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Study of Interactions between Cysteine and Cadmium(II) Ions using Automatic Pipetting System off-line Coupled with Electrochemical Analyser

Dedicated United Nation Environment Program: Lead and Cadmium Initiatives

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The sulfhydryl group of low molecular thiols including cysteine is reactive and is often found conjugated to other molecules such as nitric oxide and/or metal ions via its sulfhydryl moiety. Therefore, it is not surprising that thiols play key role in many protective mechanisms against adverse effects of metal ions. On the other, good affinity of their -SH moiety can be used as a base for heavy metal ion sensor in environmental analysis. Electrochemical measurements were performed Trace Analyzer and autosampler, using a standard cell with three electrodes and cooled sample holder (4 °C). For automated samples handling prior to their electrochemical and photometrical analysis, an automated pipetting station with computer controlling was used. Ellman reaction for –SH group detection was measured using Chemical Analyser. In this study, we primarily aimed our attention on studying of basic electrochemical behaviour of cadmium(II) ions and low molecular mass thiol cysteine. Measured concentrations of cysteine were 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1000 μM, and cadmium(II) ions 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1000 nM. Well developed peaks of cadmium(II) ions at -0.6 V and –SH moiety at –0.2 V and at coloured complex 412 nm were detected. After that we characterized the obtained differential pulse

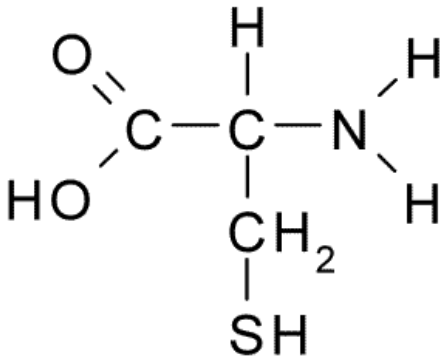
voltammograms, we followed with the studying of their mutual interactions. Each from the above mentioned thiols concentrations were mixed with 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 or 1000 nM cadmium(II) ions and incubated. Based on the affinity of –SH moiety to metal ion, cadmium-thionein complexes were formed. Samples were taken in the following time intervals: 0, 5 and 15 h and measured using differential pulse voltammetry. The changes in the signals as rapid decreasing of cadmium(II) ion peak and free thiol moiety, and occurring of Cd-S complex peak were described and mathematically and statistically evaluated. Based on the statistics, we were able to estimate mathematical calibration model, detection limits and quantification limits for determination of cadmium(II) using sensors based on cysteine.

Keywords: cysteine; electrochemical analysis; mercury electrode; differential pulse voltammetry; robotic microfluidic analysis; interaction

1. INTRODUCTION

Cadmium(II) ions interfere with biological and biochemical processes in both prokaryotic and eukaryotic organisms [1-9]. This environmental pollutant is associated with a broad spectrum of anthropogenic activities including mining and heavy industries [1,9-11]. It has been shown that areas with high concentration of cadmium(II) ions have the increased incidence of illnesses including cancer [12-14]. Entry of metal ions into an organism can be performed at different levels by food, air, through skin etc. At the cellular level the situation is more complex. Unlike gases, metals generally cannot penetrate through the plasma membrane spontaneously. Cells have evolved a variety of mechanisms for transporting of various metallic elements. Compounds rich in free –SH moieties as cysteine and reduced glutathione belong to key molecules interacting with heavy metal ions after their entering through cell transporters. Cysteine, which chemical fomula is shown in Scheme 1, is due to its chemical properties parts of many metal-binding peptides, structural proteins and enzymes [15,16]. Due to the presence of free –SH moieties, it is not surprising that this amino acid is a part of peptides and protein directly connected with the protective mechanisms in a cell against adverse effects of metal ions. Reduced glutathione (GSH), as a tripeptide containing cysteine, is a vital intra- and extra-cellular protective antioxidant, which plays a number of key or crucial roles in the control of signalling processes, detoxifying of some xenobiotics and heavy metals as well as other functions. Glutathione is found almost exclusively in its reduced form; since the enzyme, which reverts it from its oxidized form (GSSG) called glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used as a marker of cellular toxicity [17-20]. The sulfhydryl group of glutathione is highly reactive and is often found conjugated to other molecules such as nitric oxide (NO) via its sulfhydryl moiety [21-25]. In addition, due to its reactivity, GSH can be used for synthesis of phytochelatins by transferring γ-Glu-Cys moiety from a donor to an acceptor molecule. Phytochelatins (PCs, a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2 to 11)) belong to the most studied plant stress peptides [26-29] participating in the detoxification of heavy metals, because they have an ability to bind heavy metal ions via –SH groups of cysteine units and consequently transport them to vacuole [27-31], where there is no longer their toxicity.

Metallothioneins (MTs) are proteins with unusually content of cysteine moieties responsible also for protection of an organism against metal ions. Mammalian MTs are small proteins (6-10 kDa) with the high content of cysteine in its primary structure (up to 30 %) and with absence of aromatic amino acids. Main function of metallothioneins in organisms consists in transport of heavy metal ions, maintenance of oxidation-reduction status and regulation of gene expression. This protein can be considered as a marker of environmental pollution as well as some diseases including tumour ones [32-35].



Scheme 1. Chemical structure amino acid cysteine. Cysteine (abbreviated as Cys or C) is an α-amino acid with the chemical formula HO2CCH(NH2)CH2SH. It is a non-essential amino acid. Its codons are UGU and UGC. The side chain on cysteine is thiol, which is polar and thus cysteine is usually classified as a hydrophilic amino acid. The thiol side chain often participates in enzymatic reactions, serving as a nucleophile. The thiol is susceptible to oxidization to give the disulphide derivative cystine, which serves an important structural role in many proteins [36].

