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Short communication

Alert-type biological dosimeter based on enzyme logic system

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ABSTRACT

A cooperative effect of two biomarkers, α-amylase and lactate dehydrogenase, was used to analyze radiation-caused tissue damage *in vitro* in model solutions of human serum. The analytical system was based on the recently emerged biocomputing concept applying biocatalytic cascades for logic processing of biochemical input signals. The studied system resembled a Boolean NAND logic gate in which the change of the optical output signal from a high level (logic value 1) to a low level (logic value 0) confirmed the presence of both biomarkers at pathological concentrations (1,1 input signals), thus yielding the conclusion about radiation tissue damage. The system operates in a digital YES/NO format as an alert-type biosensor with a built-in Boolean logic.

1. Introduction

Radiation injury, a life threatening form of organ tissue damage, is caused by exposure to ionizing radiation. A short intensive exposure results in acute radiation syndrome [1], while chronic radiation syndrome [2] is caused by a prolonged exposure to lower intensity radiation. Immediate and effective medical treatment of persons suspected of radiation exposure requires a reliable detection approach to provide adequate diagnostic information. Traditional instrumentation – physical dosimeters – does not provide sufficient information about the physiological condition in a human organism following radiation exposure. Variations in age, gender, health, and genetic background can also affect biological responses which cannot be quantified by a standalone physical dosimeter. Biological dosimetry [3,4] based on the analysis of biomarkers in a body exposed to radiation, rather than physical measure of a radiation dose, is considered as an important tool for the effective medical management of radiation therapy for cancer [5–9], occupational radiation exposure [10] (including astronauts in manned space missions) [11–13], accidents similar to Chernobyl disaster [14], and possible radiological terror attacks [15]. Identification of biomarkers characteristic of a radiation injury is a challenging goal since radiation can result in different physiological processes, many of which are not specifically related to the direct instantaneous damage caused by radiation [16]. Potentially important radiation injury biomarkers include changes to various metabolites, proteins, RNA and DNA [16–19]. Thus, different biomedical analytical approaches have been developed to analyze their variations; among them, the most important methods are chromosome [17,18] and proteomic analysis [19]. Unfortunately, none of the presently available methods is based on a single specific biomarker, thus requiring massive analysis of multiple biomarkers with low specificity. Technically, such methods are based on sophisticated and expensive equipment, e.g., mass spectrometry for the proteomic analysis [19], which cannot be used for rapid on-site field detection of pathophysiological changes caused by radiation.

Recently emerged biomolecular information processing (biocomputing) systems [20] allow logic analysis of complex patterns of various biomarkers in novel digital biosensors [21]. Their biomedical applications [22,23] have already received high attention, particularly for the analysis of various injury conditions based on digital processing of multiple biomarkers [24–27]. It should be noted that each single biomarker used in this type of biosensor is not specific enough to derive any conclusion from the measurements, however, when measured in combination with several other biomarkers of partial specificity, an unambiguous diagnosis can be tendered. Biocomputing systems based on enzyme-catalyzed cascades mimic networked Boolean logic gates processing chemical input signals in a digital format (**0**/**1**), thus producing a **YES**/**NO** output signal operating as alert-type biosensors [21–27]. Logic processing of the biomarker inputs is performed in the biochemical domain of the systems, thus reducing the need of electronic computing systems analyzing biosensor responses. High-fidelity and easy read-out responses allow application of these novel digital

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biosensors in field conditions for various point-of-care applications. Robustness of the biomolecular information processing systems permits operation in biological fluids in the presence of numerous interferants. For example, the digital analysis of biomarkers characteristic of soft tissue injury, abdominal trauma and liver injury was successfully performed in human serum solutions [27].

The present Letter reports on the first application of an enzyme-logic system for the analysis of biomarkers corresponding to the pathophysiological changes caused by radiation. Increased levels of α-amylase and lactate dehydrogenase activate a biocatalytic cascade, resulting in the unambiguous conclusion on radiation-induced physiological processes. While the biochemical process utilized in the biosensing system is based on known biocatalytic reactions, their application for the digitized logic conclusion on the pathophysiological changes is novel. The present work extends our broad studies on the application of biomolecular logic systems for solving biomedical diagnostic problems [24–27].

