Journal of The Electrochemical Society, **164** (6) B267-B273 (2017)
0013-4651/2017/164(6)/B267/7/$37.00 © The Electrochemical Society

Molecular Screening of Blood Samples for the Simultaneous Detection of CEA, HER-1, NSE, CYFRA 21-1 Using Stochastic Sensors

Raluca-Ioana Stefan-van Staden,[a,b,*,z] Ionela Raluca Comnea-Stancu,[a,b] and Carmen Cristina Surdu-Bob[c]

[a]*Laboratory of Electrochemistry and PATLAB Bucharest, National Institute of Research for Electrochemistry and Condensed Matter, Bucharest 060021, Romania*
[b]*Faculty of Applied Chemistry and Materials Science, Politehnica University of Bucharest, Bucharest 011061, Romania*
[c]*Low Temperature Plasma Laboratory, National Institute for Lasers, Plasma and Radiation Physics (NILPRP), Magurele, 077125, Romania*

The aim of this study was to detect simultaneously four lung cancer biomarkers: CYFRA 21-1, CEA, NSE and HER-1, from blood samples. In order to do that, we have chosen stochastic senosors based on diamond paste matrices modified with different electroactive materials like dextrins and porphyrins, and ones based on three metals like Au, Cu and Ni. The stochastic sensors chosen for the screening tests of blood samples proved to be a good choice for the simultaneous detection of the four biomarkers, reaching low limits of determination of pg mL^{-1} magnitude order and high sensitivities. One main advantage of the sensors used for the simultaneous detection of the biomarkers is that the samples are analyzed as taken from the patients, without any pretreatment.
 [DOI: 10.1149/2.1621706jes]

Manuscript submitted March 2, 2017; revised manuscript received April 3, 2017. Published April 20, 2017.

According to the latest report of the American Cancer Society an estimated 224.390 new cases of lung cancer are expected in 2016, accounting for about 14% of all cancer diagnoses. Lung cancer is the second most commonly diagnosed cancer and accounts for more deaths than any other cancer in both men and women with an estimated 158.080 deaths that are expected to occur in 2016, accounting for about 1 in 4 cancer deaths.[1] Despite advances in the understanding of tumor biology in recent years, lung cancer mortality in Europe has remained largely unchanged over the past three decades, underlying the need for new treatment strategies.[2] Lung cancer at its early stage is mostly asymptomatic in most cases that make early diagnosis difficult. The current detection and diagnosis for lung cancer include physical and biochemical methods. The physical methods include X–ray, CT (Computed Tomography), PET (Positron Emission Tomography), bronchoscopy, sputum cytology and bronchial biopsy, none of which turns out to be effective in early diagnosis of lung cancer. However, ability to use these techniques is dependent on the size of tumor and some special medical equipment, leading to a increasingly cost. Several biochemical methods for the detection of lung cancer are also used clinically by detecting biomarkers released by cancer cells or lung tumors into the serum/plasma/blood. The biochemical methods are less invasive and cost-effective.[3–5]

Among the currently lung cancer biomarkers used for the screening, diagnosis and detection of lung cancer are neuron specific enolase (NSE), carcinoembryonic antigen (CEA), epidermal growth factor receptor (HER-1/EGFR), cytokeratin 19 fragment (CYFRA 21-1), tumor M2 pyruvate kinase (PKM2), pro-gastrin-releasing peptide (ProGRP), squamous cell carcinoma antigen (SCC-Ag), tissue polypeptide antigen (TPA) and tissue polypeptide-specific antigen (TPS).[6–11]

To date, the analysis of the biomarkers has been performed with enzyme-linked immunosorbent assay (ELISA),[10,12–14] electrochemiluminescence immunoassay (ECLIA),[15] radioimmunoassay (RIA),[16–18] imunoradiometric assay (IRMA),[10,19] fluorescence in situ hybridization (FISH)[20] or polymerase chain reaction (PCR).[21] These conventional well-developed methods analyze each analyte individually and require significant sample volumes for each analyte, measure single analytes per assay and are not well suited for high-throughput multiplex analysis.[22] For this reason, in the recent years the development of new methods and materials used for the assay of biomarkers has known a well defined improvement, with minimally invasive procedures and with highly sensitive and specific detection. Current systems employed for the detection of cancer biomarkers include the fabrication of different kinds of electrochemical immunosensors[23] based on gold nanocrystals (AuNCs),[24] gold nanoparticles (AuNPs),[25–27] β-cyclodextrin functionalized Cu@Ag (Cu@Ag-CD) core–shell nanoparticles,[28] or different devices like an aptamer based on gold electrodes,[29] a multi-target immunosensig lab-on-chip,[30] or a field effect transistor (FET)-based biosensor.[31] Table I summarizes the current investigations on lung cancer diagnosis using sensors. The table also lists the biomarkers tested with the corresponding detection limits and linear concentration ranges.

Our goal was to establish a method based on stochastic sensing for the simultaneous detection of four lung cancer biomarkers: NSE, CEA, HER-1 and CYFRA 21-1. For this, we proposed various stochastic sensors based on diamond paste modified with different dextrines, porphyrins, as well as stochastic sensors based on metallic materials like gold, nickel and copper.

Experimental

Reagents and materials.—Epidermal growth factor receptor (EGFR/HER-1), carcinoembryonic antigen (CEA), neuron specific enolase (NSE), natural diamond powder (DP) having paticle size of 1 μm (99.9%), maltodextrin I (MD I) with dextrose equivalent 4–7, maltodextrin III (MD III) with dextrose equivalent 16.5–19.5, 5,10,15,20-tetraphenyl-21H,23H-porphyrin (P), monosodium and disodium phosphate were purchased from Sigma Aldrich (Milwaukee, USA). CYFRA 21-1 (a human cytokeratin 19 protein) was purchased from Abcam (Massachusetts, USA). Paraffin oil and NaN_3 were purchased from Fluka (Buchs, Switzerland). α-cyclodextrin was supplied by Wacher-Chemie GmbH (Germany). Monosodium phosphate and disodium phosphate were used for preparation of phosphate buffer. Deionized water obtained from a Millipore 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 7.449 pg mL^{-1} to 0.125 mg mL^{-1}. The standard solutions of CEA and HER-1 were prepared in buffer solution pH = 7.4, containing NaN_3 0,1%. Serial dilution technique was used for the preparation of solutions of CEA in the range 16 pg mL^{-1}-16 mg mL^{-1} and HER-1 in the range 0.56 pg mL^{-1}-3.04 μg mL^{-1}. All CYFRA 21-1 standard solutions were prepared in deionized water in a range of concentrations obtained by serial dilution method from 0.002 pg mL^{-1} to 20 μg mL^{-1}. Engineered nanoporous 24 K gold microspheres, thin copper and nickel nanofilms were designed by a team of NILPRP.

