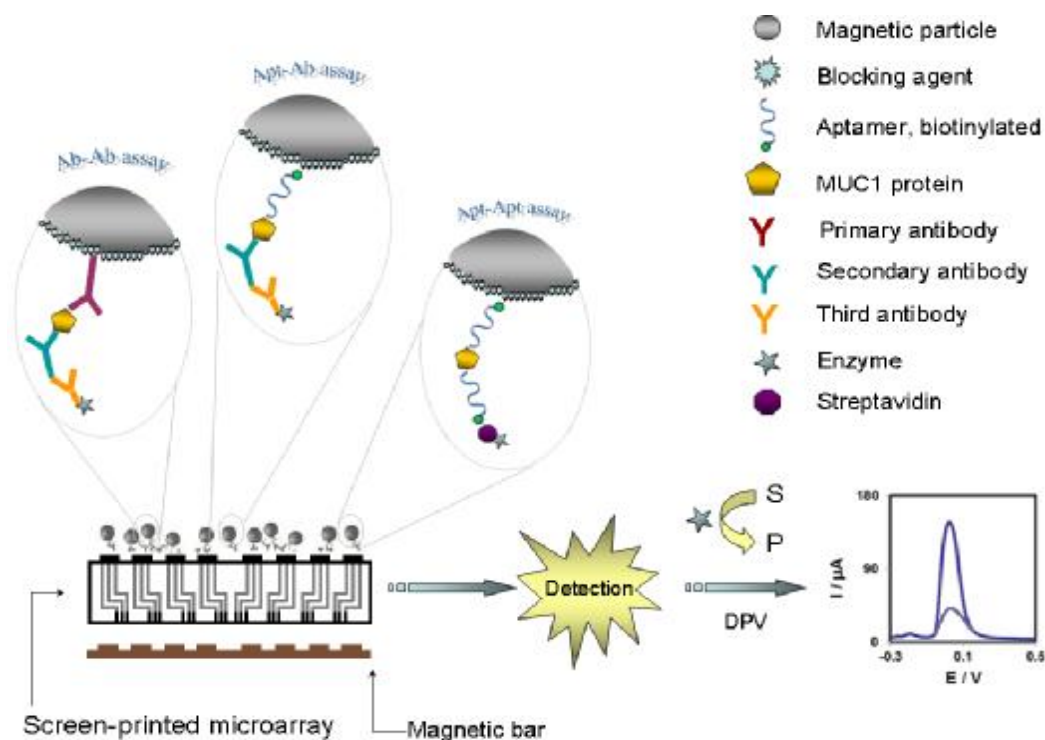


## RESULTS

Different approaches for the immunosensors development are presented

### 1. Sandwich type immunosensors using magnetic particles modified with protein G as immobilization platform and HRP as label



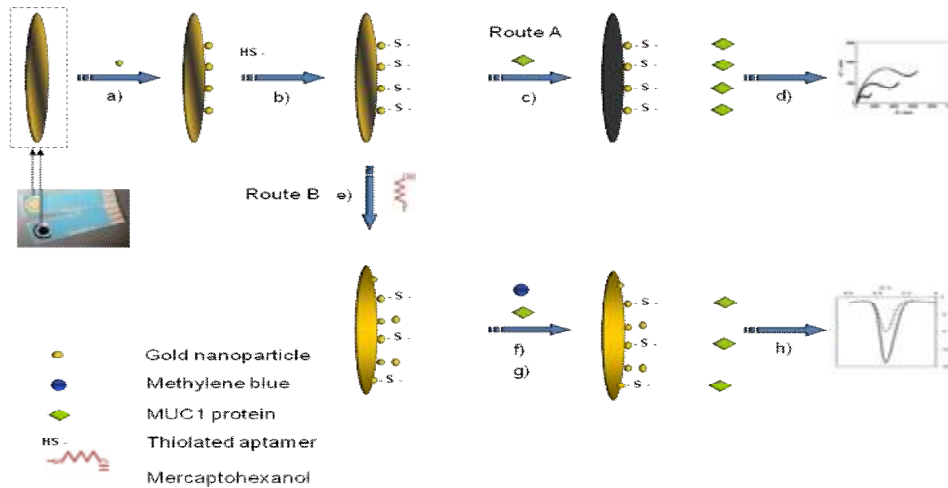
The present immunoassays present the following analytical characteristic:

