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Study of Transport of Biologically Important Compounds in Environment across Biological Membranes and Development of Methods for their Determinations

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Abstract. Different models of phospholipid membranes (PLMs) have been used for better understanding of properties and functions of real biological membranes. Mechanisms of transporting processes and properties of membranes were investigated using planar (supported) PLMs. Their electrochemical behavior was studied by electrochemical impedance spectroscopy (EIS). This technique was utilized to monitor formation, stability, and transporting process across the supported PLMs (s-PLMs). The investigated synthetic membranes were characterized by atomic force microscopy (AFM) too. The methods for isolation of protoplasts from plant cells were modified, adopted and optimized to obtain the cytoplasmic membranes containing specific transporting systems. It has not been possible to fix the protoplasts in substrate pores. Therefore, the protoplasts were destroyed and model phospholipids (lecithin) were mixed with these parts of real protoplasts, which contain real ion and other species transporters. Further, their parameters were investigated. Transporting processes of the environmentally important compounds and of supporting species (e.g., heavy metals, phytochelatin PC2, low molecular weight organic acids) across model and modified PLMs (in mixture with real parts of protoplasts) were studied. The cell penetrating peptides (transportan 10 and mastoparan X) were used to enable transfer of phytochelatin PC2. Voltammetry with hanging mercury drop electrode (HMDE) or with solid amalgam electrodes (SAEs) was utilized for detection of transported species.

Keywords: Membrane, cell, protoplast, lecithin, voltammetry, electrochemical impedance spectroscopy, heavy metals, phytochelatins, ion transporters.

1. Introduction

A huge number of xenobiotics (e.g., drugs, hazardous metals, agrochemically important compounds) are coming into contact with plants, animals or human. To start their negative (or positive) role, such compounds must be transferred into these organisms, into their cells. They are transported into different parts of organism (e.g., from plant roots to leaves) [1]. After entering cells, those species are transported into subcellular structures (e.g., from cytosol to vacuole). Each particle, which takes part in metabolic processes,

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must be first transported across the biological membranes [1]. Similar processes are running in the opposite way – out of the cells or out of several sub-cellular structures. Detailed elucidation of membrane transport mechanisms plays a key role and is prerequisite for understanding distribution of biologically important compounds (BIC) in real cells of more complex organisms (plants, animals or human) [1]-[3].

Biological membrane exists as a surface, at which the hydrophobic parts of phospholipids (PL) are protected from water, while hydrophilic ones are in contact with the aqueous medium. Only ends or edges of bilayer surface are exposed to unfavorable conditions, however, even these exposed regions can be masked by bending them underneath the surface whereby a closed edgeless structure is formed [1]. The closed bilayer is impermeable for most of water soluble molecules, as they would be insoluble in the hydrophobic bilayer core [4]. There are many different ways through which the molecules, ions or particles are being transported across the PL bilayers (PLB) [4], [5].

The use of model biological membranes for elucidation of transport processes seems to be very suitable. It is therefore necessary to form these so that their composition and structure has to be as close as possible to real membranes. The simplest model of biological membrane, which is frequently used for research purposes, is a planar lipid bilayer. Its arrangement allows easy access to both sides which represent the intracellular and extracellular media. This type of model membranes exhibits long life and is relatively stable. Their preparation time is relatively short and their size (thinness) is comparable with real phospholipid membranes (PLMs). Similarly, the plant cells from plants with large cells can be used to investigate the transport processes across real and/or mixed model membranes. Protoplasts from barley were used for this research.

