ORIGINAL ARTICLE

Impedance spectroscopy in biodynamics: Detection of specific cells (pathogens) using immune coated electrodes

Eugen Gheorghiu[1], Daniel Andreescu[2], Mariana Oporanu[3], Mihaela Gheorghiu[4], Sorin Cazacu[5], Corina Balut[6] and Alexandru Ursu[7]

Abstract

Gheorghiu, E., Andreescu, D., Oporanu, M., Gheorghiu, M., Cazacu, S., Balut, C. and Ursu, A.
Impedance spectroscopy in biodynamics: Detection of specific cells (pathogens) using immune coated electrodes
Songklanakarin J. Sci. Technol., 2002, 24(Suppl.) : 791-797

We describe the theoretical and experimental approaches for monitoring the interfacial biomolecular reaction between immobilized antibody and the antigen binding partner (the analyte, or the targeted cell) using Impedance Spectroscopy, IS. The key idea is to reveal the presence of the analyte by investigating the dynamics of the impedance changes at the interface between transducer and bulk during the process of antibody-antigen binding (coupling of specific compounds to sensor surface).

In this work, antibody-antigen (Ab-Ag) reaction was directly monitored using an impedance analyzer capable of ~ 1 measurement/second and covalent immobilization chemistry and modified electrodes in the absence of a redox probe.

The proposed approach may be applicable to monitoring other surface interfacial reactions such as protein-protein interactions, DNA-DNA interactions, DNA-protein interactions and DNA-small molecule interactions.

Key words : immunosensors, detection, bioaffinity, biodynamics, impedance spectroscopy

[1]Ph.D., Prof., Director, [2]Ph.D. (Analytical Chemistry), [4]Ph.D. (Biophysics), Senior Researcher, [5]M.Sc. (Analytical Chemistry), Junior Researcher, [6]Ph.D. (Biophysics), Senior Researcher [7]M.Sc. (Physics), Junior Researcher, International Center of Biodynamics, Bucharest 1, [3]Ph.D. (Biochemistry), Pasteur Institute, Bucharest 3, Romania
Corresponding e-mail : egheorghiu@biodyn.ro
Received, 13 December 2002 Accepted, 1 May 2003

Methodology

In recent years, different conceptual approaches have been reported for the realization of bioaffinity sensors, which are capable of detecting the specific reaction between a receptor and its ligand. The affinity of a ligand (present in solution) to an array of many different immobilized receptors (e.g. biosensors) is a potentially promising analytical tool. These biosensors are capable of detecting a single (prohibited) analyte, the complementary binding partner, such as those found in food, clinical, environmental and biological agents of mass destruction. In these biosensors, heterostructures (such as antigen, antibody, cells, DNA, enzyme-layers, and semiconductors/ thin silica layer) are immobilized at the surface of a solid electrode. The binding of the reacting partner is then verified through the detection of either a shift in impedance, or change in capacitance or admittance at the bulk of the electrode interface through impedance spectroscopy (IS). A key step of this sensing protocol is the immobilization of the bioaffinity reagents onto suitable substrates. The technique for attaching reagents to the substrates includes covalent attachment and bifunctional linkage to the adjunct layers of the polymers. The binding of the reacting partner is then verified through the detection of either a shift in impedance, or change in capacitance or admittance at the interface bulk-electrode. Impedance spectroscopy provides a rapid approach for monitoring the dynamics of biomolecular interactions and it can be used to predict important aspects of biosensors including surface reactivity, surface loading, binding constants and rates of reaction. Major fundamental issues still exist due to possible ambiguities associated with data interpretation, and the complication of analyses based on the equivalent circuits. Therefore, despite the potential advantages of using IS to study biomolecular reactions, very few of such practical biosensing systems are currently available. IS has been extensively used for biomass monitoring, as well as to study cells that are arbitrarily shaped, and to simulate the evolution of the suspension of initially synchronized budding yeast (Vranceanu & Gheorghiu 1996, Gheorghiu 1998).

Advancing recent studies on the use of IS to study Ab-Ag interactions at conducting polymer-modified electrodes (Gheorghiu, Sadik *et al.* 2002) this research aims at: (i) developing and testing immune modified electrodes against chosen pathogens, and (ii) monitoring the dynamics of the biomolecular reaction using a (differential) low frequency impedance measurement system.