Type of assay	Linear range (ppb)	LOD (ppb)	R <sup>2</sup>	Average RSD %
Antibody-Antibody	0-10 (AP)	0.69 (AP)	0.89	7
	0-10(HRP)	0.62(HRP)	0.98	7
Aptamer-Antibody	0-10	1.63	0.94	11
Aptamer-Aptamer	0-10	2.10	0.97	8

Four immunosensors were developed for the detection of cancer biomarker MUC1 using antibody and aptamer modified magnetic beads. The optimization of experimental conditions and the analytical parameters were evaluated. The assays showed good sensitivity. Aptamer-

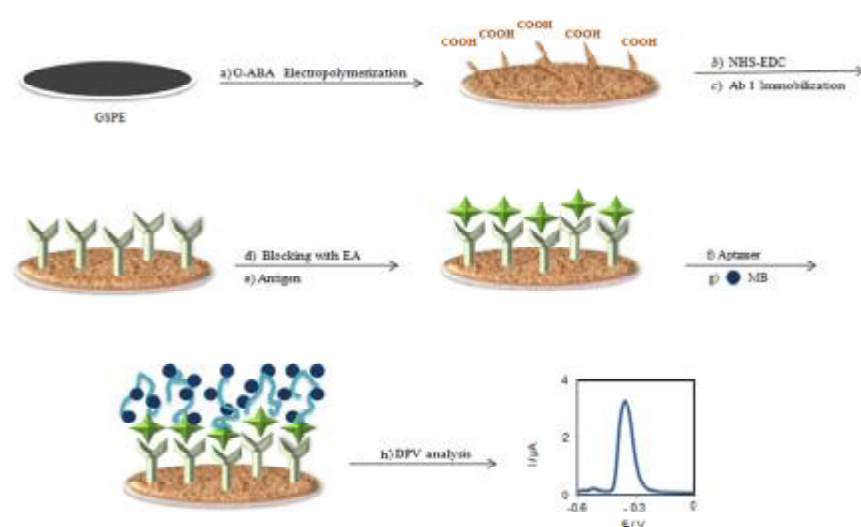
based assays showed higher selectivity for MUC1 protein and higher sensitivity in serum samples. The aptamer-based assay is suitable for the analysis of serum samples.

## 2. Label free immunosensors using gold nanoparticles as immobilization platform:



Two simple and sensitive strategies for the electrochemical detection of MUC1 protein using aptamer and Au NPs were introduced. Loosely packed aptamer were self-assembled onto SPEs surface modified with Au NPs. The interaction between aptamer and MUC1 protein was investigated by CV, EIS and DPV techniques. The estimated detection limits of the MUC1 protein are  $3.6 \text{ ng mL}^{-1}$  at AuNPs-modified graphite SPE by EIS and  $0.95 \text{ ng mL}^{-1}$  at AuNPs-modified gold SPE by DPV methods. The results demonstrate that the electrochemical method using aptamers immobilized on Au NPs is convenient, and allows quantitative detection of human MUC1 protein, providing promising perspectives for future clinical applications.

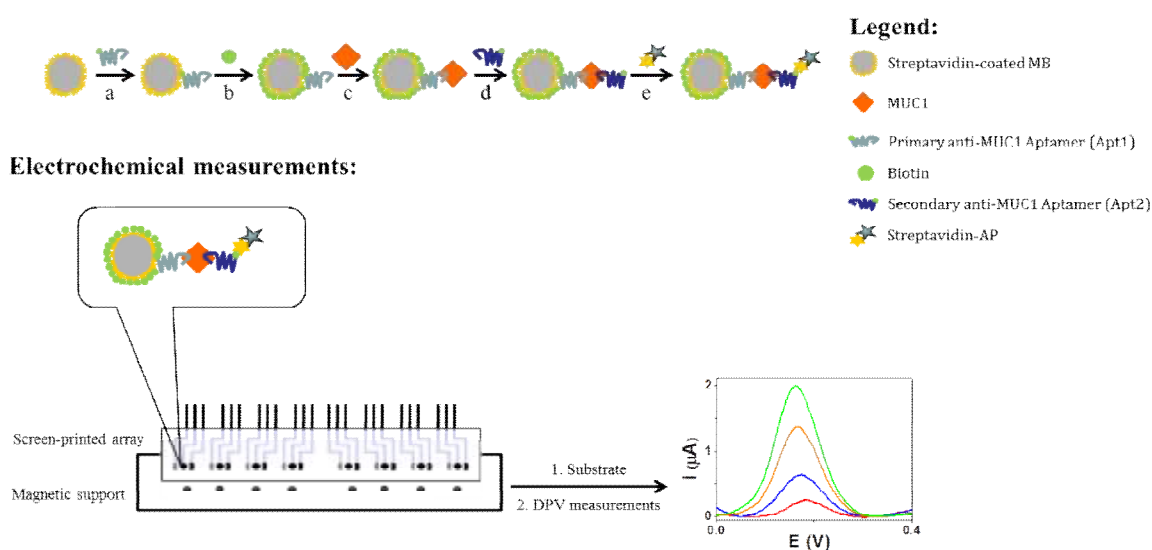
## 3. Immunosensor with Aptamer and Functionalized Polymer