1.1 The importance of thiols in metal ions intoxication

It is obvious that adverse effects of metal ions differ markedly compared plant and animals, but the role of cysteine must be considered for both groups. *Plants*. It was shown during hydroponic experiments that the uptake of Pb and Cd was enhanced in the presence of cysteine and glutathione, whereas no or very low uptake was observed in EDTA and penicillamine controls. Uptake rates were also enhanced after pre-exposure to cysteine or glutathione and inhibited in the presence of vanadate, suggesting a biological mechanism of uptake. Increasing concentrations of glutathione in solution resulted in decreasing Pb uptake rates, indicating competition for transport between free-glutathione and Pb-glutathione species. Pb uptake in the presence of increasing cysteine concentrations resulted in decreased uptake initially but linearly increasing uptake at higher concentrations [37]. It is clear that metal-thiol complexes can enhance metal uptake by roots, therefore it is important to determine whether thiols effectively solubilize these metals from the soil matrix. Vadas and Ahner conducted an experiment to study this phenomenon by combining 1 g metal ion contaminated soil and 10 ml of 10 mM thiol solution (cysteine and/or reduced glutathione) and shaking for 1 h. Both cysteine and glutathione extracted between 5 and 45% of Pb and Cd from laboratory and field-contaminated soils at pHs > 6 after 1 h. In the presence of oxygen, the half-lie of reduced cysteine was 0.1 h and dissolved

metal concentrations decreased to nearly zero over 24 h. In extractions with glutathione, both the metals and thiol were more stable, with a half-life for glutathione of 23 h, and stable dissolved metal concentrations over 24 h in the presence of oxygen. While overall cysteine was more effective than glutathione at extracting Pb from soils, its propensity to oxidize may limit its ability to increase the bioavailability of this metal to plants in toxic environments [38]. Besides uptake itself, cysteine plays a key role in detoxification mechanism in a plant. To better describe the mechanisms connected with cysteine synthesis, the experiments with modification of the connected enzymes are of great importance. O-Acetylserine lyase (OASTL, EC 2.5.1.47) catalyses the final step in cysteine biosynthesis. Transgenic plants showed markedly increased accumulation of transcripts and higher cysteine content compared with the wild-type. Results of this study demonstrate that overexpression of GmOASTL4 in tobacco can enhance cysteine levels and increase tolerance to cadmium stress [39]. The other authors investigated the interactions between Cd(II) and the C-terminal region of phytochelatin synthase using recombinant wild-type and mutant PC synthase as other enzyme closely connected with cysteine and its connection with detoxification mechanisms. They showed that site-directed mutagenesis of Cys residues at (CCXXXCXXC366)-C-358-X-359-X-363 motif decreases the number of Cd(II) and other heavy metal ions interacting with the enzyme, and that the motif binds the metals discriminatingly. The findings indicate that Cys exists as a free SH residue and that it is involved in the regulation of PC enzyme activity by transferring the metals into closer proximity with the catalytic domain [40]. In addition, the effects of cadmium on the accumulation of hydrogen peroxide and antioxidant enzyme activities in plant roots and the role of N-acetyl-L-cysteine (NAC) as a cysteine donor against Cd toxicity were investigated. The authors found involvement of NAC in the Cd tolerance mechanism through increased biosynthesis of Cd-binding proteins [41]. It follows from the mentioned results that interactions between cysteine and metal ions must be considered and play key role in high tolerance of plant cells to increased concentrations of the metal ions, but the mechanisms are still unclear [42-46].

Taking into an account the fact that animals have evolved complex mechanisms to protect themselves against metal ions based on the proteins, role of cysteine is not obvious. On the other hand, performed studies show that cysteine play also key role in detoxification of metal ions in animals. L-Cysteine given before a toxic dose of Cd resulted in high acetylcholinesterase (AChE) activity a total antioxidant status similar to the control values, and survival of the treated rats. Cd increased brain AChE activity and decreased brain total antioxidant status of adult male rats. Zinc, Ca and Cys, given just before Cd administration, modified the Cd-induced effects and restored rat brain total antioxidant status to the control levels [47]. Moreover, the influence of cysteine or N-acetyl cysteine administration on the efficacy of 2,3-dimercaptopropane-1-sulphonate (DMPS) in the treatment of cadmium intoxication was investigated in cadmium-pre-exposed rats. Cysteine, N-acetyl cysteine, DMPS, DMPS + cysteine or DMPS + N -acetyl cysteine were about equal in effectiveness in mobilizing hepatic cadmium mainly from its supernatant cytosolic fraction and both of the combinations were more effective than either of them alone in mobilizing cadmium from its nuclear mitochondrial fraction. The DMPS was apparently more effective than cysteine or N-acetyl cysteine in mobilizing renal cadmium and it was more effective than even their combinations in mobilizing cadmium from renal tissue. The treatment with cysteine or N-acetyl cysteine reduced cadmium-

induced hepatic and renal metallothionein and the treatment with DMPS reduced renal metallothionein only, probably due to removal of hepatic and renal cytosolic fraction cadmium by these agents [48]. N-(2,3,4,5,6-pentahydroxylhexyl)-L-cysteine (4) and NN'-di(2,3,4,5,6-pentahydroxylhexyl)-L-cystine (5) were synthesized. As antagonists of cadmium or lead intoxication, they were found to be effective by oral administration on repeated exposure. The bioassay *in vivo* indicated that their cadmium-mobilizing potencies are significantly superior to those of N-acetyl-L-cysteine and their lead-mobilizing properties are equivalent to those of DL-penicillamine [49].

