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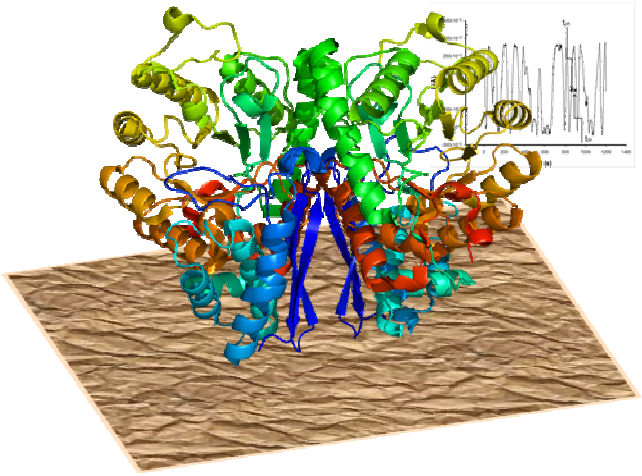
Graphical Abstract



Stochastic microsensors as screening tools for neuron specific enolase

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Abstract

Stochastic microsensors based on nanostructured materials from the classes of porphyrins and cyclodextrins, and carbon onions were used for new screening tools of whole blood samples for neuron specific enolase, a lung cancer biomarker. The neuron specific enolase was identified in whole blood sample based on its signature (t_{off} value). The best response was given by the microsensor based on the complex of Mn(III) with 5,10,15,20-tetraphenyl-21H,23H-porphyrin, that exhibit a linear concentration range between 476.75pg/mL and 7.628ng/mL, with the lowest determination limit of 51.74pg/mL. The proposed stochastic microsensors provides a fast, sensitive, reliable and lower cost assay for screening of neuron

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specific enolase from whole blood samples, without any pretreatment of whole blood samples.

Keywords: neuron specific enolase, stochastic microsensors, screening test, lung cancer

Introduction

Cancer is one of the leading causes of mortality worldwide. Lung cancer has the highest mortality of all cancers, and its incidence is gradually increasing. Statistic studies show that approximately 1.2 million new cases of lung cancer are diagnosed all around the world every year and the death rates are 17.8%. The rate of early diagnosis is very low: almost two thirds of cases are diagnosed when metastatic extension has already occurred.[1-4]

The quantitative detection of tumor biomarkers provides useful information in the early diagnosis of cancer, and its development through screening, staging, metastasis evaluation and response to pharmacologic intervention[5-7].

Neuron specific enolase (NSE) is a sensitive, specific and reliable tumor marker for lung cancer at the time of diagnosis[7]. Neuron specific enolase is the γ subunit of enolase. Enolase is a 78 kDa glycolytic enzyme that is made of three subunits: α, β, and γ, and form five different combinations each with their specific tissue[8-11]. The most frequent combinations are: the αα-homodimer (non-neuronal enolase, NNE), the ββ-homodimer (muscular enolase, MNE), and the neuron-specific enolase (NSE), which occurs in both the heterodimeric αγ form and the homodimeric γγ form[7-17]. NSE is present primarily in the cytoplasm of neurons and neuroendocrine cells[7-17]. The determination of NSE level is of great importance to process the early stage diagnostic for monitoring lung cancer[7-17].

To date, various methods have been developed for the detection of tumor biomarkers, such as ELISA (enzyme-linked immunosorbent assay)[18-22], ECLIA (electrochemiluminiscence immunoassay)[23-27], radioimmunoassay[28,29], and immunofluorescent assay[30-32]. Recently, electrochemical immunosensors has attracted wide interest in detection of neuron specific enolase due to their advantages: high sensitivity, and good reproducibility[5,6,33].

Stochastic sensors represent a good alternative to the traditional electrochemical sensors and also chromatographic methods due to their capability of determining in one run the quality and the quantity of different analytes in the sample. Their introduction as tools in biomedical analysis and nanomedicine can improve the limits of quantification of the substances of biological importance and accordingly can solve the problem of early diagnosis of different diseases[33]. Also, one of the advantages of the stochastic sensors is that they can be used for the assay of a biological molecule in a very complex matrix such as whole blood sample[33-35], because their recognition in the sample is based on the identification of t_{off} value – which is specific to a certain analyte for a given stochastic sensor; this value depends only on the size and velocity of the molecule of the analyte and on the size of the pore/channel from the membrane of stochastic microsensor.