The principles, on which the transporting processes are based, have been studied for many years in many laboratories all over the world, e.g., passive diffusion, facilitated diffusion, ion pumps and channels (e.g., in cases of Ca^{2+}, K^{+}, Na^{+}), or endocytosis and exocytosis (e.g., larger objects and particles, such as bacteria, viruses) [5]). Within the last fifteen years, a great progress has been made in identification of transporter systems in plants [5], [6], especially of transporters for ions of Mn, Fe, Cu, Zn [7]. Nevertheless, the transport of some elements or particles (e.g., heavy metals) is still poorly understood and there are many unanswered questions. The rate of diffusion of organic molecules – nonelectrolytes – depends on their lipid-water distribution coefficient [6]. The higher is the molecular solubility in fats, the faster is the diffusion rate across the membrane. Compounds insoluble in fats are transported across amphipathic proteins and can be dipped into equally oriented lipid bilayer [4]-[8]. The proteins form channels for ions and small molecules and serve for transport of bigger molecules, which would not be otherwise able to pass across the bilayer [6].

We have started studies on transportation of hazardous (heavy) metals across the cytoplasmic membrane. It has been found that only small portion of hazardous metals can be transported in ionic form. Most of them are complexed with natural ligands (low molecular weight organic acids (LMWOAs), phytochelatins) [6]-[9]. Less attention has been paid to the transport of xenobiotics (some of them belong to agrochemically important compounds), such as pesticides and their complexes with essential elements and hazardous metals, across the PLMs. Very important, but not sufficiently clarified, seems to be transport of some drugs or metabolites. It is well known that some of BIC can form complexes with essential elements and hazardous metals. However, their structure remains unknown. These reactions could be probably used by affecting (increase or decrease) their transported amounts and simultaneously, they can be advantageously used for their determination.

2. Experimental

2.1. Reagents and materials

0.1 M KCl (KCl Suprapur, Merck, Prague, Czech Republic) was used as a supporting electrolyte in our experiments. The p.a. solvents were used to dissolve the phospholipid (ethanol – 99.88%, n-heptane – 99%, both from Penta-Svec, Czech Republic). All other utilized chemicals were of analytical grade purity too. The AAS standard solution of Cd^{2+} (1000 mg dm^{-3} in 2 % HNO_3) were purchased from Analytica, Prague, Czech Republic.

The model and mixed PLMs were formed using, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, GPCho (16:0/16:0), CAS No. 63-89-8) (Avanti Polar Lipids, USA). PLMs were formed by self-assembling

in the holes of the Isopore™ Membrane Filters (Millipore, USA) polycarbonate, hydrophilic 0.05 μm, and the supporting membrane thickness amounted to 7-22 μm. The polycarbonate substrates without coverage by phospholipid membrane were first described in [10]. The polycarbonate membrane contains several defects as spikes and dips. The defects of polycarbonate substrate are covered with phospholipid bilayer when using a higher concentration of DPPC. Using Tapping Mode Atomic Force Microscopy (TM AFM) it was found that the phospholipid membrane prepared using higher concentration covers almost all defects of the membrane.

Phytochelatin PC_2 - $(\gamma\text{-Glu-Cys})_2$-Gly) was obtained from company Genosphere Biotechnologies, Paris, France. Cell penetrating peptides mastoparan X (14 amino acids, INWKGIAAMAKKLL-NH_2) and TP10 (21 amino acids, AGYLLGKINLKALAALAKKIL-NH_2) were prepared in the Institute of Organic chemistry and Biochemistry of the AS CR, v.v.i. in Prague, Czech Republic. All their solutions have been freshly prepared [11].

2.2. Apparatus

The electrochemical impedance spectroscopy (EIS) measurements were realized using the potentiostat PGSTAT302N + FRA2 module, equipped by software Nova 1.10 (Metrohm, Czech Republic), with CHI 650C Electrochemical Analyzer/Workstation, Software: CHI v 8.1 (IJ Cambria Scientific, UK) and Potentiostat No. 283 and FRA No. 1025, No. 5210 (Princeton Applied Research, USA). The electrochemical impedances were determined using silver/silver chloride electrodes (silver wire, diameter 1 mm, electroplated by silver chloride). Platinum wire, diameter 1 mm, served as the auxiliary electrode. In our EIS measurement (the dependence of the imaginary part (Z'') on the real part (Z') of impedance recorded in 0.1 M KCl) the system provided satisfactory results in the frequency range of 0.1 – 100 000 Hz, amplitude 0.005 V. Because we wanted to investigate the transporting processes under conditions very similar to those which are common in the real biological systems, the voltage -0.1 V has been used in all EIS-measurements described in this paper (this value is relatively close to the plant membrane potential). On the other hand, a shift to negative bias voltages could lead to a significant change of membrane resistance, possibly due to the increasing number of pores or defective structures in the lipid bilayers [12], [13].

