

## INVESTIGATION AND OPTIMIZATION OF REACTIVATION OF UREASE BIOSENSOR FOR HEAVY METALS INHIBITION ANALYSIS

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Received 14.09.2015

Research was aimed at the optimization of the urease biosensor for analysis of heavy metals and determining the opportunities of its reactivation. A differential pair of gold interdigitated electrodes deposited on a ceramic substrate was used as the conductometric transducer. As a bioselective membrane served urease, coimmobilized with bovine serum albumin by glutaraldehyde cross-linking on the surface of conductometric transducer. 1.0 mM urea was selected as an optimal substrate concentration for the inhibition analysis of heavy metals. The biosensor was tested for its sensitivity to different heavy metals, the calibration curves were plotted. The proposed biosensor was shown to have high reproducibility of signals prior and after inhibition, the measurement error was less than 3%. It was proved a possibility of reactivation of the bioselective membrane after urease irreversible inhibition by heavy metals, using the ethylenediamine tetraacetic acid solution. The optimum conditions of reactivation, i.e. the dependence of its level on time and concentration of heavy metals, were determined. The additional stage, post-inhibition reactivation, was shown to increase significantly the selectivity of biosensor determination of heavy metals.

**Key words:** conductometric transducer, biosensor, urease, heavy metals, inhibition analysis, enzyme reactivation.

At present, one of the most acute environmental problems is the contamination with waste products [1]. Among the various pollutants, heavy metals and their compounds are notable by abundance, high toxicity, ability to accumulate in living organisms [2]. They are widely used in manufacture, therefore, despite the clean-up, the content of heavy metals in industrial wastewater is rather high.

They also enter the environment with sewage, smoke and dust from industrial enterprises. Many metals form stable organic compounds, high solubility of which facilitates the migration of heavy metals in natural waters. More than 40 chemical elements are referred to as heavy metals, but significantly fewer number should be controlled taking into account toxicity, persistence, abundance, an ability to accumulate in the environment [3, 4].

In the aquatic environment, microparticles of heavy metals are present as solutions,

suspensions and colloids. Free hydrated ions, simple inorganic and organic complexes are soluble [5–9]. Currently, there are many methods, which are widely used for the determination of heavy metals, such as atomic absorption spectrometry, plasma mass spectrometry, etc. However, these methods utilize sophisticated appliances and are unsuitable in the field [10]. Electrochemical biosensor methods in general are more reasonable in the field because they do not require complex equipment [11–13].

Biosensor determination of heavy metal ions is based on their inhibition effect on enzymes. For example, ions of copper, cadmium, mercury, zinc are effective inhibitors of the urease activity in the urea hydrolysis. An important stage in the running of urease biosensor is a procedure of its reactivation after inhibition by heavy metal ions. An exposure of the inhibited biosensor in

buffer solution for a long time does not restore the enzymatic activity of its biomembranes whereas EDTA solution acts as efficient reactivator [14].

## Materials and Methods

### Materials

In the work, the enzyme urease from soya beans with activity of 66 U/mg was used (Fluka, Germany). Bovine serum albumin (BSA, fraction V), 50% aqueous solution of glutaraldehyde (GA) and urea were from Sigma-Aldrich Chemie (Germany).

The compounds for the preparation of buffers, inhibitors, reactivator and other inorganic compounds used in the work were of domestic production and had a chemical purity grade.

### Conductometric transducers

Conductometric transducers were produced in Lashkaryov Institute of Semiconductor Physics (Kyiv, Ukraine) in accordance with our recommendations. They consist of the sital substrate (fused  $\text{Al}_2\text{O}_3$ )  $5 \times 30$  mm in size with a pair of gold interdigitated electrodes. The  $0.1 \mu$  thick titanium sublayer is used for better adhesion of metal to the substrate. More information on the transducers used is in the previous paper [15].