Materials and Methods

Reagents and materials

Neuron specific enolase, natural diamond powder having particle size of 1 μm (99.9%), the complex of Mn(III) with 5,10,15,20-tetraphenyl-21H,23H-porphyrin (Mn(III)P), monosodium and disodium phosphate were purchased from Sigma Aldrich (Milwaukee, USA), paraffin oil was purchased from Fluka (Buchs, Switzerland), α-cyclodextrin was supplied by Wacher-Chemie GmbH (Germany). Carbon nano-onions (CNOs) were

synthesised accordingly with the method proposed by Kuznetsov et al.[36-38]. Monosodium and disodium phosphate were used for the preparation of phosphate buffer pH=7.04. Deionised water obtained from a Milipore Direct-Q 3 System (Molsheim, France) was used for the preparation of all solutions. The standard solutions of NSE were all prepared in phosphate buffer solution (pH=7.04). The range of concentrations was obtained by serial dilution method from 7.449pg/mL to 0.125mg/mL. All solutions were fresh prepared before measurements.

Apparatus

For all chronoamperometric measurements a PGSTAT 302 and software Ecochemie version 4.9 were used. The electrochemical cell was assembled with a conventional three electrode cell: the working electrode, an Ag/AgCl (0,1mol/1KCl) as reference electrode and a Pt counter electrode.

Microsensors design

The microsensors were designed accordingly with the method (e.g., optimized ratio between components) proposed by Stefan-van Staden et al previously[33]. A modified diamond paste (DP) was prepared as follows: natural monocrystaline diamond powder was mixed with paraffin oil to form a diamond paste. 50μL from the solution of the electrochemical active compound (α-cyclodextrin- CD - and the complex of Mn(III) with 5,10,15,20-tetraphenyl-21H,23H-porphyrin – Mn(III)P) was added to 100mg diamond paste. The modified paste was placed into a plastic tube.

The carbon nano-onions paste was prepared by mixing the carbon nano-onions powder with paraffin oil. The diameter of the sensors was about 25μm.

Electric contact was obtained by inserting an Ag/AgCl wire into the modified paste. The surface of the sensors was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before using. When not in use, the sensors were stored in a dry state at room temperature.

Recommended procedure - Direct amperometry

The technique used was chronoamperometry, with a potential applied versus Ag/AgCl of 125 mV. The electrodes were dipped into a cell containing a solution of biomarker of interest. Equations of calibration $1/t_{on}$=f(Conc.) are determined using statistics (linear regression equation). The unknown concentration was determined from the equation, by inserting the value of $1/t_{on}$.

Sample preparation

Whole blood samples were obtained from the Universitary Hospital in Bucharest (Ethics committee approval nr. 11/2013 from ten healthy patients and from 12 confirmed patients with lung cancer. Informed consent was obtained from all subjects.) and they were used for the screening of NSE without any pretreatement. The apparatus cell was filled with whole blood samples and the current developed was measured. The unknown concentration of NSE in whole blood samples were determined by inserting the values of $1/t_{on}$ in the equation of calibration.

Recovery tests

Recovery tests were performed as first step of method validation. After testing one whole blood sample from a healthy patient with all three microsensor, tests proving that the NSE

was not found in the sample, the sample was divided in three, the volumes were measured, and small volumes of a $4.00x10^{-7}$g/mL NSE solution were added with a micropipette to give the final concentrations of $5.00x10^{-8}$, $8.00x10^{-8}$ and $8.00x10^{-9}$g/mL for the microsensors based on α-CD/DP and CNO, and the final concentrations of $5.00x10^{-10}$, $5.00x10^{-11}$ and $8.00x10^{-11}$ g/mL for the microsensor based on Mn(III)P/DP. The concentrations determined from the calibration graphs (when 5 concentrations were used for each calibration graph) accordingly with the proposed recommended procedure – direct amperometry, were compared with the theoretical concentrations of the spiked whole blood samples.

Results and Discussions

Response characteristics of the stochastic microsensors

The principle of stochastic sensors is based on channel conductivity. While the molecule is flowing through a channel under a potential of 125mV, and it is blocking the channel, the current is dropping to zero value (the time of channel blocking is called t_{off} and represents the signature of the analyte – in this case NSE), after the blocking process the molecule of NSE is binding on the channel wall – the time of equilibration for binding process is called t_{on} and can be used for the quantitative assessment of the analyte (Figure 1) as its value is related to the concentration of the analyte through the equation: $1/t_{on}$= a + b x Conc.