The voltammetric determinations of cadmium and copper ions or its complexes were carried out by the PC-controlled voltammetric analyzer ECO-TRIBO polarograph (Polaro-Sensors, Prague, Czech Republic), equipped with POLAR.PRO software v. 5.1 and with MultiElChem v. 3.1 software (J. Heyrovský Institute of Physical Chemistry of AS CR, v.v.i., Czech Republic). Pen-type electrode – hanging mercury drop electrode (HMDE) [14]-[16] was used as the working electrode. Mercury meniscus modified silver solid amalgam electrode (m-AgSAE) [9]-[20] and mercury meniscus modified copper solid amalgam electrode (m-CuSAE) [20]-[25] were used for determination of some species, e.g., phytochelatin PC2. Ag/AgCl/KCl (3 mol L^{-1}) electrode to which all potentials are referred to and platinum wire served as a counter electrode (both Elektrochemické Detektory, Turnov, Czech Republic) in voltammetric determinations.

The measurements were performed at laboratory temperature (23 ± 2 °C). The values of pH were measured using pH-meter Jenway 3505 (Bibby Scientific Limited, UK).

For determination of CO_2, gas ion selective electrode ISE 12-23 (Monokrystaly Turnov, Czech Republic) was used.

2.3. Protoplast preparation

A few different plants have been used as source of plant protoplast across which the species have been transported: tobacco, penny crest and barley. The methods of their use are described in detail in [1]-[26].

It is well known that protoplasts exhibit spherical shape after removing their cell walls [26]. Protoplasts can be obtained from all types of actively growing young and healthy tissues. The most convenient and widely used source of plant protoplasts is the leaf [1]-[27].

Barley was cultivated hydroponically in culture vessels. The other plants were grown up in common soil. Different barley cultivations were used for the present experiment. They were grown in air conditioned rooms with artificial lightning. Plant roots were immersed into the Knop nutrient solution. For comparison of

cells cultivated in contaminated und uncontaminated soil, cadmium solution has been added to the nutrient solution. It enabled to characterize creation of specific transport mechanisms.

The cut leaves of barley were stored in an enzyme solution mixture of 1% cellulasa Onozuka R10 (BioTech, Czech Republic) and 0.25 % macerozyme R10 (BioTech, Czech Republic) (6 cm^3) dissolved in W5 solution ($CaCl_2$, glucose, KCl, 2-(*N*-morpholzino)ethanesulfonic acid (MES), NaCl, and pH (5.8) was adjusted by KOH; all from Sigma-Aldrich Czech Republic) in Petri dishes (diameter 55 mm). Release of protoplasts was carried out in the dark at 25 °C for 18 hours. Mixture of enzyme solution was filtered through a sieve 70-90 μm and then pipetted into a centrifuge tube, centrifuged for 5 minutes at 100g (800 rpm, radius14 cm). Supernatant was poured away and the sediment was resuspended in W5 solution (5 cm^3). It was centrifuged for 5 minutes at 100g once more [1]. Supernatant was poured away and the sediment was resuspended in 4 cm^3 of 20% sucrose (Sigma-Aldrich, Czech Republic) and overlaid by 2 cm^3 of W5 (not mixed). Centrifuged for 5 minutes at 100 g, with the micropipette removed the floating protoplasts into a clean centrifuge tube and resuspended them in W5 solution (volume 4 cm^3). It was necessary to repeat the centrifugation procedure until the density of protoplasts $1\text{-}2\times10^5$ protoplasts in 1 cm^3 media was achieved. The density of protoplasts was counted using the Burger cell. It was necessary to work very carefully without shocks and sudden movements, because the protoplasts are not protected by the cell wall [1].