Theory

Impedance measurements of the modified electrodes in a differential arrangement offers as the main advantage, in comparison with the single channel approach high amplification capabilities and enhanced signal to noise ratios by eliminating influences of the background - temperature and electrolyte composition, or nonspecific coupling and compensation of electrode/bulk impedance effects. From a basic electrical scheme, with impedance elements within the interface in parallel, the equivalent impedance of a probe, Z_{probe} is given by:

$$Z_{probe}(t) = Z_{electrode} + Z_{interface}(t) + Z_{bulk} \; ;$$

$$Z_{probe}(0) = Z_{electrode} + \frac{Z_0}{N} + Z_{bulk} \qquad (1)$$

where ***N* is the number of possible binding sites**. Assuming no nonspecific coupling, ***N*** corresponds to the amount of antibody Ab present at the electrode surface. The term $Z_{electrode}$ in equation 1 represents the impedance of the sensor that is not affected by the Ag-Ab coupling. It consists in the impedance of the electrode, polypyrrole layer and of entrapped Ab not affected by Ag coupling. Let us consider a reference (blank) and an active sensor (where the Ab-Ag reaction takes place) both exhibiting similar values of the $Z_{electrode}$. In the differential arrangement, the major contribution is given by the $Z_{interface}$, i.e. the impedance of the electrode–bulk interface that "hosts" the immobilized Ab reagent. We assume that the voltage drop across the interface is the same all

over the surface of the working electrode.

During the process of Ab-Ag interaction, we consider that the impedance, Z_1, of any newly bound antigen, replaces the impedance, Z_0, of the electrolyte that has previously occupied that locus. The basic assumption of our theory consists in the existence of a relation (the same whenever the same immuno sensor is used) between the admittance (impedance) of the coupled Ag or Ac and the admittance (impedance) of the bulk that has been withdrawn from the interface. In the particular case of a one to one binding scheme, for **N_1, the number of active Ab present at the surface of the working electrode**, and n(t) denoting the number of Ag bounded at time t, the impedance, $Z_{interface}$ and admittance, $Y_{interface}$ of the interface, are given by:

$$\frac{1}{Z_{\text{interface}}(t)} = \frac{n(t)}{Z_1} + \frac{N_1 - n(t)}{Z_0} \Leftrightarrow Y_{interface}(t)$$
$$= n(t)Y_1 + (N_1 - n(t))Y_0$$
$$dY_{interface}(t) = dn(Y_1 - Y_0) \qquad (2)$$

Therefore, the larger the real and imaginary components of the difference of $Y_1 - Y_0$, the better the sensitivity of this method. Assuming the following time dependence:

$$\frac{dn}{dt} = K(N_1 - n(t)) \Rightarrow n(t) = N_1\left(1 - e^{-K \cdot t}\right)$$
$$\text{if, } K = \frac{N_V - n(t)}{V} \Rightarrow n(t) =$$
$$N_1 = \frac{1 - e^{\frac{N_V - N_1}{V} \cdot t}}{1 - \frac{N_1}{N_V \cdot e^{\frac{N_V - N_1}{V} \cdot t}}} \qquad (3)$$

where **N_V/V is the volume concentration of antigens in solution**, one can derive the dynamics of the process of binding in relation to the concentrations of Ag/Ab in the experimental set-up. When a differential measurement of the working electrode (WE) versus a blank electrode (BK) is carried out, the relative, differential admittance is given by:

$$\Delta Y = \frac{Y_{BK}(t)}{Y_{WE}(t)} - 1 = \frac{Z_{WE}(t) - Z_{BK}(t)}{Z_{BK}(t)}$$
$$= \frac{Y_0}{Y_1} \cdot \frac{1}{n(t)/N_1 + \left(1 - n(t)/N_1\right) \cdot \frac{Y_0}{Y_1}} - 1 \qquad (4)$$

$$\frac{Z_1}{Z_0} = \frac{Y_0}{Y_1} = \frac{\frac{1}{R_0} + I \cdot w \cdot C_0}{\frac{1}{R_1} + I \cdot w \cdot C_1} \Rightarrow \frac{Y_0}{Y_1}$$
$$= \frac{\frac{1}{R_0 \cdot R_1} + w^2 \cdot C_0 \cdot C_1 + I \cdot w \cdot \left(\frac{C_0}{R_1} + \frac{C_1}{R_0}\right)}{\left(\frac{1}{R_1^2} + (w \cdot C_1)^2\right)} \qquad (5)$$

