Elucidation and Simulation of Transporting Processes of Heavy Metals across the Model Phospholipid Membranes

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Abstract—Transport of heavy metal cations from model solutions across the model phospholipid membranes, which simulated the real cell membrane, has been investigated in our experiments. The transporting processes were affected by the presence/absence of ionophores (calcimycin, nisin, etc.) and by the presence/absence of selected low molecular weight organic acids (LMWOAs) (oxalic, malic or citric acid) at different pH values, which are present in the rhizosphere. The model supported phospholipid membranes were prepared in pores of the polycarbonate membrane. Electrochemical impedance spectroscopy has been used to monitor the formation and stability of phospholipid membrane, whereas voltammetric methods were used for detection of species, which are transferred across the membrane. The structure of transported complexes has been investigated using electrospray ionization mass spectrometry.

Keywords—Cell membrane, Electrochemistry, Heavy metals, Ionophores, Phospholipid membranes.

I. INTRODUCTION

THE problem of contamination of waters, soils, plants, foods, generally environment, by heavy metals (e.g., lead, cadmium copper) has been solved in almost all countries of the world for many years. Simultaneously, the levels of these contaminants have been determined in different parts of human bodies. However, the ways how these metals are transported from environment into plant and living cells have remained unelucidated. Some of them are essential micronutrients required for a variety of physiological processes. Their increased, or on the opposite, decreased levels represent serious danger for the living organisms. Moreover, some metals, e.g., Cd, Pb, Hg, are toxic at almost all concentrations [1], [2].

The transporting processes have to be elucidated in microscopic or more precisely in nanoscopic scale, because to start their activity in metabolic pathways of plants or human bodies, the elements, the compounds and the other species, which are present in polluted environment, must be transported into these organisms, more precisely, into their cells. Because each living cell is surrounded by a cell membrane, each particle, which takes part in metabolic processes, must be transported across it. Such processes are realized in the opposite way – out of the cells - as well as into and out of any sub cellular structure [3], [4]. The knowledge of transporting mechanisms seems to be essential for understanding of distribution of pollutants in real cells and for their possible control in the future [5]-[8].

As it is generally known, the real membranes are very complicated. Therefore, it seems to be more suitable to utilize the model membranes for elucidation of basic transporting steps. In experiments described in this article, we have utilized the model phospholipid membranes (PLMs) for these purposes. PLMs (or lipid membranes (LMs)) are thin, flat membranes consisting of two layers of lipid molecules, with their hydrophobic parts, usually fatty acid tails, directed towards the center of the membrane, and with hydrophilic parts located at the inner and outer borders of the cell [9]. The basic building elements for the formation of the real LMs are the phospholipids (as diacylphosphatidylcholine or diacylphosphatidylinositol, etc.), which are composed of two fatty acid tails, phosphate group and choline, inositol, serine, etc. groups [10], [11]. The membranes separate the cells and the sub-cellular compartments mutually and keep ions and a great variety of molecules at places, where they are needed,
and prevent them from diffusing into areas, where they should not be [12]. PLMs are ideally suited for this role, even though they are only a few nanometers thick (about 6-10 nm), because they are impermeable to most water-soluble (hydrophilic) molecules [11], [13]. The principles, on which the transporting processes are based, have been studied for many years in many laboratories all over the world. They are based on many principles, such as passive diffusion, facilitated diffusion, ion pumps and channels (e.g., in cases of Ca²⁺, K⁺, Na⁺), or endocytosis and exocytosis (e.g., larger objects and particles, such as bacteria, viruses) [11]). In spite of a certain progress in this field of research (e.g., identification of transporter systems in plants [14]-[17] especially transporters of ions of Mn, Fe, Cu, Zn [18], [19]), the transport of some elements or particles (e.g., heavy metals) is still poorly understood and there are many unanswered questions (e.g., [20]).

By means of appropriate techniques, it is possible to prepare an artificial membrane system (e.g., [6], [9], [10], [21]-[23]). The achieved know how in this field can be very important. It gave us the basic knowledge of physical-chemical properties of studied PLMs [26] and of principles of heavy metals transport across them (e.g., [6], [9], [10], [21]).

Low molecular weight organic acids (LMWOAs) are encountered mainly as important root exudates, influencing processes in the rhizosphere. They can form different complexes with metals and affect solubility, mobilization, and uptake by plants [8], [20], [27]-[30]. Apart of this, citrate, malate, and oxalate have been implicated in metal transport through the xylem and vacuolar metal sequestration [2]. [31], [32].

The electrochemical methods seem to be highly suitable for determination of many elements, and of inorganic [33] as well as of organic compounds (e.g., [34], [35]) under physiological conditions (e.g., [36]-[38]). Particularly, voltammetry proved to be a very suitable method for elucidation and determination of the formed complexes of cadmium, lead and copper with oxalic acid (OA), malic acid (MA) and citric acid (CA) [20], [29], [39].

