

Phytochelatin Modified Electrode Surface as a Sensitive Heavy-Metal Ion Biosensor

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Abstract: Electrochemical biosensors have superior properties over other existing measurement systems because they can provide rapid, simple and low-cost on-field determination of many biological active species and a number of dangerous pollutants. In our work, we suggested a new heavy metal biosensor based on interaction of heavy metal ions (Cd^{2+} and Zn^{2+}) with phytochelatin, which was adsorbed on the surface of the hanging mercury drop electrode, using adsorptive transfer stripping differential pulse voltammetry. In addition, we applied the suggested technique for the determination of heavy metals in a biological sample – human urine and platinum in a pharmaceutical drug. The detection limits (3 S/N) of Cd(II), Zn(II) and *cis*-platin were about 1.0, 13.3 and 1.9 pmole in 5 μl , respectively. On the basis of the obtained results, we propose that the suggested technique offers simple, rapid, and low-cost detection of heavy metals in environmental, biological and medical samples.

Keywords: phytochelatin, adsorptive transfer stripping, differential pulse voltammetry, mercury, cadmium, zinc, heavy metal sensor, human urine, *cis*-platin.

Introduction

Industries produce a number of undesirable species such as pesticides, toxic organic compounds, heavy metals and so on [1-5]. An increasing concentration of heavy metals in the environment is a serious problem for human and animal health protection and production of foodstuffs in many countries around the world [6-8]. That is why easy and quick detection of heavy metals at very low concentrations levels in environmental and biological samples is necessary for assurance against acute intoxications and, first of all, against long-time exposure that may lead to many diseases and death [9-10]. Several analytical methods such as atomic absorption spectrometry [11-13], inductively coupled plasma with mass spectrometry [14-16] as well as electrochemistry [17-21], have been developed for these purposes. Electrochemical biosensors have superior properties over the other existing measurement systems because they can provide rapid, simple and low-cost on-field determination of many biological active species and number of dangerous pollutants [22-29]. In addition, biosensor technology is a powerful alternative to conventional analytical techniques, combining the specificity and sensitivity of biological systems in small devices. A number of recently published papers describe the determination of heavy metals using electrochemical biosensors based on their interactions with DNA [26,29-33], enzymes (first of all urease) [34-38], bacteria [39-41] and proteins [42-43].

Besides high molecular species – proteins such as metallothionein – it is possible to use low molecular heavy metal binding compounds such as phytochelatins (PCs) for construction of biosensors. PCs, cysteine-rich small peptides, consist of 4-23 amino acids abounding in plants as a response on heavy metal stress [44-47], participate in the detoxification of heavy metals, because they have an ability to transport heavy metal ions to vacuole [45,48], where an immediate toxicity do not menace yet. Phytochelatins have a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2$ to 11) and with the presented heavy metals (M) form M-PC complexes, in which the metal is bind via SH group of cysteine unit [48-49]; see Figure 1A. PCs are synthesized from glutathione, which is catalysed by PC synthase (γ -glutamylcysteine dipeptidyltranspeptidase, EC 2.3.2.15) activated by an increased concentration of the heavy metal (Cd, Cu, Hg, As or Pb) in a plant cytoplasm [47]. Reduced glutathione (GSH) itself plays the important role in cell protection against heavy metals, and reactive oxygen species (ROS) that are able to oxidize GSH to GSSG (oxidized glutathione; disulfide glutathione) [50]. The GSH:GSSG ratio was found as an indicator of cell damage and some diseases [50,51].

The aim of this paper was to suggest a new heavy metal biosensor based on interaction of heavy metal (cadmium and zinc) with phytochelatin using adsorptive transfer stripping (AdTS) differential pulse voltammetry (DPV). The basic scheme of the proposed heavy metals biosensor is shown in Figure 1B.

