

Assessment of cadmium and lead mobility in the rhizosphere using voltammetry and electrospray ionization mass spectroscopy

Ivana Šestáková, Jana Jaklová Dyrtrtová, Michal Jakl, and Tomáš Navrátil*

Abstract—Phytoremediation belongs to relatively modern and frequently used methods of possible treatment of environmental problems (mainly caused by human activities). It consists in mitigating pollutant concentrations in contaminated soils with plants, which are able to accumulate or eliminate metals and various other contaminants. Nevertheless, the fundamental principles of this technique, i.e., transport of pollutants from soils into the plant cells have not been elucidated. These processes can be studied using model solutions of cadmium, lead and oxalic acid and the conditions in the rhizosphere can be studied within the artificially prepared phospholipid bilayer membrane on polycarbonate support. Electrochemical impedance spectroscopy (EIS) is used to monitor the formation and stability of phospholipid membrane, whereas voltammetric methods can detect species, which are transferred across the membrane.

Keywords—Cadmium, Electrochemical impedance spectroscopy (EIS), Oxalic acid, Phospholipid bilayers, Rhizosphere, Voltammetry.

I. INTRODUCTION

CADMIUM and lead are considered as hazardous (or heavy) metals and environmental pollutants in industrial countries and their circulation through soil, water and air has greatly increased. Major sources of atmospheric emission are mining, different types of metal production and combustion of fossil fuels. The disposal of residues from coal combustion and disposal of commercial products on land and phosphate

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*T. Navrátil is with the J. Heyrovský Institute of Physical Chemistry of AS CR, v.v.i., Department of Biophysical Chemistry, Dolejškova 3, 182 23 Prague 8, Czech Republic (corresponding author: +420 266053886; fax: +420 286582307; e-mail: tomas.navratil@jh-inst.cas.cz, WWW: <http://www.jh-inst.cas.cz/www/department.php?p=37&d=4>).

I. Šestáková is with the J. Heyrovský Institute of Physical Chemistry of AS CR, v.v.i., Department of Biophysical Chemistry, Dolejškova 3, 182 23 Prague 8, Czech Republic (e-mail: ivana.sestakova@jh-inst.cas.cz).

J. Jaklová Dyrtrtová is with the Institute of Organic Chemistry and Biochemistry of AS CR, v.v.i., Spectroscopy and Physical Organic Chemistry, Flemingovo náměstí 2, 160 00 Prague 6, Czech Republic (e-mail: dyrtrtova@uochb.cas.cz).

M. Jakl is with the Czech University of Life Sciences, Faculty of Agrobiology, Food and Natural Resources, Department of Agro-Environmental Chemistry and Plant Nutrition, Kamýcká 129, 165 21 Prague – Suchbátka, Czech Republic (e-mail: jakl@af.czu.cz).

fertilizers in case of cadmium are further sources of soil contamination. The main routes of human exposure to those toxic metals are acute exposure in the working environment (inhalation of dusts and fumes, occasionally oral intake) and acute and chronic exposure of the population through food, air, and water.

Uptake of metals by plant roots is the main entry pathway into animal and human food chain. Generally, the uptake by plants depends on the concentration of metal in the soil, but overall, there is no correlation between soil metal content and plant metal content [2]. Other factors, as pH and the presence of organic and inorganic ligands, influence the size of plant available metal fraction. There is a great difference between cadmium and lead: Whereas cadmium ion is up to pH 7 present as Cd^{2+} or hydrated species, in case of lead, Pb^{2+} is predominant only to pH lower than 6, at higher pH different forms of hydroxylated species prevail, some of them with very low solubility [3]. That is the reason why only Cd is of particular concern in most studies.

The subject of this article was presented on the International Conference on Development, Energy, Environment, Economics (DEEE '10), which was held in Puerto De La Cruz, Tenerife, Spain, in December 2010 [4]. Similarly as the other contributions presented during this and other conferences, which are organized by WSEAS/NAUN/IEEE, and which has been published in corresponding journals, proceedings, and books, they belong to the scientific papers, which should help to improve the human environment and simultaneously decrease the negative consequences of human activities (industrial production, mining etc.) (e.g., [5]-[16]).

