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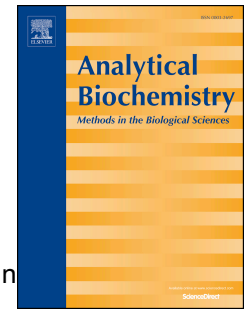

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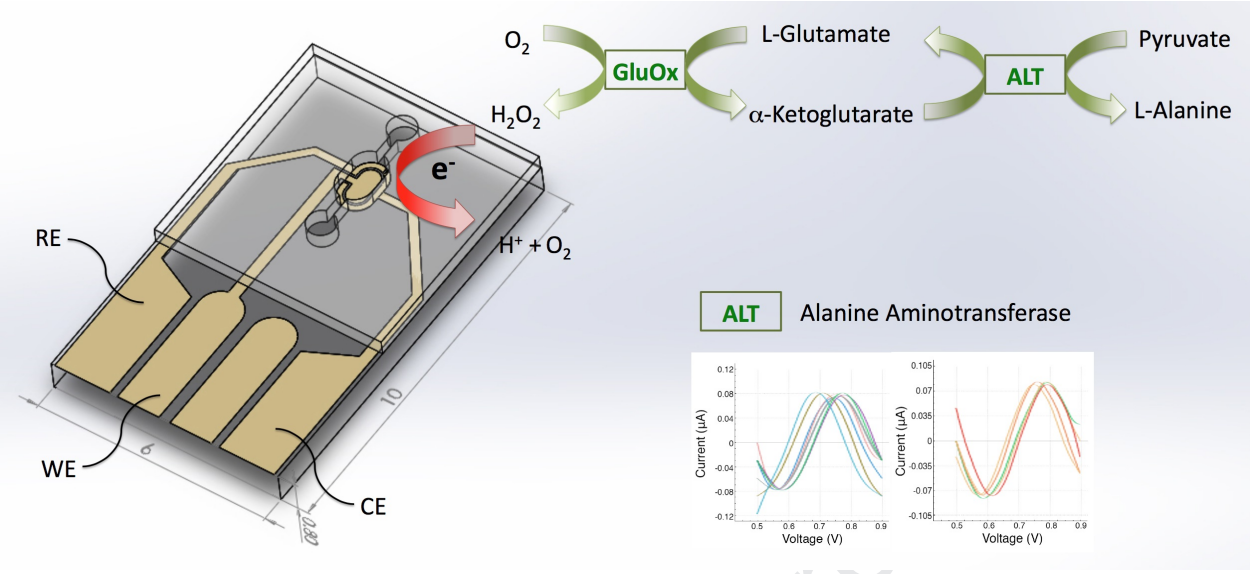

Quantitative determination of H_2O_2 for detection of alanine aminotransferase using thin film electrodes

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Abbreviations: ALT, alanine aminotransferase; **H_2O_2**, hydrogen peroxide; **DPV**, differential pulse voltammetry; **GluOx**, glutamate oxidase; **Pt**, platinum; **POC**, point-of-care; **MEMS**, microelectromechanical systems; **PBS**, phosphate buffer saline; **WE**, working electrode; **RE**, reference electrode; **CE**: counter electrode; **AFE**, analog-front-end; **DAC**, digital to analog converter; **LSB**, least significant bit

Abstract

The abnormal concentrations or absence of biomolecules (e.g., proteins) in blood can further be used in diagnosis of a particular pathology at an early stage. Current studies are intensely focusing on the analysis of interaction and detection of biomolecules via point-of-care systems (POCs), allowing miniaturized and parallelized reactions, simultaneously. Recent developments have shown that the collaboration of electrochemical sensing techniques and POCs to overcome challenging problems in health-care settings provides new approaches in diagnosis and treatment of diseases. The aim of this study was to adapt the alanine aminotransferase (ALT) enzyme to the platinum (Pt) thin film electrode system and quantitatively determine the enzyme levels via enzymatically generated H_2O_2 with differential pulse voltammetry (DPV). A simple potentiostat architecture with expanded sweep range utilizing dual LMP91000 devices was developed and adapted to the needs of the biosensor. In order to calibrate the system, known concentrations of H_2O_2 were also tested. Moreover, signals associated with the other electroactive species coming from the ALT reaction were eliminated. Resulted potential range has been achieved between +500 mV and +900 mV and the linear range was found to be 0.05 M-0.5 M for H_2O_2, whereas 5 UL^{-1} to 120 UL^{-1} for ALT enzyme.