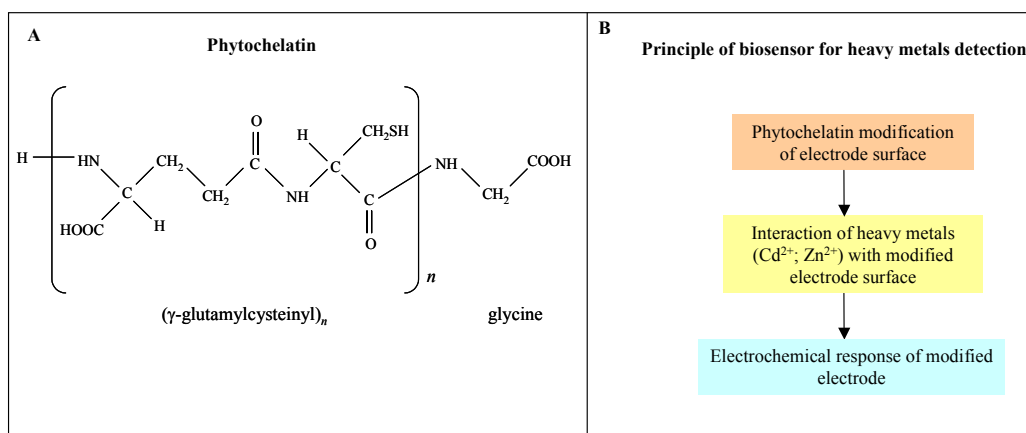


Figure 1. Chemical structure of phytochelatin (A). Scheme of basic principle of biosensor for heavy metals detection (B).

Materials and methods

Chemicals

Phytochelatin ($\gamma\text{-Glu-Cys}$)₂-Gly (PC₂) was synthesized in Clonestar Biotech; purity over 90% (Brno, Czech Republic). Tris(2-carboxyethyl)phosphine is produced by Molecular Probes (Eugen, Oregon, USA). Sodium chloride, cadmium nitrate, zinc nitrate and other used chemicals were purchased from Sigma Aldrich. The stock standard solutions of PC₂ at 10 $\mu\text{g}\cdot\text{ml}^{-1}$ were prepared by ACS water (Sigma-Aldrich, USA) and stored in the dark at $-20\text{ }^\circ\text{C}$. Working standard solutions were prepared daily by dilution of the stock solutions. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by personal computer program (MultiLab Pilot; Weilheim, Germany). The pH electrode (SenTix- H, pH 0–14/3M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

Electrochemical measurements

Electrochemical measurements were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 . The reference electrode was an Ag/AgCl/3M KCl electrode and the auxiliary electrode was a graphite electrode. The supporting electrolyte was prepared by mixing buffer components. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 240 s.

-Adsorptive transfer stripping (AdTS) differential pulse voltammetry (DPV) of phytochelatin

The amount of PC₂ was measured using AdTS DPV. The samples of the PC₂ were reduced before each measurement by 1 mM tris(2-carboxyethyl)phosphine addition according to [52]. The supporting electrolyte (sodium chloride: 0.5 M NaCl, pH 6.4) from Sigma Aldrich in ACS purity was purchased. DPV parameters were as follows: an initial potential of -1.2 V , an end potential -0.3 V , a modulation

time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV. All experiments were carried out at room temperature. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed.

Real samples

-Preparation of human urine

Human urine (obtained from healthy laboratory staff) was filtered through a Teflon disc filter (0.45 μm and 13 mm diameter, Alltech Associates, Deerfield, IL, USA) and 10 times diluted with 0.5 M sodium chloride (pH 6.4) before measurements. Moreover, we added to 10 times diluted solution of human urine Cd(II) and/or Zn(II) at 25, 50, 100, 225, 400, 600 and/or 50, 100, 200, 400, 600, 800 μM concentrations, respectively.

-Preparation of cis-platin – pharmaceutical drug

cis-Platin was synthesized and provided by Pliva-Lachema (Brno, Czech Republic) [53]. The stock standard solutions of *cis*-platin at 10 $\mu\text{g}\cdot\text{ml}^{-1}$ were prepared by sodium chloride solution (0.5 M, pH 6.4) and stored in the dark at $-20\text{ }^{\circ}\text{C}$. Working standard solutions were prepared daily by dilution of the stock solutions.