*Electrochemical Society Member.
[z]E-mail: ralucavanstaden@gmail.com

Table I. Examples of different platform sensors for lung cancer biomarkers.

Biomarker	Method	Sensor	Linear concentration range	Detection limit	Reference
NSE	Linear sweap voltammetry (LSV)	MWNTs modified GCE	5–100 ng/mL	0.18 ng/mL	23
	Cyclic voltammetry (CV)	anti-NSE/Au–Gra/NiHCFNPs/AuNCs/GCE	0.001–100 ng/mL	0.3 pg/mL	24
	Field effect transistor	anti-NSE immobilized FET biosensor	1–1000 ng/mL	100 ng/mL	31
ProGRP	Linear sweap voltammetry (LSV)	MWNTs modified GCE	50–1000 pg/mL	10 pg/mL	23
CEA	Electrochemical impedance spectroscopy (EIS)	poly-OAP/CEAAb–AuNP/Au	0.5–20 ng/mL	0.1 ng/mL	25
	Surface-enhanced fluorescence (SEF)	AgNCs, AuNPs and CEA– aptamer	0.01–1 ng/mL	3 pg/mL	26
	Cyclic voltammetry (CV)	ADA-Ab2-Cu@Ag-CD/CEA/BSA/ ADA-Ab1/CD-GN/GC	0.0001–20 ng/mL	20 fg/mL	28
HER-1	Electrochemical impedance spectroscopy (EIS)	Ab/PG/PDITC/Cys/AuNPs/Au	1 pg/mL–1 μg/mL	0.34 pg/mL	27
	Cyclic voltammetry	Ab/PG/PDITC/Cys/AuNPs/Au	-	0.93 pg/mL	27
	Microfluidic system integrated	polydimethylsiloxane (PDMS) Lab-on-chip	-	3–8 ng/mL	30
	Cyclic voltammetry (CV)	EA/anti-EGFRab/DTSP/Au	1 pg/mL–100 ng/mL	1 pg/mL	32
CYFRA 21-1	Field effect transistor	anti-CYFRA 21-1 immobilized FET biosensor	1–1000 ng/mL	1 ng/mL	31

Instrumentation.—For all chronoamperometric experiments was used a PGSTAT 12 potentiostat/galvanostat connected to a three-electrode cell, and linked to a computer via an Eco Chemie (Utretch, The Netherlands) software version 4.9. The electrochemical cell was assembled with a conventional three electrode cell: the working electrode, an Ag/AgCl (0,1 mol/1 KCl) as reference electrode and a Pt counter electrode. For the pH measurements was used a Cyberscan PCD 6500 pH/mV-meter from Eutech Instruments.

Design of the stochastic sensors.—Modified diamond pastes (DP) were prepared as follows: 200 mg of natural monocrystalline diamond powder was mixed with 20 μL paraffin oil to form a paste. 100 μL from the 10^{-3}mol/L solution of maltodextrin I (dextrose equivalent 4–7) or maltodextrin III (dextrose equivalent 16.5–19.5) was added to the diamond paste. 50 μL from the 10^{-3}mol/L solution of the electrochemical active compound (α-cyclodextrin or 5,10,15,20-tetraphenyl-21H,23H-porphyrin) were added to each 100 mg of paste. The modified paste was placed into a plastic tube with a diameter of 25 μm. Electric contact was obtained by inserting an Ag wire into the modified paste. The surface of the sensor was wetted with deionized water and polished with alumina paper before using. When not in use, the sensors were stored in a dry place at room temperature.

One nanoporous gold microsphere of a diameter of 300 μm was placed in a plastic tube so that hath of the sphere was out and the other hath inside the tube. Electrical contact was done using a silver wire.

The textile sensors based on Cu, and Ni nanofilms, were prepared using a piece of nickel or copper coated textile with a length of 2.5 cm and a width of 1.0 mm connected directly to the external circuit.

Stochastic method.—For the stochastic sensing, a chronoamperometric technique was selected for the measurements of t_{on} and t_{off} when a potential of 125 mV versus Ag/AgCl was applied. The electrodes were dipped into a cell containing standard solutions of different concentrations of of analyte. Equations of calibration $1/t_{on}$ = f(Conc.) are determined using statistics (linear regression equation). The unknown concentrations of the four biomarkers in blood samples were determined by inserting the value of $1/t_{on}$ in the equation of calibration.

Sample preparation.—Universitary Hospital from Bucharest provided us whole blood samples from patients that were diagnosed with lung cancer, or from patients that were suspected of lung malignancies (Ethics committee approval nr. 11/2013, informed consent was obtained from all subjects) in order to use them for the screening of

Table II. Response characteristics of the stochastic sensors based on diamond paste for the assay of the lung biomarkers.

Analyte	t_{off} (s)	Linear concentration range (mg mL^{-1})	Sensitivity (s mg^{-1} mL)	Equation of calibration*
		P-DP		
NSE**	2.5	4.77×10^{-7}– 7.636×10^{-6}	4.8×10^3	$1/t_{on} = 0.044 + 4.8 \times 10^3 xC$; r = 0.9922
CEA**	2.7	1.6×10^{-9} – 1.6×10^{-7}	1.76×10^5	$1/t_{on} = 0.070 + 1.76 \times 10^5 xC$; r = 0.9998
HER-1**	7.2	7×10^{-9} – 9.72×10^{-7}	1.44×10^4	$1/t_{on} = 0.041 + 1.44 \times 10^4 xC$; r = 0.9941
CYFRA 21-1	2.1	2×10^{-12} – 2×10^{-8}	3.52×10^6	$1/t_{on} = 0.020 + 3.52 \times 10^6 xC$; r = 0.9995
		α-CD-DP		
NSE**	1.5	7.63×10^{-6} – 1.22×10^{-4}	95.58	$1/t_{on} = 0.040 + 95.58 xC$; r = 0.9946
CEA**	2.1	1.6×10^{-9} – 1.6×10^{-7}	3.19×10^5	$1/t_{on} = 0.08 + 3.19 \times 10^5 xC$; r = 0.9968
HER-1**	6	5.6×10^{-1} – 1.4×10^{-9}	2.51×10^7	$1/t_{on} = 0.015 + 2.51 \times 10^7 xC$; r = 0.9982
CYFRA 21-1	2.7	2×10^{-4} – 2×10^{-2}	2.71	$1/t_{on} = 0.027 + 2.71 xC$; r = 0.9999
		MDI/DP		
NSE**	2.5	4.88×10^{-4} –3.12×10^{-2}	5.31	$1/t_{on} = 0.031 + 5.33 xC$; r = 0.9985
CEA**	3.5	1.6×10^{-7} – 1.6×10^{-5}	2.21×10^3	$1/t_{on} = 0.023 + 4.24 \times 10^2 xC$; r = 0.9934
HER-1**	5.2	2.80×10^{-10}-7.00×10^{-9}	1.41×10^6	$1/t_{on} = 0.055 + 1.41 \times 10^6 xC$; r = 0.9995
CYFRA 21-1	1.6	2×10^{-5} – 2×10^{-3}	5.38×10^3	$1/t_{on} = 0.039 + 5.38 \times 10^3 xC$; r = 0.9987
		MDIII/DP		
NSE**	4	2.98×10^{-8} – 1.91×10^{-6}	5.31	$1/t_{on} = 0.031 + 5.33 xC$; r = 0.9985
CEA**	3.5	1.6×10^{-7} – 1.6×10^{-5}	1.15×10^7	$1/t_{on} = 0.015 + 1.15 \times 10^7 xC$; r = 0.9999
HER-1**	2.5	1.94×10^{-7}-2.43×10^{-5}	1.91×10^3	$1/t_{on} = 0.021 + 1.91 \times 10^3 xC$; r = 0.9967
CYFRA 21-1	2.1	2×10^{-5} – 2×10^{-3}	2.34×10^3	$1/t_{on} = 0.023 + 2.34 \times 10^3 xC$; r = 0.9983

