ENANTIOANALYSIS OF LYSINE IN WHOLE BLOOD – A TEST THAT MAY CONTRIBUTE TO EARLY DIAGNOSIS OF BREAST CANCER

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Enantioanalysis of amino acids in biological materials is essential in cancer metabolomics. For the enantioanalysis of lysine in whole blood, a 3D stochastic platform with a 3D printed sensor made of PbO_2-rGO@Pd(0) paste modified with 4 tert-butyl-calix[4]arene was presented. The working concentration range for L-lysine was 1×10^{-18} to 1×10^{-9} mol L^{-1}, whereas for D-lysine was 1×10^{-18} to 1×10^{-2} mol L^{-1}. The sensitivities of L- and D-lysine were at magnitude orders of 10^9 s^{-1} mol^{-1} L. L- and D-lysine recoveries above 98.00% were achieved in whole blood samples using the platform.

Keywords: lysine, 3D stochastic platform, electrochemical sensor, breast cancer, whole blood

1. Introduction

The metabolism of amino acids is an essential component in the process of cell proliferation, as well as in the individualized therapy of cancer and the early identification of the disease [1]. A number of important processes, including redox, energy management, biosynthesis, and the preservation of homeostasis, are all dependent on amino acids [2]. Lysine (Lys), an important amino acid that is involved in the formation of proteins and the functioning of cells, has lately been recognized as a potential marker in the identification of breast cancer. The presence of high levels of lysine has been linked to multiple phases of the progression of cancer, highlighting the significance of early detection in the process of diagnosis [3].

Breast cancer is a significant global health issue, with its occurrence rising in populations across the globe [4]. It is crucial to promptly identify and precisely

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monitor biomarkers associated with the advancement of breast cancer in order to enhance patient outcomes.

Various conventional techniques can be employed to determine the concentration of the L-lysine enantiomer, such as titrimetry [5], colorimetry [6], and high-performance liquid chromatography (HPLC) [7].

Electrochemical sensors possess versatility, affordability, and portability, together with a high level of sensitivity and selectivity for a broad spectrum of analytical applications [8, 9, 10]. The manufacturing of the sensor electrode, which functions as the transducer element, is a crucial and challenging stage.

To date, stochastic electroanalysis is the sole method that is capable of performing reliable enantioanalysis when used for biological samples [11, 12]. Not only is it essential to get an understanding of the enantiomers (L- or D-) that are present in the samples which are being analyzed, but it is also essential to have an understanding of the quantity of these enantiomers that are present in the biological sample. With the use of this method, it is possible to distinguish between the L- and D- isomers of amino acids, and also to perform high reliable quantitative analysis of them.

Palladium-doped reduced graphene oxide (rGO@Pd(0)) and lead oxide (PbO_2) have a significant impact on enhancing electrochemical properties. Graphene-based materials, including reduced graphene oxide (rGO), have remarkable electrical conductivity and improves the movement of electrons and increases conductivity, resulting in enhanced electrochemical performance. Palladium (Pd) is widely acknowledged as a highly efficient catalyst in numerous electrochemical reactions owing to its unique electronic structure and catalytic capability [13]. It has the ability to improve electrochemical processes by attracting molecules involved in the reaction and decreasing the energy needed for the reactions to occur. Lead oxide has remarkable electrocatalytic capability and durability, rendering it highly suitable for a diverse array of electrochemical applications. It possesses the capability to enhance the oxidation or reduction of specific compounds, therefore enhancing the effectiveness of electrochemical processes [14].

4-tert-butyl-calix[4]arene is a constituent of the calixarene group, which consists of macrocyclic compounds made up of phenolic units linked by methylene bridges [15]. Calixarenes possess a distinctive concave structure that arises from the organization of aromatic rings [15, 16]. This particular type of calixarene was selected due to its ability to provide the essential channel (cavity) for the stochastic signal [15, 17].

The innovation is in the design of a portable mini-platform which enables the use of a three-dimensional sensor. This sensor was developed through the modification of a matrix consisting of rGO@Pd(0) and PbO_2 with 4-tert-butyl-

calix[4]arene. The detection of L- and D-lysine in whole blood samples may be carried out using this platform, which enables reliable and precise outcomes.