A. Soil Solution

This is the liquid part within the soil and can be handled in different ways [17]-[21]. Different sampling methods yield soil solution retained with different matrix potential and thus provide different qualities of the soil solution [22].

It would seem that the choice of sampling technique is dependent only on accessibility of the soil solution; if it is possible to get the sample directly or not. Nevertheless, with the soil solution sampling it is essential to deal with changes of thermodynamical balances in soil as well as with sorption on sampling devices, precipitation, preconcentration, dilution, and soil structure disruption during the sampling device

installation. Additionally, another important problems with soil solution sampling are heterogeneity of stands, microbial degradation of soil solution samples, impact of current conditions (temperature, precipitation, leaching) and others. In case of speciation studies namely interaction of heavy metals with LMWOAs is very contentious the thermodynamical balance interruption during the sampling.

In studies performed by our team [23] soil solution samples were taken using nylon suction cups (DI Gottfried Wieshammer, Wien, Austria) (Fig. 1) [24]. Most interesting is soil solution sampled in proximity to plant roots, in the rhizosphere. Here are metal ions in hydrated or complexed forms and variety of organic compounds. Root cells are in the first contact with metal ions and actively excrete low-molecular weight compounds that can function as metal chelators, namely low molecular weight organic acid, such as malate, citrate, oxalate [23]-[26].

B. Biological Membrane

Each real cell is surrounded by a membrane, which is mainly composed of phospholipids. Additionally, it contains a wide variety of biological molecules, primarily proteins and lipids, which take part in many cellular processes, such as ion channels, conductance, cell signaling etc. To elucidate such transport processes, synthetically prepared model phospholipid bilayers (PLBs) are utilized, in the form of black membranes, vesicles or supported membranes [5], [27]-[30] with ion channels incorporated (either artificially synthesized or obtained from real living cells).

The biological membrane exists as a surface, at which the hydrophobic parts of phospholipids (PL) are protected from water, while the hydrophilic ones are in contact with the aqueous medium. Only the ends or edges of the bilayer surface are exposed to unfavorable conditions, however, even these exposed regions can be eliminated by bending them underneath the surface whereby a closed edgeless structure is formed.

The closed bilayer is impermeable for most of water soluble molecules, as they would be insoluble in the hydrophobic bilayer core [29]-[32]. The rate of diffusion of organic molecules – nonelectrolytes – depends on their lipid-water distribution coefficient. The higher is the molecular solubility in fats, the faster is the diffusion rate across the membrane. Compounds insoluble in fats are transported across amphiphatic proteins and can be dipped into equally oriented lipid bilayer [5], [27]-[29]. 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.

Several classes of proteins have been implicated for cell membrane transport of metals such as Cu^{2+} , Zn^{2+} , Mn^{2+} , Fe^{2+} , Ni^{2+} and Co^{2+} , which are essential micronutrients for plant metabolism [33], [34].

Over the past ten years it was assumed that for Cd^{2+} as non essential metal ion, there would be no specific uptake mechanism and Cd^{2+} would enter plant cells via uptake

systems for essential cations. Mostly indirect evidences are available now that Cd is taken up into plant cells by Fe^{2+} , Ca^{2+} and Zn^{2+} transporters/channels (LCT1, ZIP family of metal transporters, Nramp family). Only exception is *Thlaspi caerulescens*, where evidences for a Cd specific uptake system were described, e.g., [23], [35]-[39] (Fig. 2).