2. Experimental

2.1. Chemicals and reagents

α-Amylase from porcine pancreas (Amy, E.C. 3.2.1.1), hexokinase from *Saccharomyces cerevisiae* (HK, E.C. 2.7.1.1), α-glucosidase from baker's yeast (α-Glu, E.C. 3.2.1.20), pyruvate kinase from rabbit muscle (PK, E.C. 2.7.1.40), glucose oxidase from *Aspergillus niger* (GOx, E.C. 1.1.3.4), lactate dehydrogenase from porcine heart (LDH, E.C. 1.1.1.27), serum from human male AB plasma, β-nicotinamide adenine dinucleotide reduced (NADH), phospho(enol)pyruvate (PEP), adenosine 5′-triphosphate (ATP, from bacterial source), glycyl-glycine (Gly-Gly), starch (soluble) and other standard organic/inorganic chemicals were purchased from Sigma–Aldrich and 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. Composition and operation of the irradiation injury detection system

Gly-Gly buffer, 50 mM, containing 6.7 mM $Mg(CH_3CO_2)_2$ was titrated with KOH to the pH value of 7.4 (note that Mg^{2+} and K^+ cations are essential for activation of PK). The following components of the biocomputing "machinery" system were dissolved in the Gly-Gly buffer to perform **NAND** (**N**ot-**AND**) logic operation upon activation with biomarker-input signals: NADH 0.3 mM, HK 10 U mL^{-1}, ATP 5 mM, PK 2 U mL^{-1}, PEP 0.5 mM. The "machinery" solution was deaerated by bubbling with Ar for 15 min prior to its use. Amy and LDH were applied as biomarker-input signals activating the biocomputing "machinery" system. Logic '**0**' and '**1**' levels of Amy (0.25 and 2.5 U mL^{-1}) [28] and LDH (0.15 and 1.0 U mL^{-1}) [29] input signals were applied in serum to realize meaningful circulating levels of these biomarkers under normal physiological and pathological conditions caused by radiation, respectively. In order to eliminate interference of glucose present in serum the following procedure was applied. Glucose was depleted from serum by pre-treatment with GOx (10 U mL^{-1}) for 60 min. Then GOx dissolved in serum was deactivated by removing oxygen with flow of Ar for 15 min. After this pretreatment, starch (0.2 mg mL^{-1}), α-Glu (0.5 U mL^{-1}) (both are the parts of the system "machinery") and the first biomarker-input – Amy (**0** or **1** logic levels) were dissolved in glucose-free serum and incubated for 10 min. Then the second biomarker-input – LDH (**0** or **1** logic levels) was dissolved in glucose-free serum. The serum solutions containing the biomarker-inputs in four different logic combinations (**0**,**0**; **0**,**1**; **1**,**0** and **1**,**1**) were mixed with the biocomputing "machinery" system dissolved in the oxygen-free Gly-Gly buffer (1:1 v/v ratio of serum and buffer). Optical absorbance measurements (Shimadzu UV-2450 UV-Vis spectrophotometer with a TCC-240A temperature-controlled holder) were performed continuously at λ = 340 nm following the decreasing concentration of NADH upon the biocatalytic reaction activated by the biomarker-input signals. The conclusion on the pathophysiological changes mimicked in the model system was derived upon comparison of the optical output signal with the experimentally determined threshold value, Abs_{340} = 1.9, separating digitized output values **0** and **1**. When the absorbance was measured below 1.9 (at the reaction time of 350 s), the output signal was considered as logic **0**, thus signaling on the radiation-caused tissue damage. All reactions and optical measurements were performed at 37 ± 0.2 °C mimicking physiological conditions.