### Scheme of experimental setup for conductometric measurements

The changes in conductivity of near-electrode layer of conductometric transducer were determined by the conductometric measuring unit. The differential mode of measurement was used to increase the sensor sensitivity and minimize noise caused by nonspecific effects. The scheme and function of this conductometric setup have been described earlier [16, 17].

### Preparation of bioselective elements

To prepare working urease-based bioselective membranes, the solution containing 5% of urease, 5% of BSA and 10% of glycerol in 20 mM phosphate buffer, pH 6.5, was used. The mixture for the reference membrane was prepared in the same way, but no enzyme was taken, only BSA of the final concentration equivalent to the amount of protein in the membrane, i.e. 10%. The solutions prepared were deposited onto the transducer working parts by micropipettes to cover completely the working electrode surface. The transducers with deposited membranes were incubated in saturated

glutaraldehyde (GA) vapor for 25 min and dried for 5 min in air at room temperature. Then the biosensors were washed for 6 min in the working buffer to remove excess unbound GA and other components of the membrane (changing the buffer every 2 min).

### Methods of measurement

Measurements were carried out at room temperature in an open cell filled with 5 mM phosphate buffer, pH 6.5, with constant stirring. The specified substrate concentration in the working cell was obtained by adding the aliquots of substrates stock solutions. For inhibition analysis, the responses to substrate were evaluated before inhibition, then the biosensor was incubated for 20 min in a solution of heavy metal ions of different concentrations and washed from excess inhibitor, and the response to substrate was re-assessed. In this way, a level of the enzyme inhibition, which corresponds to the concentration of heavy metal ions in the sample, was determined. Reactivation of the inhibited bioselective elements was performed by incubation in 10 mM EDTA solution for 30 min. Then they were washed from excess reactivator, and the response to the substrate was measured again.

All experiments were conducted in 3–5 series. Non-specific changes in the output signal associated with electrical noise and fluctuations in environmental temperature and pH were avoided due to use of a differential mode of measurements. In the statistical analysis of the results obtained, the values of arithmetic mean and its standard deviation were calculated; the data were considered significant at  $P < 0.05$ .

## Results and Discussion

### Study of main analytical characteristics of biosensor in direct substrate determination

The biosensor operation is underlain by the enzymatic reaction in the membrane containing urease deposited on the surface of conductometric transducer:



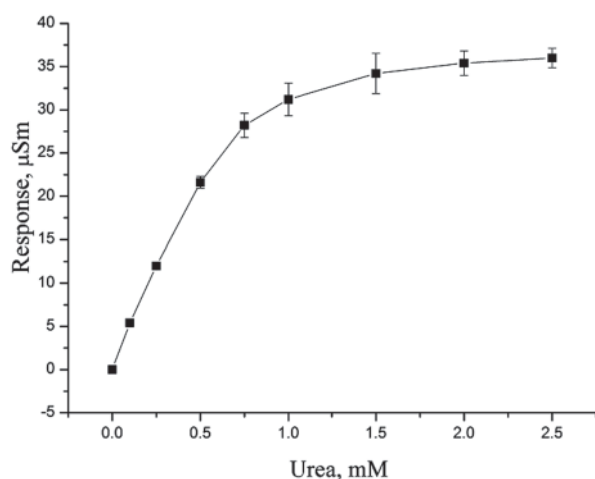
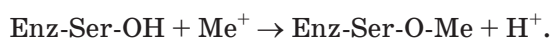
In the course of the reaction, protons are absorbed, which leads to changes in pH and generation of additional ions ( $\text{NH}_4^+$  and  $\text{HCO}_3^-$ ) in the working membrane [18]. This changes the solution conductivity, which is registered by the conductometric transducer.