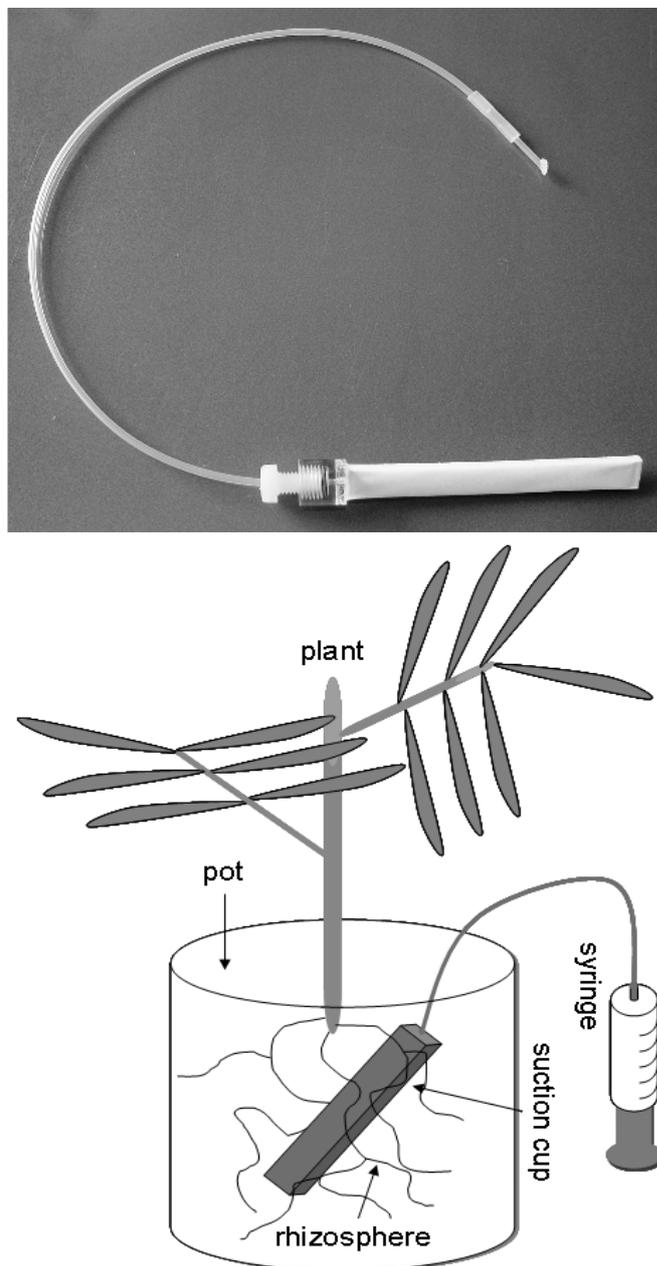


Fig. 1 suction cup and soil solution sampling design

Metal ions taken up into cells are bound by phytochelatins, synthesized in response to Cd^{2+} exposure. Additionally Cd-ligand complexes can be transported into the vacuole or other organelles.

C. Phytochelatin

Phytochelatin is important in metal sequestration, which first take place in vacuoles of root cells. Phytochelatin (PCs) are peptides of general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2\text{--}11$) and were discovered first in fission yeast *Schizosaccharomyces pombe* [40] and then in plants [41]. Phytochelatin is non-ribosomally synthesized from glutathione in a transpeptidase reaction catalyzed by enzyme