2. Experimental

2.1 Materials and reagents

The chemicals obtained from Sigma Aldrich (Milwaukee, USA) include L-lysine, D-lysine, 4 tert-butyl-calix[4]arene, L-serine, D-serine, carbohydrate antigen 19–9 (CA 19–9), cancer antigen 15–3 (CA 15–3), tumor protein p53 (p53), monosodium phosphate, disodium phosphate, and lead (IV) oxide. The paraffin oil was bought from Fluka, (Buchs, Switzerland). The reduced graphene oxide @Pd(0) was bought from Nano Innova Technologies (Toledo, Spain). The L- and D-lysine stock solutions were prepared in phosphate buffer solution (pH=7.4), at a concentration of 10^{-2} mol L^{-1}. For the rest of the dilutions (10^{-3} mol L^{-1} -10^{-18} mol L^{-1}), the serial dilution method was used alongside phosphate buffer solution and deionized water. The deionized water was obtained from a Millipore direct-Q3 system (Molsheim, France). The L-lysine solutions were stored at ambient temperature while not in use, whereas the D-lysine solutions were stored at -20°C as per the producer's guidelines.

2.2. Apparatus

The measurements were performed using a stochastic platform that integrated the 3D stochastic sensor. The platform was coupled to a Mini Potentiostat EmSTAT Pico (software PsTrace 5.9 PalmSens, Houten, Netherlands), which was connected to a laptop. The examination of lysine was carried out utilizing the stochastic approach.

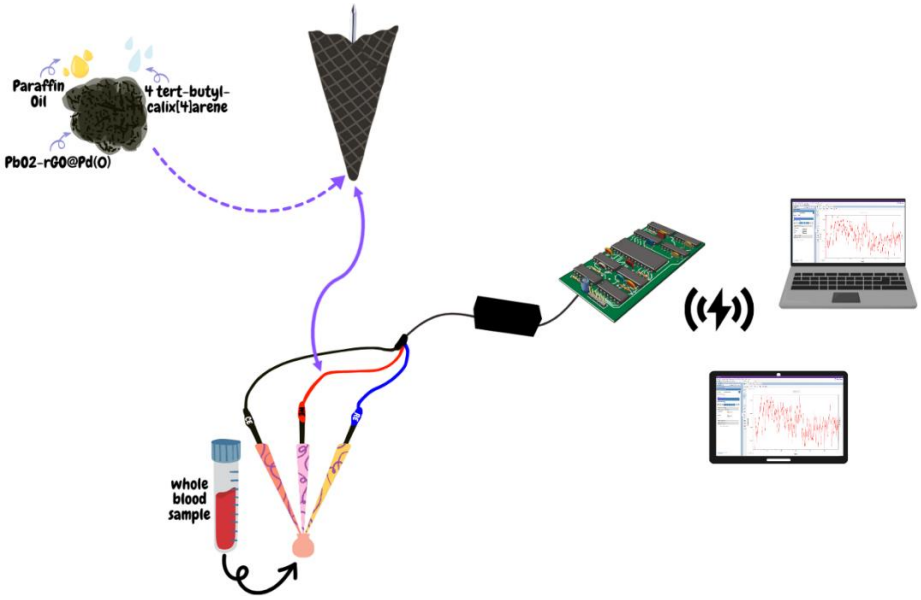
The paste's morphology was analyzed using scanning electron microscopy (SEM) with World-Class Performance on the Cube-II (Tabletop SEM) (EmCrafts Co., Ltd. Korea).

The electrochemical cell of the stochastic platform consists of a reference electrode (Ag/AgCl, 0.1 mol L^{-1} KCl), a counter electrode (platinum wire), and a 3D printed stochastic sensor utilized as the working electrode. In the research facility, 3D tubes were manufactured using a Stratasys Objet 24 printer (Rehovot, Israel). The utilized material is vero white plus, which is a durable white opaque polymer. These tubes have an interior diameter of 25 µm and a length of 1 cm.

2.3. 3D sensor and stochastic platform design

In order to acquire the 3D sensor, a matrix composed of PbO_2-rGO@Pd(0) was utilized. To enhance the electrochemical signal, a modifier was introduced by adding droplets of a solution containing 4 tert-butyl-calix[4]arene at a concentration of 10^{-2} mol L^{-1}. In order to homogenize the mixture, paraffin oil was additionally used. The homogenized paste was subsequently inserted into a custom-made three-

dimensional plastic tube, manufactured in our laboratory. In order to establish a connection between the paste contained within the tube and the external circuit, a silver wire was introduced into the tube. The platform was constructed by incorporating the sensor into the electrochemical cell and coupling it to a miniature potentiostat. After each use, the 3D sensor was cleansed with deionized water and dried. Subsequently, it was stored at room temperature and shielded from light when not being utilized. Scheme 1 depicts the design layout of the 3D sensor and the stochastic platform.