In inhibition analysis, first it was necessary to determine an optimal concentration of urea as a substrate, i.e. to choose the urea concentration, at which the biosensor sensitivity to heavy metal ions is maximal. It is known that at irreversible inhibition the largest biosensor sensitivity to toxins can be reached if the substrate concentrations are within the range of biosensor saturation with the substrate when each enzyme molecule is involved in the conversion of this substrate. The graph of dependence of biosensor responses on the urea concentration in solution (Fig. 1) shows that above 1.0 mM complete biosensor saturation with the substrate is observed. Therefore, in further experiments on inhibition analysis 1.0 mM substrate was used.

It was necessary to confirm that a decrease in the biosensor response to the substrate after its incubation in a solution of heavy metals occurs due to the inhibition of bioselective element, but not because of the measurement error. Therefore, at the next stage of work the biosensor operational stability and reproducibility of its signal were tested. For this, the biosensor responses to the same substrate concentration were measured with an 12.5 min interval over one working day. The biosensor was characterized by relatively high signal reproducibility, standard deviation 2.93%.

#### *Research on sensitivity of developed biosensors to heavy metal ions*

Heavy metal ions can inhibit the biological activity of urease through the interaction with sulfhydryl groups of the enzyme active site:



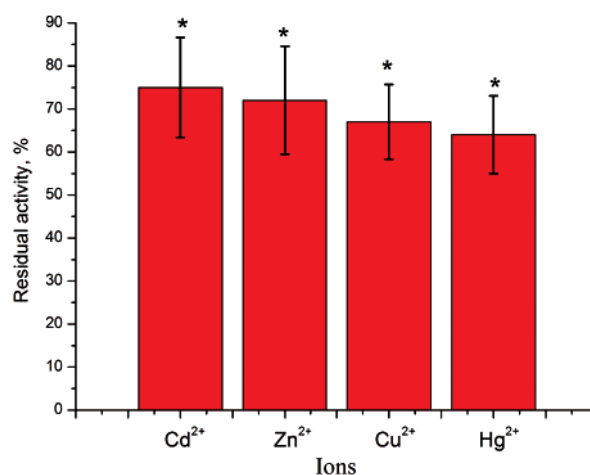
**Fig. 1. Dependence of biosensor response from urea concentration in solution.**  
Measurement was performed in 5 mM phosphate buffer, pH 6.5

The enzyme inhibition by heavy metal ions causes a decrease in the amount of ions of ammonium and bicarbonate formed as a result of enzymatic reaction of the substrate conversion, and thus — a decrease of the biosensor response [19–21].

Using selected optimal concentration of urea, it was investigated the level of inhibition of urease biosensor by various heavy metals of the same concentration (Fig. 2). Initially, the solutions of nitrates of divalent metals zinc, copper, mercury and cadmium of the same concentration (100 μM) were taken, so, the concentration of all ions was also equal. The results showed that inhibition depends on the kind of ion and its ability to inhibit the enzyme described by the inhibition constant [22, 23].

Next, sensitivity of the developed biosensor to different concentrations of heavy metals ions was studied taking copper ions as an example. The calibration curve of the residual activity of urease-based bioselective element after inhibition (Fig. 3) showed its strong dependence on the concentration of copper ions in the measurement environment.

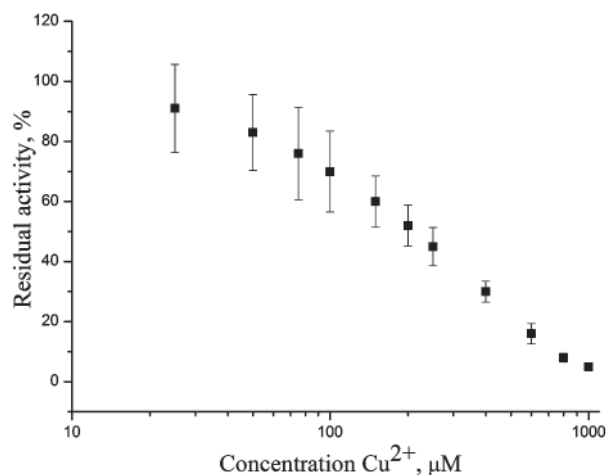
The stability of the biosensor inhibition after its incubation in the solution by heavy metal ions was also investigated (Fig. 4). For this, the responses of a number of biosensors to copper ions of the same concentration were measured after incubation under identical conditions. It was shown that the developed biosensors were characterized by relatively high signal reproducibility after inhibition with standard deviation of 2.6%.