Statistical analysis

STATGRAPHICS® (Statistical Graphics Corp®, USA) was used for statistical analyses. Results are expressed as mean \pm S.D. unless noted otherwise. A value of $p < 0.05$ was considered significant.

Results and discussion

Papers concerning the construction of heavy metals biosensors, where fungi, bacteria, proteins or peptides served as biological part of these sensors, were recently published [3,30,33-34,38,40-43,54-62]. Due to increasing interest in heavy metals biosensor development, we engaged with electrochemical determination of heavy metals by means of their interaction with a phytochelatin (PC₂)-modified mercury electrode. Basic electrochemical behaviour of heavy metal binding thiols (e.g., glutathione, phytochelatin, metallothionein) has already been studied by many techniques such as chronopotentiometric stripping analysis [23,24,63-64], cyclic voltammetry [18,25,65-66] and differential pulse voltammetry [67-70]. Primarily, it was necessary to observe in detail the electrochemical behaviour of PC₂ on the surface of hanging mercury electrode (HMDE) by differential pulse voltammetry in combination with adsorptive transfer stripping technique (AdTS DPV) with the view to use them for construction of a heavy metal biosensor because the AdTS DPV technique has not been used for these purposes yet.

Adsorptive transfer stripping technique as a base of electrochemical biosensor

Adsorptive transfer stripping technique (AdTS) was developed as a suitable tool for electrochemical detection of biomolecules such as proteins, peptides and/or DNA [29,71-79]. Principle of the technique

is in an adsorbing of studied analyte on surface of the working electrode – in our case of HMDE at open electrode system; see Figure 2A₂.