1.2 Cysteine-cadmium complexes and nanotechnologies

It is clear that cysteine and cadmium(II) ions can form complexes. It is not surprising that these complexes are being investigated. Isothermal titration calorimetry was used to study the binding of Cd(II) by phytochelatins and their selected fragments including cysteine in order to understand the influence of the chain length on the complex stabilities. Different complexes are formed with glutathione and its fragments, Cys, Cys-Gly and gamma Glu-Cys, and their stabilities depend on the corresponding pK(a) value of the thiol group in the ligands. The stability of Cd-PC(n) complexes increases moving towards higher PC(2-5), as well as the complexing capacity expressed as the number of metal ions that can be bound by one ligand molecule. The affinity of Cd(II) for the PC(n) can be described by the following GSH < PC(2) < PC(3) <= PC(4) <= PC(5) sequence [50]. To compare binding between cadmium(II) ions and various amino acids, thermogravimetric measurements were carried out. The obtained results indicated that the main step of the thermal degradation of all amino acid adducts is connected to the rupture of the metal-ligand bonds, giving the associated activation energies values of 77, 44, 55 and 41 kJ mol^{-1} for $CdCl_2$.nL, n=1.0 and 1.5, for histidine and cysteine, respectively [51]. Studying of the complex formation between cadmium(II) and the ligands cysteine and penicillamine in aqueous solutions reveals differences between cysteine and penicillamine as ligands to the cadmium(II) ion that can explain why cysteine-rich metallothionines are capable of capturing cadmium(II) ions, while penicillamine, clinically useful for treating the toxic effects of mercury(II) and lead(II) exposure, is not efficient against cadmium(II) poisoning [52].It was also described that the presence of chiral carbons in molecules containing cysteine markedly influence their affinities to zinc(II) and cadmium(II) ions [53].

Affinity of cadmium and cysteine can be also used for synthesis of some cadmium-based nanoparticles. A one-pot synthesis was reported of water-soluble cadmium sulphide nanoclusters capped with cysteine ester, with an average size of 2.0 nm and fluorescing in the blue region [54]. Cadmium(II) cysteinate compounds have recently been recognized to provide an environmentally friendly route for the production of CdS nanoparticles, used in semiconductors. Stretching modes indicates monodentate or strongly asymmetrical bidentate coordination of a cysteine carboxylate group in the CdS(3)O units. The combined results are consistent with a "cyclic/cage" type of structure for both the amorphous solids 1 and 2, composed of CdS(4) and CdS(3)O units with single thiolate (Cd-S-Cd) bridges, although a minor amount of cadmium(II) sites with CdS(3)O(2-3) and CdS(4)O coordination geometries cannot be ruled out [55].

1.3 Analytical methods based on cysteine-cadmium interaction

It is clear that above demonstrated affinity of cadmium(II) ions is considerable, therefore, it can be used for analytical instrument fabrications. Screen-printed carbon electrodes were fabricated with amino acid functionality by using *in situ* co-deposition of mercury and cysteine. The three-electrode configuration (graphite carbon working electrode, carbon counter electrode and silver/silver chloride reference electrode) incorporating a cysteine-modified working electrode exhibited good sensitivity towards cadmium(II). The stripping chronopotentiometric response for cadmium(II) was linear in the concentration range 0.4-800 μg/l when a deposition time of 2 min was used [56]. Another promising modified electrode was fabricated by polymerization a conductive polymer film of dipicolinic acid (DPA) onto gold nanoparticle (AuNP)-cysteine-gold electrode. This chemically modified electrode was used for electrochemical determination of cadmium and zinc in aqueous media using differential pulse anodic stripping voltammetry [57]. In addition to cysteine, an electrochemical sensor for the detection of cadmium(II) ions was described using immobilized GSH as a selective ligand. First, a self-assembled monolayer of 3-mercaptopropionic acid (MPA) was formed on a gold electrode. The carboxyl terminus then allowed attachment of GSH via carbodiimide coupling to give the MPA-GSH modified electrode. A cadmium(II) ion formed a complex with glutathione via the free sulfhydryl group and also to the carboxyl groups. The complexed ion was reduced by linear and Osteryoung square wave voltammetry with a detection limit of 5 nM. The effect of the kinetics of accumulation of cadmium on the measured current was investigated and modelled. The increasing temperature of accumulation and electrochemical analysis caused an increase in the voltammetric peak of approximately 4% per degrees C around room temperature. The modified electrode could be regenerated, being stable for more than 16 repeated uses and more than two weeks if used once a day [58]. Poly(L-cysteine) was successfully immobilized on controlled-pore glass and used in a now injection system incorporating a microcolumn for separation of Cd from synthetic solutions. Stability constants governing the Cd adsorption by Poly(L-cysteine) were obtained by a nonlinear least-squares analysis of the Cd binding data and revealed at least four classes of binding sites were present and that the stable Cd complexation observed for the free molecule was retained by immobilized Poly(L-cysteine) [59]. L-cysteine functionalized multi-walled carbon nanotubes (MWCNTs-cysteine) were synthesized The capability of MWCNTs-cysteine for selective separation and preconcentration of heavy metal ions were statically and dynamically evaluated with Cd(II) as a model heavy metal ion. Unlike MWCNTs, the sorption of Cd(II) onto MWCNTs-cysteine was not influenced by ionic strength in a wide range [60]. Developed a colorimetric assay for the highly sensitive and selective detection of Cd(II) using gold nanoparticles (AuNPs) cofunctionalized with 6-mercaptonicotinic acid and L-cysteine through the formation of an Au-S bond. In the presence of Cd(II), the aggregation of functionalized AuNPs occurred by means of a metal-ligand interaction that led to visible colour changes [61]. There were also suggested other methods for analysis of metal ions [62-64].

In this study, we primarily aimed our attention on studying of basic electrochemical behaviour of cadmium(II) ions and low molecular mass thiol cysteine. Measured concentrations of cysteine were 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1000 μM, and cadmium(II) ions 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1000 nM. The changes in the signals as

rapid decreasing of cadmium(II) ion peak and free thiol moiety, and occurring of Cd-S complex peak were described and mathematically and statistically evaluated. Based on the statistics, we were able to estimate mathematical calibration model, detection limits and quantification limits for determination of cadmium(II) using sensors based on cysteine.

2. EXPERIMENTAL PART

2.1 Chemicals

Cysteine, cadmium(II) nitrate, sodium acetate, acetic acid, water and other chemicals were purchased from Sigma Aldrich in ACS purity unless noted otherwise. Pipetting was performed by certified pipettes Eppendorf (Germany). Working standard solutions were prepared daily by diluting the stock solutions. Stock solutions of 1 mg/ml of Cd was prepared by dissolving appropriate amount of cadmium(II) nitrate in water and diluted to 1000 ml volumetric flask. Acetate buffer of pH 5 was prepared with 0.2 M acetic acid and 0.2 M sodium acetate and diluted with water and used as a supporting electrolyte. High purity deionised water (Milli-Q Millipore 18.2 MΩ/cm, Bedford, MA, USA) was used throughout the study.