**Fig. 2. Residual activity of urease biosensor after inhibition by different heavy metal ions.**

Ions concentration — 100 μM.

Measurement was performed in 5 mM phosphate buffer, pH 6.5; \*  $P < 0.05$  in comparison with initial activity of biosensor

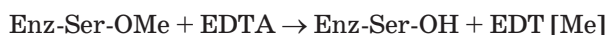


**Fig. 3. Residual activity of urease biosensor after inhibition by Cu<sup>2+</sup> ions with different concentrations. Inhibition time — 20 minutes.**

Measurement was performed in 5 mM phosphate buffer, pH 6.5

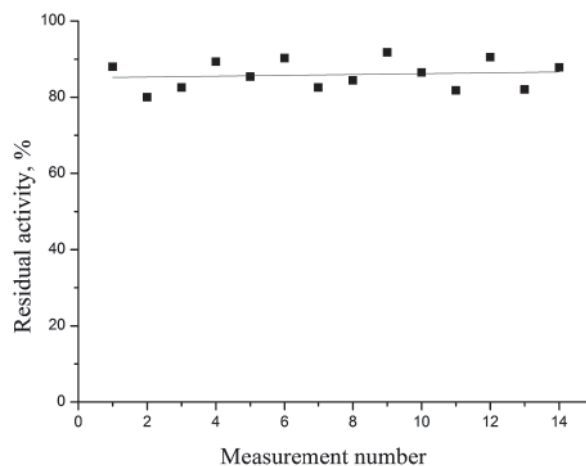
#### *Research and optimization of conditions of biosensor reactivation using EDTA*

The urease inhibition by heavy metal ions is known to be irreversible, which is a cause of inefficiency of urease-based biosensors due to its one-time usage. Therefore, it was proposed to use a stage of biosensor reactivation for its repetitive employ, namely, to explore the possibility of reactivation of the developed biosensor after its inactivation by heavy metal ions. The initial urease activity after inhibition can be recovered by the reactivator EDTA, which displaces the heavy metal ion bound to serine residue in the enzyme. Thus the enzyme is reactivated, i.e. its ability to interact with the substrate is restored [14, 24].



The reactivation efficiency depends strongly on the time of reactivation, reactivator concentration, and other parameters. Therefore, the conditions of reactivation by EDTA should be first optimized. For this purpose, it was chosen an optimal time of biosensor incubation in the reactivator solution, which corresponds to the highest possible level of recovery of the enzyme membrane activity after inhibition. The optimal time of reactivation by 10 mM EDTA was 30 min.

It was also studied an efficiency of reactivation of urease biosensor after its inhibition during constant time (30 min) in the solution of copper ions of different



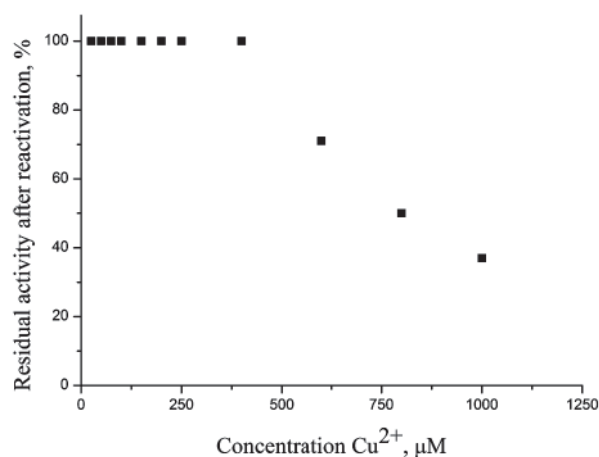
**Fig. 4. Reproducibility of urease biosensor after inhibitory analysis. Inhibitor concentration — 50 µM Cu<sup>2+</sup>. Inhibition time — 20 minutes.**

Measurement was performed in 5 mM phosphate buffer, pH 6.5

concentrations. Fig. 5 shows that reactivation under selected optimum conditions provided complete restoration of the enzymatic activity of membrane inhibited in the solution of copper nitrate of concentrations up to 400 µM.

