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Bio-logic analysis of injury biomarker patterns in human serum samples

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ABSTRACT

Digital biosensor systems analyzing biomarkers characteristic of liver injury (LI), soft tissue injury (STI) and abdominal trauma (ABT) were developed and optimized for their performance in serum solutions spiked with injury biomarkers in order to mimic real medical samples. The systems produced 'Alert'-type optical output signals in the form of "YES-NO" separated by a threshold value. The new approach aims at the reliable detection of injury biomarkers for making autonomous decisions towards timely therapeutic interventions, particularly in conditions when a hospital treatment is not possible. The enzyme-catalyzed reactions performing Boolean **AND**/**NAND** logic operations in the presence of different combinations of the injury biomarkers allowed high-fidelity biosensing. Robustness of the systems was confirmed by their operation in serum solutions, representing the first example of chemically performed logic analysis of biological fluids and a step closer towards practical biomedical applications of enzyme-logic bioassays.

1. Introduction

Rapid development of chemical [1–6] and particularly biochemical [7–12] systems switchable between two distinct states resulted in the formulation of molecular assemblies performing Boolean logic operations (e.g. **AND**, **OR**, **NAND**, **NOR**, **XOR**, etc.) and their networks mimicking digital computing processes. These systems, originally aiming at solving computing problems [13,14] (e.g. complex combinatorial problems [15]), were recently considered as potential multi-signal analyzing biosensors [16] with built-in Boolean logic resolved by chemical means without involvement of electronic computers. Analysis of various combinations of biochemical signals through biomolecular-implemented logic is particularly promising in biomedical applications [17–19]. The digital nature of the output signals generated in the form of YES-NO separated by a threshold allows alert-kind high-fidelity biosensing along with a proper therapeutic intervention. The use of enzymes as logic gates performing sensing that harnesses a Boolean logic architecture is a novel concept. This approach is extremely attractive since enzymatic systems can accept multiple biochemical signals and upon biochemical processing of the input information they can generate an output signal to activate an electrochemical transducer and/or a chemical actuator (i.e. drug-releasing membrane). The biomolecular logic analysis of multiple biomarkers appearing in complex combinations requires concerted operation of several biocatalytic steps, while each one may require different conditions. Optimization of the biocatalytic cascades [20], and particularly minimization of the signal-noise amplification [21–23], is a challenging goal in the development of novel digital biosensors with built-in logic. A particularly difficult task is the transition of the multi-component biomolecular systems from an *in vitro* test-tube analysis where they operate in a clean model solution to real-life biomedical samples with many different interferants present. Biosensing in real biological fluids containing many potential interferants is a major problem for relatively simple single-enzyme biosensors [24,25] (particularly in case of implantable sensing devices [26]), while it is becoming even more challenging for multi-enzyme biocatalytic cascades used in multi-analyte biosensors.

Biochemical logic systems composed of enzyme-based logic gates offer great promise for the reliable detection of injury biomarkers and for making autonomous decisions towards a timely therapeutic intervention during trauma or shock events, particularly in the case when a hospital treatment is not immediately possible, for example for injured soldiers on a battlefield. Our efforts focus on providing a biochemical logic-based assessment of the overall physiological condition of a soldier during injury. The biochemical logic network systems are composed of enzyme-based logic gates performing specific biocatalytic reactions for the reliable diagnosis of an injury and eventually will enable the automated treatment of injured soldiers. Such systems will be activated by different biochemical input signals corresponding to various injury scenarios (brain injury, trauma, shock, fatigue, stress, etc.). Parallel activation of different gates would lead to distinct logic operations, reflecting the nature and severity of the injury, hence providing a reliable diagnosis essential for correct decision-

