns of different chemical signals and generating final output signals with the encoded information dependent on the entire set of input signals (Strack et al., 2008b). Experimental evaluation and theoretical analysis of enzyme logic gates predicted that an acceptable noise level and error-free information processing could be achieved upon concatenation of at least 10 logic gates, processing many different combinations of input signals (Privman et al., 2008). The enzyme information processing systems were integrated with various electrochemical analyzing interfaces to transduce the resulting output signal from a chemical to electrical form (Zhao et al., 2009). Enzyme biocomputing systems were also shown to operate as logic switches for complex bioelectronic devices (Amir et al., 2009) and nano-structured signal-responsive materials performing various chemical actuation functions (Tokarev et al., 2009).

Such integrated multi-functional systems composed of the biochemical information processing units, electronic transducers and chemical actuators would allow fast autonomous and reliable analysis of pathophysiological changes originating from an injury, identification of the type of injury, process the medical state in real time and performing proper drug-delivery intervention when physiologically appropriate. Application of enzyme logic systems coupled with signal-responsive materials and electronic transducers would thus allow for the analysis, decision-making and therapeutic intervention performed by pure chemical means without sophisticated electronic equipment, and hence could lead to improved survival rate among injured civilians and soldiers.

The present paper describes a novel approach to the biochemical analysis and data processing based on biocomputing with enzyme-based logic gates that eventually could be used for real-time field monitoring of injured people. Specifically, biocomputing systems analyzing biochemical markers, characteristic of different pathophysiological conditions, have been developed here to illustrate the potential biomedical application of biocomputing. The paper represents the advanced step of our recent research (Pita et al., 2009) with significant improvement of the signal processing applying a new set of logic operations which allow better discrimination of different pathophysiological conditions. Our data illustrate the ability to generate distinct patterns of output signals from different combinations of biochemical input signals, characteristic of various normal and abnormal physiological conditions. We anticipate that such biochemical logic gates will create high fidelity diagnosis for a greatly improved decision-making, leading to an optimal timely therapeutic intervention and to improved survival of injured soldiers and civilians. Keeping in mind this ultimate goal and aiming at minimally invasive enzyme logic gate based sensing device, we stepped forward from optical analysis of the enzyme logic operations to electrochemical transduction of the output signals. Both signal transduction methods (optical and electrochemical) were employed and compared, demonstrating good consistency of the results.

2. Experimental

2.1. Chemicals and reagents

Lactate oxidase (LOx) (E.C. 1.1.3.2) was purchased from Genzyme Corp. and was purified as follows. About 100 µL of LOx (0.5 units µL⁻¹) in a 50 mM phosphate buffer solution (pH 7.4) was taken in 100 kDa Centrisart ultra-filtration tube and centrifuged at 7000 rpm for 15 min at 4 °C. The sediment was washed with 80 µL of phosphate buffer and centrifuged. The process was repeated five times. All other chemicals and enzymes were purchased from Sigma–Aldrich and used as supplied without any further purification: β-nicotinamide adenine dinucleotide (NAD⁺), d- (+)-glucose, (l)-norepinephrine (NE), l- (+)-lactic acid. Other enzymes used were horseradish peroxidase (HRP) type VI (E.C. 1.11.1.7) and glucose dehydrogenase (GDH) from Pseudomonas sp. (E.C. 1.1.1.47). Ultra-pure water (18.2 MΩ cm) from NANOpure Diamond (Barnstead) source was used in all of the experiments.

2.2. Instruments

The optical measurements were performed using a Shimadzu UV-2450PC spectrophotometer. The electrochemical measurements were performed using a CH Instrument Model CHI660C with an electrochemical cell consisting of a glassy carbon working electrode, Ag/AgCl (3 M NaCl) reference and a platinum wire counter electrode.
2.3. Composition of logic gates and input signals