*$<C>$ = mg mL^{-1}, $<t_{on}>$ = s.
**Refs. 33–37.

Table III. Response characteristics of the stochastic sensors based on metals for the assay of the lung biomarkers.

Analyte	t_{off} (s)	Linear concentration range (mg mL^{-1})	Sensitivity (s mg^{-1} mL)	Equation of calibration*
		Au		
NSE**	2	1.22×10^{-4}– 1.95×10^{-3}	8.22	$1/t_{on} = 0.034 + 8.22xC$; r = 0.9950
CEA**	3	1.6×10^{-7}– 1.6×10^{-5}	2.92×10^{3}	$1/t_{on} = 0.02 + 2.92 \times 10^{3}xC$; r = 0.9995
HER-1**	1.4	2.80×10^{-10}–7.00×10^{-9}	2.15×10^{6}	$1/t_{on} = 0.018 + 2.15 \times 10^{6}xC$; r = 0.9967
CYFRA 21-1	2.5	2×10^{-5} – 2×10^{-3}	8.97×10^{3}	$1/t_{on} = 0.041 + 8.97 \times 10^{3}xC$; r = 0.9999
		Cu		
NSE**	3	1.91×10^{-6}–3.05×10^{-4}	1.11×10^{3}	$1/t_{on} = 0.019 + 1.11 \times 10^{3}xC$; r = 0.9987
CEA**	3.5	1.6×10^{-7}– 1.6×10^{-4}	4.24×10^{2}	$1/t_{on} = 0.023 + 4.24 \times 10^{2}xC$; r = 0.9934
HER-1**	2	7.00×10^{-9}- 1.94×10^{-7}	3.45×10^{5}	$1/t_{on} = 0.009 + 3.45 \times 10^{5}xC$; r = 0.9983
CYFRA 21-1	1.6	2×10^{-8} – 2×10^{-6}	2.75×10^{4}	$1/t_{on} = 0.035 + 2.75 \times 10^{4}xC$; r = 1
		Ni		
NSE**	3	7.63×10^{-6}– 4.88×10^{-4}	1.05×10^{2}	$1/t_{on} = 0.031+1.05 \times 10^{2}xC$; r = 1
CEA**	2.5	1.6×10^{-11}– 1.6×10^{-8}	4.30×10^{6}	$1/t_{on} = 0.026 + 4.30 \times 10^{6}xC$; r = 0.9950
HER-1**	3.5	2.80×10^{-10}- 7.00×10^{-9}	4.41×10^{6}	$1/t_{on} = 0.027 + 4.41 \times 10^{6}xC$; r = 0.9998
CYFRA 21-1	2.1	2×10^{-5} – 2×10^{-2}	4.48	$1/t_{on} = 0.029 + 4.48xC$; r = 0.9999

*$<C>$ = mg mL^{-1}, $<t_{on}>$ = s.
**Refs. 36,37.

the four biomarkers. The samples were used for the screening of the lung cancer biomarkers without any pretreatment. The apparatus cell was filled with whole blood sample and the unknown concentration of the biomarker in whole blood samples were determined using the stochastic method.

Results and Discussion

Response characteristics of the stochastic sensors proposed for the screening of the lung cancer biomarkers.—The principle of stochastic sensors is based on the alteration of electrical current by