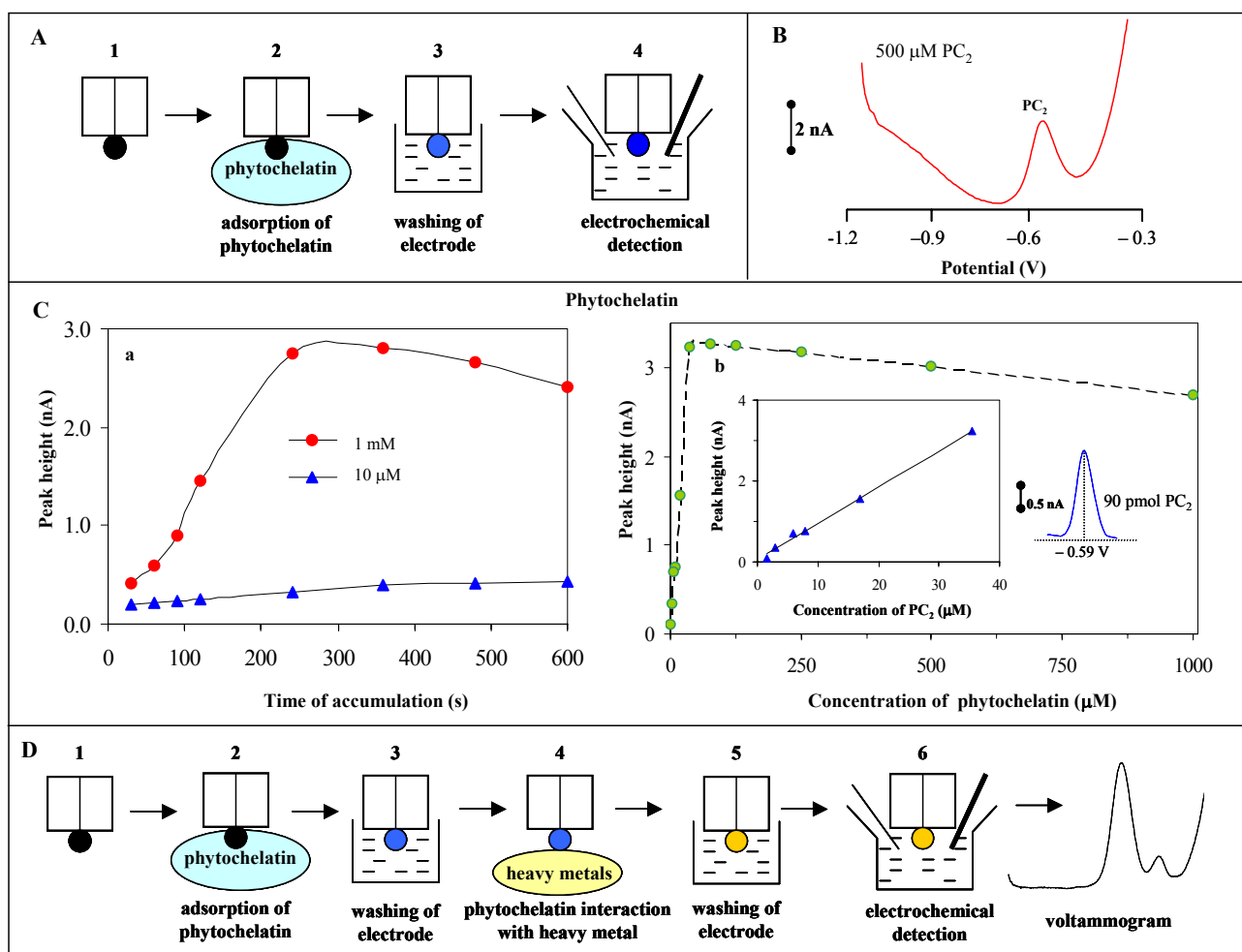


Figure 2. Scheme of adsorptive transfer stripping technique used for the detection of peptide – phytochelatin; (1) renewing of hanging mercury drop electrode (HMDE) surface; (2) adsorbing of PC₂ in a drop solution onto HMDE surface; (3) washing electrode in sodium chloride (0.5 M, pH 6.4); (4) measurement of PC₂ by differential pulse voltammetry (DPV) in 0.5 M sodium chloride, pH 6.4 (A). Typical DPV voltammograms of 500 μM PC₂ (B) obtained by AdTS DPV technique. DPV parameters were as follows: an initial potential of –1.2 V, an end potential –0.3 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV, an accumulation time of 120 s. Dependence of PC₂ peak height on accumulation time at its two different concentrations – 1 mM and 10 μM (Ca). Influence of phytochelatin concentrations on PC₂ peak height (Cb and inset in Cb). Inset in Figure Cb: peak of PC₂ (17 μM – 90 pmol of PC₂ in 5 μl drop) after baseline correction. Scheme of adsorptive transfer stripping technique used for the detection of heavy metals; (1) renewing of hanging mercury drop electrode (HMDE) surface; (2) adsorbing of PC₂ in a drop solution onto HMDE surface; (3) washing electrode in sodium chloride (0.5 M, pH 6.4); (4) interaction of heavy metal (cadmium and/or zinc) in a drop solution with peptide modified HMDE surface; (5) washing electrode in sodium chloride (0.5 M, pH 6.4); (6) measurement of PC₂ by DPV in 0.5 M sodium chloride, pH 6.4 (D).

After the absorbing, the electrode is removed from the solution and redundancy of analyte is washed from the surface of the working electrode in buffer (Figure 2A₃). The adsorbed analyte is finally detected in the presence of an indifferent electrolyte (Figure 2A₄). It was proved that during the described process running on the surface of HMDE, only one assembled layer of the adsorbed analyte, which can be bio-macromolecules species and/or compounds capable of adsorbing on the electrode surface, could form [80]. On the base of the above-mentioned description of the transfer technique, we were concerned with the possibility of using of a peptide (PC₂) modified HMDE surface for heavy metals determination. Primarily, we focused on the optimisation of the modification of the electrode surface by phytochelatin.