Consequently, the progress of the Ab-Ag reaction can be revealed directly by the relative, differential admittance. Rearranging Equation 4 one obtains:

$$\frac{n(t)}{N_1} = \frac{\Delta Y(t)}{(1 + \Delta Y(t)) \cdot \left(1 - Y_1/Y_0\right)} \qquad (6)$$

where ΔY is the measured data and Y_0 and Y_1 are the admittances of the bulk electrolyte and the related, newly bound Ag respectively. The ratio Y_1/Y_0 depends on the chosen pair Ab-Ag, as well on the entrapment procedure (providing different orientations of the active sites of the Ab). One should expect different values of Y_1/Y_0 for Ag with different molecular weights (e.g. *IgG* and *Escherichia coli*). The left hand side of Equation 6 represents the ratio of bounded Ag versus surface-bound Ab, while right hand side comprises an expression, ΔY depending merely on measured admittance values provided by EIS. The sign of ΔY is related to the ratio Y_1/Y_0 whether below or above unity.

Figure 1 represents the time series of the impedance changes in relation with the amount of (added) antigen provided by equation 4 assuming the time dependency given in equation 3.

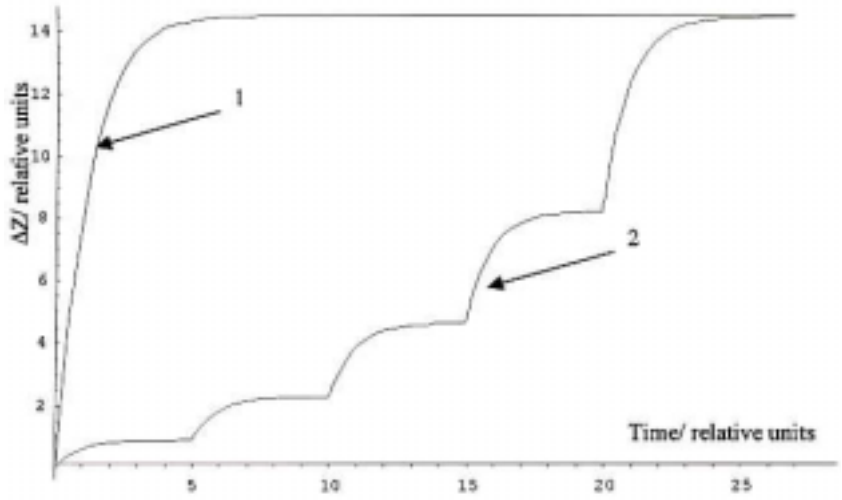

Figure 1. Evolution of impedance changes in relation with the amount of (added) antigen provided by equation 4 assuming the time dependency given in equation 3; Curve 1: the entire amount is added at start and Curve 2: the antigen is subsequently added

Experimental protocol

Reagents

The supporting electrolyte was phosphate buffer saline solution (PBS) containing 0.067 M KH_2PO_4 (Chimopar S.A.), 0.067 M Na_2HPO_4 $12H_2O$ (Chimopar S.A.) and 150 mM NaCl (Chimopar S.A.). The pH of solution at 26 °C was 7.4.

The antibodies used were anti-Salmonella (0.07 mg/ml) and anti-Escherichia coli (0.06 mg/ml) produced by Pasteur Institute – Bucharest, Romania. *Salmonella typhimurium* and *Escherichia coli* stock cultures were also obtained from Pasteur Institute.

1-(2-cyanoethyl)pyrrole (99%), acetonitrile (99%) and $LiClO_4$ were purchased from Aldrich. Glycerol (99%) used for saturating the non-specific sites was obtained from Sigma.

Electrochemical set-up

Galvanostatical electropolymerization process has been accomplished in a three-electrode cell consisting of a platinum or gold wire as working electrode (area 0.1 cm^2), saturated calomel electrode (SCE) as reference electrode and a platinum sheet (in cilindrical form) as the auxiliary electrode. Cell volume was 5 ml. Electropoly-merization has been carried out using a galvano-stat (and related software) designed and produced by the International Center of Biodynamics (ICB) – Bucharest Romania.