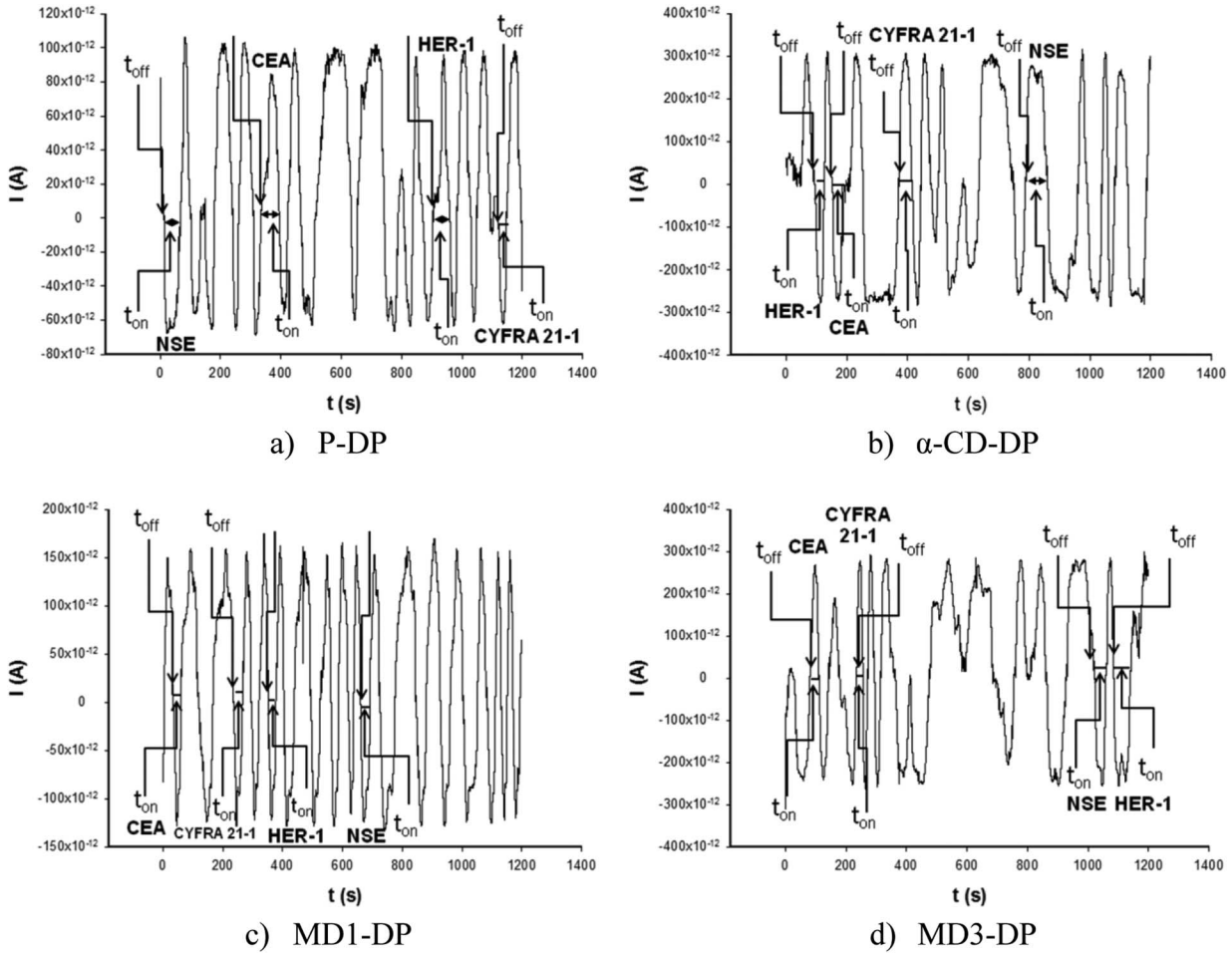

Figure 1. Examples of diagrams recorded for the simultaneous detection of NSE, CEA, HER-1 and CYFRA 21-1 from blood samples using stochastic sensors based on diamond baste modified with: a) porphyrin; b) α-cyclodextrin; c) maltodextrin I; d) maltodextrin III.

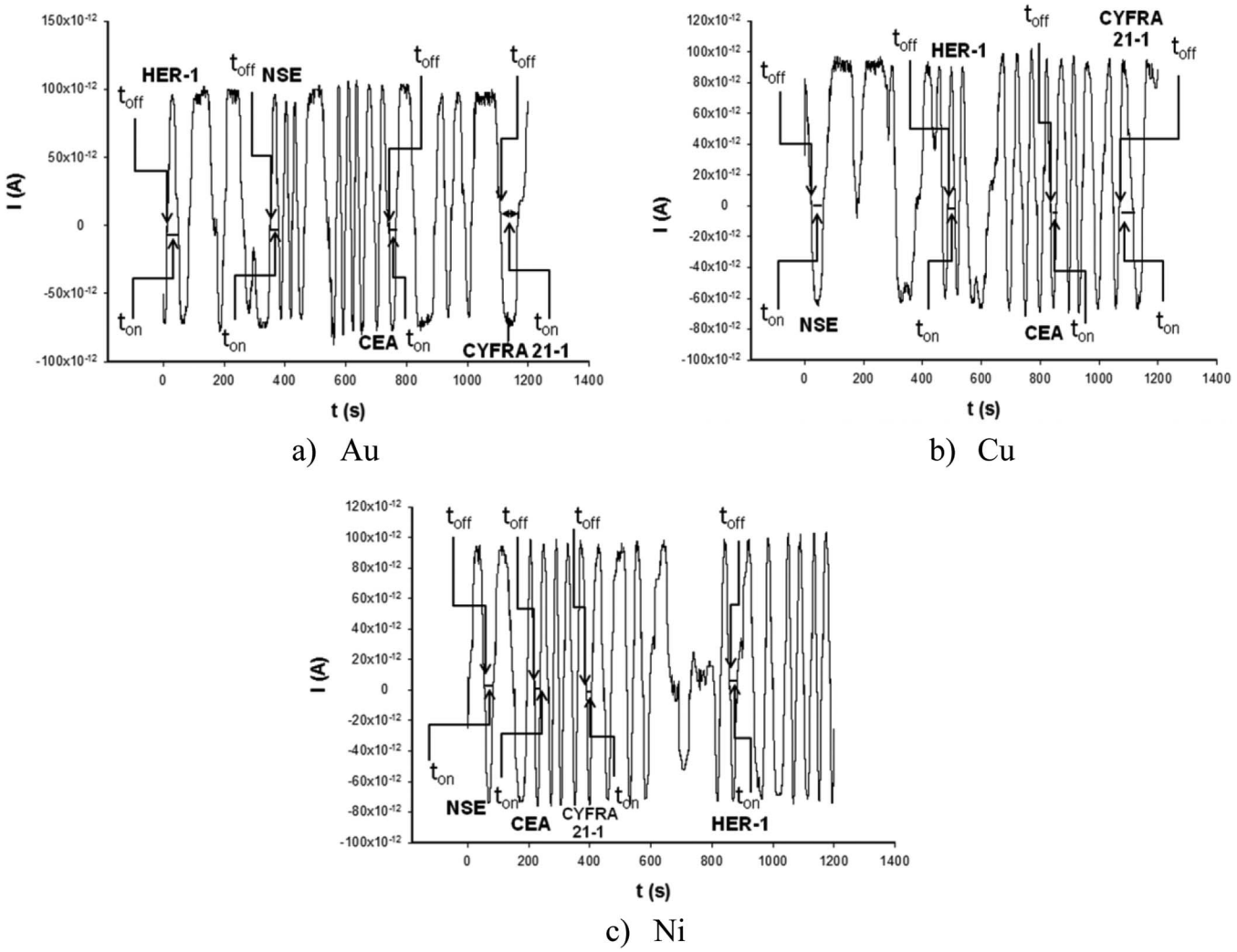

Figure 2. Examples of diagrams recorded for the simultaneous detection of NSE, CEA, HER-1 and CYFRA 21-1 from blood samples using stochastic sensors based on: a) Au; b) Cu; c) Ni.

distinct analytes that interact transiently or permanently with a functional recognition group located within a nanopore. In the absence of compounds, the nanopore is always open and a constant ionic current is observed. In the presence of an analyte, when this is entering the pore, is blocking partially or totally, and the intensity of the current is also dropping to zero (t_{off}) – the value measured for the time off is giving the qualitative identification of the analyte, while the time spend in the pore for binding and redox processes (t_{on}) is giving the concentration of the analyte. In the case of the lung cancer biomarkers the following reactions are taking place:

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

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

$$Ch_{(i)} + HER-1_{(i)} \Leftrightarrow Ch \bullet HER-1_{(i)}$$

$$Ch_{(i)} + CYFRA-21-1_{(i)} \Leftrightarrow Ch \bullet CYFRA-21-1_{(i)}$$

where *Ch* represents the channel and *i* the interface.