#### *Determination of number of cycles of reactivation of urease-based biosensor*

At the next stage, the possibility of multiple reactivation of the developed biosensor was estimated with a purpose of its repetitive application for the inhibition analysis of heavy metal ions. In this case the biosensor can be used several times when working with irreversible inhibitors.



**Fig. 5. Dependence of biosensor reactivation level after inhibition by Cu<sup>2+</sup> ions with different concentrations.**

Measurement was performed in 5 mM phosphate buffer, pH 6.5

The experimental results are shown in Fig. 6. First, the basic responses to urea were received (the first column). Next, the biosensors were incubated in a copper ions solution to obtain a certain residual activity of the enzyme (the second column). Further step was the biosensor reactivation in EDTA solution up to restoration of the urease activity to its original level (the third column). This procedure described was repeated until the bioselective membrane activity was completely restored.

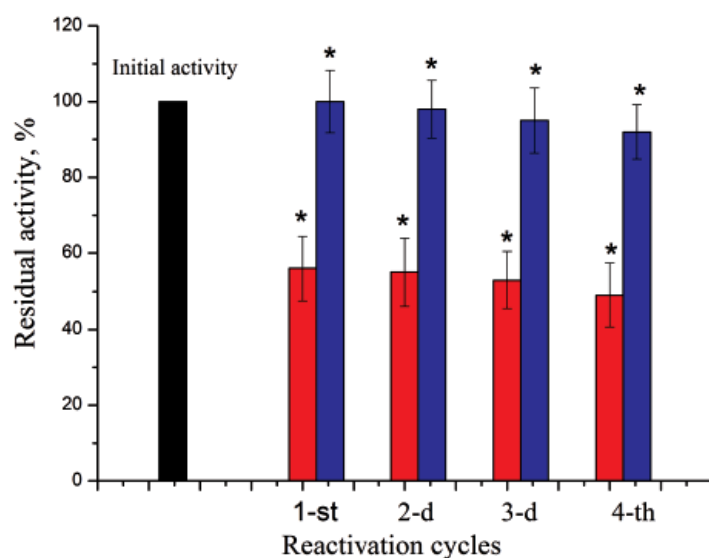
The next cycles of inhibition and reactivation did not lead to complete restoration of the enzyme activity, but the sensitivity of biosensor was sufficient to reuse it for the analysis of heavy metal ions in the solution.

In the work, the urease-based biosensor is optimized for determination of heavy metal ions. The biosensor sensitivity was tested, the calibration curves were plotted for direct urea determination and for inhibition analysis of

heavy metal ions. The developed biosensor was characterized by high signal reproducibility at determination of both substrate and inhibitor. The possibility of biosensor reactivation after inhibition by heavy metal ions was proved. The optimum conditions of the process of reactivation using EDTA were selected.

It was established that the initial level of enzyme inhibition affects the capability of biosensor to be reactivated. It is shown that application of the reactivation stage allows repetitive usage of urease-based biosensors for inhibition analysis of heavy metal ions, which indicates the suitability of the method for multiple application.

The authors are grateful for financial support from the National Academy of Sciences of Ukraine in the frame of complex scientific and technical program "Sensor devices for medical-environmental and industrial-technological needs: metrological support and pilot exploitation".