The aptamer based biosensor was designed by electropolymerization of *o*-aminobenzoic acid (*o*-ABA) conductive polymer on the surface of graphite based SPEs. The MUC1 monoclonal mouse antibody was subsequently attached on the electrode surface through covalent bonding

between the antibody and the carboxyl groups from poly(*o*-ABA). The immunoreaction between MUC1 protein and antibody was carried out, followed by immobilization of aptamers, by using MB as an electrochemical indicator. It is known that MB is reduced to a leucomethylene blue at the electrode surface by accepting two electrons. MB binds to the aptamer and to the proteins. The calibration curve for the determination of MUC1 protein was obtained with both CV and DPV measurements under optimized conditions. The aptasensor based on a conducting polymer electrode system was sensitive to concentrations of MUC1 protein down to 0.62 ppb, in a linear range between 1-12 ppb, by using DPV method.

#### 4. Studies on real samples by using the aptasensors



The target molecule is captured between the primary antibody or aptamer immobilized on the magnetic beads and a secondary antibody or aptamer. The affinity reaction is labelled with Alkaline Phosphatase (AP) in different assay configurations. All variables concerning the bioassays were optimized. The electrochemical multidetection is achieved using eight screen-printed cells, by differential pulse voltammetry (DPV), through the addition of the enzymatic substrate (1-naphthyl phosphate) and its subsequent conversion to the electrochemical active compound 1-naphthol.

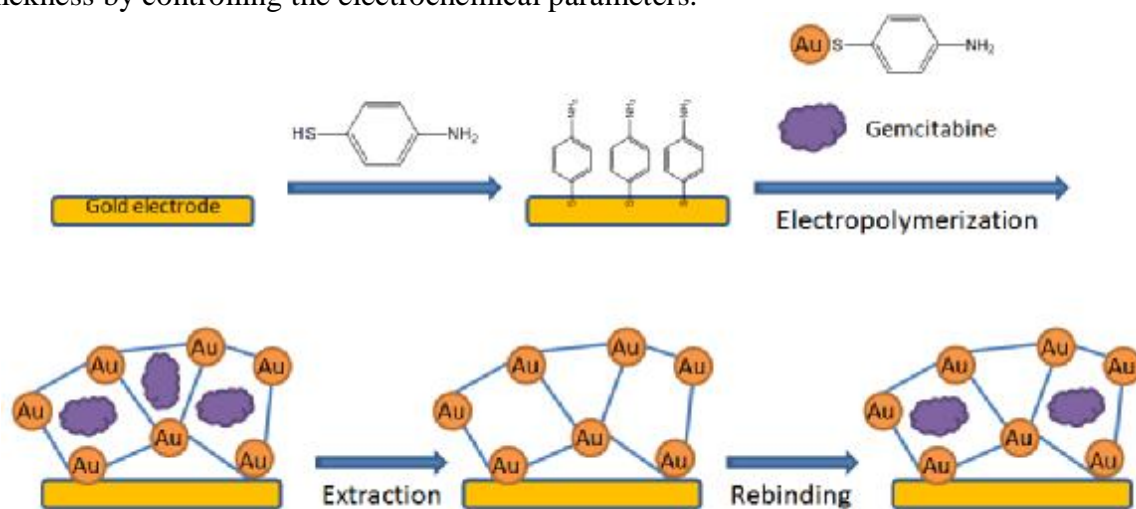
The proposed aptamer-based assay showed better sensitivity and higher sensibility for detection of MUC1 cancer biomarker respect to antibody-based assay. In particular, in optimized conditions, a linear calibration curve of MUC1 buffered solutions was obtained in a range from 0 to 10 ng/mL with detection limit ( $LOD = 3S_{blank}/Slope$ ) of 1.4 ng/mL (corresponding to the range from 0 to 0.28 nM,  $LOD = 0.037$  nM), well below the detection limits reported by other aptasensors present in literature.