The AND logic gate consisted of a 1 mL of phosphate buffer solution, 50 mM, pH 7.4, containing both LOx, 0.1 units mL$^{-1}$ and HRP, 5 units mL$^{-1}$. The biochemical input signals were NE and lactate. The input concentrations of NE were 2.2 nM and 3.5 μM selected as digital 0 and 1 signals, respectively. Lactate concentrations of 2 mM and 13 mM were defined as digital logic inputs of 0 and 1, respectively. Norepi-quinone (NQ) biocatalytically produced in the AND logic gate was defined as the output signal and its production was monitored by the optical absorbance measurements at $\lambda = 465$ nm and by electrochemical current measurements at $E = -0.25$ V. The IDENTITY gate consisted of a 1 mL of phosphate buffer solution, 50 mM, pH 7.4, containing GDH, 0.025 units mL$^{-1}$ and 20 μM NAD$^+$. This gate used only one input signal of glucose. The glucose concentrations of 4 mM and 26 mM were defined as digital logic inputs of 0 and 1, respectively. The output signal of the IDENTITY gate was defined as a concentration of the biocatalytically produced NADH, which was measured optically at $\lambda = 340$ nm and electrochemically at $E = 0.75$ V. The combined system composed of the AND and IDENTITY gates included all enzymes/cofactors operating as the biocomputing “machine” (LOx, HRP, GDH, and NAD$^+$) and it was using three chemical inputs, including glucose, lactate and NE, applied in the concentrations corresponding to their digital 0 and 1 values. The optical output signals corresponding to the formation of NQ and NADH in the same solution were measured at 465 nm and 340 nm, respectively. In the case of electrochemical measurement, the output signal of NQ and NADH were measured at $-0.25$ V and +0.75 V, respectively. All measurements were carried out at ambient temperature (23 ± 2°C).

3. Results and discussion

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/circuitries. We selected glucose, lactate and norepinephrine (NE) as physiological markers signaling on different kinds of injuries, and we applied them to demonstrate the concept of the biochemical signal processing and autonomous decision-making. Specific patterns in the concentrations of these markers can provide sufficient information to identify the type of injury occurred in a body. Abnormal increase in the concentration of glucose might originate from hemorrhagic shock (HS) (Kline et al., 1997; Zink et al., 1999), while higher than normal physiological concentration of lactate could be caused also by HS or/and trauma brain injury (TBI) (Zink et al., 1999; Prasad et al., 1994). A high concentration of NE can be indicative of any traumatic injury (Prasad et al., 1994; Rosenberg et al., 1961). Thus, glucose, lactate and NE were applied as chemical input signals for the enzyme logic circuitry. 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 NE. Abnormal high concentrations of glucose (30 mM) characteristic of HS (Kline et al., 1997; Zink et al., 1999), of lactate (13 mM) observed in case of both TBI and HS (Zink et al., 1999; Prasad et al., 1994), and of NE (3.5 μM) typical for any traumatic injury (Prasad et al., 1994; Rosenberg et al., 1961) were considered as logic 1 values for the input signals. It should be noted that only two concentrations of the chemical input signals were applied corresponding to the digital values of 0 and 1, while their intermediate concentrations were considered as digitally undefined.

As illustrated in Scheme 1C, the enzyme logic circuitry analyzing three chemical input signals (glucose, lactate and NE) included two parallel-functioning biocatalytic pathways composed of AND/IDENTITY logic gates. The AND logic gate was composed of LOx and HRP activated by the lactate and NE input signals and produced norepi-quinone (NQ) as an output signal (Scheme 1A). Lactate was oxidized by LOx in the presence of oxygen resulting in the biocatalytic formation of H$_2$O$_2$, which activated the oxidation of NE by HRP. The oxidation of NE yielding norepi-quinone (NQ) proceeded only in the presence of both input signals (lactate and NE), mimicking a Boolean AND logic operation, while the absence of any of these two inputs results in the inhibition of the biocatalytic chain reaction. The IDENTITY gate was based on GDH which translated the glucose input signal into a NADH output signal, Scheme 1B.
difference between the present system and traditional biosensors lies in the simultaneous operation of all biocatalytic reactions in a single solution with the readout of only two output signals for three applied inputs logically processed by the enzyme system.