II. EXPERIMENTAL

A. Apparatus

The electrochemical impedance spectroscopy (EIS) measurements were realized using CHI 650C Electrochemical Analyzer/Workstation, Software: CHI v 8.1 (IJ Cambria Scientific, UK). The electrochemical impedances were measured in the three electrode system. Silver/silver chloride electrodes (silver wire, diameter 1 mm, electroplated by silver chloride) served as working and reference electrodes. Platinum wire, diameter 1 mm, served as the auxiliary electrode.

The impedances were measured in the frequency range from 1000 to 0.1 Hz with amplitude of 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 contribution (this value is relatively close to the plant membrane potential) [1]. 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 [7], [9], [40].

The voltammetric determinations of heavy metals (cadmium and copper ions or their complexes, transported across the PLMs) 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. 2.4 software (J. Heyrovský Institute of Physical Chemistry of the AS CR, v.v.i., Czech Republic). Pen-type electrode – hanging mercury drop electrode (HMDE) (e.g. [41]-[43]) was used as the working electrode, Ag/AgCl/KCl(3 mol L⁻¹) was the electrode to which all potentials are referred to and platinum wire served as a counter electrode (both Elektrochemické Detektorý, Turnov, Czech Republic).

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 CO₂, the gas ion selective electrode ISE 12-23 (ISE) (Monokryskly Turnov, Czech Republic) was used.

B. Voltammetric Determination of Heavy Metal Cations

The way used for determination of heavy metal cations was described in detail in [1], [7]. For the determination of Cu²⁺, Cd²⁺, and Pb²⁺ ions, the sample was diluted with 0.1 M KCl and acidified by addition of HNO₃, Suprapur (Merck, Czech Republic), to pH 1. Differential pulse anodic stripping voltammetry (DPASV) was performed at conditions: accumulation potential (Eacc) -800 mV, accumulation time (tacc) chosen according to the determined concentration level (from 30 to 240 s), initial potential (Ein) -800 mV, final potential (Efin) +150 mV, scan rate 20 mV s⁻¹, pulse amplitude 50 mV. A new drop was used for each record. Oxygen was removed from the measured solutions by bubbling nitrogen (purity class 4.6; Messer Technogas, Prague, Czech Republic) for 10 minutes.

C. Voltammetric Determination of Low Molecular Weight Organic Acids

To differentiate between transport of free metal ions and their complexes, it was necessary to be able to determine the complexes of investigated metals with LMWOAs [1], [44], [45]. The heavy metal complexes with OA were detected in model solutions using differential pulse anodic stripping voltammetry and differential pulse cathodic stripping voltammetry. When the pH of the model solutions were adjusted to pH 7 with sodium hydroxide, a mixed complex consisting of Cd, Pb and OA was found, its peak potential varied from -600 to -542 mV (vs. Ag/AgCl/KCl(3 mol L⁻¹)) and depended on the Pb:Cd or Cd:Pb ratio [6]. According to
the calculated stability constants [39], the highest values were obtained for a mixed complex Cd-OA-Pb, in comparison with “single” complexes of OA with Pb and Cd. The existence of all focused metal complexes was recorded in neutral or weakly acidic medium. In acidic medium (pH 2) do not exist any Pb or Cd complex with OA. Therefore, Cd and Pb were present in free ionic forms [20], [29], [39]. The formation of mixed Cd-OA-Pb complex was utilized for detection of transport of OA acid across the phospholipid membrane, for which the adjustment of electrolyte in the electrochemical cell to pH 7.5 was important. Under such conditions OA was transported across PLM, and its presence has been proved also by electrospray ionization mass spectrometry (ESI-MS). On the other hand, in absence of OA, cadmium ions by themselves at pH 7.5 were not transported [7], [12], [46].

D. Electrical Equivalent Circuits

The main aim of application of EIS is the construction of electrical equivalent circuits (EECs) which consist of different components (resistors, capacitors, etc.). Each of them can characterize a different part of the membrane, support, etc.

Two types of EECs were utilized in the present work to characterize the formed supported PLMs (SPLMs) bilayers and the corresponding transport processes (Fig. 1).

![Fig. 1 Electrical equivalent circuits used for characterization of model SPLMs formed on polymer surfaces](image)

The simpler one (Fig. 1A - composed of one resistor in serial combination with parallel combination of a resistor and a capacitor) was applicable for characterization of the free polycarbonate membranes. The other one (Fig. 1B) was more suitable for characterization of SPLMs formed on the polycarbonate membrane pores. This circuit was similar to the simpler one, but additionally, a parallel combination of one capacitor and one resistor was added to the first capacitor (series-connected) [4].