Keywords: alanine aminotransferase; differential pulse voltammetry; electrochemical sensing; hydrogen peroxide; potentiostat; thin-film electrodes

1. Introduction

Real-time monitoring of the specific target analytes in the health and environmental sectors as well as food industry has a widespread interest [1–3]. Near-patient monitoring systems also called point-of-care systems (POCs), are of paramount importance in timely management of modern health-care settings as they are rapid, low-cost and highly sensitive for detection of biomolecules [4–6]. Recently, advances in electrochemical sensing play a key role in POCs and gaining a reputation for monitoring and diagnosis of different diseases and infections [7–11]. Having simple and easy-to-manage detection modality, such sensing models do not require bulky components which are mainly used in colorimetric-based detection strategies [7]. Currently, thick and thin-film electrode microelectromechanical systems (MEMS) have taken their places among POCs by offering many advantages such as high-sensitivity, performance, portability and minimal consumption of sample and reagents as well as high-throughput for biosensing [12–16]. Moreover, a variety of simple and miniaturized potentiostats have been developed and demonstrated for their performances on electrochemical sensors and biosensors with emphasis on their low cost and small size [17–19].

The fact that it is possible the rapid and accurate detection of many biomolecules causing serious diseases has been proved with recent outlines of the studies in which electrochemical sensing techniques have been employed for monitoring of many vital biological indicators, heavy metals or enzyme levels (e.g., liver enzymes) in blood [15,20–22]. For instance, hydrogen peroxide (H_2O_2), a member of reactive oxygen species family, has a significant role as signal transduction molecules [23]. Acting as a critical messenger in fundamental biological systems, the abnormal generation of H_2O_2 may be associated with oxidative stress, chronic inflammation, diabetes, learning disorders and eventually Alzheimer's disease [24–

26]. To this respect, real-time determination of H_2O_2 has a great importance in clinical, environmental and food analyses. Thus, several techniques have been used for H_2O_2 detection including spectrophotometry, fluorescence, chemiluminescence and finally electrochemical sensors. For example, researchers have demonstrated a multiple biosensor for the electro-chemiluminescent detection of enzymatically produced H_2O_2 [27]. As the H_2O_2 is the by-product of many enzymatic reaction such as glutamate oxidase, enzymatically produced H_2O_2 was analyzed to determine L-glutamate [28,29]. Moreover, nanoparticles and nanowires was used in many studies to modify electrode surfaces to increase conductivity and sensitivity of the electrochemical H_2O_2 sensors [30–32]. For instance, Fan *et al.* [33] demonstrated the peroxidase activities of platinum-ferritin nanoparticles via H_2O_2 substrates under different physical conditions.

In the present study, we report electrochemical sensing approach that integrates an enzymatic reaction and platinum (Pt) thin-film electrode systems. Alanine aminotransferase (ALT) enzyme was selected as the model enzyme as the H_2O_2 is a by-product of its enzymatic reaction [15,34]. Electrochemical measurements using differential pulse voltammetry (DPV) method have been studied and reaction optimization of ALT enzyme has been carried out via enzymatically-produced H_2O_2.

2. Materials and Methods

2.1. Materials and Reagents

ALT (E.C.2.6.1.2, 90 U/mg, 1 kU from porcine hearth) was purchased from Lee Biosolutions, L-glutamate oxidase (EC 1.4.3.11 from Streptomyces sp., 1.75 U/ 0.05 mg), α-ketoglutarate, L-alanine, potassium ferrocyanide and potassium ferricyanide were purchased from Sigma Aldrich. The enzymes were dissolved and diluted with 0.01 M phosphate buffer saline (PBS)

(pH 7.4) and the substrate solution for ALT contains 500 mM L-alanine, 75 mM α-ketoglutarate, 5 mM potassium ferrocyanide: potassium ferricyanide (1:1) mixture.