2.4. Applied electrochemical cells

In last few years, our research team has developed a few cells for investigation of transport mechanism across biomimetic membranes (U-type, V-type, Insert, etc.) [6, 13, 28, 29]. Nevertheless, in the last year the most frequently used and the most successful is the by us developed "Glass cell" [1, 26, 30-32]. The cell is composed of two glass cylindrical compartments (they are denoted as "Electrolyte 1", "Electrolyte 2", volume of each amounts to 3 cm^3). These compartments represent intracellular and extracellular milieu. The compartments are separated by two Teflon® parts with holes (0.07 cm^2), between which the polycarbonate membrane was inserted.

The electrodes were placed into the holes on the top of glass compartments (two silver wires coated with silver chloride as working electrodes and platinum wire as auxiliary electrode). The same supporting electrolyte (0.1 mol dm^{-3} KCl) was inserted in both compartments. Transported cadmium ions were placed in the compartment "Electrolyte 1". The added cadmium ions (final concentration amounted to 0.1 mmol dm^{-3}) were too low to influence substantially the difference of osmotic pressures between both compartments [1, 31, 33].

2.5. Voltammetric analyses

The transported species across model and mixed PLMs were quantitatively and qualitatively analyzed with application of voltammetry. Voltammetric measurements of heavy metal ions were performed in 0.1 mol dm^{-3} KCl. Standard addition method was applied for calculation of concentrations. Voltammetric behavior of PCs was studied in 0.1 mol dm^{-3} KCl or in borate buffer pH 8.4 (prepared from Suprapur sodium tetraborate acidified by HNO_3 Suprapur, Merck, Czech Republic). Voltammetric determinations of oxalates were performed in Britton-Robinson buffer. Britton-Robinson buffer was prepared by mixing the proper amounts of 0.2 mol L^{-1} NaOH (Lachema, Czech Republic) with acidic component containing 0.04 mol L^{-1} H_3PO_4, 0.04 mol L^{-1} H_3BO_3 and 0.04 mol L^{-1} CH_3COOH (all Lachema, Czech Republic).

A new drop was used for each record on HMDE. Mercury meniscus modified silver solid amalgam electrode (m-AgSAE) and mercury meniscus modified copper solid amalgam electrode (m-CuSAE) were prepared, activated and electrochemically cleaned before and after each record (at chosen regeneration potential (E_{reg}) and for chosen regeneration time (t_{acc})) according to the literature [34-36]. Standard addition method was applied for calculation of concentrations. Deionized water from a Milli-Q Gradient system, Millipore, Czech Republic (conductivity < 0.05 μS cm^{-1}) was used. Methanol was analytical grade quality from Penta-Svec, Czech Republic. All measurements have been realized in nitrogen atmosphere. Oxygen was removed from the measured solutions by bubbling nitrogen (purity class 4.6; Messer Technogas, Czech Republic) for 5 minutes.

3. Results and Discussion

3.1. PLM characterization

For characterization and modelling of electrochemical properties of model, real and mixed PLMs we have used EIS. We have successfully applied EIS for characterization of sPLM on porous substrates and at agar surface (e.g., [1, 4, 6, 13, 29, 31, 33, 37]) since it is non-invasive and offers results on-line. This technique can characterize the state and the changes of investigated PLMs during the transport of e.g. hazardous metals and their complexes across them. The results of EIS are used for construction of equivalent electrical circuits (EEC). In this framework it was necessary to propose EECs for better and reliable characterization of the studied transporting processes: different components (to add capacitances, resistances, Warburg impedances, etc.). Another non-invasive method, which we have used for characterization of formed PLMs, is atomic forces microscopy (AFM). During last few years we have developed a procedure of visualization of formed supported or tethered (anchored) PLMs on surfaces which will be used as substrates (e.g., polycarbonates) [1]. The plant cells and prepared protoplasts can be observed using optical microscopy (their diameters will amount from 1 to 100 μm). Unfortunately, this process is complicated by the fact that the cytoplasmic PLM is invisible in optical microscopy. However, we can utilize marked chloroplasts inside the prepared protoplasts.