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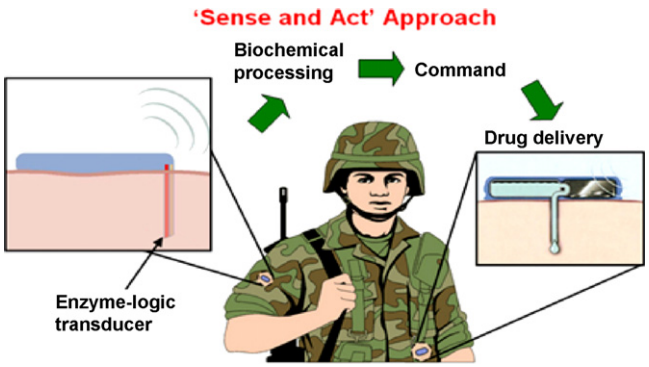

Fig. 1. 'Sense-and-Act' concept for the analysis of battlefield injury conditions followed by an automatic medical treatment.

making and automated therapeutic intervention according to the novel "Sense-and-Act" concept (Fig. 1). This approach was applied recently to the analysis of biomarkers characteristic of various injuries using model buffer solutions [27–30]. Among pervasive battlefield injuries, acute liver injury (LI), soft tissue injury (STI) and abdominal trauma (ABT) are the most common sustained by soldiers in combat [31–34]. The present paper reports on the first application of digital biosensors with the built-in logic for the analysis of the LI, STI and ABT biomarkers in serum solutions mimicking the operation of future *in vitro* and *in vivo* multi-parameter biosensors.

2. Materials and methods

2.1. Chemicals and reagents

Alanine transaminase from porcine heart (ALT, E.C. 2.6.1.2), pyruvate kinase from rabbit muscle (PK, E.C. 2.7.1.40), creatine kinase from rabbit muscle (CK, E.C. 2.7.3.2), lactate dehydrogenase from porcine heart (LDH, E.C. 1.1.1.27), serum from human male AB plasma, β-nicotinamide adenine dinucleotide dipotassium salt (NAD^+), β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), L-alanine (Ala), α-ketoglutaric acid (α-KTG), L(+)-lactic acid (Lac), creatine anhydrous (Crt), phospho(enol)pyruvate monopotassium salt (PEP), adenosine 5′-triphosphate disodium (ATP, from bacterial source), glycyl-glycine (Gly–Gly), magnesium acetate ($MgAc_2$), potassium hydroxide (KOH) were purchased from Sigma–Aldrich and were used as supplied without any pretreatment or purification. Ultrapure water (18.2 MΩ cm) from NANOpure Diamond (Barnstead) source was used in all of the experiments.

2.2. Instrumentation and measurements

A Shimadzu UV-2450 UV–vis spectrophotometer (with a TCC-240A temperature-controlled holder and 1 mL PMMA cuvette) was used for all optical measurements. A Mettler Toledo SevenEasy s20 pH-meter was employed for the pH measurements. All optical measurements were performed in temperature-controlled cuvettes at $37 \pm 0.2\,^{\circ}C$ mimicking physiological conditions and all reagents were incubated at this temperature prior measurement.

2.3. Composition and operation of channels for the analysis of injuries

2.3.1. Liver injury (LI)

Pure human serum was used as a background solution. Ala (200 mM), α-KTG, (10 mM) and NADH (150 μM) were dissolved in this solution to perform the **NAND** logic operation upon activation with the biomarker inputs. Logic '**0**' and '**1**' levels of ALT (0.02 and 2 U mL^{-1}) and LDH (0.15 and 1 U mL^{-1}) input signals were applied to the system in order to realize circulating levels of these biomarkers at a normal physiological level and an elevated pathological concentration corresponding to the severe liver injury, respectively [35]. In an additional set of experiments (see Supplementary data) another level of the input signal '**1**' for ALT was applied, 0.2 U mL^{-1}, corresponding to a mild liver injury condition. The output signal corresponding to the decreasing concentration of NADH was measured optically at λ = 340 nm [36].