2.2. Equipment

Electrochemical measurements were performed with GalvanoPlot GX201 device, a compact electrochemical interface developed by Solar Biotec, Turkey for amperometric and voltammetric techniques. MEMS electrode chips of 10 x 6 x 0.75 mm sized Pt thin-film electrodes on the glass substrate (ED-SE1-Pt from Micrux Technologies, Asturias, Spain) were used together with a drop-cell platform (ED-Drop-Cell from Micrux Technologies, Asturias, Spain) which can be integrated to the GalvanoPlot device. Experiments were performed through GalvanoPlot Controller software, a dedicated PC interface for GalvanoPlot device.

2.3. Voltammetry Protocol and Signal Processing

DPV was the technique of choice to measure H_2O_2 concentrations as well as ALT enzyme activity. Voltage bias was swept in the range of 200 to 900 mV with rectangular wave pulses of 2mV amplitude, 10msec width and 20msec period. Current flows through the working electrode was amplified through the built in transimpedance amplifier (TIA) of LMP91000 analog front-end (AFE) integrated circuit with gain settings of 3.5 kΩ and 350 kΩ (with ±5% tolerance) for H_2O_2 and ALT experiments, respectively. Also, a digital low pass filter of 3dB cutoff at 2 Hz was applied to current signals in both experiments.

2.4. Hydrogen Peroxide Calibration

It was aimed to calculate ALT concentration via enzymatically-produced H_2O_2. Thus, known concentrations of H_2O_2 (0.05M, 0.1M, 0.15M, 0.2M, 0.25M, 0.3M, 0.35M, 0.4M, 0.45M and 0.5M) were prepared in PBS solution and tested in order to obtain cross-calibration of H_2O_2. Following the optimization of potential range to be applied, different concentrations of H_2O_2

samples were prepared by 5 mM potassium ferrocyanide: potassium ferricyanide (1:1) solution in order to increase electrochemical response. The current values belonging to each H_2O_2 concentration were read through GalvanoPlot Controller software.

2.4. Enzymatic Reaction and Measurement

To determine the ALT activity, a cross-calibration was carried out by calibrating H_2O_2 followed by known concentrations of ALT samples, which were set as 5 UL^{-1}, 15 UL^{-1}, 20 UL^{-1}, 30 UL^{-1}, 40 UL^{-1}, 80 UL^{-1}, 100 UL^{-1}, and 120 UL^{-1} for the enzymatic reactions with total volumes of 100 µL. About 2 µL of the reaction medium was transferred to electrodes (**Fig. 1**) and the current values of the corresponding enzymatic reactions were noted after 15 minutes at room temperature (298 K).

3. Results and Discussion

Among the microfluidic technologies, the POCs allow paralleled simultaneous reactions in a miniaturized setting. The studies on the applications of microfluidic systems with both free and immobilized enzymes have been increasing not only in the biocatalytic field but also for the diagnosis of diseases. In the following section, signal processing, quantitative analysis of H_2O_2 concentration produced by a serial of alanine aminotransferase enzymatic reactions are presented.

3.1. Differential Pulse Voltammetry Utilizing Dual LMP91000 Devices

GalvanoPlot GX201 instrument is the smallest electrochemical interface on the market (48x37x23mm), which recruits the low voltage potentiostat technology for on-chip voltammetry and chronoamperometry. Instrument relies on the AFE integrated circuit LMP91000 (Texas Instruments, TX, US) for application of a programmable voltage bias between working (WE) and reference electrodes (RE) of the MEMS style sensor through a

counter electrode (CE) feedback network and reading the signal of current flowing over the WE [35]. Although LMP91000 is primarily designed to serve for chronoamperometry studies for chemical sensing gas sensors, it is also preferred AFE in biosensor studies, where successful amperometric and cyclic voltammetry applications were demonstrated [36–38] . In these studies, AFE was programmed in a way that they could sweep the +-0.6 V range in 50mV steps for a Cyclic Voltammetry (CV) and signal converted and amplified over the built-in TIA of AFE was measured through an Analog to Digital Converter (ADC), while the instrument was controlled over BeagleBone, Arduino Unos, or Raspberry Pi.

Limits of the programmable bias of the device is defined by the voltage reference (VREF) of the voltage divider in the AFE. The potential difference between WE and RE is equal to VREF times Bias Amount, which can take values between 1% to 24% in 2% increments. Most reliable voltage reference devices have standard output potentials and the highest values available below the maximum supply voltage of the AFEs are 4.5V and 5.0V. Given that always a headroom will be necessary for the common supply standard of 5.0V, choice of VREF is practically limited to 4.5V ceiling, which will bring the device range limited to ±1.08V.