Response characteristics of the stochastic sensors proposed for the detection of NSE, CEA, HER-1 reported previously[33–37] as well as the response characteristics obtained for CYFRA 21-1 are shown in Tables II and III. The sensors based on diamond paste showed good response characteristics, high values of sensitivity and low limits of determination. The sensor based on P/DP has exhibit the best response among all the diamond paste sensors with limits of determination of pg mL^{-1} magnitude order for all studied biomarkers: 477 pg mL^{-1} for NSE, 1.6 pg mL^{-1} for CEA, 7 pg mL^{-1} for HER-1 and for CYFRA 21-1 has reached a limit of determination of 0.002 pg mL^{-1} (Table II). Among the sensors based on metals, the one based on Ni gave the best response for CEA and HER-1, the calibration plots showed a good linear relationship between the values of $1/t_{on}$ and the analyte concentrations in the ranges from 0.016 to 16 pg mL^{-1} for CEA and 0.28-7 pg mL^{-1} for HER-1, respectively. The Cu based sensor exhibited a good linear response in the range from 1.91 to 488 ng mL^{-1} for NSE and 20 pg mL^{-1}-2 ng mL^{-1} for CYFRA 21-1 respectively (Table III).

All the sensors proposed were stable over a period of six months, when used daily for measurements. No significant differences in stability were recorded for the proposed sensors, proving that in this case the type of modifier, matrix or metal used did not influenced the stability. Furthermore, five sensors of each category were constructed and used daily for one month, when the RSD(%) values for their slopes did not varied with more than 1.00%, proving the reliability of the design.

The selectivity of the sensors was proved by the different signatures (t_{off} values) obtained for each biomarker when the same sensor was

Table IV. Results of screening tests of whole blood samples for NSE, CEA, HER-1 and CYFRA 21-1.

Sample Nr	Microsensor based on	CEA (ng/mL)	NSE (ng/mL)	HER-1 (ng/mL)	CYFRA 21-1 (ng/mL)
Control	P/DP	-	-	-	-
	αCD/DP	-	-	-	-
	MDI/DP	-	-	-	-
	MDIII/DP	-	-	-	-
	Au	-	-	-	-
	Cu	-	-	-	-
	Ni	-	-	-	-
1	P/DP	10.14 ± 0.04	213.12 ± 0.07	52.00 ± 0.07	27.52 ± 0.02
	αCD/DP	10.08 ± 0.06	213.74 ± 0.04	52.10 ± 0.07	27.48 ± 0.05
	MDI/DP	10.16 ± 0.08	213.64 ± 0.04	51.28 ± 0.08	27.25 ± 0.05
	MDIII/DP	10.59 ± 0.07	214.00 ± 0.09	52.32 ± 0.06	27.02 ± 0.03
	Au	10.30 ± 0.07	213.86 ± 0.03	51.60 ± 0.08	26.90 ± 0.03
	Cu	10.78 ± 0.07	214.00 ± 0.08	52.00 ± 0.07	27.00 ± 0.04
	Ni	9.98 ± 0.08	214.00 ± 0.07	52.40 ± 0.07	27.49 ± 0.04
2	P/DP	71.20 ± 0.06	34.81 ± 0.07	14.33 ± 0.03	70.26 ± 0.05
	αCD/DP	70.52 ± 0.06	34.40 ± 0.05	14.99 ± 0.07	70.52 ± 0.08
	MDI/DP	71.02 ± 0.08	34.28 ± 0.08	14.06 ± 0.08	70.50 ± 0.05
	MDIII/DP	70.00 ± 0.08	34.25 ± 0.05	14 .49 ± 0.05	70.07 ± 0.08
	Au	70.38 ± 0.06	35.21 ± 0.07	14.80 ± 0.08	70.20 ± 0 .03
	Cu	71.90 ± 0.05	34.90 ± 0.07	14.00 ± 0.09	69.20 ± 0.07
	Ni	70.60 ± 0.05	36.20 ± 0.05	14.01 ± 0.08	69.27 ± 0.04
3	P/DP	10.20 ± 0.02	14.45 ± 00.08	21.30 ± 0.02	8.67 ± 0.03
	αCD/DP	10.00 ± 0.05	14.10 ± 0.08	21.09 ± 0.03	8.01 ± 0.03
	MDI/DP	9.97 ± 0.03	13.47 ± 0.07	21.38 ± 9.93	8.05 ± 0.06
	MDIII/DP	11.60 ± 0.09	14.00 ± 0.04	20.57 ± 0.05	7.78 ± 0.07
	Au	10.07 ± 0.03	14.14 ± 0.06	21.58 ± 0.02	8.78 ± 0.07
	Cu	10.67 ± 0.05	14.25 ± 0.06	21.96 ± 0.02	8.78 ± 0.07
	Ni	10.30 ± 0.05	14.05 ± 0.06	21.90 ± 0.02	8.13 ± 0.05
4	P/DP	17.60 ± 0.03	21.86 ± 0.02	54.45 ± 0.03	154.97 ± 0.04
	αCD/DP	16.10 ± 0.03	21.80 ± 0.03	54.85 ± 0.05	155.04 ± 0.07
	MDI/DP	16.90 ± 0.05	21.55 ± 0.03	54.56 ± 0.05	155.17 ± 0.07
	MDIII/DP	16.20 ± 0.05	20.86 ± 0.04	54.10 ± 0.07	155.37 ± 0.08
	Au	16.47 ± 0.07	21.24 ± 0.04	54.70 ± 0.04	155.52 ± 0.06
	Cu	16.31 ± 0.02	21.40 ± 0.05	54.25 ± 0.04	155.04 ± 0.06
	Ni	17.33 ± 0.02	21.03 ± 0.03	54.70 ± 0.05	155.00 ± 0.07
5	P/DP	38.21 ± 0.05	34.00 ± 0.06	20.47 ± 0.03	108.06 ± 0.05
	αCD/DP	38.02 ± 0.05	34.65 ± 0.07	21.19 ± 0.07	108.12 ± 0.06
	MDI/DP	37.72 ± 0.05	33.87 ± 0.07	21.32 ± 0.04	107.56 ± 0.06
	MDIII/DP	37.64 ± 0.03	33.90 ± 0.05	20.47 ± 0.08	108.06 ± 0.08
	Au	38.10 ± 0.04	34.18 ± 0.05	20.70 ± 0.05	107.86 ± 0.05
	Cu	38.60 ± 0.04	34.02 ± 0.05	20.28 ± 0.05	108.13 ± 0.05
	Ni	37.26 ± 0.04	34.20 ± 0.04	21.19 ± 0.03	107.36 ± 0.04
6	P/DP	52.10 ± 0.02	41.75 ± 0.07	21.79 ± 0.03	15.08 ± 0.03
	αCD/DP	52.02 ± 0.03	40.25 ± 0.08	21.89 ± 0.04	15.00 ± 0.02
	MDI/DP	51.81 ± 0.03	40.53 ± 0.05	21.01 ± 0.06	15.44 ± 0.02
	MDIII/DP	52.00 ± 0.02	40.75 ± 0.05	22.10 ± 0.08	15.65 ± 0.05
	Au	52.68 ± 0.04	41.80 ± 0.01	22.60 ± 0.02	14.64 ± 0.07
	Cu	51.80 ± 0.04	42.60 ± 0.03	22.84 ± 0.03	15.09 ± 0.03
	Ni	51.90 ± 0.04	41.48 ± 0.03	21.61 ± 0.03	15.24 ± 0.03
7	P/DP	18.23 ± 0.03	21.91 ± 0.02	2.72 ± 0.04	21.43 ± 0.02
	αCD/DP	18.00 ± 0.03	22.10 ± 0.02	2.81 ± 0.01	21.30 ± 0.04
	MDI/DP	17.80 ± 0.05	22.00 ± 0.01	2.35 ± 0.02	21.45 ± 0.05
	MDIII/DP	18.50 ± 0.05	22.27 ± 0.01	2.80 ± 0.05	21.77 ± 0.08
	Au	19.80 ± 0.03	22.12 ± 0.01	2.69 ± 0.04	20.68 ± 0.03
	Cu	19.19 ± 0.03	21.75 ± 0.07	2.53 ± 0.03	20.62 ± 0.03
	Ni	19.00 ± 0.03	21.80 ± 0.03	2.74 ± 0.03	21.56 ± 0.07
8	P/DP	20.90 ± 0.04	48.50 ± 0.01	11.57 ± 0.07	87.40 ± 0.05
	αCD/DP	20.72 ± 0.05	49.85 ± 0.03	11.93 ± 0.04	88.38 ± 0.05
	MDI/DP	20.39 ± 0.05	48.80 ± 0.03	11.69 ± 0.07	88.67 ± 0.07
	MDIII/DP	20.90 ± 0.03	48.25 ± 0.05	12.70 ± 0.09	88.30 ± 0.08
	Au	21.38 ± 0.03	49.12 ± 0.07	11.84 ± 0.03	87.79 ± 0.03
	Cu	20.00 ± 0.04	48.65 ± 0.05	11.83 ± 0.05	87.14 ± 0.02
	Ni	21.30 ± 0.04	49.08 ± 0.05	11.20 ± 0.05	88.69 ± 0.05
9	P/DP	11.93 ± 0.05	13.20 ± 0.02	2.33 ± 0.01	40.00 ± 0.03
	αCD/DP	12.00 ± 0.03	12.57 ± 0.02	3.24 ± 0.07	41.20 ± 0.04
	MDI/DP	12.67 ± 0.03	13.29 ± 0.04	2.38 ± 0.03	41.07 ± 0.06
	MDIII/DP	12.25 ± 0.04	13.22 ± 0.04	3.47 ± 0.03	40.33 ± 0.08
	Au	12.70 ± 0.04	12.38 ± 0.03	3.16 ± 0.02	40.00 ± 0.05