Each member of the circuits could be used for characterization of the system (serial resistor (R_s) correspond to the resistance of the electrolyte; parallel capacitor of the first circuit (denoted as C) and of the second circuit (C_1) correspond to the parasitic capacitance of the membrane; parallel resistors to its resistance; the other parallel combination of a capacitor (denoted C_2) and a resistor in the second type of the circuit describe the electrical properties of the SPLMs) [4], [9], [10]. There are a few other possibilities of the used circuits, applications of which are tested in these days [1], [3], [9], [12], [47].

E. Electrochemical Cell

The newly developed electrochemical cell was utilized for investigation of transporting processes. This type is called “Glass” cell [1]. It is composed of two glass columns in which 2 mL of “Electrolyte 1” and 2 mL of “Electrolyte 2”, respectively, are inserted [1]. The compartments were separated by two Teflon parts with a hole (0.07 cm²), where the polycarbonate porous membrane was inserted. The electrodes were placed into the holes in the top of glass compartments. In both compartments, the same supporting electrolyte (0.1 M KCl) was used. The transported heavy metal ions were placed in “compartment 1” always. If necessary, the solutions of LMWOAs were poured into this compartment (final concentration of LMWOAs amounted to 2 mM). pH adjustment was realized either by direct addition of 0.1 M NaOH into the “Compartment 1” or by exchange of aliquot of “Electrolyte 1” [1], [44].

III. RESULTS AND DISCUSSION

As it was published by our team earlier, the SPLM is completely formed and stabilized approximately one hour after insertion of PL solution on the porous material [9]. The results achieved using different phospholipids are almost equivalent. The structure was studied using atomic forces microscopy (AFM). The complete covering of the supporting membrane by phospholipids under absence of any LMWOA causes that the negligible amounts of metals are transported between compartments (only very small amounts are transported via the imperfections in the model membranes).

A. Transport of copper and cadmium ions in the absence of an ionophore across the SPLM

The ionophore calcimycin [1], [9], [44] proved to be suitable for transport of divalent cations [48], [49]. Hence, it is applicable for transport of Cu²⁺ and Cd²⁺ ions too. Similarly, LMWOAs can be utilized for these purposes. In the acidic pH area (about 2.5), only small amounts of heavy metals are transported (less than 1 % of Cu²⁺ was transported to the “Compartment 2” in the presence of OA, MA or CA). In this pH range, neither CA nor MA forms any stable complex with either Cu or Cd. On the contrary, the complexes with Cd²⁺ and Cu²⁺ ions are formed in the case of OA. The transport of complexes was not confirmed at these pH values in absence of
ionophores.

If pH value of “Electrolyte 1” was adjusted to the value of about 7.5 and none ionophore was present, the transported amounts of metal ions substantially increased [1]. It is explainable by decarboxylation of LMWOAs in “Electrolyte 1", which is realized in neutral and alkaline solutions [1], [44], [45]. Due to this process bubbles of CO₂ are released [1], [45] and they can partly destroy the compact SPLMs, and through these pores the cations of heavy metals are easily transported. The intensity of decarboxylation process increases: CA < MA< OA at pH 7.5 [1]. It was found that the amount of transported cadmium ions to “Electrolyte 2" increases linearly with concentration of OA in “Electrolyte 1" [45]. Because the molecules of MA and CA are larger than OA, they cannot pass through the pores formed in SPLMs. The evolution of CO₂ was proved using gas ISE. The presence of OA in “Electrolyte 2” was proved voltammetrically using formation of the mixed complex Cd-OA-Pb. Similarly, the presence of OA in “Electrolyte 2” can be proved using ESI-MS [20]. The fact that the ions are transported between electrolytes across the SPLM can be seen from the time dependence of capacitance C₂ (Fig. 1B) in applied EEC.

B. Transport of Copper and Cadmium ions in the presence of the calcimycin ionophore across SPLM

If the ionophore calcimycin was incorporated into the SPLM, the transported amounts were substantially higher than in its absence. Similarly as in the case of ionophore absence, if the transport was realized, the capacitance C₂ (Fig. 1B) of the system decreased. The principles of this transport were elucidated more in detail in [1].

The effect of simultaneous presence of LMWOAs and calcimycin ionophore was studied in detail as well. The behavior of all studied divalent cations (Cd²⁺, Cu²⁺, Pb²⁺) was similar. With increasing pH value of the “Electrolyte 1”, the portion of transported cations complexed with LMWOAs increased. However, because some part of cations is bound in complexes, smaller amounts of cations can be transported using calcimycin. However, there was a difference observed among studied LMWOAs: MA and CA complexes cannot be transported using the calcimycin ionophore, because they are not as small as those with OA and using calcimycin cannot pass across the PLM.