2.2 Preparation of deionised water and pH measurement

The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.3 Determination of cadmium by differential pulse voltammetry

Determination of cadmium by differential pulse voltammetry were performed with 746 VA Stand instrument connected to 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing VA Database 2.2 by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%). Acetate buffer (0.2 M CH3COONa + CH3COOH, pH = 5) as a supporting electrolyte was used. The supporting electrolyte was exchanged after an analysis. The parameters of the measurement were as follows: initial potential of –0.8 V, end potential 0.15 V, adsorption potential –0.8 V, deposition 240 s, deoxygenating with argon 90 s, time pulse 0.04 s, step potential 4 mV, modulation amplitude 25 mV, volume of injected sample: 100 µl, volume of measurement cell 2 ml (100 µl of sample + 1900 µl acetate buffer). Other experimental details are shown in the following papers [11,65].

2.4 Fully automated pipetting

Fully automated pipetting was carried out on automated pipetting system epMotion 5075 (Eppendorf, Germany). The pipetting provides a robotic arm with dispensing tools (TS50, TS300, TS1000), which are in the positions T1 – T4. The samples are placed in the positions B2 and C2 in Eppendorf Rack for 96 Test Tubes (diameter 10.9 mm and max. 75 mm length, no temperature control). Eppendorf Reservoir Holder with 7 × 3 0ml reservoirs (maximum filling volume: 30 ml, working volume: 25 ml, detection limit optical sensor: 3,000 µl) was located in the position B1, where acetate buffer, cadmium and cysteine solutions were available. The device was controlled by the PC software (Eppendorf). The tips were located in the A4 (epTIPS Motion, 50 µl), A3 (epTIPS Motion, 300 µl) and A2 (epTIPS Motion, 1,000 µl) positions. The experimental program is described in Results and discussion section.

2.5 Preparation of Cys-Cd complexes

The Cys-Cd complex was prepared by incubation of Cys 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1,000 µM with cadmium(II) as $Cd(NO_3)_2$ 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1,000 nM in the presence of 1000 µl of 100 mM acetate buffer pH 5.0 in dark at 20 °C. Experiment was performed in triplicates and samples were taken in the following time intervals as 0 h, 10 h and 15 h.

2.6 Mathematical treatment of data and estimation of detection limits

Mathematical analysis of the data and their graphical interpretation was realized by software Matlab (version 7.11.). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated, whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

Recently, we investigated electrochemically interaction of heavy metals with glutathione using differential pulse voltammetry Brdicka reaction [66]. The observed signal probably relates to forming of thiol-heavy metal ion complex adsorbed on the surface of the working electrode [66]. The observed affinity of glutathione to metal ions can be also related to the fact that level of these compounds can be considered as a marker of metal ion environmental pollution [67-73]. Besides thiols, cadmium as the most studied metal ions was suggested to disrupt the endocrine system, targeting in particular the oestrogen signalling pathway already at environmentally relevant concentrations. Thus far, the reports on the binding affinity of cadmium towards human oestrogen receptor alpha have been contradicting, as have been the reports on the *in vivo* oestrogenicity of cadmium [74].

3.1 Automatic pipetting system as a tool for studying of cadmium-cysteine interactions

The suggested experimental procedure can not be performed manually both because of large experimental errors and also the inability to meet well-defined time intervals. For this purpose, it seems very appropriate technology tool of fully automated pipetting robot. The system was previously validated by our group for fully automated detection of nucleic acids [75-79]. For our purposes, EP Motions robotic station with a variety of tools (suitable reservoirs, pipetting and temperature-controlled head position) was used.