Table IV. Continued

Sample Nr	Microsensor based on	CEA (ng/mL)	NSE (ng/mL)	HER-1 (ng/mL)	CYFRA 21-1 (ng/mL)
	Cu	12.03 ± 0.05	13.28 ± 0.03	3.00 ± 0.03	40.12 ± 0.05
	Ni	12.20 ± 0.05	13.80 ± 0.05	3.42 ± 0.03	41.35 ± 0.04
10	P/DP	29.07 ± 0.04	13.60 ± 0.06	18.45 ± 0.06	195.51 ± 0.08
	αCD/DP	29.80 ± 0.04	13.83 ± 0.05	18.02 ± 0.04	195.81 ± 0.08
	MDI/DP	29.67 ± 0.05	13.88 ± 0.07	18.06 ± 0.04	195.85 ± 0.06
	MDIII/DP	29.50 ± 0.05	13.00 ± 0.07	18.53 ± 0.05	195.03 ± 0.07
	Au	29.74 ± 0.07	14.39 ± 0.05	18.80 ± 0.03	194.73 ± 0.07
	Cu	29.60 ± 0.03	13.36 ± 0.05	17.57 ± 0.03	194.80 ± 0.05
	Ni	29.00 ± 0.05	14.00 ± 0.05	18.06 ± 0.04	195.90 ± 0.04
11	P/DP	11.30 ± 0.02	26.78 ± 0.03	56.45 ± 0.04	8.15 ± 0.01
	αCD/DP	11.95 ± 0.02	25.25 ± 0.03	57.28 ± 0.08	8.67 ± 0.03
	MDI/DP	11.05 ± 0.01	25.00 ± 0.04	57.20 ± 0.05	8.17 ± 0.03
	MDIII/DP	11.00 ± 0.05	27.12 ± 0.07	56.53 ± 0.07	8.61 ± 0.05
	Au	11.73 ± 0.03	26.82 ± 0.02	57.60 ± 0.05	8.63 ± 0.02
	Cu	10.97 ± 0.03	27.50 ± 0.03	57.60 ± 0.05	8.50 ± 0.02
	Ni	11.46 ± 0.05	25.60 ± 0.03	56.38 ± 0.03	8.56 ± 0.03
12	P/DP	11.30 ± 0.02	46.70 ± 0.03	17.76 ± 0.03	27.09 ± 0.03
	αCD/DP	11.90 ± 0.02	46.38 ± 0.05	18.20 ± 0.04	27.07 ± 0.05
	MDI/DP	11.47 ± 0.04	46.29 ± 0.07	17.76 ± 0.05	26.90 ± 0.05
	MDIII/DP	11.08 ± 0.03	46.64 ± 0.07	18.20 ± 0.07	26.94 ± 0.07
	Au	11.26 ± 0.03	47.60 ± 0.04	18.56 ± 0.04	26.70 ± 0.03
	Cu	11.43 ± 0.05	46.97 ± 0.03	18.36 ± 0.04	26.23 ± 0.04
	Ni	12.00 ± 0.05	47.34 ± 0.03	18.00 ± 0.05	26.69 ± 0.04

used. Ascorbic acid was also given a diferent signature, proving that it is not interfering in the mesurements.