IV. CONCLUSION

The simulation of transporting processes across model SPLMs can be realized using a two-electrolyte arrangement with artificial SPLM which is formed in direct pores of a polycarbonate membrane.

On the basis of results achieved by us, which are present in this contribution, it can be concluded that the ionophore calcimycin proved to be a suitable transporter of diveral heavy (hazardous) metals (Cd²⁺, Cu²⁺). The ionophore calcimycin must be incorporated in the SPLM to realize the transport. It was proved that the transporting processes are substantially affected by pH and by absence or presence of LMWOA(s). The destruction of LMWOAs at pH about 7.5 and higher causes formation of CO₂ bubbles (proved using an ion selective electrode). These can cause the disintegration of the consistent phospholipid membrane and the particles can be transported across it. The highest intensity of particle transport was observed in the case of OA in comparison with MA and CA. The realized transporting processes were recorded using EIS measurement and the transported species were subsequently determined voltammetrically.

Combination of voltammetry, EIS, ISE, AFM and ESI–MS proved to be successful in detection and characterization of transported species.

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Abstract—With mercury modified copper solid amalgam electrode (inner diameter 1.5 mm), the voltammetric behavior of thiram was studied. Differential pulse voltammetry (DPV) and direct current voltammetry (DCV) were utilized for this purpose. The reaction mechanism was investigated using DCV and elimination voltammetry with linear scan (EVLS). The optimum conditions for DPV determination of thiram were found in Britton-Robinson (BR) buffer of pH = 5, initial potential \( E_{in} = +100 \) mV and accumulation potential \( E_{acc} = -200 \) mV vs. Ag/AgCl/3M KCl, scan rate 20 mV.s\(^{-1}\). Applying prolonged accumulation time (100 s), the limit of detection \( 3 \times 10^{-9} \) mol.L\(^{-1}\) was reached.

Keywords—Thiram, Copper, Solid amalgam electrode, Voltammetry.

I. INTRODUCTION

THIRAM (tetramethylthiuram disulfide) belongs to wide group of organosulfur compounds (dithiocarbamates) and is used as protective pesticide [1]-[3]. Pesticides are commonly used in our society. At present, the effort is to reduce their usage.

Dithiocarbamates have strong metal binding capacity [4]. Thiram introduces very high chemical and biological activity [5], [6]. The biological activity is given by dithiocarbamate group [7], [8]. This group can react with sulphur-containing enzymes and coenzymes [9], [10]. Thiram is complexing agent for metals, the metal-pesticide interaction is very important because it could change the toxicity of the appropriate compounds [5]. Thiram has capability to form a strong metal complex with copper [11]-[13].

It is used as fungicide forestalling fungal diseases on crop, vegetables, fruit trees and ornamentals from damage by rabbits and deer [5], [10], [14], [15]. Thiram is used as animal repellent, to control scab, as vulcanization agent in rubber industry [3], [15].

It is hazardous for the environment. The major problem is persistence of thiram in the soils. Its destiny is defined by physicochemical processes, chemical or biological transformation. Thiram is irritating to human skin, eyes and respiratory mucous membrane [16]. It is highly toxic for fish [5].

This contribution deals with method which was developed for determination of thiram in various matrices using mercury meniscus modified solid amalgam electrode (m-CuSAE).

II. EXPERIMENTAL

A. Reagents

The stock solution of thiram ( Sigma-Aldrich, Czech Republic) with concentration \( 6.5 \times 10^{-3} \) mol L\(^{-1}\) was prepared by dissolving 23.4 mg in 15 ml ethanol (Penta-Svec, Czech Republic). The analyzed solutions were prepared daily by its dilution in ethanol. Britton-Robinson (BR) buffers of pH values from 3 to 12 were prepared by mixing the proper amounts of alkaline component with acidic component. All the other chemicals used were of analytical grade. Deionized water from Milli-Q-Gradient (Milipore, Prague, Czech Republic) was utilized.

B. Instrumentation

Voltammetric measurements were realized with the computer controlled Eco-Tribo Polarograph (Polaro-Sensors, Prague, Czech Republic), equipped with MultiElChem 2.4 software for windows 7 (J. Heyrovský Institute of Physical Chemistry of the AS CR, v.v.i., Czech Republic). The copper solid amalgam electrode was used with meniscus modification (m-CuSAE). The working surface of this newly developed electrode was 3 mm\(^2\) (the repeatability less than \( \pm 5\) %). As reference electrode, Ag/AgCl/3M KCl was utilized and platinum wire was used as an auxiliary electrode (both from Electrochemical detectors, Turnov,