Initially, we analyzed the performance of each logic gate separately, finally aiming to apply them simultaneously. The formation of NQ upon the operation of the AND gate (Scheme 1A), was analyzed for four different combinations of the two input signals (lactate and NE): 0, 0; 0, 1; 1, 0 and 1, 1 being at their logic values of 0 and 1. The formation of NQ was detected by optical absorbance measurements at λ = 465 nm (Fig. 1 left), and by electrochemical current measurements at the potential of −0.25 V (Fig. 1 right). Such detection potential was selected based on square wave voltammetric measurements (not shown). The built up of the output signal, corresponding to the formation of NQ, above the threshold values of 2 mOD (Fig. 1 left, inset), and 40 nA (Fig. 1 right, inset) for the optical and electrochemical measurements, respectively, was defined as the logic value 1 for the output signal of the AND gate. Otherwise, the output signal was defined as a logic 0 value. Only a 1, 1 combination of the input signals resulted in the output signal 1 resembling the features of the AND logic gate and signaling about the formation of NQ above the threshold value. The obtained optical and electrochemical results show good consistency. It should be noted that NQ was also produced in a lower concentration (below the threshold value) for the 0, 0, 0, 1 and 1, 0 combinations of the input signals, because the logic 0 values of the input signals were not physically zero, but corresponded to the normal physiological concentrations of lactate and NE.

The second logic gate was composed of a single enzyme (GDH) and was aimed to discriminate between normal and pathological concentrations of glucose. The input of glucose was applied at two different levels of 4 mM and 26 mM being considered as logic 0 and 1 values, respectively (Fig. 2). The biocatalytic formation of NADH as the result of the biocatalytic reaction (Scheme 1B) was considered as the output signal. The production of NADH was monitored by optical absorbance measurements at λ = 340 nm (Fig. 2 left), and by amperometric analysis carried out at +0.75 V (Fig. 2 right). The exact potential for the oxidation of NADH was determined using square wave voltammetry of NADH (not shown). The NADH output signal was defined as a logic 0 value. Both methods, optical and electrochemical, resulted in the consistent data demonstrating good agreement. It should be noted that the logic 0 value of the input signal was not physically zero, but rather corresponded to a physiologically normal glucose concentration, 4 mM, thus resulting in a relatively high level background signal.

The most important experiments included the simultaneous operation of both logic gates upon concerted operation of GDH, LOx and HRP activated by three input signals (glucose, lactate and NE) in connection to eight different input combinations. The definition of logic 0 and 1 values of the input signals was the same as described above for the individual logic gates, and the analysis of the output signals was performed by the optical and electrochemical means similarly to the procedures described above. Fig. 3 shows the optical (top) and electrochemical (middle) detections for the output signal produced by the AND gate in the form of NQ. The high level signals corresponding to the NQ production were observed only for the 1, 1 combination of the lactate and NE input signals, regardless of the logic value of the glucose input (in other words, input signal combinations of 0, 1, 1 and 1, 1, 1, where the first logic value is reserved for glucose, resulted in logic 1 output value of NQ). Fig. 3(bottom)
Fig. 3. Time-dependent signals corresponding to the formation of NQ generated by the combined AND–IDENTITY logic system upon application of various combinations of the input signals (glucose, lactate and NE) measured by optical means (top) and amperometrically (middle). (Bottom) Bar diagram featuring the combined AND–IDENTITY logic operation of the optical and electrochemical systems. Absorbance measurements were performed at λ = 465 nm. Electrochemical measurements were performed at −0.25 V. Dash line shows the threshold values separating digital 0 and 1 output signals produced by both systems.

summarizes the output optical and electrochemical signals for all combinations of the input signals. The threshold values of 1 mOD and 40 nA were applied to separate the logic 0 and 1 values of the NQ output signal.

Fig. 4 displays the optical (top) and electrochemical (middle) detection for the output signal produced by the IDENTITY gate in the form of NADH. The high level signals of the NADH production were observed only for the logic value 1 corresponding to the glucose input regardless of the logic values of the lactate and NE inputs (in other words, for input signal combinations of 1,0,0; 1,0,1; 1,1,0 and 1,1,1, where the first logic value is reserved for glucose, resulted in logic 1 output value of NADH). Fig. 4(bottom) summarizes the output signals obtained by both methods for all combinations of the input signals. The threshold values of 35 mOD and 110 nA were applied to separate logic 0 and 1 values of the NADH output signal.

The resulting data demonstrate good agreement between the optical and electrochemical analysis of the logic circuitry operation. Note also that the enzyme logic “machinery” operated in the same solution without any visible cross-reactivity.