3.2. Preparation and optimization of procedures for protoplast isolation

Firstly it is necessary to successfully isolate protoplasts from plant tissue with an optimized technique. For protoplast preparation a few different methods were tested and modified in the framework of experiments [1]-[32]. The successful isolation, presence, appropriate shape and size of protoplasts were confirmed by inverse microscope IX51 and CCD camera DP71 and fluorescence method with fluorescein diacetate (FDA) was applied for determination of cell viability [1].

3.3. Transport of heavy metal ions across model membrane

The simulation of transporting processes across PLMs was realized using a two-electrolyte arrangement with phospholipid membrane which was created directly in pores of a polycarbonate substrate. Calcimycin was proved as a suitable transporter for divalent heavy (hazardous) metals (Cd^{2+}, Cu^{2+}). Calcimycin must be incorporated in the s-PLMs to realize the transport ionophores. Transport of heavy metal ions is substantially influenced by presence or absence of LMWOA(s). It was proved that pH plays a very important role for transporting processes in presence of LMWOAs [6].

Membrane containing real part of the plant tissues was utilized for experiments with transported cadmium ions. The blank samples were tested to ensure that Cd^{2+} were not released from the extracted protoplasts. When PLMs with added extracted parts of protoplasts were applied and simultaneously cadmium ions were added to the “Electrolyte 1”, cadmium ions were established in the compartment “Electrolyte 2”. From the fact that Cd^{2+} transports across model biomembranes with protoplasts, we can conclude that some biotransporters from cells were incorporated into model membrane. Also the influence of used protoplasts from different types of plant tissues (from roots and leaves of barley) was tested and the effect or non-effect of cadmium presence in nutrient medium was examined.

3.4. Voltammetric detection of phytochelatin transported across model and protoplast modified phospholipid membranes

The realized experiments utilized for investigation of transport of phytochelatin PC_2 have been performed in pores of hydrophilic polycarbonate substrate. Model and by protoplasts modified phospholipid membranes were created as described above. Such PLMs were characterized with use of EIS as compact and stable. We successfully incorporated the real transporters present in barley roots and leaves into the model PLMs. Therefore, the transport of phytochelatin PC2 could be demonstrated across such modified membranes.

Three different electrodes were utilized for quantitative and qualitative characterization of transported phytochelatin PC2: HMDE, m-CuSAE and m-AgSAE were proved as suitable and very useful for determination of this interesting compound [22], [23].

Two cell penetrating peptides CPPs (TP10 and mastoparan X) were used as artificial phytochelatine transporters across the model phospholipid membrane. Not any PC2 could be transported across pure model

PLM formed of phospholipid only. It was found that TP10 is several times more efficient in PC2 transport than mastoparan X.

It was proved that naturally present phytochelatin transporters are mainly present in root protoplasts of barley and then in its leaves.

3.5. Separation of particular PLMs

In real cells the species must be transported not only across the cytoplasmic membrane, but also from cytoplasm into vacuole, etc. To understand these processes, it is necessary to study such processes separately on particular subcellular membranes. Therefore, the isolation of particular membranes is necessary to realize for this purpose. In recently started experiments our research team has focused to solve this task using differential centrifugation (sucrose gradient centrifugation). Nevertheless, we intend to apply some other proper methods for these purposes [38], [39].

3.6. Histoimmunochemical experiments for transporter investigation

Identification of presence and of quantity of selected protein transporters have been realized using proper histoimmunochemical determination. The used method must be adopted according to the particular transporter type. The protoplast is fixed in paraformaldehyde–buffer solution. Then the samples of protoplasts must be incubated with a primary antibody against specific protein transporter. Then, the protoplasts is washed and incubated with a secondary antibody conjugated with e.g., fluorescein-5-isothiocyanate, and their nuclear structures will be visualized. The protoplasts can be mounted on slides in a drop of glycerol and analyzed by a laser confocal microscope. In our preliminary experiments we tried to confirm presence and quantity of cadmium transporters in barley protoplasts (Fig. 1).