#### 5. MIP based sensors for gemcitabine detection

Establishing personalized therapeutic schemes depending on the treatment response of each patient is extremely important for good chemotherapy management. Gemcitabine (GMT), chemically called 4-amino-1-(2-deoxy-2,2-difluoro- $\beta$ -D-erythro-pentofuranosyl) pyrimidin-2(1*H*)-on, is a nucleoside analog used for the treatment of various carcinomas, such as non-small cell lung cancer, pancreatic cancer, metastatic breast cancer and advanced recurrent ovarian cancer. Monitoring the concentration of antineoplastic drugs is important for the optimization of therapy and management of side effects, as at too low doses the effects can be

reduced or even lost, while at too high dosage side effects and toxicity can occur. Therefore, the determination of GMT in biological samples and pharmaceuticals is of paramount importance.

The molecular imprinting technique has been widely used in recent years for the preparation of polymeric materials with special molecular recognition abilities. The method consists in polymerizing functional monomers in the presence of template molecules and subsequently extracting the template from the resulting polymer matrix, which generates cavities complementary in shape and size with the template. A material with specific recognition properties towards template molecules is thus obtained, with the advantages of physical and chemical stability, ease of preparation, low cost, and possibility of use in harsh environmental conditions. The complex preparation process and the low-rate mass transfer and poor site accessibility associated with the conventional bulk method for the preparation of molecularly imprinted polymers (MIP) has focused attention on the electropolymerization technique for the deposition of imprinted films. Electrodeposition of polymers consists in the adsorption of an electropolymerized material at the electrode surface and, unlike bulk polymerization, eliminates the need for the rigorous synthesis and film preparation typically required by spin- or solvent-casting techniques. Moreover it has the advantage of ease of control over the film thickness by controlling the electrochemical parameters.



The peak current density ratio (signal-blank)/blank versus logarithm function of GMT concentration exhibited a linear response over a range from 3.8 fM to 38 nM, with a limit of detection of 3 fM. The limit of detection was calculated by interpolating the mean current obtained for the blank for three replicates plus three times its standard deviation in the linear equation. Blank is considered to be the signal obtained after extraction of the template, upon analysis of a solution containing 10 mM [Fe(CN)<sub>6</sub>]<sup>-3/-4</sup> in PBS pH 7.2 that does not contain any GMT.

### 1. Analysis in real samples

Several configurations of immuno/biosensors were tested on real samples (serum) for MUC1 and MUC 4 detection as well as for the detection of gemcitabine (an antineoplastic agent used in ovarian and breast cancer treatment).

Once verified the suitability of the aptamer assay to detect MUC1 in standard solutions, preliminary experiments using normal human serum samples were carried out. With this aim, batches of non-pathologic serum samples were spiked with MUC1 in order to have different final concentrations in the range from 0–1.4 nM. Thus, each spiked sample was filtered and then diluted 1:200 before incubation with functionalized beads in order to reduce the matrix effect. The obtained results showed an increase of the current between serum alone and serum

added with different concentration of MUC1. The serum samples of different pathological conditions were analyzed. The results are shown in Table 1 from 1 to 4 samples. A higher MUC1 concentration in the sample 3 respect to others was detected. This could be due to the fact that both ovaries are affected by the tumor. Moreover, the MUC1 lowest value obtained for sample 4 is related to the presence of a non-malignant pathology. The serum samples shown in the Table 1 from 5 to 9 are obtained from patients affected by invasive ductal breast carcinoma.

**Table 1.** MUC1 determination in cancer serum samples from cancer patients performed with aptamer-based bioassay. Results were referred to undiluted cancer serum samples.