Some application of electrochemistry required sensor bias voltages swept over the range limit of LMP9100, that is ±0.6V with 2.5V internal reference of evaluation board and 1.08V typical maximum with a 4.5V external reference, so we implemented a new design of the electronics architecture. In order to build a device with a comparable performance to the basic design, we kept the LMP91000 AFE as primary component and added another LMP91000 to interoperate with the first one. This novel architecture of the AFE was capable of reaching ±2.0V, almost doubling the original design **(Fig 2)**. In this design, the first device controls CE and RE, while the second controls WE. VREF pins of each device are connected to their individual Digital Analog Converter (DAC) channels, allowing WE and RE to be

programmed separately, so in a much more flexible way. MCP4922 is a DAC that has 12 bits of resolution and 2 individual channels. When this dual DAC device is supplied through the REF5045, a low noise, low temperature drift (3ppm/°C) and 0.05% accuracy voltage reference of 4.5V output, RE electrode will have a value of 3.015V (4,500mVx%67), setting the high limit of range of the electrodes. Although DAC can provide voltages down to zero, the minimum VREF value of LMP91000 is limited to 1.5V, setting the low limit of range to 1.005V(1,500mVx%67). Output resolution of the DAC is at about 1 mV (V_{LSB}=4,500mVx2^{-12}= 1.099mV), setting the resolution of programmable voltage bias in first LMP91000 circuit to sub-milivolt levels (1.099mVx67%=736µV). Second circuit, which controls the potential on WE, as well as measures the current flows through it was preferably set to the same proportion of the supply (67% of VREF, as internal zero) to make programming of the WE possible in a range of 1.005V to 3.015V, likewise. As shown in the inset of **Fig 2,** being able to control each channel of DAC individually, WE-RE bias could be controlled in a wider range of ±2.01V.

It was observed during the experiments that, some signals with high positive values saturate the A1 Opamp that holds the CE-RE potential balance. To avoid this, CE needs to have negative voltage values in some instances, and this is provided by integrating an external Opamp with negative supply to the RE output of first LMP9100 circuit. A negative voltage source with internal voltage regulator, LM2687, is used for supplying negative voltages to this Opamp. LM2687 is a coil-less switched capacitor integrated circuit that has a switching frequency of 100KHz, so switching noises at high frequency is easily filtered out from the signal.

AFE provides the transamplified signal in voltage form through the voltage output (VOUT) pin. We utilized a Sigma-Delta ADC of 16 bits, ADC161S626 to digitalize the signal. Last bit of the ADC is the sign bit, bringing the reading resolution to about 137µV (V_{LSB}4,500mVx2^{-}

197 [15]) which corresponds to 391pA (137µV/ 350,000Ω) and 39.1nA (137µV/ 3,500Ω), at 350KΩ
198 and 3.5KΩ gain settings of the TIA, respectively.

199 3.1. Determination of Voltage and Concentration Ranges for Detection of Hydrogen Peroxide

200 According to the main ALT reaction, in presence of oxygen, water and glutamate oxidase
201 (GluOx), L-glutamate turns into α-ketoglutarate, ammonia and H_2O_2 (Eqs. (1),(2) and (3)
202 [39].

203 (1) $L-alanine+\ \alpha-ketoglutarate \xrightarrow{ALT} pyruvate+\ L-glutamate$

204 (2) $L-glutamate+O_2+\ H_2O \xrightarrow{GluOx}\ \alpha-ketoglutarate+NH_3+\ H_2O_2$