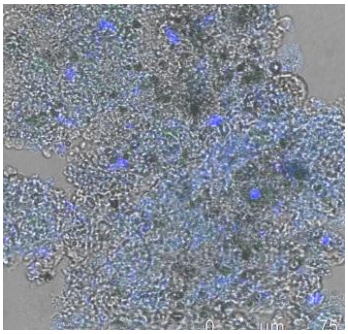

Fig. 1: Histoimmunochemical determination of presence and of quantity cadmium protein transporters. Laser confocal microscope picture. Blue areas correspond to the cadmium transporters in barley protoplasts.

4. Conclusions

In our research we have focussed on physicochemical aspects of transport of selected xenobiotics across the biomimetic membranes. We have combined the results achieved using modern electrochemical methods (voltammetry, chronopotentiometry, etc.), high performance liquid chromatography with fluorescence, with those obtained by histoimmunochemical methods, physiology, microscopy, mathematical methods and electrospray ionization mass spectrometry (ESI-MS). Such “multidimensional inside” can help to elucidate the existing forms of investigated and transported species in different parts of the cell (inside or outside the cell, transported form, etc.). Simultaneously, we have concentrated our attention on complex characterization of structures of the transporters.

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6. References

[1] K. Novakova, T. Navratil, I. Sestakova, M. P. Le, H. Vodickova, B. Zamecnikova, R. Sokolova, J. Bulickova, and M. Gal. Characterization of cadmium ions transport across model and real biomembranes and indication of induced damage of plant tissues. *Monatsh. Chem.* 2015, 146 (5): 819–829.

[2] S. Clemens. Evolution and function of phytochelatin synthases. *J. Plant Physiol.* 2006, 163 (3): 319-332.

[3] T. Navratil, I. Sestakova, and V. Marecek. Transport of heavy metals across the supported phospholipid bilayers. In*t. J. Energy Env.* 2011, 5 (3): 337-346.

[4] T. Navratil, I. Sestakova, J. Jaklova Dytrtova, M. Jakl, and V. Marecek. Study of charged particles transport across model and real phospholipid bilayers. *WSEAS Trans. Environ. Dev.* 2010, 6 (3): 208-219.

[5] R. K. Murray, D. Bender, K. M. Botham, P. J. Kennelly, V. W. Rodwell, and P. A. Weil, Harper's Illustrated Biochemistry, McGraw-Hill Lange, Columbus, 2012.

[6] M. Parisova, T. Navratil, I. Sestakova, J. Jaklova Dytrtova, and V. Marecek. Influence of low molecular weight organic acids on transport of cadmium and copper ions across model phospholipid membranes. *Int. J. Electrochem. Sci.* 2013, 8 (2): 27-44.

[7] T. Navratil, I. Sestakova, J. Jaklova Dytrtova, M. Jakl, and V. Marecek. Study of charged particles transport across model and real phospholipid bilayers. In, M. Otesteanu, S. Celikyay, N. Mastorakis, S. Lache, F.K. Benra (Eds.). *Proc. 7th WSEAS International Conference on Environment, Ecosystems and Development*. Puerto de la Cruz, SPAIN, 2009 pp. 212-217.

[8] L. Rose and A. T. A. Jenkins. The effect of the ionophore valinomycin on biomimetic solid supported lipid DPPTE/EPC membranes. *Bioelectrochemistry* 2007, 70 (2): 387-393.

[9] J. Jaklova Dytrtova, I. Sestakova, M. Jakl, and T. Navratil. Electrochemical detection of cadmium and lead complexes with low molecular weight organic acids. *Electroanalysis* 2009, 21 (3-5): 573-579.