Scheme 1. The design of the 3D sensor and the stochastic platform.

2.4. Recommended procedure

The stochastic method employed the chronoamperometric technique conducted at a potential of 0.2 V versus the Ag/AgCl reference electrode. The L- and D-lysine analytes were identified in whole blood samples based on their signatures; these are also known as t_{off} values. The stochastic platforms were calibrated using a range of solutions with varying concentrations of L- and D-lysine. The calibration equations for these biomarkers were derived using the proposed stochastic platform. These equations were based on determining the t_{on} value, which is the value obtained after reading between two consecutive t_{off} values on the diagram (Fig. 1.). The a and b parameters in the calibration equations ($1/t_{on}=a+bxConc._{biomarker}$) were determined by using the linear regression method. The analytes of interest were identified in the assayed whole blood samples based on their specific signatures. Afterwards, the t_{on} values were measured and used in

the calibration equation to determine the concentration of L- and D-lysine in the biological samples.

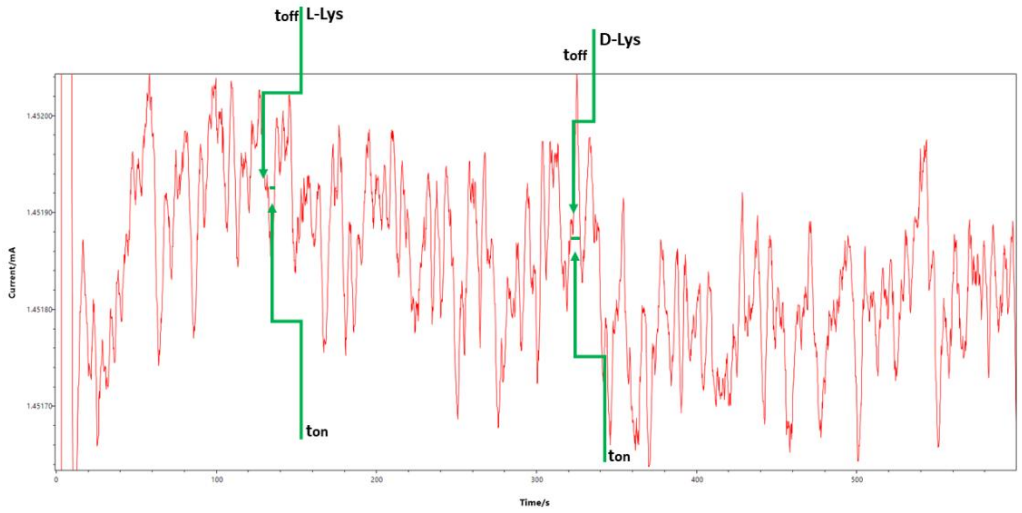


Fig. 1. One of the diagrams obtained after the screening of whole blood samples for L- and D-lysine.

2.5. Samples

A total of 20 blood samples (10 blood samples from breast cancer patients and 10 blood samples from healthy volunteers) at the University Emergency Hospital Bucharest, following the approved methodology by the University of Medicine and Pharmacy "Carol Davila" in Bucharest, as per Ethics Committee number 75/2015. Prior to the collection of these samples, the patients were not receiving any form of medical intervention. Additionally, the samples were not subjected to any pre-treatment before to analysis. Every participant engaged in this research study gave written consent after being fully informed. The blood samples were analyzed using the electrochemical platform that was developed, and a corresponding schematic depiction was recorded. In the molecular recognition diagrams, the t_{off} values for L- and D-lysine signatures were initially established.

3. Results and discussions

3.1. Structural characterization of the active side of the 3D stochastic sensor.

SEM analysis (Fig. 2) shows that the modified paste has a good morphology. In the SEM micrograph, it can be seen that the Pd nanoparticles are uniformly dispersed over the entire surface of the reduced graphene structure. Observations from SEM image analysis show that the lead oxide particles have a fine structure and the morphology of the particles is rod-like. It can also be seen that 4-tert-butyl-

calix[4]arene showed a consistent and uniform pattern of square-shaped cells. The particles are rigid and developed in a direction perpendicular to the surface.