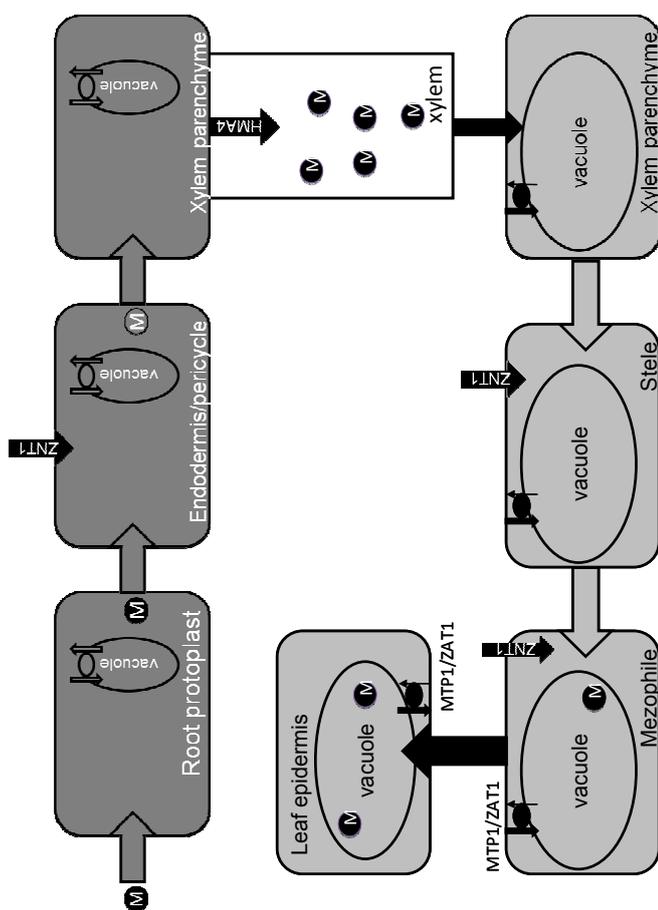


Fig. 2 transport of metal (M) from soil into root cells (dark gray) and via xylem into Leaf (light gray) (on the basis of [1]) Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system

phytochelatin synthase. Instead of Gly, some other amino acid residues might be present [42]. Phytochelatin synthase is activated in the presence of metals, and deactivated when the metals have been bound by the newly synthesized PC [43], [44]. Phytochelatin synthase adds a $\gamma\text{-Glu-Cys}$ fragment from glutathione ($\gamma\text{-Glu-Cys-Gly}$) to another glutathione (to form a PC with $n = 2$ (Fig. 3)) or to another phytochelatin chain to increase the repeating unit from n to $n+1$ (e.g., PC_3 ($\gamma\text{-Glu-Cys}$)₃-Gly (Fig. 4)). Plants and algae produce mixtures of the different PCs [45], whose composition depends, among other factors, on the level of metal contamination.

Cd^{2+} ions are the most potent activators of phytochelatin synthesis. Also different metal ions can activate the synthesis

of phytochelatin, e.g., Pb^{2+} , Zn^{2+} , Cu^+ , Ag^+ and others. However, mainly cadmium and (in much lesser extent) copper or silver were found as bound to phytochelatin [46], [47]. Lead is considered as not forming complexes with phytochelatin [47], and oxidized forms of phytochelatin were proved in plants cultivated with lead [48].

Another pathway of metal uptake regulation is biosynthesis and release of peptides and related substances by microorganisms, many of these compounds include cysteine-containing tripeptides similar to glutathione and phytochelatin [49], [50] and cyclic peptides as rhodotorulic acid and siderophores [51]-[53]. From the ability to bind metals or transfer them across biomembranes arise the antibiotic or antifungal properties of these cyclic peptides [54]. These substances create stable complexes with metals and their particular charges and ionic radii are highly specific. The transport proteins known from biological membranes such as antamanide and enniatins [55] have similar structure as $\text{cyclo}(\text{Pro-Gly})_3$, a synthetic cycloprotein [56].

For studies of phytochelatin complexes, voltammetric

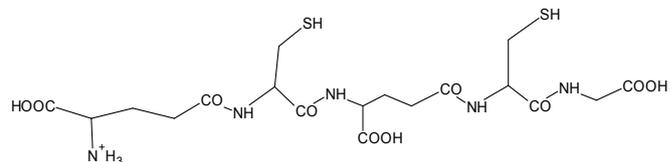


Fig. 3 phytochelatin PC_2 , ($\gamma\text{-Glutamyl-cysteinyl}$)₂glycine, ($\gamma\text{-Glu-Cys}$)₂Gly; molecular mass 427.54 $\text{g}\cdot\text{mol}^{-1}$