3. Results and discussion

Among many known biomarkers [16] characteristic of radiation-caused damage, we selected enzyme inputs able to operate in a biocatalytic cascade performing a logic operation based on the recently developed enzyme logic concept [20,21]. It should be noted that the biomarker selection is a challenging problem – many important biomarkers cannot be integrated in a single biocatalytic cascade to yield an integrated logic circuitry. The presently selected biomarkers, α-amylase (Amy) and lactate dehydrogenase (LDH) are convenient because they can operate in a single biocatalytic cascade producing an output signal dependent on the presence of both of them. An increased concentration of Amy in blood, urine and saliva was reported as a result of irradiation exposure [16,28,30–33]. Amy release originates mainly from gland tissue damaged by radiation. Another imminent effect of irradiation is tissue breakdown and hemolysis causing massive release of LDH into blood stream [34–36]. Since Amy and LDH can be combined in a biocatalytic cascade resulting in consumption of NADH, we selected these two enzymes as biomarkers of the radiation damage. The biocatalytic cascade, Fig. 1, includes a multi-step biocatalytic reaction which can be completed only in the presence of all biocatalytic species, including both the biomarkers – Amy and LDH. The reaction cascade starts with the decomposition of starch upon a cooperative biocatalytic effect of Amy and α-Glu yielding glucose (Glc) as a product. Then, Glc is converted to glucose-6-phosphate (G6P) in the presence of HK and ATP, resulting in the formation of adenosine 5′-diphosphate (ADP), which allows the next reaction step producing pyruvate (Pyr) from PEP in the presence of PK. Finally, the *in situ* produced Pyr allows oxidation of NADH to NAD^+ biocatalyzed by LDH to yield decrease of the optical absorbance at λ = 340 nm as the final output signal. The reactions biocatalyzed by HK and PK are needed to couple the Amy and LDH biomarker-input signals in a single biocatalytic cascade. Therefore, the NADH concentration decreases as a result of the completed reaction and should be observed only in the presence of both biomarkers, Amy and LDH. The absence of both or either input signals should result in the incomplete biocatalytic chain, thus inhibiting the NADH consumption. For the digital operation of the enzyme logic system, the presence of Amy and LDH at normal physiological concentrations in blood was defined as logic input **0**, while their elevated pathological concentrations were considered as logic input **1** (see specific concentrations in Section 2). Therefore, the **1**,**1** (Amy, LDH) input signal combination resulted in the highest activity of the biocatalytic cascade with the rapid decrease of NADH concentration. It should be noted however, that **0** logic concentrations of the input signals correspond to the normal physiological concentrations of Amy and LDH rather than their complete absence. Thus, **0**,**0**; **0**,**1** and **1**,**0** combinations of the input signals still allow the biocatalytic reaction to proceed,

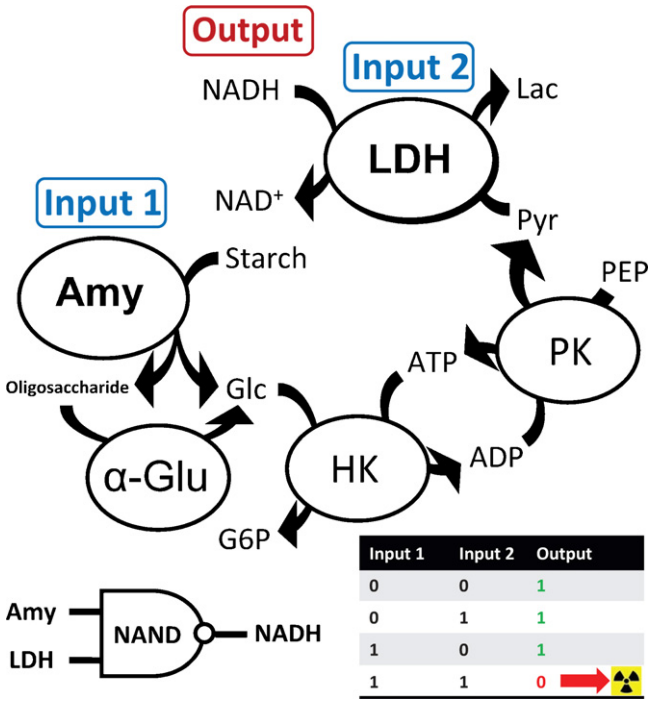

Fig. 1. Multi-enzyme biocatalytic cascade for the analysis of irradiation damage. Biomarkers Amy and LDH are applied as the input signals; NADH is the output signal. Other products of the biocatalytic cascade are the following: glucose (Glc), glucose-6-phosphate (G6P), pyruvate (Pyr) and lactate (Lac). Note that for simplicity the scheme does not include some reacting cofactors, promoters and byproducts – for the full composition of the system refer to Section 2. Schematic representation of the **NAND** logic gate and the corresponding truth table are shown at the bottom.