All preparation steps were controlled by impedimetric measurements using an Agilent 4294A impedance analyzer.

Sensor preparation. Antibodies immobilization

Platinum and/or gold wire electrodes were polished with 0.3 μm alumina on a polishing pad and rinsed with distilled water. Following that, the electrodes were purged for 15 minutes in ($^1/_4$ H_2O_2 35% + $^3/_4$ H_2SO_4 96%) solution for chemical cleansing. Electrodes were rinsed with deionized water to eliminate the impurities, then dried under nitrogen stream.

The electrogeneration of poly(cyanoethylpyrrole) membranes was achieved by galvanostatical electro-oxidation of the monomer (5 mM) in acetonitril solution containing 0.1M $LiClO_4$ (lithiumperchlorate) as a supporting electrolyte.

The applied current density was 3 mA/cm^2. For this current densities, the chronopotentiome-

tric curve of the poly(cyanoethylpyrrole) production on the gold electrode is similar to that observed on platinum electrode.

Reaching a potential value of around 0.8 V ensures the formation of electroplymerised film on the electrode surface (Figure 2). The charge passed during the poly(cyanoethylpyrrole) formation was 180 mC/cm^2.

The antibodies (anti-Salmonella and anti-Escherichia coli) were immobilised via electrostatic interactions between the cyano-groups of the conductive polymer (poly cyanoethil-pyrrole) and their hydroxyl groups using an activation protocol similar with the one described by Oureghi *et al*. 2001.

The poly(cyanoethylpyrrole) chemically modified electrodes were incubated, at 4°C, for 12 hours, in solutions of antibodies: anti-Salmonella and anti-Escherichia coli having concentrations of 0.07 mg/ml. After incubation, the electrodes were rinsed with a PBS solution in order to eliminate the loosely bounded antibodies and to saturate the free cyan groups and incubated overnight at 4°C, in a glycerol solution. A final PBS rinsing step removed the glycerol in excess.

Results and Discussion

The immune coated electrodes were used to detect using impedance spectroscopy. Using the custom made low frequency (ν = 10Hz÷1kHz) impedance spectrometer designed and developed at ICB and matched coated electrodes, we were able to detect *Salmonella and Escherichia coli*, specifically, when their concentration was above a certain threshold level. After sensor stabilization in the measurement chamber, known amounts of pathogens (matching the bounded antigen) are added stepwise.

The measured impedance changes, due to the antibody-antigen (*Ab-Ag*) complex formation were able to be discerned from the background, and reveal the interaction of the antibody attached on the electrode with different antigen concentrations present in the solution. The extent of the interaction was found to depend on the surface loading (antibody concentration). For each new addition, after ~45 minutes a plateau (equilibrium) value of impedance is reached. The relative amount of bound antigen at equilibrium was evaluated with respect to the impedance changes; an absolute value determination might only be possible when knowing the antibody loading at the surface from prior calibration procedures.

Comparative measurements of the characteristic changes in impedance (either modulus, real or imaginary parts) during Ab-Ag binding were performed using an Agilent 4294 A, impedance analyzer, connected to a personal computer revealing good agreement with the recordings of