2.3.2. Soft tissue injury (STI)

Gly-Gly buffer, 50 mM, containing 6.7 mM $MgAc_2$ was titrated with KOH to the pH value of 8.5 (note that Mg^{2+} and K^+ cations are essential for activation of CK and PK, respectively). The following components of the biosensing system were dissolved in the Gly-Gly buffer: NADH (0.2 mM), ATP (1 mM), PEP (0.5 mM), PK (2 U mL^{-1}), Crt (15 mM) to perform the **NAND** logic operation upon activation with the biomarker inputs. Then the biosensing system prepared in Gly-Gly buffer was diluted (1:1, v/v) with human serum to mimic biological samples. Logic '**0**' and '**1**' levels of CK (0.05 and 0.355 U mL^{-1}) and LDH (0.075 and 0.5 U mL^{-1}) input signals were applied to the system in order to realize meaningful circulating levels of these biomarkers under normal physiological and pathological injury conditions, respectively [37]. Immediately following the mixing, optical absorbance measurements were recorded continuously at λ = 340 nm, monitoring the decreasing concentration of NADH.

2.3.3. Abdominal trauma (ABT)

Gly-Gly buffer, 50 mM, containing 6.7 mM $MgAc_2$ was titrated with KOH to the pH value of 8.5. Then NAD^+ (10 mM) was added to the solution to perform the **AND** logic operation upon activation with the biomarker inputs. Then the biosensing system prepared in Gly-Gly buffer was diluted (1:1, v/v) with human serum to mimic biological samples. Logic '**0**' and '**1**' levels of Lac (0.8 and 3.0 mM) and LDH (0.075 and 0.5 U mL^{-1}) input signals were applied to the system in order to realize circulating levels of these biomarkers characteristic of normal physiological and pathological injury conditions, respectively [37,38]. The output signal corresponding to the NADH formation was measured optically at λ = 340 nm.

3. Results and discussion

3.1. Liver injury (LI)

Two enzymes, ALT and LDH, were applied as biomarkers characteristic of liver injury [35]. Their simultaneous increase in concentration, from normal to pathological levels (Table 1), provides an evidence of LI conditions. The biochemical cascade catalyzed in the presence of the both enzyme-biomarkers results in the oxidation of NADH to NAD^+ (Fig. 2A), thus yielding the corresponding absorbance decrease (Fig. 2B). The logic value of the output signal changes from the high '**1**' value to the low '**0**' value only upon the cooperative work of the both enzymes (logic inputs combination '**1**,**1**'), thus mimicking **NAND** (**N**ot-**AND**) logic operation. It should be noted that the physical value of the output signal (decrease of the NADH absorbance) is also affected by other combinations of the input signals ('**0**,**0**'; '**0**,**1**'; '**1**,**0**') reflecting their non-zero physical values corresponding to the logic level '**0**'. Still a threshold at the absorbance of 0.65 measured at the sampling time of 20 s allows clear separation of the logic '**0**' and '**1**' output values measured below and above the threshold, respectively (Fig. 2C). Thus, the optical output signal measured below an absorbance of 0.65 allows the diagnosis of the liver injury (note that the ALT '**1**' input in the present experiment corresponds to 2 U mL^{-1} charac-

Table 1
Physiological (logic input **0**) and pathological (logic input **1**) levels of clinically relevant biomarkers for each logic gate with the output compound indicated.

	Injury	Biomarkers	Physiological (**0**)	Pathological (**1**)	Output	Ref.
1	Liver injury (LI)	Alanine transaminase (ALT) Lactate dehydrogenase (LDH)	0.02 U/mL 0.15 U/mL	0.2[b] (2)[c] U/mL 1 U/mL	NADH decrease	[35]
2	Soft tissue injury (STI)	Creatine kinase (CK) Lactate dehydrogenase (LDH)	0.05 U/mL[a] 0.075 U/L[a]	0.355 U/mL[a] 0.5 U/L[a]	NADH decrease	[37,38]
3	Abdominal trauma (ABT)	Lactate (Lac) Lactate dehydrogenase (LDH)	0.8 mM[a] 0.075 U/mL[a]	3 mM[a] 0.5 U/mL[a]	NADH increase	[37–39]

[a] Note that the concentrations of the biomarkers were used at their half-values to reflect the dilution of serum with Gly-Gly buffer in the 1:1 (v/v) proportion.
[b] Pathological concentrations corresponding to a mild LI.
[c] Pathological concentrations corresponding to a severe LI.

teristic of severe LI). A similar experiment performed with the ALT '**1**' input of 0.2 U mL^{-1} typical of mild LI allows the correct diagnosis as well due to the well-separated '**0**' and '**1**' output signals (see Figure S1, Supplementary data). Note that all experiments were performed in human serum solutions mimicking real biomedical samples. A sampling time of 20 s was selected to yield the best separation between the logic '**0**' and '**1**' output signals.