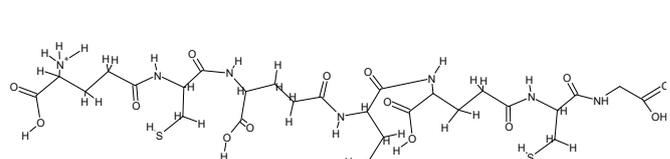


Fig. 4 phytochelatin PC_3 , ($\gamma\text{-Glutamyl-cysteinyl}$)₃glycine, ($\gamma\text{-Glu-Cys}$)₃Gly; molecular mass 772.84 $\text{g}\cdot\text{mol}^{-1}$

methods were applied, both for detection in plant extracts [57]-[59] or characterization of complexes of synthetically prepared peptides with metal ions. Several types of working electrodes were employed, ranging from hanging mercury drop electrode (HMDE) to amalgam electrodes or carbon paste electrodes [60]-[63]. Most successful approach is the combination of voltammetry and multivariate curve resolution with alternating least squares method [64]-[71]. For detection of phytochelatin in real plant extracts electrospray ionization mass spectrometry (ESI-MS) is optimal, especially in combination with liquid chromatography [72], [73] size-exclusion chromatography (with ICP-MS detection [74]) or capillary zone electrophoresis [75].

II. EXPERIMENTAL

A. Apparatus

The electrochemical impedance spectroscopy measurements were realized using 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.

The voltammetric determinations of cadmium or lead ions 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. 2.1 software (J. Heyrovský Institute of Physical Chemistry of AS CR, v.v.i., Czech Republic). Pent-type electrode – HMDE [76]-[78] was used as the working electrode, 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). For the determination of cadmium and lead ions, the sample was acidified by addition of HNO₃, Suprapur (Merck, Czech Republic), to pH 1 and analyzed using differential pulse anodic stripping voltammetry (DPASV) at conditions: E_a = -850 mV, E_{in} = -700 mV, E_{fin} = -200 mV, scan rate 10 mV·s⁻¹, pulse amplitude 50mV. A new drop was used for each record; measurement has been performed in nitrogen atmosphere. pH was measured by digital pH/mV meter MPH 61 with combined electrode TYPE 01-29 (all Monokrystal, Turnov, Czech Republic).

Determination of hazardous (heavy) metal ions (Cd²⁺, Pb²⁺, etc.) and their complexes, transport of which across the PLBs is presented in this manuscript, and of many other organic as well as of inorganic compounds using voltammetric methods with various working electrodes, was many times described in literature and our research group has been developing such analytical methods over many decades (e.g., with hanging mercury drop electrode (e.g., [63], [79]-[88]). Nevertheless, it has been reported that the solid electrodes can be successfully used for voltammetric determinations of mentioned transported environmentally important species. Our research team has been dealing with development and application of such types of electrodes, e.g., solid amalgam electrodes (silver, copper, etc.) (polished, film, mercury meniscus modified etc.) (e.g., [8], [89]-[98]), composite solid electrodes (silver, graphite, gold, etc.) (e.g., [91], [99]-[108]), and solid amalgam composite electrodes for many years (e.g., [91], [104], [109], [110]).

The experiments described in this manuscript were realized with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (lecithin, DPPC, GPCho (16:0/16:0), CAS No. 63-89-8) (Avanti Polar Lipids, Alabaster, USA) (Fig. 5). The PLBs were formed by self-assembling in the holes of the Isopore™ Membrane Filters (Millipore, USA) polycarbonate, hydrophilic 8.0 μm, and the supporting membrane thickness amounted to 7-22 μm. The area of one pore amounted to 50 μm², the experimentally found porosity of the membranes was about 25-45 %.

B. Electrospray Ionization Mass Spectrometry

ESI-MS was used to probe the complexes formed from the solution. Oxalic acid was studied separately with divalent cadmium and lead salts in the solvent system methanol/water (1:1). These solutions were studied using an ion-trap mass spectrometer fitted with an electrospray ionization source of a Finnigan LCQ device (ThermoFinnigan, San Jose, CA, USA) operated in positive-ion mode [111]. Nitrogen was used as the nebulizer gas. The operating conditions were set as follows: spray voltage 4.5 kV, capillary voltage 3 V, heated capillary temperature 75 – 100 °C, tube lens offset 50 V, sheath gas flow rate and auxiliary gas flow rate 30 arbitrary units. The samples were introduced into the ESI source via a needle at a flow rate of 5 μL min⁻¹. Mass spectra were recorded from *m/z* 50 to 2000.