Serum numbers	Histological Diagnosis	MUC1 concentration ( $\mu\text{g/mL}$ )
1	Left ovarian carcinoma	34 $\pm$ 3
2	Right ovarian tumor	55 $\pm$ 3
3	Bilateral ovarian carcinoma	100 $\pm$ 7
4	Right ovarian cyst	5.2 $\pm$ 0.3
5	Invasive ductal breast carcinoma	24 $\pm$ 1
6	Invasive ductal breast carcinoma	128 $\pm$ 5
7	Invasive ductal breast carcinoma	21 $\pm$ 1
8	Invasive ductal breast carcinoma	47 $\pm$ 2
9	Invasive ductal breast carcinoma	40 $\pm$ 2
10	Non pathological serum	1.60 $\pm$ 0.08

The samples were characterized by the Nottingham Prognostic Index (NPI), which is used to determine the prognosis following surgery for breast cancer. NPI is calculated using three criteria: the size of the lesion, the number of involved lymph nodes and the grade of tumor. A 93% 5-year survival rate is associated with score 2, whereas for score 3 the 5-year survival rate is 85%. Comparable MUC1 concentrations were obtained in the samples 5 and 7 corresponding to serum samples of patients with 2 NPI value. On the contrary, a higher MUC1 concentration could be detected in the samples 6, 8 and 9 with 3 NPI value. An increase of CV values, respect to standard MUC1 solutions assay, were found. This fact can be addressed to the variability of the matrix due to the other physiological or pathological patients conditions that can influence MUC1 serum over-expression.

***Highly sensitive molecularly imprinted electrochemical sensor based on an electropolymerized microporous metal organic framework for detection of gemcitabine***

To further demonstrate its potential for practical applications, the newly developed sensor was tested for the detection of gemcitabine in spiked serum samples and drug formulations.

For the analysis of serum samples, commercially available calf serum (Sigma-Aldrich) was diluted 1:1000 and filtered through a cellulose membrane (Millex-GV, 0.22  $\mu\text{M}$ ) in order to reduce the matrix effect. The samples were then spiked with various concentrations of GMT

and analyzed according to the procedure previously described. The results are shown in Table II. Good recoveries, ranging between 96.38% and 102.03%, were obtained, with good reproducibility, validating the practicability of the developed sensor for biological fluids. In addition, this novel sensor was applied to the detection of GMT in a pharmaceutical formulation. Gemcitabine TEVA 1000mg, a powder for infusion solution, was assayed with the developed sensor. After dissolution of the powder in water according to the labeling, the concentration of GMT in the solution was 38mg/ml, so it was diluted to reach the linear range of the sensor. The standard addition method was used, and then the concentration of GMT found after the analysis with the proposed sensor was 36.89 mg/ml, with an RSD of 1.34%.

**Table II.** Detection of GMT in commercial serum samples by MIP sensor

Added-log [C] (M)	Found -log [C] (M)	Recovery %	RSD% (n=3)
14	14.19	101.40	2.37
12	11.56	96.38	5.72
10	10.20	102.03	3.28

#### ***Immunosensor for MUC4 detection by using aryldiazonium salt electrochemistry***

*p*-Aminophenylacetic acid was used as aryl diazonium substrate and was electrochemically immobilized on the carbon based screen printed electrodes by chronoamperometry in 0.5 M chloride acid solution. Electrochemical impedance spectroscopy and cyclic voltammetry were used in order to characterize and optimize the electrografting process. The optimized platform was further employed for the development of an electrochemical immunosensor and applied for the detection of Mucin4 (MUC4) protein, a useful biomarker for endometriosis, pancreatic, esophageal and breast cancer.

The in situ surface modification through diazotation technique enables the covalent binding of the antiMUC4 antibody (AB) within the amidic interactions which is then followed by the affinity reaction with the MUC4 antigen. The immunoassay was optimized with respect to several parameters, such as diazonium salt concentration and electrografting conditions (method, duration, electrode type, and carboxyl activation), the concentration and incubation time of blocking agent, monoclonal antibody, and antigen. The performance of the impedimetric immunoassay was also studied.

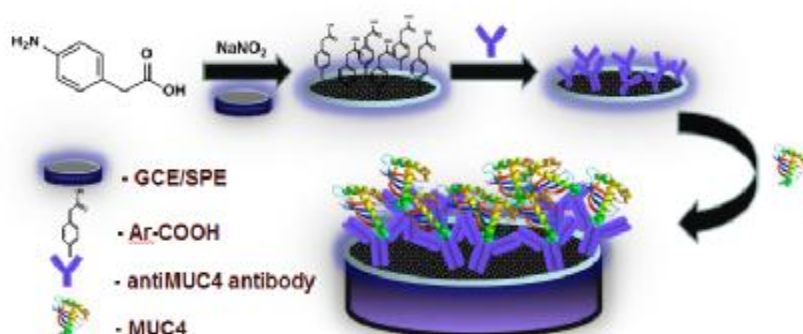


Fig.1. Immunosensor design



The electrochemical label-free immunosensor was electrochemically characterized and optimized by using cyclic voltammetry and electrochemical impedance spectroscopy. A LOD of 330 pg  $\mu\text{L}^{-1}$  was calculated based on the calibration data and the performances of the immunoassay will be further tested on real samples.