3.2. Soft tissue injury (STI)

Two enzymes, CK and LDH, were applied as biomarkers characteristic of soft tissue injury [37,38]. Their simultaneous increase from normal to pathological concentrations (Table 1) provides an evidence of STI conditions. The biochemical cascade catalyzed in the presence of the both enzyme-biomarkers (note the biocatalytic operation of PK being a part of the logic gate "machinery") results in the oxidation of NADH to NAD$^+$ (Fig. 3A), thus yielding the corresponding absorbance decrease (Fig. 3B). The logic value of the output signal changes from the high '**1**' value to the low '**0**' value only upon the concerted work of the both enzyme-inputs (logic inputs combination '**1**,**1**'), thus mimicking **NAND** logic operation. Since the logic '**0**' values of the input signals are not physical zero concentrations (they rather correspond to the normal physiological concentrations of the enzymes), the NADH absorbance is also changing upon other combinations of the inputs ('**0**,**0**'; '**0**,**1**'; '**1**,**0**'). However, a threshold at the absorbance of 2.1 measured at the sampling time of 350 sec allows good differentiation of the logic '**0**' and

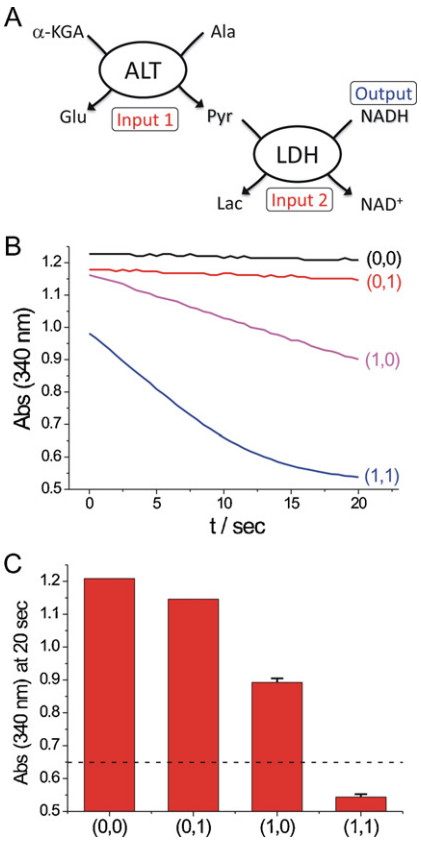

Fig. 2. Logic analysis of the liver injury (LI) conditions. (A) The biocatalytic cascade applied for the realization of the **NAND** logic gate activated by ALT (Input 1 – corresponding to severe LI) and LDH (Input 2). (B) Absorbance changes corresponding to the consumption of NADH upon operation of the logic system in the presence of different combinations of the input signals. (C) The bar chart showing the NADH absorbance at 340 nm after 20 s of the biocatalytic reaction activated with different combinations of the biomarker-input signals. The dashed line is the threshold separating the logic '**0**' and '**1**' output values. The normal '**0**' and pathological '**1**' concentrations of the input signals are summarized in Table 1.