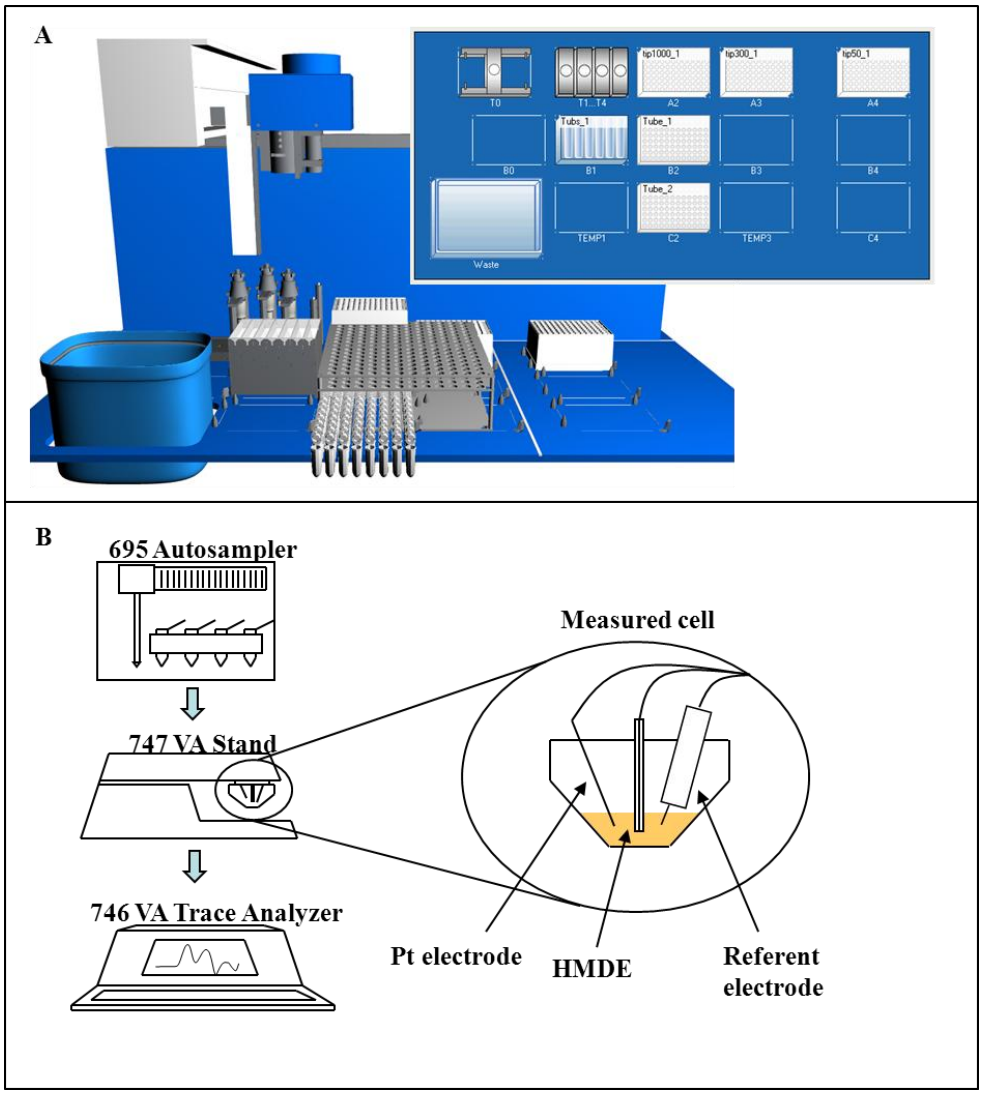


Figure 1. Technological linking of (**A**) pipetting robot and (**B**) electrochemical analyser. In the pipetting part, there are mixed solutions in given time intervals according to the programs. Subsequently, the mixed solutions are transferred to the electrochemical sampler and analysed electrochemically in well-defined time intervals.

The entire system is also fully programmable with a detailed review and testing software package. In our experimental arrangement the pipetting station was off line connected to the

electrochemical analyser (Figs. 1A and B). In pipetting space there were prepared all the necessary solutions (cadmium, cysteine and acetate buffer) to prepare an appropriate reaction mixture. Samples for interaction cadmium and cysteine were prepared from storage solutions (2 mM Cys, 2 μM Cd and acetate buffer pH = 5). The aim was to prepare mixtures of these concentrations of cadmium(II) ions 0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1,000 nM) with the following concentrations of cysteine (0, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1,000 μM). The program was as follows: A) Dosage of cadmium: reagent transfer (pipet tool: TS_300; transfer type: pipette; aspirate from bottom; liquid type: water). B) Dosage of acetate buffer: reagent transfer (pipet tool: TS_300; transfer type: pipette; aspirate from bottom; liquid type: water). C) Dosage of cysteine: reagent transfer (pipet Tool: TS_1000 + TS_300 + TS_50; transfer type: pipette; Aspirate from bottom). D) Mix after dispensing: No. of cycles: 3, speed: 10.4, volume: 50 μl, liquid type: water). E) Electrochemical analysis by differential pulse voltammetry. The samples were obtained in 4 minutes long time intervals. This time exactly followed the electrochemical analysis of each of the samples.

3.2 Electrochemical detection of cysteine-cadmium interactions

Electrochemical detection of cadmium ions on mercury electrode is of great interest [80-87], in spite of the fact that there are some hidden and undiscovered processes. In this study, cadmium(II) ions was accumulated at -0.9 V onto surface of hanging mercury drop electrode for 240 s. Formed Cd(Hg) was then electrochemical stripped and typical peak appeared (Fig. 2). Not-surprisingly, low concentrations of cadmium(II) ions gave well-developed peaks at -0.61±0.02 V (n = 5) (Fig. 2A). Further, we measured the dependence of the peak height on the concentration of cadmium(II) ions. The obtained dependence was strictly linear with the following equation $y = 0.0043x$; $R^2 = 0.9990$ (inset in Fig. 2A). Relative standard deviation did not exceed 3 %.

Under same conditions, cysteine was also analysed. This aminoacid has free –SH moiety, which has high affinity to mercury electrode. Formed Cys-(Hg) complex was stripped by forming typical signal at –0.28±0.03 V. It clearly follows from the results obtained that signals were well developed (Fig. 2B) and relative standard deviation did not exceed 3.5 %. The signals were shifting to more negative values with the increasing concentration of cysteine for 0.1 V per 100 μM. the cyclic voltammetric behaviour of L-cysteine at different pH values in the absence and presence of ions such as chloride from hydrochloric acid or sodium chloride, bromide from sodium bromide, and phosphate from the buffer made from potassium dihydrogen phosphate and sodium hydroxide were investigated. In the active region, the cathodic current depended on the starting potential as well as the L-cysteine concentration. Also if the starting potential was more anodic, the cathodic and anodic active regions extended much more into both cathodic and anodic regions. The admittance data indicated strong interactions between alpha-COO(-) and alpha-NH(3)(+) groups of L-cysteine [88].

Recently, The interactions between cysteine and cadmium(II) ions immediately after their mixing are shown in Fig. 2C. Concentration of cadmium(II) ions was constant and concentration of cysteine changed as 10, 25, 50, 75, 100, 150, 200 and 250 μM. In voltammograms, there is indicated

clearly the shift of cysteine signal (–0.34±0.02 V). In addition, the presence of cadmium(II) ions led to the change of the shape of cysteine signals. The decreasing part of voltammogram decreases less steeply and there is a distinct shift of the peak end with a slight increase with the increasing concentration of cysteine. Moreover, new signal appeared at –0.27 V, which clearly corresponded to Cys-Cd complex. The formation of this complex is clearly indicated in voltammograms in Fig. 2D, which were measured after 5 hours long interactions.