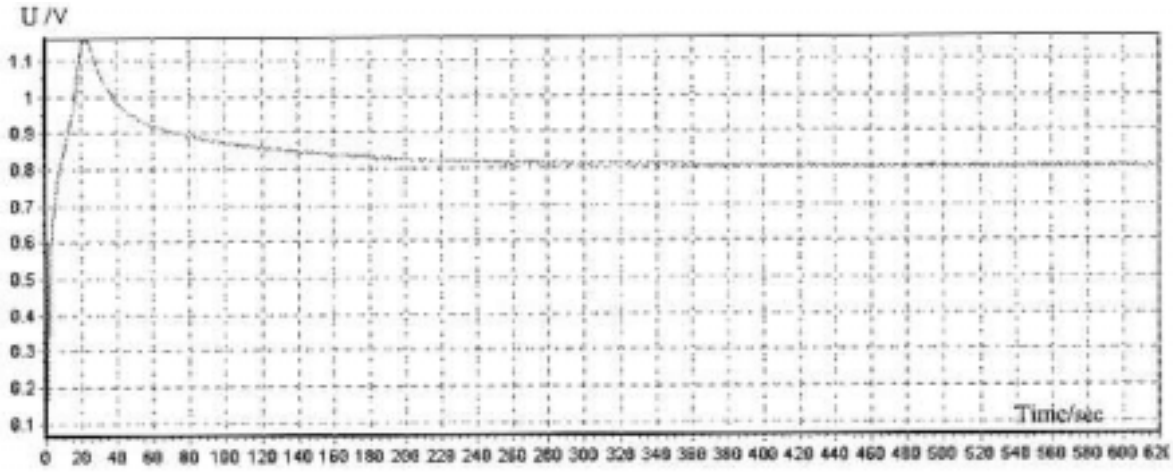

Figure 2. Galvanostatic behaviour of poly-cyanopolypyrrole deposition on a golden wire at 3 mA/cm^2

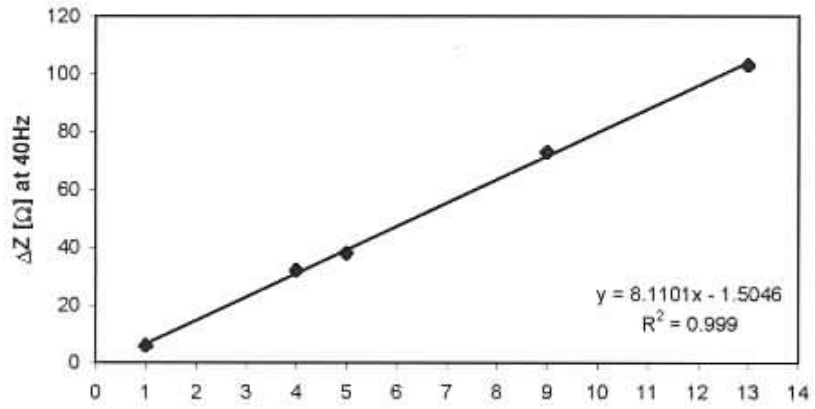

Figure 3. Calibration curve: Impedance modulus at 40 Hz as function of *E.coli* concentration.

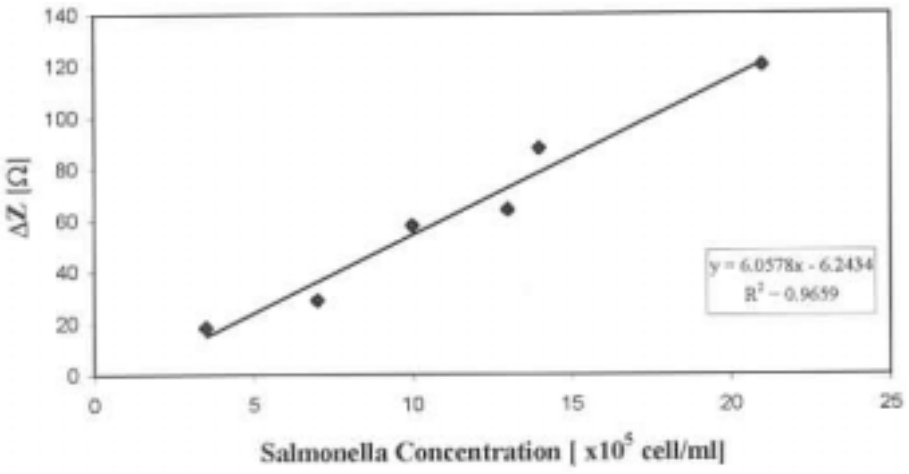

Figure 4. Calibration curve: Salmonella concentration – Impedance modulus at 40 Hz

the custom-built analyzer. The advantage of using the last set-up is the possible adaptation of a differential measurement design with far less expenditure and greater versatility.

Figure 3 presents the characteristic calibration curve obtained: Impedance modulus at 40 Hz versus the concentration of added *Escherichia coli*.

Starting level of impedance before first injection with Escherichia coli: 4370 Ω and phase: -82°. For the evaluation unit used (i.e. gold electrodes with A-Escherichia coli bound at the surface), when injecting the pathogen (*Escherichia coli*) we have obtained, at 40Hz, a sensitivity of ~8 Ω/10^5 cell/ml.

Similar evolutions were also recorded in the case of Salmonella present in the solution as revealed in Figure 4.

A linear relation between ΔZ_{re} and antigen (*Salmonella and E. coli*) concentration was obtained between 5 10^5 – 10^7 cell/ml. The detection limit was ~ 10^5 cell/ml.