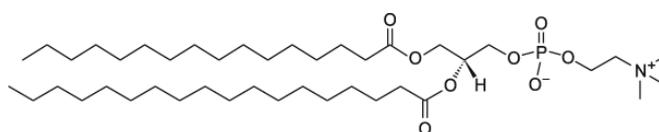


Fig. 5 structure of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (lecithin, DPPC, GPCho (16:0/16:0), CAS No. 63-89-8) - building stone for construction of PLB in described experiments

Collision-induced dissociation (CID) of the mass-selected precursor was achieved through rf-excitation of the ions within the helium buffer gas present in the ion trap as the collision partner. The collision energy was optimized for each experiment and is expressed in terms of the manufacturer's normalized collision energy (%), where the range from 0 to 100 % corresponds to a resonance excitation *a.c.* signal of 0 – 2.5 V (zero-to-peak) at the secular frequency of the ion of interest. This energy depends on the *m/z* value of the parent ion, and that is the reason why this value is not expressed directly in voltage units.

III. RESULTS AND DISCUSSION

A. Phospholipid Membrane Preparation

The first step of model biological membrane investigations consists in formation of such membranes. Of course, the simplest way is to form such a bilayer on some supporting material (supported phospholipid bilayers; SPBL), e.g., a metallic substrate (mercury [112], gold, or on some gel surface), or in the form of a self-supporting membrane, e.g., as a bubble at a Teflon cap [113], filling a small micro-holes in a plate [114], or in a membrane [115], [116].

This, last mentioned way of preparation was used by our research group:

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine monohydrate (Fig. 5) was used for preparation of PLB on porous membranes: The capacity of membranes increases with time after the bilayer formation, until a steady-state value attained some 20-30 min. later [113], [115]. In our experiments, there were the PLBs formed as self-assembled in the holes of the supporting membrane (Isopore™ Membrane Filters, Millipore,

USA; polycarbonate, hydrophilic, 2.0 or 8.0 μm , and thickness 7-22 μm) by injection of 10 μL of phospholipid solutions in heptane on the membrane surface. These membranes (supports) were glued on the plastic cup(s) of the polypropylene electrochemical cell, which was together with electrolyte 1 and working electrode inserted into second part of the cell, containing electrolyte 2, reference and auxiliary electrode [30]. Such type of the electrochemical cell is called "Insert" type [5], [29], [117]. Nevertheless, similar result can be achieved by "U-Cell": The part of polycarbonate membrane (alone or with lecithin solution) is placed between two parts of glass U-shaped cell, which were then filled with a proper electrolyte or sample [117].

Formation of PLB was monitored by ESI measurement. After steady state has been reached, changes of electrolyte 1 were realized (change of pH, addition of metal ions, oxalic acid) and to them the PLB was exposed exactly 1 hour. Subsequently, voltammetric analysis of the electrolyte 2 has been performed.

B. Voltammetric Analysis

The Cd and Pb complexes with oxalic acid (OA) were detected in model solution of sodium perchlorate, 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 varies from -582 to -542.5 mV (vs. Ag/AgCl/KCl sat.) and depends on the Pb:Cd or Cd:Pb ratio. According to calculated stability constants [24], highest values were obtained for mixed complex Cd-OA-Pb, in comparison with "single" complexes of OA with Pb and Cd (Pb-OA and Cd-OA). The existence of all focused metal complexes is confined to neutral or weakly acidic medium. In acidic medium (pH 2) in model and soil solution does not exist any Pb or Cd complexes, all Cd and Pb were presented in free ionic forms.