[10] R. Sokolova, J. Bulickova, M. Parisova, T. Navratil, and M. Gal. The Adsorption of Phospholipids at the Interface. In, T. Navratil, M. Fojta, K. Peckova (Eds.). *Proc. Modern Electrochemical Methods XXXIII*. Jetrichovice, 2013 pp. 187-190.

[11] K. Novakova, Study of transport of biologically important compounds in environment across biological membranes and development of methods for their determinations, Institute of Environmental and Chemical Engineering, Univewrsity of Pardubice, Pardubice, 2015, pp. 84.

[12] G. Laputkova, M. Legin, and J. Sabo. Agar working electrode as a support for bilayer lipid membrane: effects of direct current bias voltages. *Chem. Listy* 2010, 104 (5): 353-359.

[13] T. Navratil, I. Sestakova, K. Stulik, and V. Marecek. Electrochemical measurements on supported phospholipid bilayers: preparation, properties and ion transport using incorporated ionophores. *Electroanalysis* 2010, 22 (17-18): 2043-2050.

[14] L. Novotny, and T. Navratil. Effect of surfactants and related biological active substances on the O-2/H2O2 voltammetry and its utilization for determination of the total surfactant content. *Electroanalysis* 1998, 10 (8): 557-561.

[15] T. Navratil, and L. Novotny. Detection of bioactive surfactants in aqueous solutions on the basis of H2O2-voltammetry. *Fresen. J. Anal. Chem.* 2000, 366 (3): 249-253.

[16] J. Skopalova, and T. Navratil. Application of elimination voltammetry to the study of electrochemical reduction and determination of the herbicide metribuzin. *Chem. Anal.-Warsaw* 2007, 52 (6): 961-977.

[17] R. Selesovska, L. Bandzuchova, and T. Navratil. Voltammetric Behavior of Methotrexate Using Mercury Meniscus Modified Silver Solid Amalgam Electrode. *Electroanalysis* 2011, 23 (1): 177-187.

[18] J. Barek, D. Cabalkova, J. Fischer, T. Navratil, K. Peckova, and B. Yosypchuk. Voltammetric determination of the herbicide Bifenox in drinking and river water using a silver solid amalgam electrode. *Environ. Chem. Lett.* 2011, 9 (1): 83-86.

[19] D. Cabalkova, Voltametricke stanoveni herbicidu Bifenoxu na stribrne pevne amalgamove elektrode, BSc. Faculty of Science, Department of Analytical Chemistry, Charles Univesity in Prague, Prague, 2006.

[20] T. Navratil, B. Yosypchuk, and J. Barek. A multisensor for electrochemical sequential autonomous automatic measurements. *Chem. Anal.-Warsaw* 2009, 54 (1): 3-17.

[21] B. Yosypchuk, T. Navratil, A.N. Lukina, K. Peckova, and J. Barek. Solid amalgam composite electrode as a new sensor for the determination of biologically active compounds. *Chem. Anal.-Warsaw* 2007, 52 (6): 897-910.

[22] K. Novakova, T. Navratil, J. Jaklova Dytrtova, and J. Chylkova. Application of copper solid amalgam electrode for determination of fungicide tebuconazole. *Int. J. Electrochem. Sci*. 2013, 8 (1): 1-16.

[23] K. Novakova, T. Navratil, J. Jaklova Dytrtova, and J. Chylkova. The use of copper solid amalgam electrodes for determination of the pesticide thiram. *J. Solid State Electrochem*. 2013, 17 (6): 1517-1528.

[24] J. Barek, J. Fischer, T. Navratil, K. Peckova, B. Yosypchuk, and J. Zima. Nontraditional electrode materials in environmental analysis of biologically active organic compounds. *Electroanalysis* 2007, 19 (19-20): 2003-2014.

[25] J. Barek, J. Fischer, T. Navratil, K. Peckova, and B. Yosypchuk. Silver solid amalgam electrodes as sensors for chemical carcinogens. *Sensors-Basel* 2006, 6 (4): 445-452.