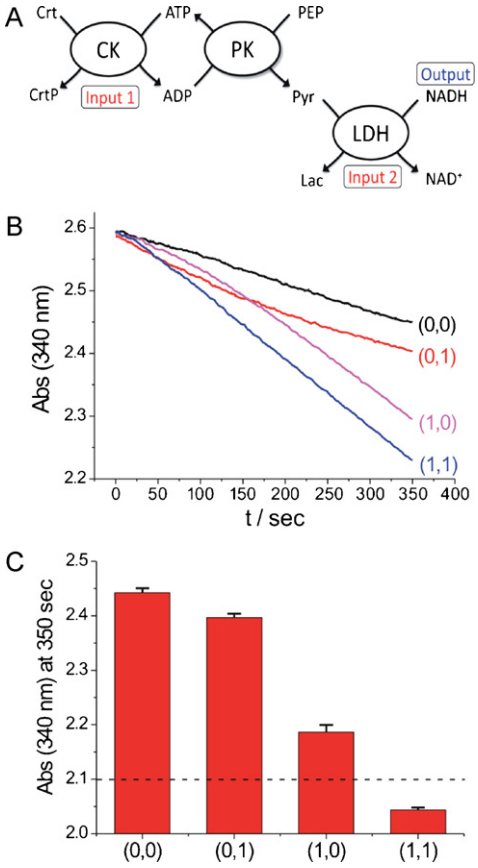

Fig. 3. Logic analysis of the soft tissue injury (STI) conditions. (A) The biocatalytic cascade applied for the realization of the **NAND** logic gate activated by CK (Input 1) and LDH (Input 2). (B) Absorbance changes corresponding to the consumption of NADH upon operation of the logic system in the presence of different combinations of the input signals. (C) The bar chart showing the NADH absorbance after 350 sec of the biocatalytic reaction activated with different combinations of the biomarker-input signals. The dashed line is the threshold separating the logic '**0**' and '**1**' output values. The normal '**0**' and pathological '**1**' concentrations of the input signals are summarized in Table 1.

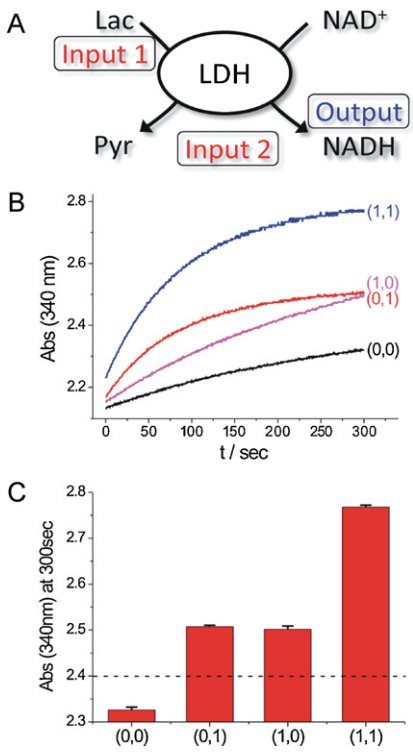

Fig. 4. Logic analysis of the abdominal trauma (ABT) conditions. (A) The biocatalytic reaction applied for the realization of the **AND** logic gate activated by Lac (Input 1) and LDH (Input 2). (B) Absorbance changes corresponding to the formation of NADH upon operation of the logic system in the presence of different combinations of the input signals. (C) The bar chart showing the NADH absorbance after 300 s of the biocatalytic reaction activated with different combinations of the biomarker-input signals. The dashed line is the threshold separating the logic '**0**' and '**1**' output values. The normal '**0**' and pathological '**1**' concentrations of the input signals are summarized in Table 1.

'**1**' output values measured below and above the threshold, respectively (Fig. 3C). Thus, the optical output signal measured below an absorbance of 2.1 allows the diagnosis of the soft tissue injury. The sampling time of 350 s was optimized to yield the best separation between the logic '**0**' and '**1**' output signals. It should be noted that we were not able to realize the logic system operation in a pure serum solution; a mixture (1:1, v/v) of human serum with a Gly-Gly buffer was thus employed with the optimized pH and added Mg^{2+} and K^{+}. In future sensing devices, this could be accomplished by mixing the real serum samples with the optimized buffer solution in a microfluidic system.