Oxalic acid was the most abundant low-molecular-weight organic acid (LMWOA) in soil solution from rhizobag experiment with smith willow and alpine penny-cress and in those real soil solutions (diluted by sodium perchlorate) voltammetric analysis revealed peak of mixed Cd-OA-Pb complex and PbOA complex. When amount of lead in biomass of willow and alpine penny-cress was compared, higher uptake of lead compared to willow could be explained by invoking a co-transport of Pb with Cd in the "mixed" complex with oxalic acid. This result suggests that the transport of whole complexes across the plant membrane is preferred over that of single of the cadmium or lead ions as was observed for aluminium [2], [118].

After confirmation of the mixed complex Cd-OA-Pb formation in solution of potassium chloride, which is used as electrolyte in ESI measurement, we could prove passage across PLB of both, cadmium and lead when electrolyte 1 contained 0.1 mol·L⁻¹ KCl at pH 7.5 (addition of NaOH), oxalic acid, Cd²⁺ and Pb²⁺.

In the next experiment, only oxalic acid was added to electrolyte 1 under steady state of phospholipid bilayer formed:

A: 0.1 mol·L⁻¹ KCl, pH 7.5, 30 $\mu\text{mol}\cdot\text{L}^{-1}$ OA;

B: 0.1 mol·L⁻¹ KCl, pH 5.7, 30 $\mu\text{mol}\cdot\text{L}^{-1}$ OA.

After 1 hour of PLB membrane exposition to electrolyte 1, only in case A oxalic acid can be detected in electrolyte 2 by means of formation of mixed complex Cd-OA-Pb, as is documented on Fig. 6. In case of experiment B no mixed complex was detected in electrolyte 2. From those experiments conclusion can be made that the contact of oxalic acid in solution of pH 7.5 influences permeability of PLB. It should be noted, that in strongly contaminated soils pH of soil solutions quite often reached such pH value.

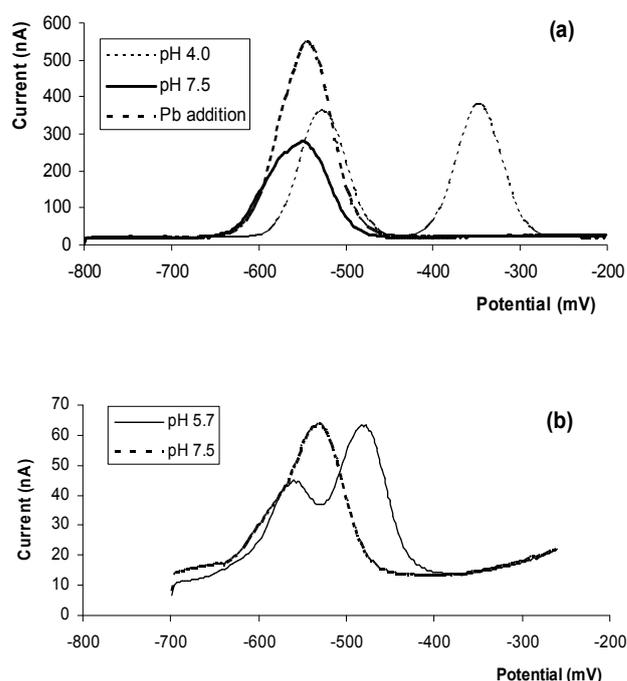


Fig. 6 (a) model solution of 0.1 mol·L⁻¹ KCl with 1·10⁻⁴ mol·L⁻¹ OA, 1·10⁻⁶ mol·L⁻¹ Cd²⁺ and 5·10⁻⁷ Pb²⁺; "Pb addition" – addition of 1·10⁻⁶ mol·L⁻¹ Pb²⁺; (b) electrolyte 2 from experiment A, after addition of Cd²⁺ and Pb²⁺ ions

C. "Mixed" Cd/Pb complexes determined by ESI-MS

Two „mixed“ complexes of cadmium, lead and oxalic acid were found in the model solutions as well as in the soil solution from willow (*Salix smithiana*) planted on Cd and Pb contaminated fluvisol from the Litavka River region in the Czech Republic. The designed structures are: [CdPb(OAH)₂(H₂O)₄]⁺ and [CdPb(OA)(H₂O)₃(CH₃OH)]⁺, where OA means C₂O₄²⁻ and OAH means C₂O₄H⁺.