Analytical applications.—In order to evaluate the practical application of the stochastic sensors proposed for the simultaneous detection of NSE, CEA, HER-1 and CYFRA 21 we analyzed a total of 13 whole blood samples. Thus, we identified all the four biomarkers from the whole blood samples based on the signatures of the biomarkers (t_{off} values), and their concentration was calculated based on the t_{on} values (Figs. 1, 2). The values of t_{on} were measured and calculated from the calibration graph in order to determine the concentration of the four biomarkers (Table IV). The control sample was obtained from a healty patient; none of the biomarkers was identified in the whole blood sample of the patient.

Recovery tests.—A recovery test was performed using the proposed sensors for CYFRA 21-1. The concentrations determined from the calibration graphs accordingly with the proposed method, were compared with the theoretical concentrations of the spiked whole blood samples. As it was showen in Table V the recoveries were higher than 93.00%. Recoveries for NSE, CEA, and HER-1 were demonstrated previously.[33–37]

Conclusions

In this study CYFRA 21-1 was added to the previously tested biomarkers in order to get a complete screening test for lung cancer. The sensors proposed showed very good analytical performances for the measurement of NSE, CEA, HER-1 and CYFRA 21-1 with wide concentration linear range, low determination limits, good sensitivity, reproducibility and stability, thus providing promising tools for the simultaneous detection in early clinical diagnosis. All the sensors have been successfully applied for the simultaneous detection of NSE, CEA, HER-1 and CYFRA 21-1 from whole blood samples. Compared with the sensors proposed previously in the literature, one can find the following advantages of using the stochastic sensors: the sample did not need any processing, the limits of determinations are far lower, the sensitivity is higher, and all four biomarkers can be determined using the same sensors in one run.

Table V. Recovery of CYFRA 21-1 in whole blood samples.

Microsensor based on	CYFRA 21-1, Recovery, %
P/DP	97.48 ± 0.02
αCD/DP	99.93 ± 0.05
MDI/DP	96.45 ± 0.03
MDIII/DP	99.84 ± 0.07
Au	99.70 ± 0.03
Cu	99.53 ± 0.04
Ni	93.25 ± 0.04

Acknowledgments

The project was supported by the Ideas project, contract nr 123/2011. I.R. Comnea-Stancu is thankful for the funding from the Sectorial Operational Programme Human Resources Development 2007–2013 of the Ministry of European Funds through the Financial Agreement POSDRU/159/1.5/S/132395.

References

1. American Cancer Society. Cancer Facts & Figures 2016. Atlanta: American Cancer Society; 2016.
2. R. Stahel, S. Peters, P. Baas, E. Brambilla, F. Cappuzzo, D. De Ruysscher, W. E. E. Eberhardt, E. Felip, D. Fennelli, A. Marchetti, L. Paz-Ares, and A. A. Adje, Strategies for improving outcomes in NSCLC: A look to the future, *Lung Cancer*, **82**, 375 (2013).
3. A. Lu and L. Zhang, Tumor-Dependent and -Independent Serum/Plasma Biomarkers for Early Diagnosis of Lung Cancer, *Transl Med*, **6**, 160 (2016).
4. A. K. Greenberg and M. S. Lee, Biomarkers for lung cancer: clinical uses, *Curr Opin Pulm Med*, **13**, 249 (2007).
5. H. J. Sung and J. Y. Cho, Biomarkers for the lung cancer diagnosis and their advances in proteomics, *BMB Reports*, **41**, 615 (2008).
6. T. Xiao, L. Bao, and H. Ji, Finding biomarkers for non-small cell lung cancer diagnosis and prognosis, *Front Biol*, **7**, 14 (2012).
7. J. Kulpa, E. Wojcik, M. Reinfuss, and L. Kołodziejski, Carcinoembryonic Antigen, Squamous Cell Carcinoma Antigen, CYFRA 21-1, and Neuron-specific Enolase in Squamous Cell Lung Cancer Patients, *Clinical Chemistry*, **48**, 1931 (2002).
8. M. P. Pavlou and E. P. Diamandis, The cancer cell secretone: A good source for discovering biomarkers?, *Journal of Proteomics*, **73**(10), 1896 (2010).
9. F. Buttitta, L. Felicioni, M. Del Grammastro, G. Filice, A. Di Lorito, S. Malatesta, P. Viola, I. Centi, T. D'Antuono, R. Zappacosta, S. Rosini, F. Cuccurullo, and A. Marchetti, Effective assessment of EGFR mutation status in bronchoalveolar lavage and pleural fluids by next generation sequencing, *Clin Cancer Res*, **19**, 691 (2013).

