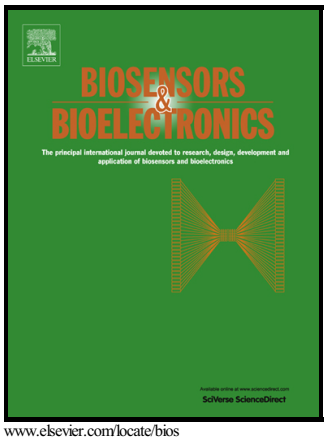

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ENZYME BIOSENSOR SYSTEMS BASED ON POROUS SILICON PHOTOLUMINESCENCE FOR DETECTION OF GLUCOSE, UREA AND HEAVY METALS

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ABSTRACT

A phenomenon of changes in photoluminescence of porous silicon at variations in medium pH is proposed to be used as a basis for the biosensor system development. The method of conversion of a biochemical signal into an optical one is applied for direct determination of glucose and urea as well as for inhibitory analysis of heavy metal ions. Changes in the quantum yield of porous silicon photoluminescence occur at varying pH of the tested solution due to the enzyme-substrate reaction. When creating the biosensor systems, the enzymes urease and glucose oxidase (GOD) were used as a bioselective material; their optimal concentrations were experimentally determined. It was shown that the photoluminescence intensity of porous silicon increased by 1.7 times when increasing glucose concentration in the GOD-containing reaction medium from 0 to 3.0 mM, and decreased by 1.45 times at the same increase in the urea concentration in the urease-containing reaction medium. The calibration curves of dependence of the biosensor system responses on the substrate concentrations are presented. It is shown that the presence of heavy metal ions (Cu^{2+}, Pb^{2+}, Cd^{2+}) in the tested solution causes an inhibition of the enzymatic reactions catalyzed by glucose oxidase and urease, which results in a restoration of the photoluminescence quantum yield of porous silicon. It is proposed to use this effect for the inhibitory analysis of heavy metal ions.

Keywords:
biosensor
luminescence
porous silicon
enzyme
heavy metal

1. Introduction

In recent years, semiconductor sensors and sensor arrays for the detection of chemical and biological substances have drawn much attention (Kohl, 2001; Sailor, 2012). The ultimate goal of the research in this field was to fabricate sensors that can determine the presence of a wide range of substances at the relevant concentration levels with sufficient selectivity and sensitivity. The research would produce the apparatus that could be applicable in many segments of human activity including food processing, environmental remediation, agriculture, medical diagnostics and defense. The main requirements to the devices developed, together with selectivity and sensitivity, are fast response, low fabrication costs, reliability and portability (Masrournia et. al., 2011).

The development of highly sensitive, low-cost, reliable glucose sensors having an excellent selectivity has been the subject of concern for decades, not only in medical science but also in the food industries (Newman, et al, 2005). Urea is widely distributed in nature and its analysis is of considerable interest in the clinical chemistry, agro-food chemistry, and environmental monitoring. Previously, series of enzyme-based biosensors were developed for glucose and urea detection, i.e. potentiometric, amperometric, impedimetric and conductometric (Pizzariello, et al, 2001; Eggenstein et al, 1999; Rahman et al, 2010).

Well-known, the most dangerous polluting substances are heavy metals (Golovanov, 2008; Zayets et. al., 2008). In humans and herbivorous animals, heavy metals come from plant food, and to plants - from the soil. The mechanism of the harmful effects of heavy metals is the formation of covalent bonds with the radicals of biologically active molecules, particularly due to binding with (-SH) groups of amino acid residues that are a part of active sites of enzymes (Soldatkin et. al., 2008; Kycherenko et. al., 2009). As a result, the three-dimensional structure of enzyme is broken, reducing its activity (irreversible inhibition).

Heavy metals affect the physiological functions of the body, violate the acid base balance of blood, alter the activity of enzymes, etc. (Amine et. al., 2006). At present, the toxicity of mixtures of heavy metals is of particular importance because of the anticipated increase in heavy metal concentrations in soil caused by sewage entering groundwater and organisms (Preston et. al., 2000; Yadav, 2010). Therefore, the development of biosensors, simple, easy-to-use and cheap devices for environmental monitoring, is an important task of modern biotechnology development (Bontidean et. al., 2003; Blake et. al., 2001; Corbisier et. al., 1999). Indirect determination of heavy metal ions can be ensured by inhibition of the reactions catalyzed with glucose oxidase (GOD) and urease (Chey et al, 2012; Upadhyay, 2012) that can be realized in enzyme biosensors.