Cross reactivity assessment

Using the same measuring cell (with the active electrode containing Antibody against *E. coli*) we have injected alternatively different amounts of *Escherichia coli* and *Salmonella*.

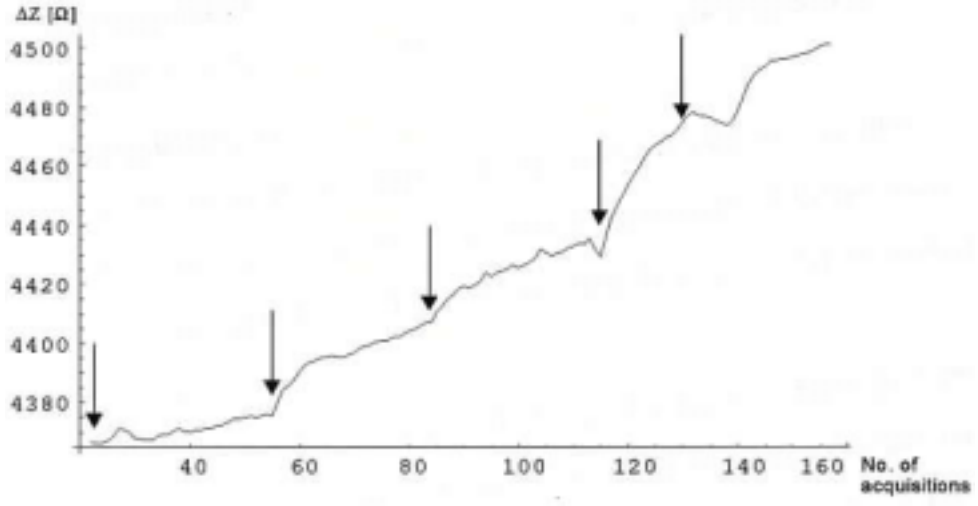

Figure 5. Time series of the impedance changes in relation with the amounts of added

Escherichia coli and *Salmonella*:1.- *E. coli* 10^5 cell/ml (20µl); 2- *E. coli* 4 10^5 cell/ml (60µl); 3- *Salmonella*. 2 10^6 cell/ml (40µl); 4- *E. coli* 4 10^5cell/ml (60µl); 5- *E. coli* 4 10^5cell/ml (60µl);

Figure 5 reveals the time series of the impedance modulus as function of the added amounts of *Escherichia coli* and *Salmonella*.

When injecting 2×10^6 cell/ml of *Salmonella* we obtained an increase of Impedance modulus of ~35 Ω ! corresponding to an interference of ~20%. This is due to the fact that Escherichia antigens are also found in some members of other genera: e.g. Salmonella, Shigella, Citrobacter and Proteus. Therefore it is not possible to asses only the amount of *E.coli* but of other pathogens as well.

The response of the electrodes to other potential interferences such as proteins or compounds with similar structures was tested. The electrodes had shown no response for: 100 ppm BSA.

Acknowledgments

The support of European Union through EU Project G6RD-CT-2000-00420 is gratefully acknowledged.

References

Vrinceanu, D., and Gheorghiu, E. 1996. Bioelectrochem. Bioener, 40(167).

Gheorghiu, E. 1998. Nonlinear Analysis of Synchronized Cell Suspensions; Experimental and Theoretical Aspects, Experimental Chaos IV, Eds. W.Ditto, M.Spano, L.Pecora, World Scientific Press, 185.

Sadik, O.A., Gheorghiu, E., Xu, H., Andreescu, D., Balut, C., Gheorghiu, M., and Bratu, D. 2002. Differential impedance spectroscopy for monitoring protein immobilization and antibody-antigen reactions", Analytic. Chem., 74 : 3142-3150.

Oureghi, O., Senillou, A., Jaffrezic – Renault, N., Martelet, C., Ben Ouada, H., Cosnier, S. 2001. J. Electroanal. Chem., 501 : 62-69

Romanian Patent No. A/01228/2000: " Method for detecting target analytes in liquid media": Gheorghiu E., Gheorghiu, M., Balut C., Bratu D.

Romanian Patent No. A/01229/2000: " Fast, high accurate method to measure ac impedances", Gheorghiu E, Bratu D., Gheorghiu M., Balut C.