however, with a lower rate compared to the **1**,**1** combination of the biomarker-inputs. Fig. 2 shows the NADH absorbance decrease upon application of four different combinations of the input signals: **0**,**0**; **0**,**1**; **1**,**0** and **1**,**1**. Fig. 2, inset, shows a bar chart for the optical output signals generated by the enzyme logic system obtained after 350 s of the biocatalytic reaction in the presence of various combinations of the biomarker-input signals. A threshold absorbance value of 1.9 allows clear separation of a low output signal generated upon the cooperative effect of elevated concentrations of Amy and LDH (**1**,**1** inputs) from a high signal for the inhibited reaction when either or both input signals were applied at the logic **0** level (**0**,**0**; **0**,**1**; **1**,**0** inputs). The output signal pattern

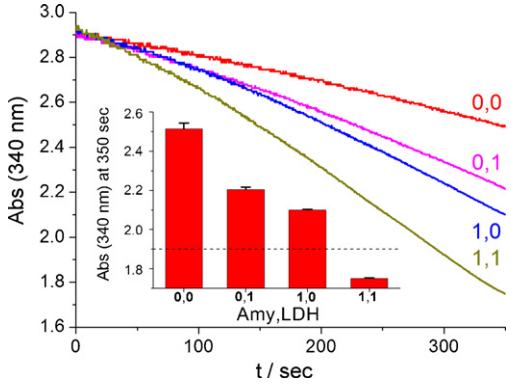

Fig. 2. Optical detection of the output signal generated by the logic system obtained upon different combinations of the biomarker input signals (Amy, LDH). Inset: bar chart for the optical output signals generated by the enzyme logic system obtained after 350 s of the biocatalytic reaction. The radiation damage diagnosis corresponds to the output signal below the decision threshold (dashed line). The logic system composition and the input signal concentrations are specified in Section 2.

corresponds to the Boolean logic operation **NAND** which results in the output signal **0** (low signal) only for the **1**,**1** combination of the inputs [37], Fig. 1, bottom. It should be noted that the time point cutoff to determine **0**/**1** output signals was optimized experimentally to allow the best separation between logic **0** and **1** values. Increasing the gap separating **0** and **1** output signals is a very interesting and challenging goal. This could be achieved by introducing an additional filter system into the logic circuitry for converting the biochemical response to the input signals from convex, as typical for most biochemical processes, to sigmoidal, as desired for digital systems [38,39]. This work is in the progress and will be reported elsewhere soon.

Numerous compounds present in the serum samples can potentially interfere with the enzymatic "machinery" of the developed analytical system. Particularly, glucose, pyruvate and ADP are intermediate products in the biocatalytic cascade and their natural presence in serum could result in a false positive signal even in the absence of the Amy input signal. It should be noted that the depletion of naturally existing serum glucose prior to the measurements was essential for the discrimination between the outputs generated by **0**,**1** and **1**,**1** (Amy, LDH) signals. On the other hand, the system operation was possible in the presence of pyruvate and ADP naturally existing in serum. The system operation was examined using different samples of human 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 system always allowed convenient discrimination of **0** and **1** output signals, thus providing reliable diagnostics of the pathological conditions. It should be noted that the advantage of the present approach is in the use of simultaneous concentration changes of two biomarkers, while traditional biosensor approach is based on a single biomarker analysis. The probability of a false-positive signal is significantly reduced by coupling of two biomarkers in the integrated logic circuitry.

4. Conclusion

Since the biomarkers, Amy and LDH, reveal relatively low specificity for the physiological changes caused by radiation only the presence of both of them at elevated concentrations can provide evidence of radiation damage. Our proof-of-concept study clearly demonstrates that the optical changes observed in the presence of both the biomarkers appearing at pathological concentrations can be easily discriminated from the output when at least one of the selected biomarkers exists at its normal physiological concentration. The digitally operating enzyme logic system demonstrated robust performance in serum solutions in the presence of various naturally existing interferants, thus allowing the unambiguous alert-type conclusion in the YES/NO form about the presence of the radiation-caused tissue damage. The developed digitized logic system for the analysis of the radiation-caused tissue damage could be integrated in a modular design composed of logic units analyzing other injuries [25], such as soft-tissue injury, traumatic brain injury, liver injury, abdominal trauma, hemorrhagic shock, and oxidative stress. The complex multifunctional system will be generating a digital "injury code" for the rapid and reliable assessment of varied and complex forms of injury in circumstances where access to a clinical laboratory is not viable. Practical future applications would benefit from further miniaturization and/or integration into a 'reagentless' solid-state optical or electrochemical device.

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