Using the adsorptive transfer stripping technique for determination of phytochelatin

An electrochemical behaviour of the phytochelatin 2 (key plant peptide binding heavy metals; PC₂) was studied on the surface of the HMDE by differential pulse voltammetry (DPV) in combination with adsorptive transfer stripping technique (AdTS). The voltammogram of 500 µM PC₂ accumulated on the HMDE surface during the time of 120 s and analysed in 0.5 M NaCl (pH 6.4) is shown in Figure 2B. On the obtained record, we observed the signal at potential -0.57 V, which probably correspond to adduct of the PC₂ with mercury on the surface of the HMDE (HS-peptide + Hg = HgS-peptide) [81-82].

It was necessary to know the way of probable interaction of peptide with the working electrode surface with the view to use the modified HMDE as a suitable toll for detection of heavy metals. On the most important index of status of electrode, the double-layer is dependent on the current response on the accumulation time [80,83]. An influence of the accumulation time of PC₂ at 1 mM and 10 µM concentrations on the electrochemical response (current height of PC₂ signal) was studied. The observed dependence at 1 mM PC₂ concentration steeply increased up to 240 s and resembled to the Langmuir isotherm (Figure 2Ca). From the obtained results it follows that the signal of PC₂ increased up to 240 s of the peptide accumulation time, which is probably connected with sequent filling up of the electrode surface. The maximum of the presented curve at 240 s probably relates with needed time for filling up of the HMDE electrode surface by one layer of PC₂ – surface assembled monolayer (SAM) [84]. After 240 s, the signal of adsorbed peptide did not increase, contrariwise decreased, which probably relates with formation of the poly-layer of the PC₂ on the HMDE surface – decreasing the possibility of the detection of adsorbed molecules. In the case of lower tested PC₂ concentration (10 µM), we observed the increase of PC₂ peak height with increasing accumulation time at all tested values (Figure 2C(a)). In addition, we indeed observed very low increase of the peak height (about 4 %) from the accumulation time of 360 s. Due to using of PC₂ at 1 mM concentration for the determination of heavy metals, we used the accumulation time of 240 s, because up this time the surface assembled monolayer is formed.

The next important index of behaviour of phytochelatin on the HMDE surface was the change of PC₂ current response according to its different concentrations. At the accumulation time of 240 s, concentrations of PC₂ varying from 2.5 to 1000 µM were tested (Figure 2C(b)). The obtained dependence set from the obtained PC₂ current responses according to its different concentrations was linear in the concentration range 0 – 40 µM ($y = 0.0894 + 0.0621x$; $R^2 = 0.9969$, inset in Figure 2C(b)).

In addition, we observed a decrease of the current responses from the PC₂ concentration of 100 µM. This phenomenon probably relates with a forming of poly-layer on the electrode surface [80,83,85].

Modification of the HMDE surface by phytochelatin

For our purposes, we used the HMDE as the physical-chemical part and phytochelatin 2, which is able to bind heavy metals [68,70,86-93], as the biological part of the suggested heavy metals biosensor. That is why we could suggest following experiments: i) on the HMDE surface adsorb PC₂; ii) remove redundant PC₂; iii) expose the adsorbed PC₂ to interaction with heavy metal; iv) detect changes in the signals of PC₂ (Figure 2D). We selected for our purposes two heavy metals – cadmium and zinc.

That is why we were interested if free ions of selected heavy metal are able to adsorption and transfer on the HMDE surface. If we accumulated (120 s) only free ions of Cd(II) and/or Zn(II) without MT on the surface of the mercury working electrode, we did not observe any signal corresponding to heavy metal species (not shown). The described effect prove that free ions of heavy metals are not able to transfer and consequently to detect.