205 (3) $H_2O_2 \xrightarrow{Oxidation}\ O_2+2H+2\,e^-$

206 As the H_2O_2 can be easily detected electrochemically, they can be used as glutamate sensors
207 [40,41]. To date, various oxidase enzymes such as GluOx, cholesterol oxidase, with various
208 modifications were used to monitor enzymatically-generated H_2O_2 [27,40,42]. Herein, to
209 determine the specific potential range of H_2O_2, the non-specific peaks generated by other
210 reactants (potassium ferro-/ferri-cyanide and PBS) in the reaction medium were separately
211 analyzed and subsequently eliminated from the total potential range [27]. Following the
212 elimination of non-specific peaks, 500 mM H_2O_2 was injected onto thin film electrode surface
213 and potential range has been achieved between +500 mV and +900 mV. Similarly, Marquette
214 *et al.* [27], reported the applied potential as +850 mV in between the glassy carbon electrode
215 and a platinum pseudo-reference locating at the multifunctional biosensing chip designed for
216 electrochemiluminescent detection of enzymatically produced H_2O_2, whereas the classical
217 potential of H_2O_2 has been reported as +425 mV in previous biosensor studies related to
218 cholesterol and choline determinations [43,44]. Therefore, non-covalent immobilization has
219 been regarded to have a significant effect on electrode systems which is particularly
220 highlighted in enzymatic biosensors [27]. Subsequently, the detection sensitivity testing was

performed for different concentrations of H_2O_2 (0.05 M to 0.5 M). Electrochemical responses were obtained by plotting the corresponding H_2O_2 sensing currents (μA) vs. applied potentials (V). For H_2O_2, the linear region was 0.05-0.5 M, and an equation of "*y = 96.926x + 17.48*" was obtained by linear regression with R^2 = 0.978. As can be seen from **Fig. 3A** to **3J**, the increasing concentrations of H_2O_2 resulted in increasing current values from 18.56 μA to 63.49 μA.

3.2. Monitoring of ALT Level via Hydrogen Peroxide Detection

Pt thin-film electrodes were tested with different concentrations of ALT enzyme (5-120 UL^{-1}) **(Fig. 4)** including physiological (5- 40 UL^{-1}) and critical (≥96-UL^{-1}) levels in human blood which might be associated with a pathology [39]. After a duration of 15 minutes, oxidation potential interval of 500-900 mV was applied to demonstrate direct electro-oxidation of H_2O_2 produced from the enzymatic reaction as described in Eqs. (1) (2) and (3). The data from these measurements resulted in a calibration curve according to the changes in ALT activity. The increasing rate of the signal obtained in proportional to ALT concentration was 1.72 nA/UL^{-1}. The linear range was found to be 5–120 UL^{-1} and equation of "*y = 0.0003x + 0.0775*" was obtained by linear regression with R^2 = 0.908. The increasing levels of ALT in the reaction resulted in increasing current values from 0.078 μA to 0.106 μA as shown in **Fig. 4A** to **4H**. In another study, Jamal *et al*. [34] demonstrated GluOx modified Pt electrodes as electrochemical biosensors and used both ferrocene carboxylic acid mediated and unmediated reactions of ALT. Unmediated systems, having the same reaction mechanism with the current study, demonstrated direct electro-oxidation of H_2O_2 at 500-700 mV with a linear range of 10-1000 UL^{-1} and limit of detection value as 3.29 UL^{-1}. In the latter case related to similar results obtained in the recent study, Song *et al.* [45] used polydimethylsiloxane microchannel incorporated with Pt electrodes to developed an amperometric biosensor for ALT detection and obtained a linear range between 1.3 UL^{-1} to

250 U L^{-1}. Comparable results indicate that actual potential applied and the scanning range of the biomolecules can be shifted depending on the selection and the modifications on electrode system [46]. Although a system utilizing Pt thin-film electrodes has proved to function for monitoring of ALT level via H_2O_2 detection, a relatively higher potential range was required to overcome the interaction of metal surface by oxygen and proton. Hence, further surface modifications like additional mediators, nanoparticles or anti-interference membranes that lower the oxidation potential will be necessary to minimize the potential oxidation of other species in real biological samples.

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Conflicts of Interest

Authors have declared no conflict of interest.