3.3. Abdominal trauma (ABT)

The enzyme LDH and its substrate Lac appearing together at elevated concentrations (Table 1) can be used as biomarkers of ABT [37–39]. The biocatalytic reaction activated by the enzyme and the corresponding substrate, results in the concomitant reduction of NAD^{+} cofactor (Fig. 4A), thus leading to increased absorbance at $\lambda = 340$ nm corresponding to the formation of NADH (Fig. 4B). The logic value of the output signal changes from the low '**0**' value to the high '**1**' value only upon the concerted work of the both inputs (logic inputs combination '**1**,**1**'), thus mimicking **AND** logic operation (Fig. 4C). Since the logic '**0**' values of the input signals are not physical zero concentrations (they correspond to the normal physiological concentrations of the enzyme and its substrate), the NADH absorbance is also changing upon other combinations of the inputs ('**0**,**0**'; '**0**,**1**'; '**1**,**0**'). However, a threshold at the absorbance of 2.4 measured at the sampling time of 300 s allows good differentiation of the logic '**0**' and '**1**' output values measured below and above the threshold, respectively (Fig. 4C). Thus, the optical output signal measured above absorbance of 2.4 allows the diagnosis of the abdominal trauma. Despite the fact that the system is rather simple its low-noise operation is challenging and requires optimization [40]. The sampling time of 300 s was optimized to yield the best separation between the logic '**0**' and '**1**' output signals. Similarly to the STI system, we were not able to realize the logic gate operation in a pure serum solution and a mixture (1:1, v/v) of human serum with a Gly-Gly buffer with the optimized pH has been applied.

3.4. Interferants in serum

Numerous compounds present in the serum samples can potentially interfere with the enzymatic "machinery" of the analytical systems described above. Pyruvate is an intermediate product in the LI and STI detection systems. In the LI system pyruvate is generated as an intermediate product by the first system input, ALT enzyme. In the STI system it is also produced by CK/PK biocatalytic cascade. In both systems the pyruvate production corresponds to the presence of one of the biomarker inputs, ALT or CK, respectively. However, pyruvate is present in blood serum (approx. 40 μM) [41] and it could provide a false positive signal even in the absence of the first inputs. Similar complication may occur for ADP in the STI system. Since lactate is a common blood constituent, with its concentration significantly elevated by majority of traumatic injuries [42,43], it can prevent conversion of pyruvate to lactate and readout of the output signal corresponding to the decrease of the NADH concentration. In order to suppress the LDH-induced biocatalytic conversion of lactate to pyruvate for the proper performance of the STI system, the solution pH value should be optimized. Another potential complication can originate from the presence of various ions in human serum samples. CK enzyme, which is a key component for the STI system, can be inhibited by many bivalent cations (Ca^{2+}, Zn^{2+} and Cu^{2+}) and anions (Cl^{-} and PO_4^{3-}) [44]. In order to achieve an adequate performance of the enzymes for the STI gate, Mg^{2+} ions were added to the solution for preventing the CK inhibition. Additionally, K^{+} ions were added to the reaction mixture to enhance the PK and CK activities. This was the major reason for applying the 1:1 buffer–serum mixture for the analysis of STI and ABT.

The operation of all three logic systems presented in this study was examined using different samples of serum. Some minor sample-to-sample absorbance variations were observed, mostly due to the difference in the transparency of the serum samples. However, the robust operation of the bioanalytical systems always allowed convenient discrimination of '**0**' and '**1**' output signals, thus providing reliable diagnostics of the injury conditions.

4. Conclusions

The obtained results demonstrated for the first time the reliable operation of multi-analyte biosensor systems with the digital output in human serum solutions mimicking real biomedical samples. These results provide necessary background for transferring the enzyme-logic experiments to the *in vivo* analysis of the injury biomarkers. By offering a unique "decision" making (YES/NO) feature, the present systems can potentially compete with established immunoassays, without any need of labeled reactants. Additional work is still required to allow the next step in the development of the implantable bioanalytical system, which will transfer the analytical methods from optical to electrochemical techniques in connection with minimally invasive amperometric sensing. The coupling of such on-body digital biosensor systems with on-demand drug-delivering chemical actuators could also be realized using real biological fluids. Though there are still many

technological challenges to be overcome before this becomes a reality, such autonomous loop-based individualized integrated (sensing/release) medical systems would eventually have an enormous impact upon the treatment and survival of injured soldiers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.10.057.

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