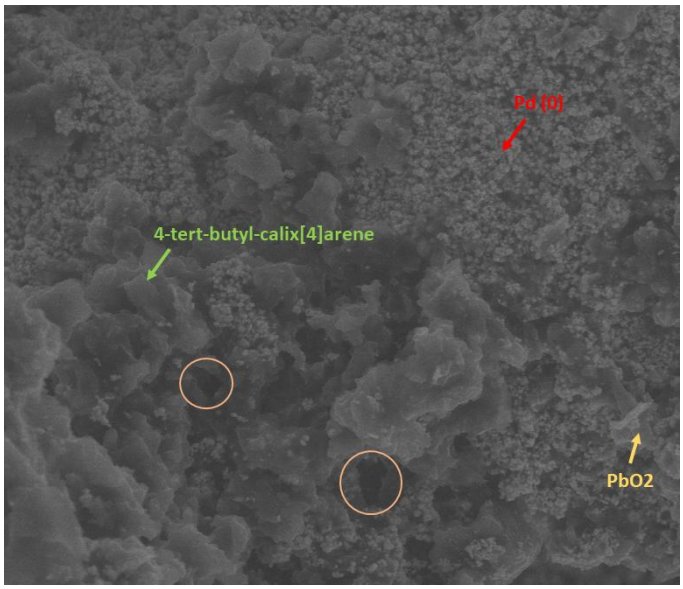


Fig. 2. SEM image for the 3D sensor integrated into the stochastic platform. Circles indicate the presence of the pores in the paste.

3.2. Response Characteristics of the Stochastic Platform

The response characteristics were obtained using the stochastic mode referred to previously. The identification of each enantiomer (L- and D-lysine) was achieved through qualitative analysis utilizing the value of t_{off} from Table 1, referred to as the enantiomer's signature. The distinct signatures (t_{off} values) observed for both enantiomers confirm that they can be simultaneously assayed in whole blood samples. Table 1 displays the results of quantitative analysis, which was conducted by measuring t_{on} values and assessing associated factors such as sensitivity, limit of determination, and working concentration range for each enantiomer and platform. Both enantiomers exhibited an identical lower limit of determination. Regarding the sensitivity obtained for the assay of the enantiomers, it was obtained the same magnitude order of 10^9 s mol L^{-1} for both of them.

Wide working concentration ranges were obtained for both enantiomers. No discrepancies were observed in the calibration of the suggested platforms when it was performed in synthetic solutions and in whole blood samples.

Table 1

The response characteristics of the stochastic platforms when used for the enantioanalysis of lysine.

Analyte	Linear concentration range (mol L^{-1})	Calibration equation and correlation coefficient (r)*	t_{off} (s)	Sensitivity (s^{-1} mol^{-1} L)	LOQ (mol L^{-1})
L-lysine	1.00×10^{-18} – 1.00×10^{-9}	$1/t_{on}=0.57+1.10\times10^{9}\times C$; r=0.9999	0.6	1.10×10^{9}	1.00×10^{-18}
D-lysine	1.00×10^{-18} - 1.00×10^{-2}	$1/t_{on}=0.08+5.88\times10^{9}\times C$; r=0.9999	1.6	5.88×10^{9}	1.00×10^{-18}

*C - concentration = M; t_{on} = s; LOQ - limit of quantification.

Repeatability studies were conducted on the stochastic platforms. 10 platforms were built using the procedure described above. The evaluation process for each platform was conducted uniformly, and the sensitivities were calculated and compared while each platform was immersed in solutions of L- and D-lysine. The measured RSD values for the sensitivities were below 0.10% throughout the testing of the stochastic platform. The values demonstrated the replicability of the platform's design. The stability of the platform was assessed by doing the following checks: A total of 30 platforms were designed and stored, following the aforementioned instructions. Each day, an entirely new platform was taken out of storage and placed in different solutions containing varied concentrations of L- and D-lysine. The sensitivities of each measurement were recorded for comparison once all of the 3D stochastic sensors were used up in 30 days. The data obtained at the conclusion of the time period demonstrated the remarkable durability of the platforms, as the fluctuations in sensitivity over time were less than 1.00%, regardless of the modifier employed in the platform design.