10. S. Cedres, I. Nunez, M. Longo, P. Martinez, E. Checa, D. Torrejon, and E. Felip, "Serum tumor markers CEA, CYFRA 21-1, and CA-125 are associated with worse prognosis in advanced non-small-cell lung cancer (NSCLC)", *Clinical Lung Cancer*, **12**(3), 172 (2011).
11. J. M. Ahn and J. Y. Cho, Current serum lung cancer biomarkers, *J Molecular Biomarkers and Diagnosis*, **4**, 1 (2013).
12. A. E. Erbaycu, A. Gunduz, O. Batum, Z. U. Zeren, F. Tuksavul, and S. Z. Guclu, "Pretreatment and treatment-Induced Neuron-specific Enolase in Patients with Small-Cell Lung Cancer: An Open Prospective Study", *Arch Bronconeumol*, **46**(7), 364 (2010).
13. S. Maramottia, M. Paci, F. Miccichč, A. Ciarrocchia, A. Cavazza, M. De Bortoli, E. Vaghic, D. Formisano, L. Canovi, G. Sgarbi, and I. Bongarzone, Soluble epidermal growth factor receptor isoforms in non-small cell lung cancer tissue and in blood, *Lung Cancer*, **76**, 332 (2012).
14. J. S. Ha, H. Y. Sung, Y. K. Seon, H. M. Lim, H. K. Kim, and S. S. Park, XAGE-1a and XAGE-1d are potential biomarkers of lung squamous cell carcinoma, *Clinica Chimica Acta*, **413**, 1226 (2012).
15. S. Kischkel, W. Miekisch, A. Sawacki, E. M. Straker, P. Trefz, A. Aman, and J. K. Schubert, Breath biomarkers for lung cancer detection and assessment of smoking related effects – confounding variables, influence of normalization and statistical algorithms, *Clin Chim Acta*, **411**, 1637 (2010).
16. A. Ono, T. Naito, I. Ito, R. Watanabe, T. Shukuya, H. Kenmotsu, A. Tsuya, Y. Nakamura, H. Murakami, K. Kaira, T. Takahashi, T. Kameya, T. Nakajima, M. Endo, and N. Yamamoto, Correlations between serial pro-gastrin-releasing peptide and neuron specific enolase levels, and radiological response to treatment and survival of patients with small-cell-lung cancer, *Lung Cancer*, **76**, 439 (2012).
17. Y. Wu, Y. Wu, J. Wang, Z. Yan, L. Qu, B. Xiang, and Y. Zhang, An optimal tumor marker group-coupled artificial network for diagnosis of lung cancer, *Expert Syst. Appl*, **38**, 11329 (2011).
18. T. Hanagiri, M. Sugaya, M. Takenaka, S. Oka, T. Baba, Y. Shigematsu, Y. Nagata, H. Shimokawa, H. Uramoto, M. Takenoyama, K. Yasumoto, and F. Tanaka, Preoperative CYFRA 21-1 and CEA as prognostic factors in patients with stage I non-small cell lung cancer, *Lung Cancer*, **74**, 112 (2011).
19. K. Charalabopoulos, A. Karakosta, G. Bablekos, C. Golias, A. Charalabopoulos, E. Tsanou, D. Peschos, L. Zoganas, and A. Batistatou, CEA Levels in Serum and BAL in Patients Suffering from Lung Cancer, *Medical Oncology*, **24**, 219 (2007).
20. K. Furugaki, T. Iwai, Y. Moriya, N. Harada, and K. Fujimoto-Ouchi, Loss of an EGFR-amplified chromosome7asanovelmechanismof acquired resistance to EGFR-TKIs in EGFR-mutated NSCLC cells, *Lung Cancer*, **83**, 44 (2014).
21. T. K. F. Yung, K. C. Allen Chan, T. S. K. Mok, J. Tong, K. F. To, and Y. M. D. Lo, Single-Molecule Detection of Epidermal Growth Factor Receptor Mutations in Plasma byMicrofluidics Digital PCR in Non Small Cell Lung Cancer Patients, *Clin Cancer Res*, **15**, 2076 (2009).
22. Y. Wang, F. Fang, C. Shi, X. Zhang, L. Liu, J. Li, X. Zhou, J. Yao, and X. Kang, Evaluation of a method for the simultaneous detection of multiple tumor markers using a multiplex suspension bead array, *Clinical Biochemistry*, **45**, 1394 (2012).
23. Z. Zhong, N. Peng, Y. Qing, J. Shan, M. Li, W. Guan, N. Dai, X. Gu, and D. Wang, An electrochemical immunosensor for simultaneous multiplexed detection of neuron-specific enolase and pro-gastrin releasing peptide using liposomes as enhancer, *Electrochimica Acta*, **56**, 5624 (2011).
24. J. Han, Y. Zhou, Y. Q. Chai, Y. L. Yuan, and R. Yuan, Novel electrochemical catalysis as signal amplified strategy for label-free detection of neuron specific enolase, *Biosensors and Bioelectronics*, **31**, 399 (2012).
25. H. Tang, J. Chen, L. Nie, Y. Kuang, and S. Yao, A label-free electrochemical immunoassay for carcinoembryonic antigen (CEA) based on gold nanoparticles (AuNPs) and nonconductive polymer film, *Biosensors and Bioelectronics*, **22**, 1061 (2007).
26. X. Yang, Y. Zhuo, S. Zhu, Y. Luo, Y. Feng, and Y. Xu, Selectively assaying CEA based on a creative strategy of gold nanoparticles enhancing silver nanoclusters' fluorescence, *Biosensors and Bioelectronics*, **64**, 345 (2015).
27. R. Elshafey, A. C. Tavares, M. Siaj, and M. Zourob, Electrochemical impedance immunosensor based on gold nanoparticles-protein G for the detection of cancer marker epidermal growth factor receptor in human plasma and brain tissue, *Biosensors and Bioelectronics*, **50**, 143 (2013).
28. J. Gao, Z. Guo, F. Su, L. Gao, X. Pang, W. Cao, B. Du, and Q. Wei, Ultrasensitive electrochemical immunoassay for CEA through host–guest interaction of β-cyclodextrin functionalized graphene and Cu@Ag core–shell nanoparticles with adamantine-modified antibody, *Biosensors and Bioelectronics*, **63**, 465 (2015).
29. A. Ilyas, W. Asghar, P. B. Allen, H. Duhon, A. D. Ellington, and S. M. Iqbal, Electrical detection of cancer biomarker using aptamers with nanogap break-junctions, *Nanotechnology*, **23**, 275502, 1 (2012).
30. S. S. Kallempudi, Z. Altintas, J. H. Niazi, and Y. Gurbuz, A new microfluidics system with a hand-operated, on-chip actuator for immunosensor applications, *Sensors and Actuators B: Chemical*, **163**, 194 (2012).
31. S. Cheng, S. Hideshima, S. Kuroiwa, T. Nakanishi, and T. Osaka, Label-free detection of tumor markers using field effect transistor (FET)-based biosensors for lung cancer diagnosis, *Sensors and Actuators B*, **212**, 329 (2015).
32. A. Vasudev, A. Kaushik, and S. Bhansali, Electrochemical immunosensor for label free epidermal growth factor receptor (EGFR) detection, *Biosensors and Bioelectronics*, **39**, 300 (2013).
33. R. I. Stefan-van Staden, I. R. Comnea-Stancu, C. C. Surdu-Bob, and C. Stanciu-Gavan, Pattern recognition of neuron specific enolase and carcinoembryonic antigen in whole blood samples, *J. Mol. Recognit.*, **28**, 103 (2015).
34. I. R. Comnea-Stancu, R. I. Stefan-van Staden, J. F. van Staden, and C. Stanciu-Gavan, Stochastic sensors based on maltodextrins for screening of whole blood for neuron specific enolase, carcinoembryonic antigen and epidermal growth factor receptor, *Microsystem Technologies*, **22**, 25 (2016).
35. R. I. Stefan-van Staden, I. Moldoveanu, and C. Stanciu-Gavan, Pattern recognition of HER-1 in biological fluids using stochastic sensing, *J Enzyme Inhib Med Chem.*, **30**, 283 (2014).
36. R. I. Stefan-van Staden, I. Moldoveanu, C. C. Surdu-Bob, and C. Stanciu Gavan, Engineered nanoporous gold microspheres for stochastic sensing, *RSC Advances*, **4**, 54140 (2014).
37. R. I. Stefan-van Staden, I. R. Comnea-Stancu, C. C. Surdu-Bob, and M. Badulescu, Nanostructured materials detect epidermal growth factor receptor, neuron specific enolase and carcinoembryonic antigen, *Nanoscale*, **7**, 15689 (2015).