Electrochemical behaviour of phytochelatin-modified HMDE in presence of Cd(II) and Zn(II)

Phytochelatin 2 (1 mM) was adsorbed on the HMDE surface for the duration of 240 s. Then, the modified electrode was washed in the basic electrolyte solution and consequently, interacted with 500 µM of Cd(II) and/or Zn(II) for the duration of 300 s that was established as the most effective. The obtained voltammograms are shown in Figure 3A (Cd) and 4B (Zn). In the presence of Cd(II) we recorded except original signal PC₂ also another two signals that we named CdPC₂ (−0.76 V) and PC₂(Cd) (−0.45 V). In addition, we observed a linear decrease of PC₂ signal and increase of CdPC₂ and PC₂(Cd) signals according to the rise of Cd(II) added concentration. The equations of the mentioned rising linear curves were in case of CdPC₂: $y = 0.0128x + 0.2085$; $R^2 = 0.9918$; and PC₂(Cd): $y = 0.0999x - 4.8325$; $R^2 = 0.9997$. The detection limit (3 S/N) of Cd(II) calculated from increase of PC₂(Cd) peak was about 1.055 pmole in 5 µl (0.211 µM); see Figure 3C.

In the case of Zn(II) determination by PC₂ modified HMDE, we observed only one additive signal, which was named ZnPC₂: −1.09 V, in comparison with control detection of PC₂ without interaction with Zn(II). The signal of PC₂ linear decreased and ZnPC₂ signal linearly increased ($y = 0.8496x - 18.598$, $R^2 = 0.9961$) according to rising Zn(II) concentration. The detection limit (3 S/N) of Zn(II) calculated from increase of ZnPC₂ peak was about 13.30 pmole in 5 µl (2.66 µM); see Figure 3D.

Determination of Cd(II) and Zn(II) by PC₂ modified HMDE in biological matrix

We decided to test our peptide-modified heavy metal biosensor by means of detection of Cd(II) and/or Zn(II) in the presence of the biological matrix (human urine). In the concrete, PC₂ (1 mM) was adsorbed (240 s) on the HMDE surface, washed (0.5 NaCl) and then the modified electrode interacted with Cd(II) and/or Zn(II) in the presence of human urine (10× diluted) for the duration of 300 s. Subsequently, the electrode was washed (0.5 NaCl) and placed to an electrochemical cell-containing supporting electrolyte (0.5 M NaCl; pH = 6.4). Human urine contained additions of Cd(II) and/or

Zn(II) at concentrations (25, 50, 100, 225, 400, 600 and/or 50, 100, 200, 400, 600, 800 μM , respectively). All studied signals embodied very similar electrochemical behaviour in the course of their analysis both in buffered solution and biological matrix (not shown). The only two differences between analysis in buffered and non-buffered medium, which we found out, are peak heights and their relationship. In the presence of human urine, heights of all studied signals were lower than in the buffered medium – sodium chloride (differences from 10 to 15%). This effect is probably caused by impurities included with real sample that may complex with heavy metal ions.