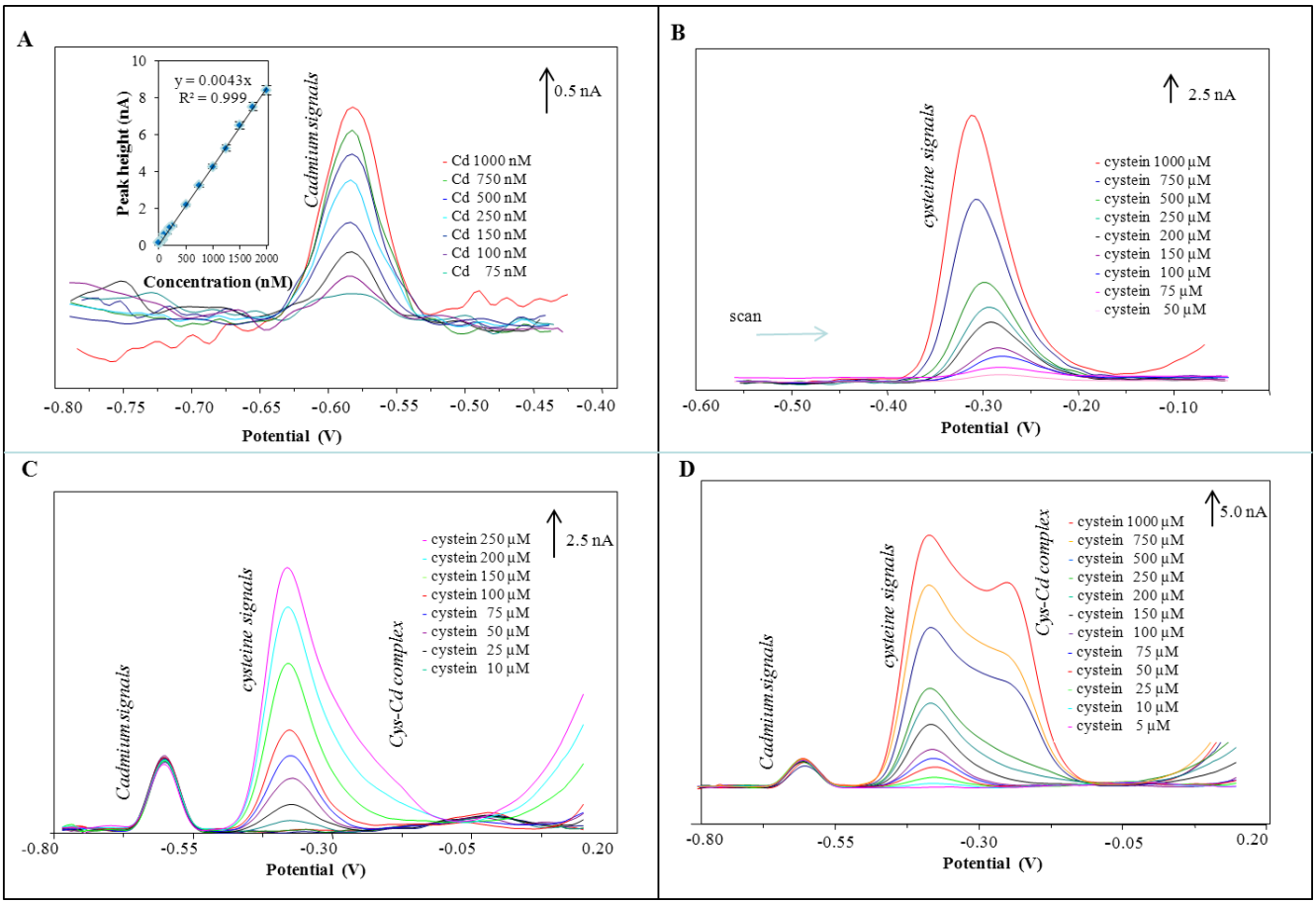


Figure 2. (**A**) Typical DP voltammograms of cadmium(II) ions (75, 100, 150, 250, 500, 750 and 1000 nM); in inset: calibration curve. (**B**) Typical DP voltammograms of (50, 75, 100, 150, 200, 250, 500, 750 and 1000 µM). (**C**) DP voltammograms of cadmium cysteine interactions; concentration cadmium(II) ions (1000 nM) and cysteine concentration (10, 25, 50, 75, 100, 150, 200 and 250 µM), time of incubation 0 h. (**D**) DP voltammograms of cadmium cysteine interactions; concentration cadmium(II) ions (1000 nM), cysteine concentration (5, 10, 25, 50, 75, 100, 150, 200, 250, 500, 750 and 1,000 µM), time of incubation 5 h.

The obtained experimental data were processed and evaluated with regard to changes in cysteine signals, which showed well observable differences (Figs. 3-5). Changes in the height of cysteine signal in the presence of cadmium(II) ions of the following concentrations as 75, 100, 150, 200, 250, 500, 750 and 1,000 are shown in Figs. 3A-H. Fig. 3A shows 1000 nM Cd; (B) 750 nM Cd; (C) 500 nM Cd; (D) 250 nM Cd; (E) 200 nM Cd; (F) 150 nM Cd; (G) 100 nM Cd and (H) 75 nM Cd.

The changes were followed in three time intervals as 0, 5 and 15 h. Cadmium(II) ions linearly decreased with the increasing concentrations of cysteine. When applying concentrations of cadmium(II) ions below 100 nM there were observed interesting effects at low concentrations of cysteine. In this area of concentration it leads to a dramatic increase in signal variability of cadmium(II) ions immediately after mixing. The signals of cadmium(II) ions showed a similar pattern as the signal cysteine. The obtained results revealed decline of the interaction dependence related to the applied concentration of cadmium (differences in the curves are most different in Figs. 3A, B, C, and least different in Fig. 3H). Changes in voltammetric signal corresponded to -SH groups ranged from 20 to 40% of the

Signal of complex Cys-Cd enhanced depending on the time of interaction and the concentration of cadmium(II) ions and cysteine. At low concentrations of cadmium(II) ions (below 100 nM) there was not possible to identify this signal. The amount of formed complex was associated with the interaction time and the maximum was observed after 5 hours long interaction. At longer interaction (15 hours), the signal decreased to 65-88% of the complex signal measured after 5 hours long interaction (n = 5). The interesting behaviour of Cd-Cys complex can be related to the published paper, in which the authors rationalized cadmium interactions with –SH moieties in terms of the replacement of hydrophobic GS-moieties coordinated to each mercurial by less hydrophobic Cys-moieties. Using X-ray absorption spectroscopy, the Cd-compound that eluted with a PBS-buffer that contained 5.0 mM GSH was structurally characterized as tetrahedral (GS)(4)Cd [89]. At the lowest concentrations of cadmium(II) ions 0.5, 1, 2.5, 5 and 10 nM very small changes were observed (Figs. 4A-H).