[26] K. Novakova, T. Navratil, I. Sestakova, J. Langmaier, M. Heyrovsky, B. Zamecnikova, and H. Vodickova. Isolation and characterization of protoplasts and their utilization for model membrane preparation. In, T. Navratil, M. Fojta, K. Peckova (Eds.). *Proc. XXXIV Moderni Elektrochemicke Metody (Modern Electrochemical Methods XXXIV)*. Jetrichovice, 2014 pp. 114-117.

[27] N. Kaur, M. Vyvadilova, M. Klima, and M. Bechyne. A simple Procedure for mesophyll protoplast culture and plant regeneration in brassica oleracea L. and Brassica napus L. *Czech Journal of Genetics and Plan Breeding* 2006, 42 (3): 103-110.

[28] J. Jaklova Dytrtova, T. Navratil, and V. Marecek. Phospholipid layer stabilization via Yb(III) on ITIES and facilitated K(I) transport. *Collect. Czech. Chem. Commun*. 2011, 76 (12): 1917-1930.

[29] T. Navratil, I. Sestakova, and V. Marecek. Supported phospholipid membranes formation at a gel electrode and transport of divalent cations across them. *Int. J. Electrochem. Sci*. 2011, 6 (12): 6032-6046.

[30] K. Novakova, T. Navratil, I. Sestakova, V. Marecek, and J. Chylkova. Characterization of electrochemical behavior of lecithin-cholesterol mixture in formation of model phospholipid membranes. In, T. Navratil, M. Fojta, K. Peckova (Eds.). *Proc. XXXIII Moderni Elektrochemicke Metody (Modern Electrochemical Methods XXXIII)*. Jetrichovice, 2013 pp. 128-131.

[31] K. Novakova, T. Navratil, I. Sestakova, V. Marecek, and J. Chylkova. Utilization of Electrochemical Impedance Spectroscopy for Elucidation of Electrochemical Properties of Lecithin - Cholesterol Mixtures in Model Phospholipid Membranes. *Chem. Listy* 2014, 108 (3): 219-225.

[32] K. Novakova. Model Biological Membranes: Their Characterization and Utilization. *Chem. Listy* 2015, 109 (3): 166-175.

[33] J. Jaklova Dytrtova, M. Jakl, K. Novakova, T. Navratil, and V. Sadek. Binding abilities of copper to phospholipids and transport of oxalate. *Monatsh. Chem.* 2015, 146 (5): 831–837.

[34] B. Yosypchuk, and L. Novotny. Cathodic stripping voltammetry of cysteine using silver and copper solid amalgam electrodes. *Talanta* 2002, 56 (5): 971-976.

[35] B. Yosypchuk, I. Sestakova, and L. Novotny. Voltammetric determination of phytochelatins using copper solid amalgam electrode. *Talanta* 2003, 59 (6): 1253-1258.

[36] J. Fischer, L. Vanourkova, A. Danhel, V. Vyskocil, K. Cizek, J. Barek, K. Peckova, B. Yosypchuk, and T. Navratil. Voltammetric determination of nitrophenols at a silver solid amalgam electrode. *Int. J. Electrochem. Sci*. 2007, 2 (3): 226-234.

[37] I. Sestakova, J. Jaklova Dytrtova, M. Jakl, and T. Navratil. Assessment of cadmium and lead mobility in the rhizosphere using voltammetry and electrospray ionization mass spectroscopy. *Int. J. Energy Env*. 2011, 5 (3): 347-355.

[38] E. Etxeberria, and P. Gonzalez. Simultaneous isolation of tonoplast and plasmalemma from Citrus juice cells: Combination of sucrose gradient and two phase partitioning. *HortScience* 2004, 39 (1): 174-176.

[39] W.G. Szymanski, S. Kierszniowska, and W.X. Schulze. Metabolic labeling and membrane fractionation for comparative proteomic analysis of Arabidopsis thaliana suspension cell cultures. *J Vis Exp* 2013, (79): e50535.