Therefore, there are two stages: stage 1 on which the NSE extracted from the solution into the membrane-solution interface is blocking the channel, the intensity of the current recorded being 0; and stage 2 (bounding stage) when NSE is interacting with the wall of the channel, and the following equilibrium equation is taking place:

$$Ch_{(i)} + NSE_{(i)} \Leftrightarrow Ch \bullet NSE_{(i)}$$

where Ch is the channel, and *i* is the interface.

Accordingly, the diagrams obtained using stochastic microsensors can be used for the qualitative as well as quantitative analysis. The qualitative assay of NSE is given by the values of t_{off} (signature of the analyte), presented in Table 1. The signatures of NSE (values of t_{off}) were identified in the diagrams obtained for the whole blood samples (qualitative assay) (Figure 1).

Response characteristics of the stochastic microsensors are shown in Table 1. Using the values of t_{on}, one can determine the equation of calibration, sensitivity (slope of the calibration graph, or the value of b from the equation of calibration), and linear concentration range. Calibration graphs for the sensors are shown in Figure 2. All the microsensors used for the screening of NSE gave very good response characteristics with high values of sensitivity, and low limits of quantification (pg/mL magnitude order). As can be seen in Table 1 the best response characteristics in terms of sensitivity and limit of determination (the lowest concentration that can be reliable determined using the proposed microsensors which in this case is the lowest concentration from the linear concentration range) were recorded for the stochastic microsensor based on Mn(III)P/DP. The limits of determination for NSE obtained using the proposed stochastic sensors are lower than the limits of detection reported recently by using a multiplexed system with time-resolved förster resonance energy transfer (150ng/mL) [39], by those presented in the review of Harmsma et al.[40] as well as by the limit of detection of the commercial kit from Brahms/Thermo Fisher scientific for optimized standard clinical KRYPTOR system (10ng/mL). The proposed method is a direct assay of NSE, and not an indirect one like the ones proposed by Wu et al when the SOX2 DNA amounts detected are correlated with the quantity of NSE in serum samples[41] or when enzyme immunoassay is used[42]. A comparison between the best limits of determination obtained

using methods proposed to date for the assay of NSE and the limits of determination obtained using the proposed stochastic microsensors is shown in Table 2; accordingly, the microsensor based on Mn(III)P/DP is the best being able to detect the lowest amount of NSE. Furthermore, the proposed sensors are able to perform qualitative and quantitative analysis of NSE in whole blood immediately after is taken from the patients, without any pretreatment of whole blood.

Analytical application

The proposed stochastic microsensors were used as screening tools for whole blood samples. Qualitative assessment of NSE in the diagrams was done based on its signature (t_{off} values), while the quantitative analysis of NSE was done using the values of t_{on} (Figure 1).

Recovery tests of NSE in whole blood samples were performed by spiking the whole blood with 3 different concentrations of NSE. The results obtained are: 98.75±0.32% (n=10 determinations) for the stochastic microsensor based on α-CD/DP, 98.98±0.21% (n=10 determinations) for the stochastic microsensor based on CNOs, and 99.02±0.13% (n=10 determinations) for the stochastic microsensor based on Mn(III)P/DP. The results proved that NSE can be reliable assayed from the whole blood samples.

Table 3 shows the quantitative analysis of NSE in whole blood samples obtained from confirmed patients with lung cancer. Based on the paired t-test performed for 99% confidence level (all t_{calc} values, at the 99.00% confidence level, are less than the tabulated theoretical t-value: 4.032), one can conclude that there is no statistically significant difference between the results obtained using the three stochastic microsensors, and they can be successfully used for the quantification of NSE in whole blood samples. For the ten healthy patients, the t_{off}

specific for NSE was not found in the diagrams obtained for the whole blood samples, the results confirming their healthy status.

Conclusions

The proposed stochastic microsensors can reliably be used as screening tools of whole blood samples for NSE assessment. They are reproducible as design and response characteristics. The best results for the screening test were obtained using stochastic microsensors based on the complex of Mn(III) with 5,10,15,20-tetraphenyl-21H,23H-porphyrin. The advantages of using the stochastic microsensors are: they can be used as fast screening tools of whole blood for neuron specific enolase; the cost of such screening test is low, and no sample pretreatment is needed – the whole blood samples are used as taken from the patients.