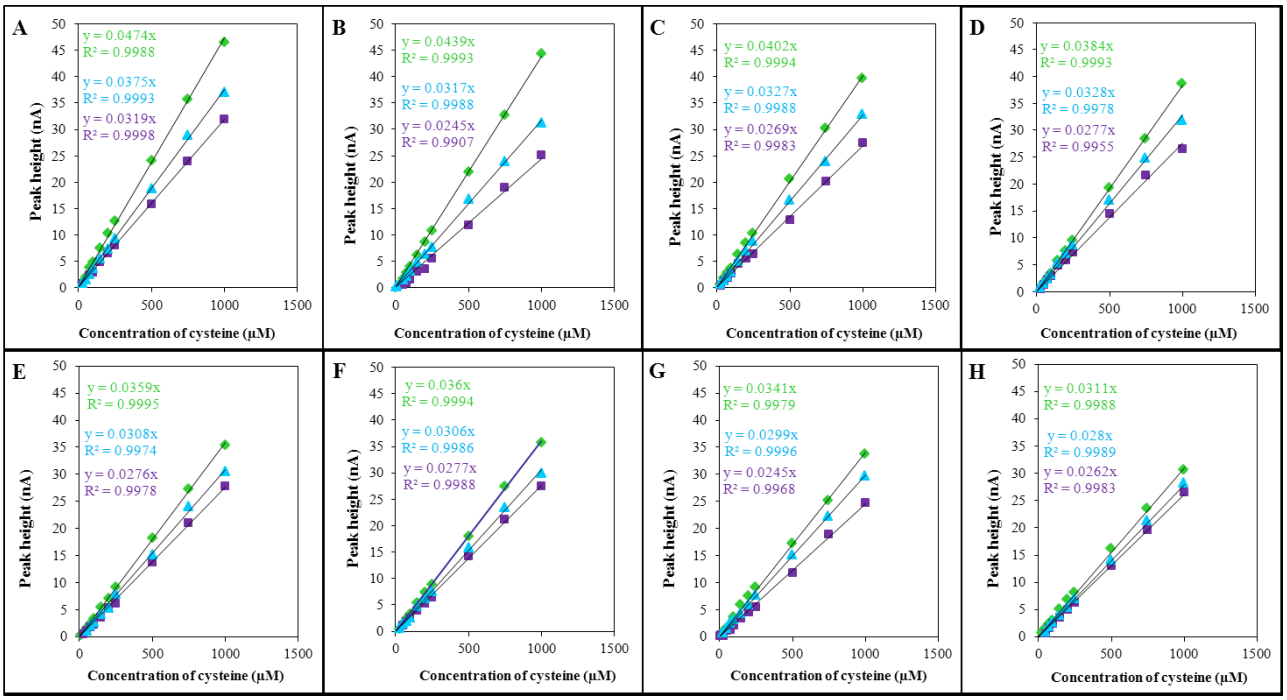


Figure 3. Cysteine signal as a function of cysteine concentration for various concentration of cadmium(II) ions (A) 1000 nM Cd; (B) 750 nM Cd; (C) 500 nM Cd; (D) 250 nM Cd; (E)200 nM Cd; (F) 150 nM Cd; (G) 100 nM Cd; (H) 75 nM Cd. Various times of the interaction are presented (-♦- 0h; -■- 5h; -▲- 15h).