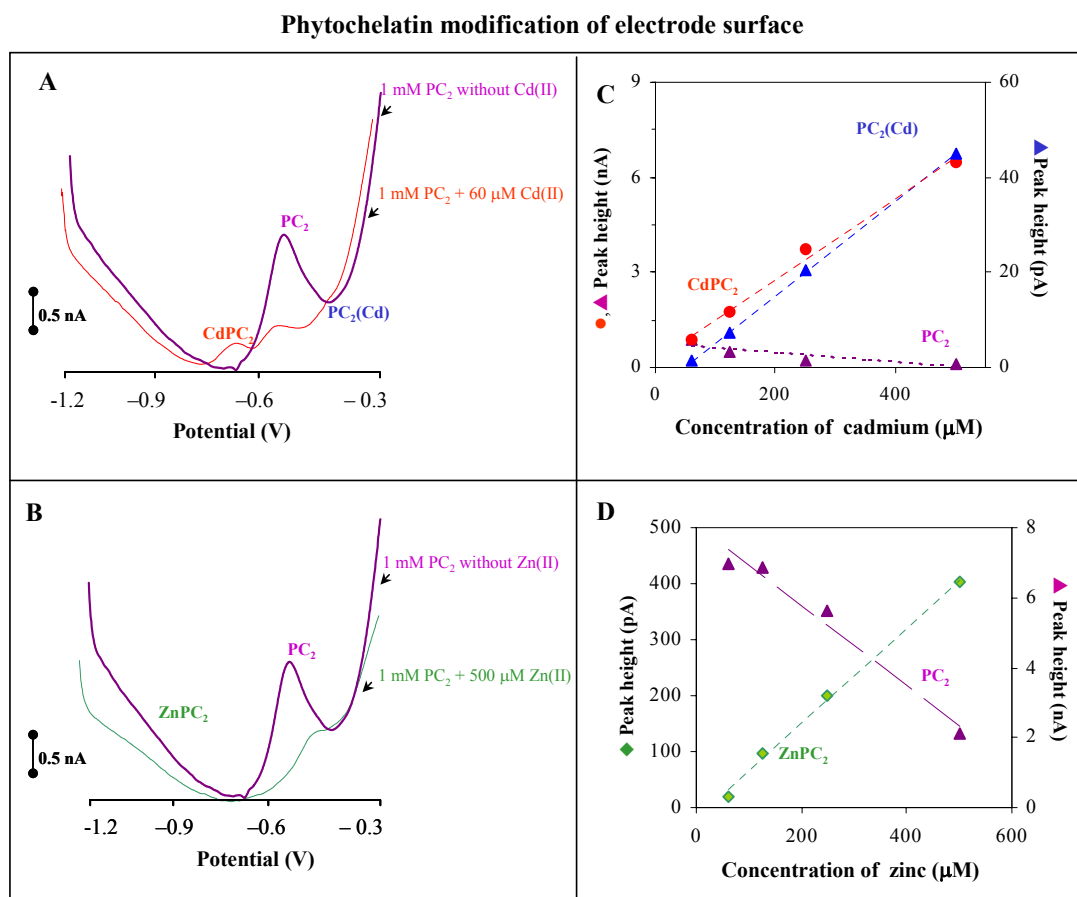


Figure 3. Phytochelatin. Typical DPV voltammograms of 1 mM PC_2 without addition of Cd(II) and 1 mM $\text{PC}_2 + 60 \mu\text{M}$ cadmium ions (A), and 1 mM PC_2 without addition of Zn(II) and 1 mM $\text{PC}_2 + 500 \mu\text{M}$ zinc ions (B). DPV parameters: time of accumulation 240 s, time of interaction 300 s. Dependence of CdPC_2 , $\text{PC}_2(\text{Cd})$ and PC_2 peak heights on interaction time (C). Dependence of ZnPC_2 and PC_2 peak heights on interaction time (D). PC_2 concentration: 1 mM. For other details, see Figure 2.

Using of peptide-modified HMDE to study of anticancer drug – cis-platin

We attempted to use our suggested peptide modified heavy metal biosensor for the determination of the anticancer drug – cis-platin $[\text{Pt}^{\text{II}}(\text{NH}_3)_2\text{Cl}_2]^0$; MW 303. A prepared solution of Pt complex interacted with PC_2 modified HMDE for the duration of 300 s. A resulting voltammogram is shown in Figure 4A. A phytochelatin-modified HMDE surface formed with presented Pt complex that we detected at potential -0.96 V and named as PtPC_2 . In addition, we studied the influence of different

durations of interaction between the PC₂-modified electrode surface and Pt on height of the presented PtPC₂ signal. We selected a duration of 300 s as most suitable for the Pt complex interaction with the PC₂-modified electrode surface (not shown). The dependence of heights of the PC₂ and PtPC₂ signals on *cis*-platin concentration is shown in Figure 4B. The PC₂ signal decreased and PtPC₂ linearly increased ($y = 0.0532x - 5.7079$; $R^2 = 0.9946$) in the studied concentration of *cis*-platin varying from 100 to 750 μM . The detection limit (3 S/N) of *cis*-platin ($[\text{Pt}^{\text{II}}(\text{NH}_3)_2\text{Cl}_2]^0$) calculated from increase of PtPC₂ peak was about 1.958 pmole in 5 μl (0.392 μM) at the interaction time of 300 s.