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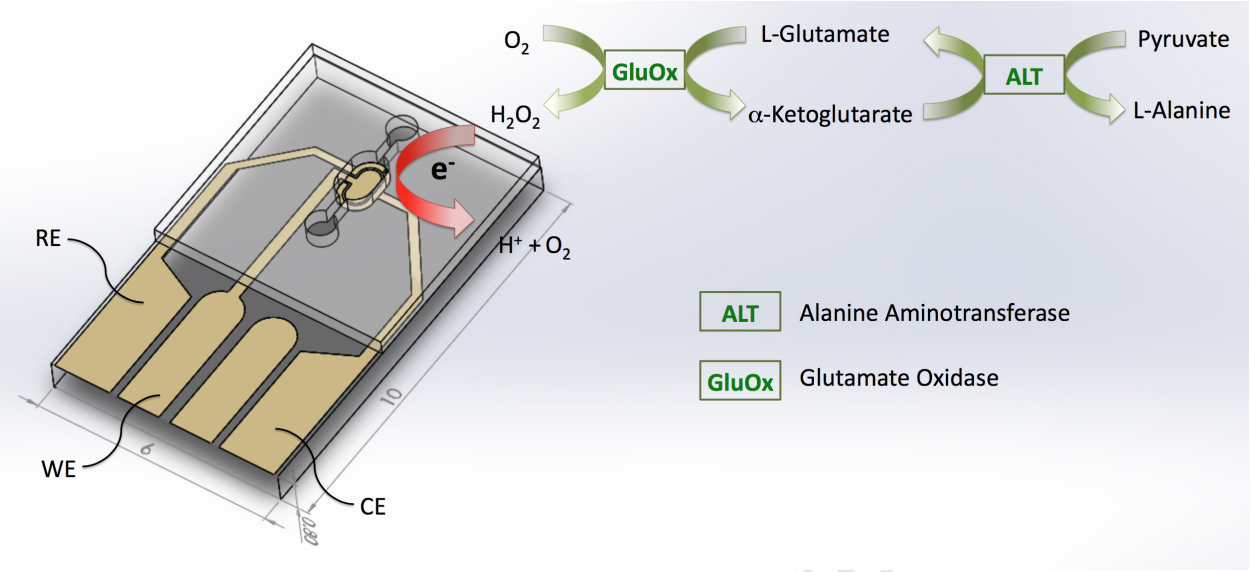
Figure captions

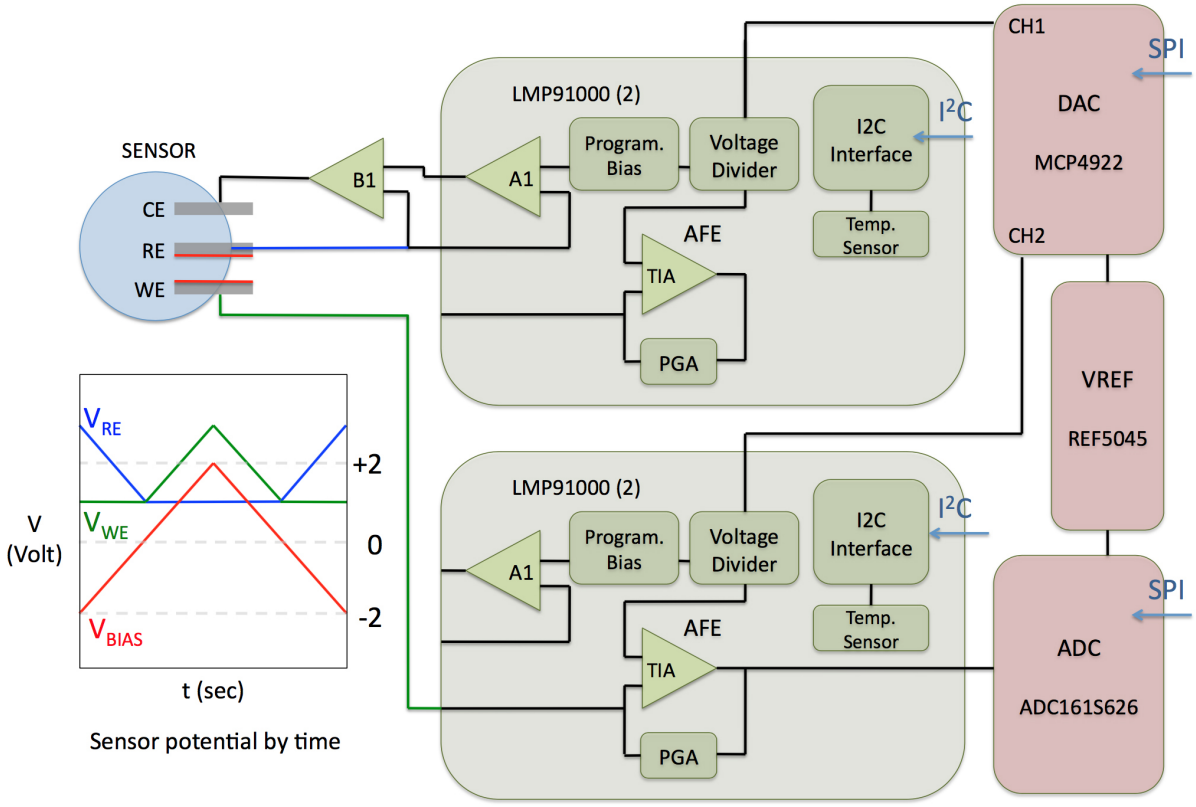
Figure 1. Illustration of the reaction for ALT detection on the Pt thin film electrode surface. Generated glutamate by ALT enzyme (right) in presence of the L- alanine and α-ketoglutarate reacts with the GluOx (left) and subsequently converted to H_2O_2.