## Disemination

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5. Mihaela Tertiş, Oana Hosu, Luminița Fritea, Cosmin Farcău, Andreea Cernat, Robert Săndulescu, Cecilia Cristea, A Novel Label-free Immunosensor Based on Activated Graphene Oxide for Acetaminophen Detection, *Electroanalysis*, 27(3) (2015) 638-647 (IF 2,502);
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8. Anca Florea, Cecilia Cristea, Robert Sandulescu, MUC1 tumor marker for the detection of ovarian cancer. A minireview, *Farmacologia*, 62(1), **2014**, p. 1-13, (IF 1,251)
9. Andrea Ravalli, Giovanna Marrazza, Anca Florea, Cecilia Cristea, Robert Săndulescu, Electrochemical immunoassay for mucin 1 detection as diagnostic tool in ovarian cancer, *Sensors and Microsystems in Lecture Notes in Electrical Engineering*, Volume 268, pp 165-168, **2014**, Ed. Springer, DOI 10.1007/978-3-319-00684-0\_31, ISBN 978-3-319-00683-3.
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2. O. Hosu, M. Tertiş, G. Melinte, R. Săndulescu, C. Cristea. Mucin4 immunosensor based on p-aminophenylacetic acid grafting on carbon electrodes as immobilization platform, *Procedia Technology special issues for Biosensors 2016 Conference. Submitted*
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### **Book Chapter**

R. Săndulescu, C. Cristea, E. Bodoki, R. Oprean, *Chapter: Recent advances in the analysis of bioactive compounds based on molecular recognition, in Frontier in Bioactive Compounds*, ed. Bentham Science Publishers. *In press*



## PhD Thesis

**Anca Ștefana Florea**, Electrochemical affinity sensors for biomedical, food and environmental applications, Joint PhD thesis with public defense in 14th of September 2015 at „Iuliu Hațieganu” U.M.Ph. Cluj-Napoca (prof. dr. Robert Săndulescu „Iuliu Hațieganu” U.M.Ph. Cluj-Napoca and prof. Nicole Jaffrezic-Renault, Université Claude Bernard Lyon 1, France)

## Participation in Symposium and Conferences with oral (o) and poster (p) presentations

1. Cecilia Cristea, Anca Florea, Robert Sandulescu, Carbon nanotubes modified screen printed electrodes for the biosensors development, 3th Regional Symposium on Electrochemistry, RSE-SEE 2012, 22-28 mai Bucuresti, Romania (p)
2. Cecilia Cristea, Veronica Sima, Anca Florea, Robert Sandulescu, Nouveaux biocapteurs pour l'analyse des médicaments, Atelier scientifique Nomares, 22 mai 2012, Bucuresti, Romania (o)
3. Cristea, Anca Florea, Robert Săndulescu, Screen-Printed Electrodes Modified with Carbon Nanotubes for the Quantification of Acetaminophen, 63th Annual Meeting of the International Society of Electrochemistry, august, Prague, Cehia (p)
4. Anca Florea, Cecilia Cristea, Andrea Ravalli, Giovanna Marrazza, Robert Săndulescu, New Electrochemical Sandwich Assay for the MUC 1 Detection, 63th Annual Meeting of the International Society of Electrochemistry, august, Prague, Cehia (p)
5. Anca Florea, Zahra Taleat, Cecilia Cristea, Mohammad Mazloum Ardakani, Giovanna Marrazza, Robert Săndulescu, Magnetic beads-base electrochemical immunosensor for the detection of MUC 1 cancer biomarker, Workshop Vinca, Beograd, Serbia (o)
6. Anca Florea, Andrea Ravalli, Cecilia Cristea, Giovanna Marrazza, Robert Săndulescu Electrochemical immunoassay for the detection of mucins cancer biomarkers, 4th Regional Symposium on Electrochemistry, RSE-SEE 2013, 26-30 May 2013, Ljubljana (o)
7. Zahra Taleat, Cecilia Cristea, Mohammad Mazloum Ardakani , Giovanna Marrazza, Robert Săndulescu, An Electrochemical Immunoassay Based on Aptamer- Protein Interaction and Functionalized polymer and Its Application in Cancer Biomarker Detection, 12th Topical Meeting of the International Society of Electrochemistry in Bochum, Germany, 17-21 March, 2013 (p)
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9. Zahra Taleat, Cecilia Cristea, Mohammad Mazloum Ardakani, Giovanna Marrazza, Robert Săndulescu, An Electrochemical Immunoassay Based on Aptamer- Protein Interaction and Functionalized polymer and Its Application in Cancer Biomarker Detection, Journées d'électrochimie, 8-12 iulie 2013, Paris, France (p)
10. B. Feier, D. Floner, C. Cristea, R. Sandulescu, F. Geneste, Modified Graphite Felt for the Detection of Copper (II), Summer School in Electrochemistry 2013, 17-22 June 2013, Cluj-Napoca Romania (poster)
11. Cecilia Cristea, Anca Florea, Ramona Galatus, Ede Bodoki, Robert Sandulescu, Dorin Petreus, Innovative immunosensors for early stage cancer diagnosis and therapy monitoring, IFMBE International Conference on Health informatics ICHI, 3-5 november 2013, Vilamoura, Portugal (poster)
12. Anca Florea, Mihaela Tertis, Robert Sandulescu, Alexandru Cristea, Cecilia Cristea', Designing polymer-based immunosensing platforms for cancer biomarker detection,