3.3. Selectivity of the stochastic platform

The distinct values obtained for L- and D-lysine, as shown in Table 1, provide further proof that the proposed 3D sensor exhibits enantioselectivity. The level of selectivity was demonstrated by analyzing various biomarkers commonly used for breast cancer detection, including L-serine (L-ser), D-serine (D-ser), p53, human epidermal growth factor receptor (HER-1), and human epidermal growth factor receptor (HER-2). The signatures observed for L- and D-lysine were distinct from those of the biomarkers indicated before, confirming the selectivity of 3D sensors (Table 2).

Table 2

Selectivity of the stochastic platform used for the assay of lysine (N=10).

3D Sensor	L-Lys	D-Lys	L-Ser	D-Ser	p53	HER-1	HER-2
4 tert-butyl-calix[4]arene/ PbO_2-rGO@Pd(0)	0.6	1.6	1.0	2.1	3.5	0.3	2.5

3.4. Utilizing the Stochastic Platform to Screen Whole Blood Samples for L- and D-lysine with the Incorporation of the 4 tert-butyl-calix[4]arene/PbO_2-rGO@Pd(0) 3D Sensor

A total of 10 whole blood samples were analyzed utilizing the suggested stochastic platform. Once both enantiomers were identified, their concentration was evaluated using the procedure outlined above. The data provided in Fig. 3a) clearly show a significant correlation between the levels of L- and D-lysine in whole blood samples obtained from patients diagnosed with breast cancer. Conversely, Fig. 3b) clearly illustrates the absence of the D enantiomer, as it was not detected in the whole blood samples collected from healthy volunteers.

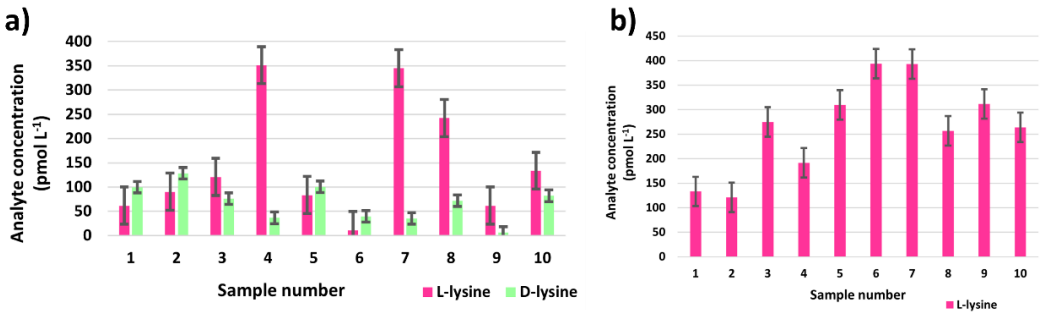


Fig. 3. a) Determination of L- and D-lysine in whole blood samples from confirmed patients with breast cancer using the proposed stochastic platform; b) Determination of L-lysine in whole blood samples from healthy volunteers using the proposed stochastic platform.

The platform was used to conduct recovery tests for L- and D-lysine in whole blood samples in order to meet the validation requirements. The initial concentrations of L- and D-lysine in whole blood samples were measured. Subsequently, varying amounts of L- and D-lysine were added to the samples, ranging from extremely small to higher amounts. New measurements were then conducted. The additional quantity was compared to the assayed quantity of L- and D-lysine in whole blood samples. The results are presented in Table 3.

Table 3

Recovery of the enantiomers of L-Lys and D-Lys present in different ratios of whole blood samples (N=10).

Enantiomers	%, Recovery, L-Lys: D-Lys				
	1:1	1:2	2:1	1:10	10:1
L-Lys	99.97±0.03	99.15±0.02	99.37±0.02	99.95±0.02	99.10±0.02
D-Lys	98.95±0.02	98.17±0.03	99.02±0.04	99.47±0.02	98.99±0.03

The recovery tests yielded excellent results, with all recoveries exceeding 98.00% and exceptionally low relative standard deviation (RSD) values, below 0.07%, based on 10 measurements. The proposed 3D stochastic sensor was highly accurate and precise in screening biological samples.

4. Conclusions

A platform incorporating a 3D stochastic sensor was developed, characterized, and validated for accurately determining the enantiomeric composition of lysine in whole blood samples. The platform can reliable perform qualitative and quantitative enantioanalysis of lysine in whole blood samples. The feature of it is its further utilization for screening tests of whole blood from patients confirmed with breast cancer and from healthy volunteers in order to prove that the enantioanalysis of lysine is a screening test able to contribute to early diagnosis of breast cancer.

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R E F E R E N C E S

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