Figure 2. Novel architecture of the potentiostat AFE depending on dual LMP91000 devices and capable of reaching ±2.0V scan range. Inset: Potential of each electrode during a full range voltage bias sweep. A1: Single supply Operational Amplifier; B1: Dual (negative and positive) supply Operational Amplifier; CH1: Channel 1; CH2: Channel 2; PGA: Programmable Gain Amplifier; TIA: Transimpedance Amplifier; VREF: Voltage Reference; ADC: Analog to Digital Converter; DAC: Digital to Analog Converter; I2C: Inter-integrated Circuit Protocol; SPI: Serial Peripheral Interface Protocol; CE: Counter Electrode; RE: Reference Electrode; WE: Working Electrode.

Figure 3. Electrochemical responses of H_2O_2 doped Pt thin film electrode under the operating potential range of 200-900 mV with a step value of 2 mV and an amplitude of 80 mV at various concentrations, 0.05M (A); 0.1 M (B); 0.15 M (C); 0.2 M (D); 0.25 M (E); 0.30 M (F); 0.35 M (G); 0.4 M (H); 0.45 M (I); 0.5 M (J).

Figure 4. Corresponding electrochemical responses of ALT reaction medium doped Pt electrode at various concentrations, 5 U L^{-1} (A); 15 U L^{-1} (B); 20 U L^{-1} (C); 30 U L^{-1} (D); 40 U L^{-1} (E); 80 U L^{-1} (F); 100 U L^{-1} (G); and 120 U L^{-1} (H).