**Fig. 6. Reproducibility application of urease based conductometric biosensor after inhibition by copper ions and reactivation by EDTA. Inhibition time in copper (II) nitrate solution (150  $\mu$ M) — 20 min, reactivation time in EDTA solution (10 mM) — 30 min. Measurement was performed in 5 mM phosphate buffer, pH 6.5; \*  $P < 0.05$  in comparison with initial activity of biosensor**



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## ДОСЛІДЖЕННЯ ТА ОПТИМІЗАЦІЯ РЕАКТИВАЦІЇ УРЕАЗНОГО БІОСЕНСОРА ЗА ІНГІБІТОРНОГО АНАЛІЗУ ІОНІВ ВАЖКИХ МЕТАЛІВ

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Метою роботи було визначення умов оптимізації роботи уреазного біосенсора під час аналізу важких металів та можливості його реактивації. Як кондуктометричний перетворювач використовували диференційну пару золотих гребінчастих електродів, нанесених на керамічну підкладку. Роль біоселективної мембрани відіграла уреазу, коімобілізовану з бичачим сироватковим альбуміном за допомогою поперечного шивання глутаровим альдегідом на поверхні кондуктометричного перетворювача. Для інгібіторного аналізу іонів важких металів підібрано оптимальну концентрацію субстрату — 1,0 мМ сечовини. Перевірено чутливість біосенсора до різних іонів важких металів та побудовано калібрувальні криві. Показано, що запропонований біосенсор характеризувався високою відтворюваністю сигналів до та після процесу інгібування з похибкою вимірювання менше 3%. Доведено можливість реактивації біоселективної мембрани розчином етилендіамінтетраоцтової кислоти після незворотного інгібування уреазу важкими металами. Підібрано оптимальні умови реактивації біосенсорів, зокрема залежність рівня реактивації від часу та концентрації іонів важких металів у розчині. Встановлено, що, використовуючи додатковий етап реактивації біосенсорів після інгібування, можна значно підвищити селективність процедури біосенсорного визначення іонів важких металів.

**Ключові слова:** кондуктометричний перетворювач, біосенсор, уреазу, важкі метали, інгібіторний аналіз, реактивація ензиму.

## ИССЛЕДОВАНИЕ И ОПТИМИЗАЦИЯ РЕАКТИВАЦИИ УРЕАЗНОГО БИОСЕНСОРА ПРИ ИНГИБИТОРНОМ АНАЛИЗЕ ИОНОВ ТЯЖЕЛЫХ МЕТАЛЛОВ

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Целью работы было определение условий оптимизации работы уреазного биосенсора при анализе тяжелых металлов и возможности его реактивации. Как кондуктометрический преобразователь использовалась дифференциальная пара золотых гребенчатых электродов, нанесенных на керамическую подложку. Роль биоселективной мембраны играла уреазу, коимобилизованная с бычьим сывороточным альбумином с помощью поперечной шивки гутаровым альдегидом на поверхности кондуктометрического преобразователя. Подобрана оптимальная концентрация субстрата для ингибиторного анализа ионов тяжелых металлов — 1,0 мМ мочевины. Проверена чувствительность биосенсора к разным ионам тяжелых металлов и построены калибровочные кривые. Показано, что предложенный биосенсор характеризовался высокой воспроизводимостью сигналов до и после ингибирования с погрешностью измерения менее 3%. Доказана возможность реактивации биоселективных мембран раствором этилендиаминтетрауксусной кислоты после необратимого ингибирования уреазу тяжелыми металлами. Подобраны оптимальные условия реактивации биосенсоров, в частности зависимость уровня реактивации от времени и концентрации ионов тяжелых металлов в растворе. Установлено, что, используя дополнительный этап реактивации биосенсоров после ингибирования, можно значительно повысить селективность процедуры биосенсорного определения ионов тяжелых металлов.

**Ключевые слова:** кондуктометрический преобразователь, биосенсор, уреазу, тяжелые металлы, ингибиторный анализ, реактивация энзима.