Cis-platin – $[\text{Pt}^{\text{II}}(\text{NH}_3)_2\text{Cl}_2]^0$; anticancer drug

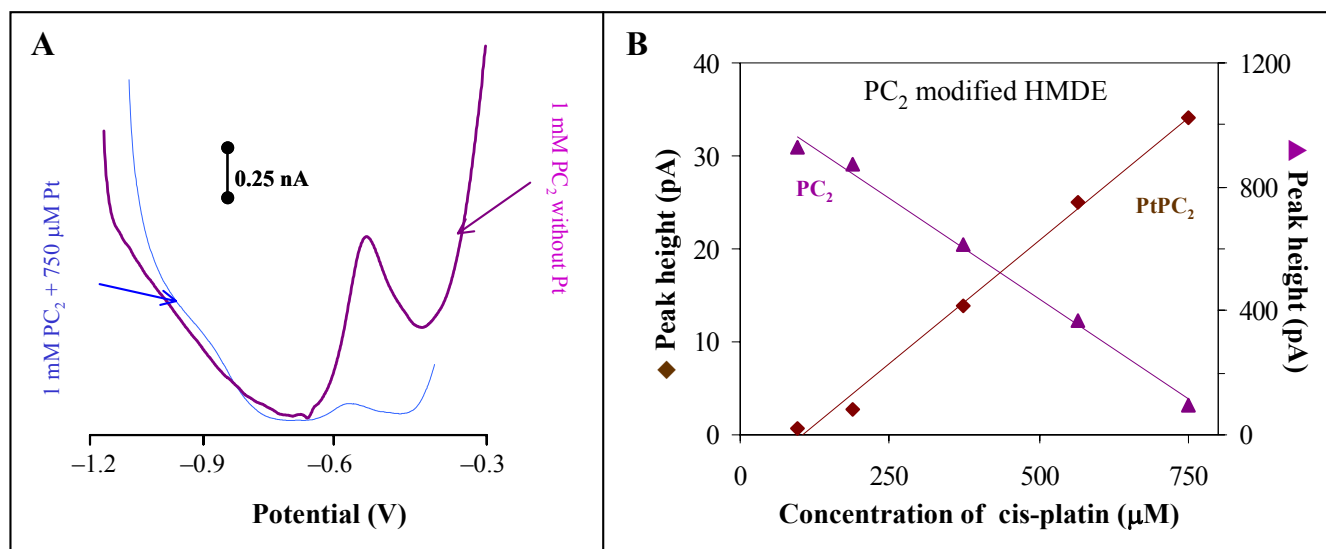


Figure 4. *Cis*-platin – $[\text{Pt}^{\text{II}}(\text{NH}_3)_2\text{Cl}_2]^0$; anticancer drug. Typical DPV voltammograms of 1 mM PC₂ without addition of *cis*-platin and 1 mM PC₂ + 750 μM of *cis*-platin (A). DPV parameters: time of accumulation 240 s, time of interaction 300 s. Dependences of PtPC₂ and PC₂ peak heights on different concentration of *cis*-platin (B). PC₂ concentration: 1 mM. DPV parameters: time of accumulation 240 s, time of interaction 300 s. For other details, see Figure 2.

Conclusions

A development of easy and rapid renewable sensors for the detection of different species is one of the most important tasks of analytical chemistry and biochemistry. We suggested a simple sensor for the determination of Cd(II) and Zn(II) using a modification of the hanging mercury drop electrode surface by phytochelatin. The main advantage of using HMDE in comparison with other solid electrodes (carbon, gold and so on) is sensitivity. On the basis of the obtained results we propose that the suggested technique offers simple, rapid, and low-cost detection of heavy metals in environmental, biological, and medical samples.

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