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CH1
SPI
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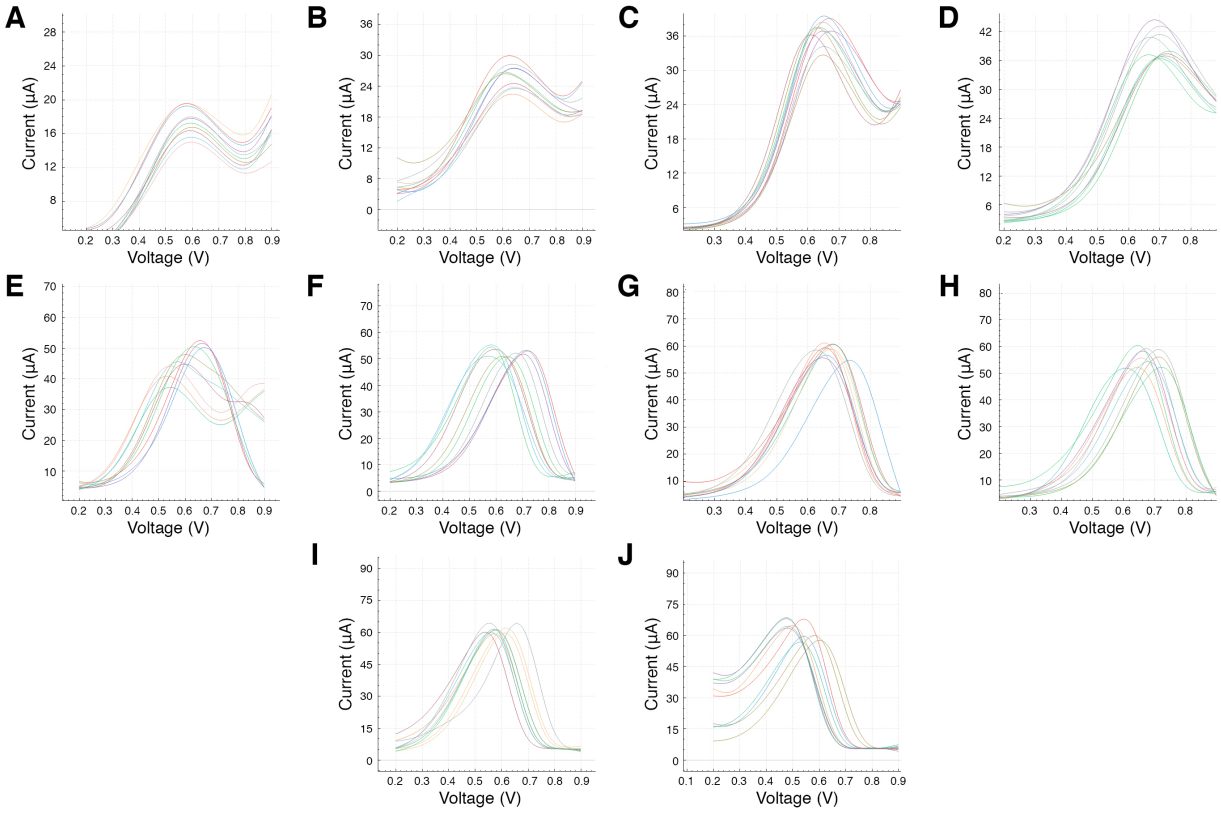
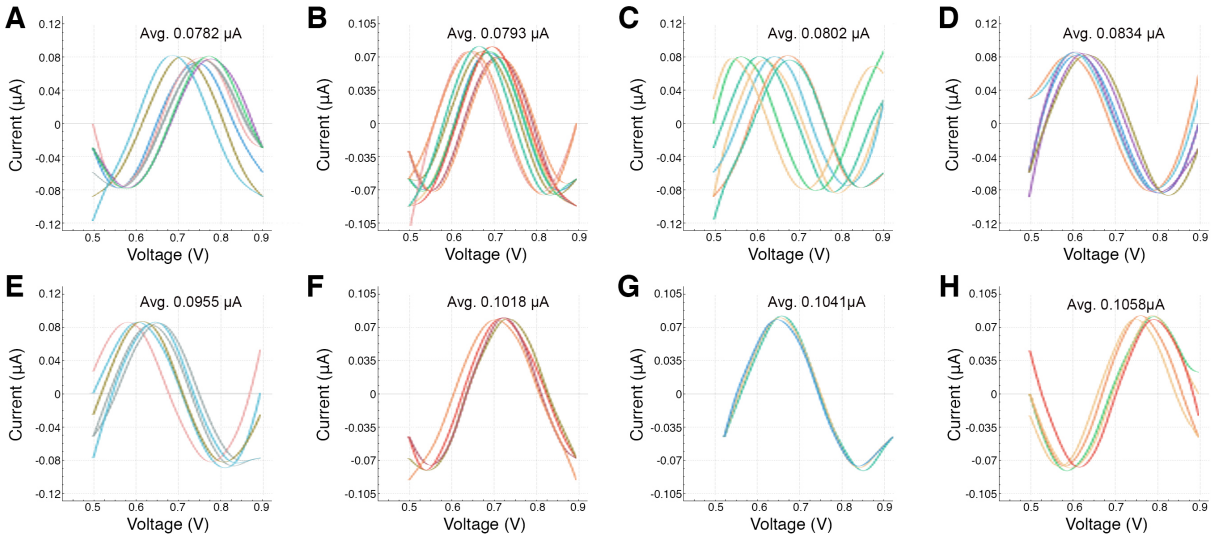
Sensor potential by time





Highlights

- A platinum thin film electrode sensor was proposed to quantify ALT enzyme levels
- Along with a simple potentiostat instrument
- Enzymatically-generated H_2O_2 concentration was determinated with DPV
- Applied potantial ranges and detection limits for both H_2O_2 and ALT enzyme were elicited

Author Statement

E.S., B.O., M.K. and E.I.A. designed the study and performed the experiments; A.D. and Y.K. contributed to voltammetry protocol and signal processing; E.S. and Y.K. wrote the paper; O.Y.C. supervised the research and edited the manuscript. All authors read and approved the final manuscript.