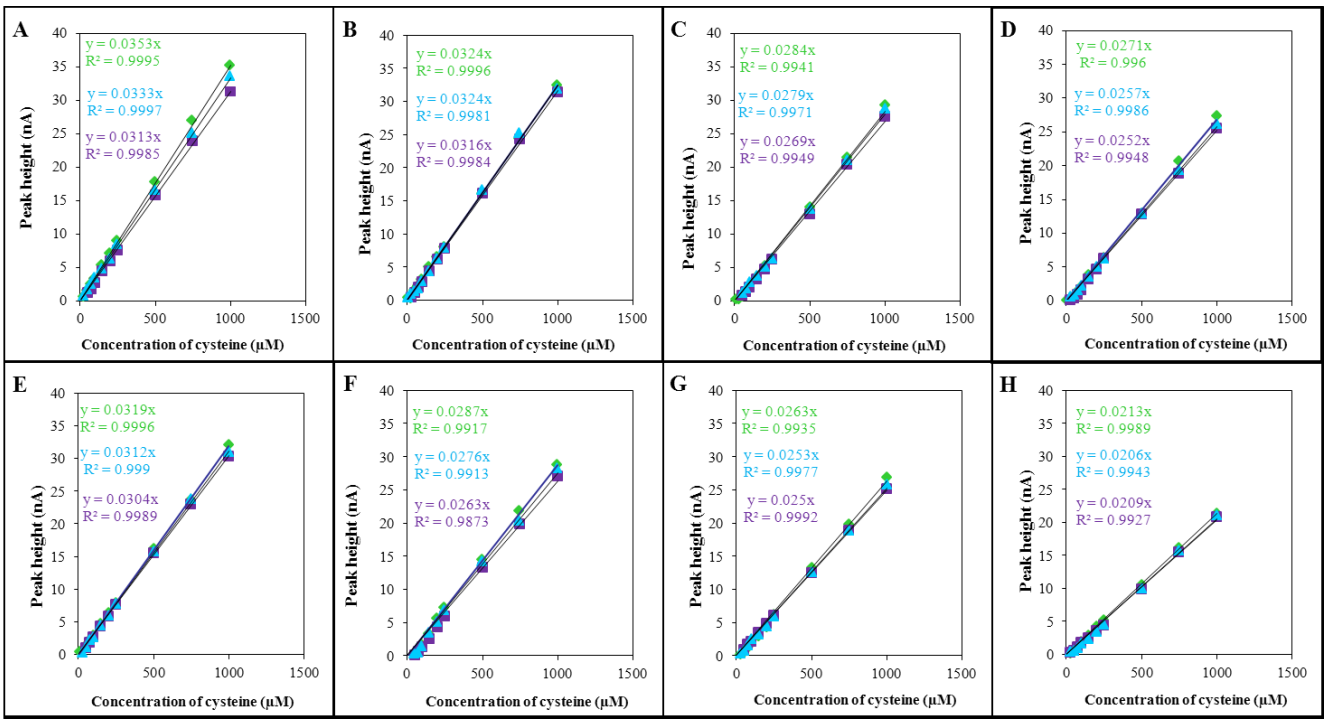


Figure 4. Cysteine signal as a function of cysteine concentration for various concentration of cadmium(II) ions (**A**) 50 nM Cd; (**B**) 25 nM Cd; (**C**) 10 nM Cd; (**D**) 5 nM Cd; (**E**) 2.5 nM Cd; (**F**) 1 nM Cd; (**G**) 0.5 nM Cd; (**H**) 0 nM Cd. Various times of the interaction are presented (-♦- 0h; -■- 5h; -▲- 15h).

For possible use of this technology there can be seen good resolution at a concentration of cadmium(II) ions from 25 to 50 nM. Furthermore, the results indicate the dynamic current imbalance in the complex formation of Cd-Cys, which is documented by shift of the signal after 15 h interaction.

3.3 Summarization of the results

The obtained experimental results were summarized in Fig. 5, where slopes of the obtained dependences shown in Fig. 3 and 4 are presented. When analysis was carried out immediately after mixing, the increased dependence on the concentration of cysteine was obtained, because the interaction between cadmium(II) ions and cysteine is not fast and content of –SH moieties is high. The obtained dependence varies from free cysteine very low (up to 20%), but free cysteine provides strictly linear relationship (R^2 = 0.999). The most significant changes were observed after 5 hours long interaction, where the signals decreased steeply also at low concentrations of both cysteine and cadmium(II) ions, and this trend could be observed throughout the experiment (Fig. 5A). After 15 hour long interactions there were detected changes in the ion balance and possibly to different changes in the structural arrangement of molecules. It clearly follows based on the obtained that there was a disclosure of some –SH moieties to the electrode and thus the observed signal increased. The slopes for dependencies of cadmium(II) ions signal on concentration of cysteine are shown in Fig 5B. The obtained experimental results revealed that cadmium(II) ions signal decreased. It is very interesting

relatively strong decrease in signal slopes immediately after mixing. At low concentrations of cadmium(II) ions after 5 and 15 hours long interaction, strong signal decrease was measured, which clearly corresponds to interaction of Cd with -SH groups of cysteine. With the increasing concentration of cadmium(II) ions signals are clearly reduced and, as in the case of –SH moieties, the difference between 5 and 15 hours of interaction was observed.

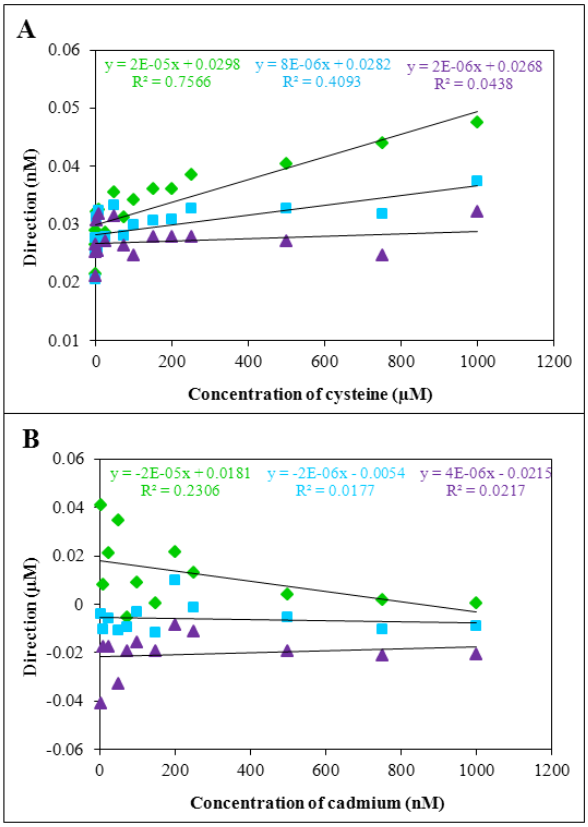


Figure 5. (**A**) Comparison of slopes of dependences of interactions cadmium(II) ions with varying concentrations of cysteine. (**B**) Comparison of slopes of dependences of interaction of constant cysteine concentration with varying concentrations of cadmium(II) ions. Various times of interaction are presented (-♦- 0h; -■- 5h; -▲- 15h).

4. CONCLUSIONS

Sorption isotherms for Cd(II) in presence of different substrates shows that Cd(II) uptake depends both on Cd(II) starting concentration and the nature of the substrate. Thermal decomposition of Cd-cysteine treated clay minerals evidences the evolution of H_2O, H_2S, NO_2, SO_2, and N_2O_3. Biophysical studies studies suggest that Cd(II) coordinates with oxygen atoms, to give monomer complexes or CdO molecules, either on the mineral surface and/or in the interlayer. For Cd-cysteine complexes the data agree with the existence of Cd-S clusters, thus suggesting a predominant role of the thiol group in the bonding of Cd with the amino acid [90]. In this study, we suggested a simple sensor for determination of Cd(II) based on the modification of the hanging mercury drop electrode surface

by thiols, which could be used for other types of the working electrodes. Based on the obtained results we assume that the suggested technique offers detection of heavy metals in the environment and can be also used for studying of biomolecules with cadmium(II) ions [8].

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