MS spectra (Fig. 7) of the system consisted of Cd(NO₃)₂ and Pb(NO₃)₂, both 1.25·10⁻⁵ mol·L⁻¹, and oxalic acid 2.5·10⁻⁵ mol·L⁻¹ elucidated presence of two types of "mixed" complexes.

IV. CONCLUSION

Formation of phospholipid bilayer within the pores of polycarbonate membrane offers possibility to combine ESI measurement in order to follow PLB formation and properties and enable variable exposition of formed PLB membrane to the presence of metal ions or their complexes, changes of pH etc. The influence of PLB composition and presence of ionophores can be also easily varied.

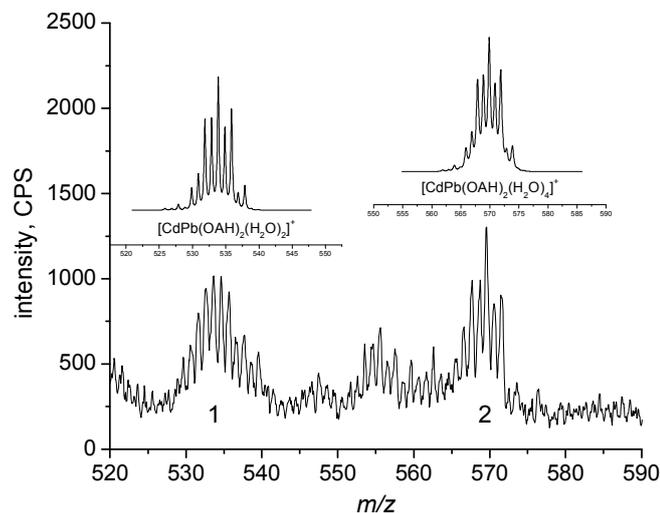


Fig. 7 ESI-MS spectra (positive-ion mode) of a solution of $\text{Pb}(\text{NO}_3)_2$, $\text{Cd}(\text{NO}_3)_2$ (both $1.25 \cdot 10^{-5} \text{ mol} \cdot \text{L}^{-1}$), and oxalic acid ($2.5 \cdot 10^{-5} \text{ mol} \cdot \text{L}^{-1}$) in methanol/water (1:1); 1 – $[\text{CdPb}(\text{OAH})_2(\text{H}_2\text{O})_2]^+$ and 2 – $[\text{CdPb}(\text{OAH})_2(\text{H}_2\text{O})_4]^+$. Computation of the isotope pattern and mass for the complexes

Voltammetric methods were applied as sensitive enough for detection of species passed across the membrane. Examples of anodic stripping voltammetry or mixed complex formation were given. Further possibility is detection of phytochelatins – using sensitive hydrogen catalytic wave in Brdička reaction on HMDE or solid amalgam electrode or application of ESI-MS (Fig. 8). Such studies are in progress.

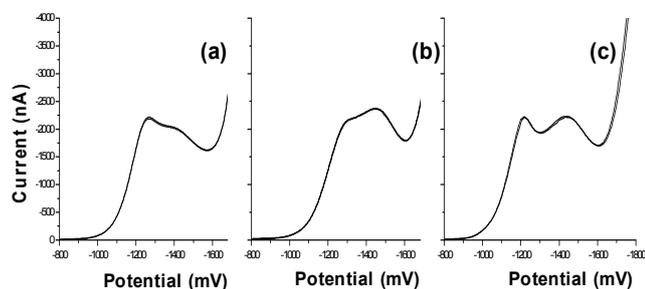


Fig. 8 determination of phytochelatins using Brdička reaction. DC voltammetry on HMDE, accumulation time 30 s at -800 mV, scan rate = $250 \text{ mV} \cdot \text{s}^{-1}$. Extracts from barley: (a) leaf, (b) stem, (c) root

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