Features of the proposed stochastic sensors are their application for a panel of biomarkers used in lung cancer diagnosis (as described by Harmsma et al[34]) such as CEA, NSE, ProGRP, CK-BB, SCC, CgA, NCAM and several cytokeratins, which will bring them closer to a diagnostic tool for lung cancer.

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Table 1. Response characteristics of the stochastic microsensors used for the screening tests of whole blood for NSE.

Stochastic microsensors based on	**Signature of NSE t_{off} (s)**	**Sensitivity (s g^{-1}mL)**	**Linear concentration range (g/mL)**	**Limit of determination (g/mL)**
α-CD/DP	1.5	$4.84x10^{4}$	$7.63x10^{-9} – 4.88x10^{-7}$	$7.63x10^{-9}$
CNOs	1.5	$1.87x10^{6}$	$1.91x10^{-9} – 3.05x10^{-8}$	$1.91x10^{-9}$
Mn(III)P/DP	2.5	$4.83x10^{6}$	$2.98x10^{-11} – 7.63x10^{-9}$	$2.98x10^{-11}$

All values are the average of 30 determinations.

Table 2 Comparison of limits of determination obtained using the proposed stochastic microsensors and the best results from the literature.

Limit of determination (ng/mL)	Reference
5.0	9
150.0	39
0.05	42
10.0	Standard method*
Proposed method based on stochastic microsensors designed with:	
7.63	α-CD/DP
1.91	CNOs
0.0298	Mn(III)P/DP

*Kit from Brahms/Thermo Fisher scientific for optimized standard clinical KRYPTOR system.

Table 3. Results of screening tests of whole blood for NSE.

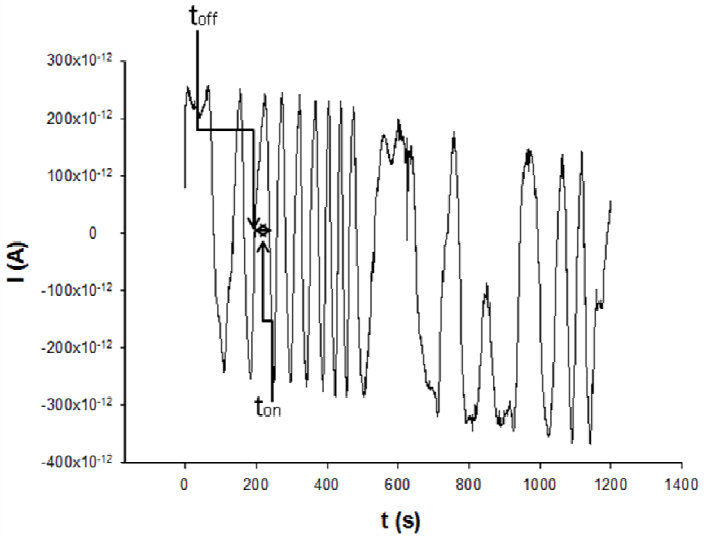
Sample nr.	NSE, ng/mL			t-test
	Microsensor based on			
	α-CD/DP	CNOs	Mn(III)P/DP	
1	8.00±0.12	7.90±0.15	8.30±0.09	2.32
2	1.00±0.21	0.92±0.19	1.20±0.10	2.40
3	2.00±0.21	1.92±0.22	2.40±0.15	2.43
4	0.84±0.06	0.89±0.03	0.91±0.02	2.23
5	1.00±0.12	0.96±0.07	1.30±0.12	2.29
6	0.028±0.001	0.026±0.001	0.028±0.002	3.29
7	0.037±0.005	0.025±0.002	0.036±0.002	3.33
8	681.00±0.14	680.00±0.31	679.00±0.11	2.83
9	206.00±0.13	208.00±0.24	205.00±0.09	2.92
10	24.50±0.12	21.80±0.20	27.80±0.02	3.01
11	6.00±0.15	6.40±0.20	7.00±0.11	3.12
12	6.00±0.23	6.50±0.34	6.80±0.12	2.74

All values are the average of ten determinations.