The final judgment on the physiological conditions in the studied model system was based on the comparison of the two output signals generated by the AND/IDENTITY gates (Table 1). Different diagnoses could be derived from various combinations of the output signals. The output signal combination 0,0 can be obtained for three different combinations of the input signals: 0,0,0; 0,0,1 and 0,1,0. The first situation (0,0,0) corresponds to all three input signals being at their normal physiological levels, thus reflecting normal (i.e., non-injury/trauma) physiological conditions. The second input pattern (0,0,1) corresponds to an increased level of NE alone, thus reflecting a stress situation without any physical injuries. The third combination of the input signals (0,1,0) corresponds to the abnormally increased level of lactate, which might originate from physical exercises, still without any evidence of injury. Three scenarios do not signal about any injury and do not require any medical intervention. The output signal combination 0,1 could theoretically originate from three different patterns of the input signals: 1,0,0; 1,0,1 and 1,1,0. However, only the first of them is physiologically pos-
Based on the application of minimally invasive on-body electrodes. Indeed, the normal concentration of lactate is impossible when there are increased levels of NE and glucose, both being signals of an injury. Similarly, a physiologically normal NE level is impossible in the presence of high concentrations of lactate and glucose, which can occur only in the case of injury. The only physiologically meaningful combination of the input signals (1,0,0) corresponds to a high level of glucose, which is not related to injuries (but rather to a diabetes condition). Therefore, the output signal combination 0,1 will be an unlikely situation, which is of little or no relevance to injury. The most valuable output of the gate system corresponds to the last combinations of the output signals: 1,0 and 1,1, which originate from the input signal combinations 0,1,1 and 1,1,1, respectively. Both of these combinations of the biomarker input indicate serious injuries. The first output signal pattern (1,0) corresponds to lactate and NE being at high concentrations reflecting a physiological situation characteristic of TBI; the second pattern (1,1) corresponds to all three markers (glucose, lactate and NE) being at high concentrations, thus indicating a likely occurrence of HS. These two combinations of output signals allow discrimination between two different forms of severe injury originating from TBI and HS and require an immediate medical intervention.

4. Conclusions

The new approach to multi-signal biosensing allows qualitative evaluation of the biochemical information in terms of YES–NO providing the background for logic analysis of complex patterns of biochemical signals. The quantitative assessment of the biochemical information is provided by the threshold values between 0 and 1 logic values. The studied in vitro model system demonstrated the applicability of enzyme logic gates to the assessment of biochemical signals and processing their combinations at physiological concentrations relevant to normal and pathophysiological conditions associated with TBI and HS. The studied systems demonstrated stable information processing and generated distinguishable patterns of the output signals from different combinations of biochemical input signals, characteristic of various normal and abnormal physiological conditions. The developed approach paves the way to the novel digital biosensors processing multiple–biochemical input signals and producing a combination of output signals dependent on the whole pattern of various input signals. The biochemical signals are processed by chemical means based on the enzyme logic system and the difference between different physiological scenarios can be directly derived from the chemically processed information, hence obviating the need for computer analysis of the biosensing information. The biochemical signals were transduced by optical and electrochemical means to demonstrate the broad applicability of the developed approach. For practical applications, particularly related to the battlefield analysis of physiological conditions of injured soldiers, we plan an electrochemical approach based on the application of minimally invasive on-body electrodes.

In addition to the analysis of the data, the output signals might be directed to chemical actuators (e.g. signal-responsive membranes) leading to an on-demand drug release. We anticipate that such biochemical logic gates will facilitate decision-making in connection to an autonomous feedback-loop drug-delivery system and will revolutionize the monitoring and treatment of injured soldiers and civilians and enhance their survival rate.

Acknowledgement

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References


Table 1

The truth table for the combination of AND/IDENTITY logic gates.

<table>
<thead>
<tr>
<th>Glucose input signal</th>
<th>Lactate input signal</th>
<th>NE input signal</th>
<th>NQ output signal</th>
<th>NADH output signal</th>
<th>Biomedical conclusions</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>Normal physiological conditions</td>
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<tr>
<td>1</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>Abnormal level of glucose—not related to injuries</td>
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<td>0</td>
<td>0</td>
<td>Stress—not related to injuries</td>
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<td>Physiologically not applicable</td>
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<tr>
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<td>0</td>
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<td>Hard physical exercise—not related to injuries</td>
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