- The 4<sup>th</sup> IEEE International Conference on E-Health and Bioengineering - EHB 2013, Grigore T. Popa University of Medicine and Pharmacy, Iași, Romania, November 21-23, 2013 (comunicare orală)
13. Anca Florea, Mihaela Tertis, Zahra Taleat, Robert Sandulescu, Cecilia Cristea, Electrochemical sensors for the detection of mucines tumor markers for cancer diagnosis, SPQ-Analitica 2014, 14-15 April 2014, Coimbra, Portugal, (comunicare orală)
  14. L. Fritea, A. Florea, M. Tertiş, A. Cristea, R. Săndulescu, C. Cristea, Polymer based nanostructures for innovative bio and immunosensors development, MediTech 2014, Advancements of Medicine and Health Care through Technology, June 5-7, 2014, Cluj-Napoca, Romania (comunicare orală)
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  16. Luminița Fritea, Mihaela Tertiş, Cecilia Cristea, Robert Săndulescu,  $\beta$ -cyclodextrin/polyethyleneimine film modified glassy carbon electrodes for the detection of some pharmaceuticals, 10th International Symposium on Drug Analysis, 25th International Symposium on Pharmaceutical and Biomedical Analysis, Liege, Belgium - June 22-25, 2014 (poster)
  17. Luminița Fritea, Mihaela Tertiş, Robert Săndulescu, Cecilia Cristea, Nanomaterial Platforms for Biosensor Design Applied in Pharmaceutical Analysis, International conference on electrochemical sensors Matrafured 2014, 15-20 June 2014, Visegrad, Hungary (poster)
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  20. Cecilia Cristea, Mihaela Tertis, Luminița Fritea, Anca Florea, Oana Hossu, Robert Săndulescu, Nanostructured platforms with Different types of Polymers for Biosensors Development, 65<sup>th</sup> Annual meeting of the International Society of Electrochemistry, 31 August - 5 September, 2014, Lausanne, Switzerland (poster)
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  22. Cecilia Cristea, Mihaela Tertiş, Oana Hosu, Luminița Fritea, Robert Săndulescu, Innovative carbon-based nanoplatfoms for biosensing design, 5<sup>th</sup> Regional Symposium on Electrochemistry-RSE-SEE, 7-11 Iunie 2015, Rio Pravets, Sofia, Bulgaria (keynote lecture);
  23. Anca Florea, Minh Huy Do, Trinh-Thi Nhu-Trang, Cecilia Cristea, Robert Săndulescu, Nicole Jaffrezic-Renault, Molecularly Imprinted Electrochemical Sensor for the Sensitive Detection of Pesticides, XXIII International Symposium on

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  26. Anca Florea, Cecilia Cristea, Robert Săndulescu, Francis Vocanson, Nicole Jaffrezic-Renault, Antineoplastic Drug Detection by Molecular Imprinting Sensor based on Electropolymerization of Microporous-Metal-Organic Framework, Journées d'Electrochimie 2015, 6-10 Juillet 2015 Roma, Italia (poster)
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