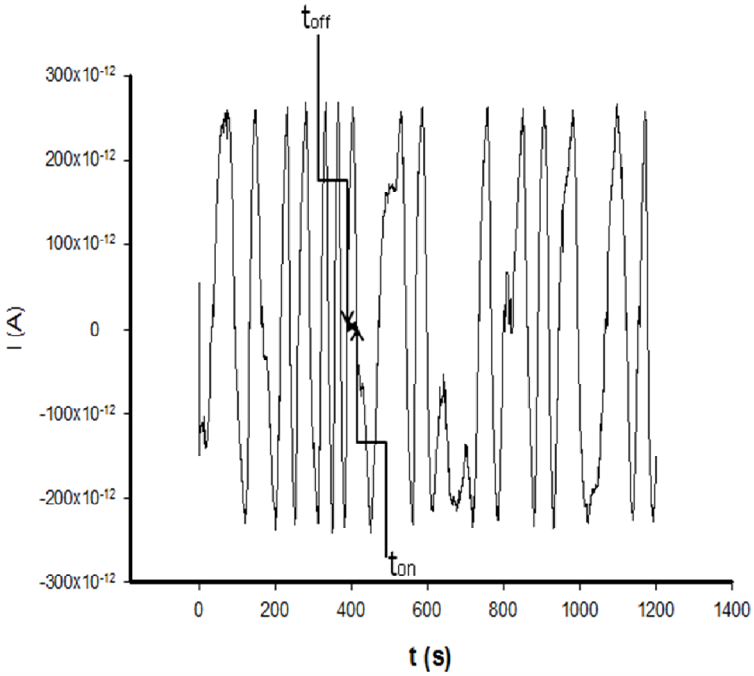
Caption to Figures

Figure 1 Examples of diagrams obtained when whole blood was screening using stochastic microsensors based on (a) α-CD/DP; (b) CNOs; (c) Mn(III)/DP.

Figure 2 Calibration graphs for the stochastic microsensors based on α-CD/DP, CNOs and Mn(III)/DP.

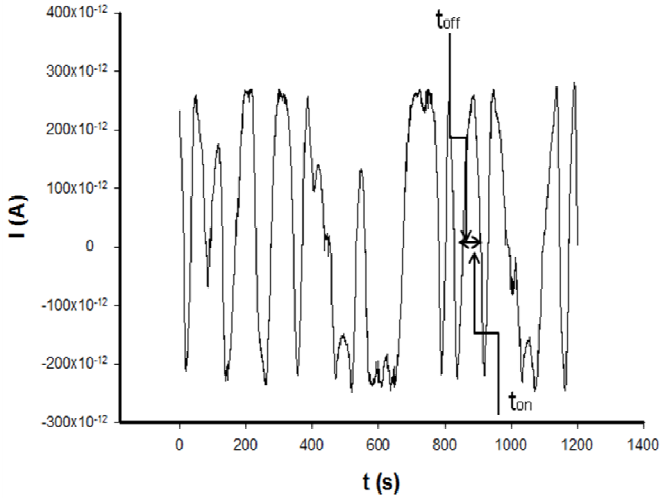


(a)



(b)

Fig. 1



(c)

Figure 1

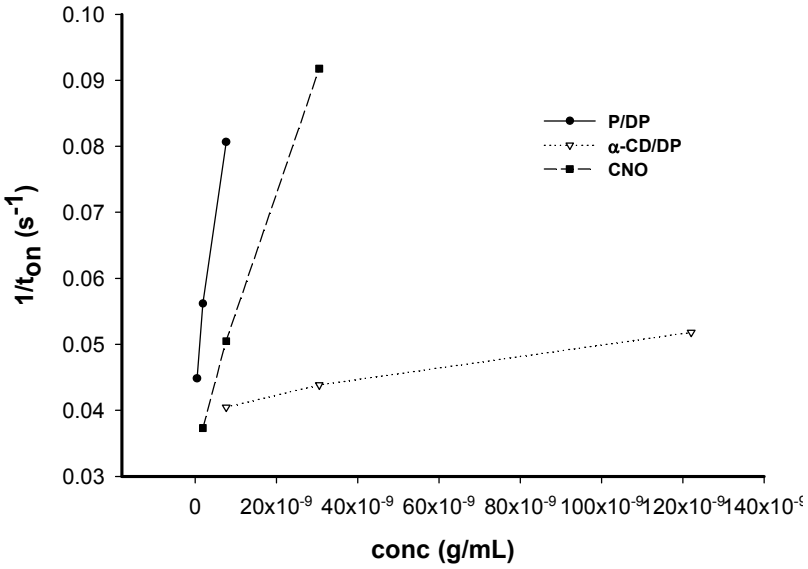


Figure 2