An increasing number of researchers have explored the application of novel nano-scale metal oxides, noble metal-doped metal oxides for enzyme-based sensors. Novel analytical devices based on nanostructured metal oxides are cost-effective, highly sensitive due to the large surface-to-volume ratio of the nanostructure, and additionally show high selectivity when coupled to biorecognition molecules with simple design. Some metal oxides such as ZnO and CeO_2 show excellent biocompatibility that enables reliable immobilization of glucose oxidase (Liu, et al, 2008).

However, there are still some disadvantages of enzyme-based glucose determination. Biosensors are characterized by complicated enzyme immobilization, critical operating conditions such as optimum temperature and pH, the chemical instability, and high cost (Reitz, et al, 2008).

The biosensor can be considered as promising when it does not require complex modification and enzyme immobilization, and the assay results can be read as soon as the probe-sample incubation is completed. Such biosensor can be created using luminescent effect. Recently, quantum dots have attracted great attention and have been intensively studied in biological analysis because of their unique optical properties. It was demonstrated that a simply assembled complex consisting of CdTe quantum dots and glucose oxidase can be used for sensitive glucose determination based on effective fluorescence quenching by H_2O_2. (Cao et al, 2008). The next glucose sensor was developed using the changes in photoluminescence (PL) intensity of colloidal solutions of silicon nanoparticles, occurring in the reaction catalyzed by glucose oxidase (Yi et. al., 2013).

In the present work we propose to use the luminescent properties of porous silicon layers on silicon wafer.

As known, electrochemically etched nanoporous silicon is considered as a promising material for luminescent chemical sensors (Sailor, 2012; Salcedo, 2004). First, under illumination of nanoporous silicon with UV light, the intense PL is observed in the visible range at room temperature. The effect is explained by the exciton recombination in quantum-sized nanoparticles (so-called S-band) (Cullis et. al., 1997). Depending on the size of silicon nanoparticles, the spectrum maximum shifts from 690 nm (for the nanoparticles with a diameter of 3.2 nm) to 390 nm (for those with a diameter of 1.5 nm) (Serdiuk et. al., 2011). The PL quantum yield can reach 8-20% depending on the method of porous silicon production and its further treatment (Gelloz et. al., 2005; Skryshevsky et. al., 1996). Second, the molecules adsorption on the porous silicon surface results in luminescence quenching. Depending on the type of adsorbed molecules it can be either reversible or irreversible (Lauerhaus et. al., 1992).

It was shown (Benilov et. al., 2007) that the porous silicon luminescence is sensitive to pH of various liquids that was revealed by the changes in intensity and decay time. High PL intensity and long decay time correspond to lesser pH (solution acidification) whereas low PL intensity and short decay - to higher pH. Thus, the luminescence effects in porous silicon can be used to monitor the changes in solution pH caused, for example, by enzyme-substrate reactions.

The purpose of this study was to show the possibility to develop novel enzyme biosensor systems for determination of glucose and urea (direct analysis) as well as heavy metal ions (inhibitory analysis) using porous silicon layers.

2. Materials and methods

Glucose oxidase and urease were selected as the model enzymes since they are relatively stable, well-studied and commonly used in biosensorics.

The preparations of lyophilized enzymes were used: urease from soybeans, activity 31 U/mg ("Fluka", Switzerland), glucose oxidase (GOD) from Penicilliumvitale, activity 130 U/mg ("Diagnosticum", Lviv, Ukraine). As substrates, urea ("Sigma-Aldrich Chemie", USA) and glucose ("Sigma-Aldrich Chemie", Germany) were used. As the inhibitors were used aqueous solutions of heavy metals nitrates [$Cu(NO_3)_2$, $Pb(NO_3)_2$, $Cd(NO_3)_2$] of domestic production. The compounds for buffer preparation and other inorganic substances used were of domestic production and of analytical grade.

The porous silicon layers up to 1 μm thick were obtained by the etching of p-Si wafer of (100) orientation and 4.5 Ohm.cm resistivity in the mixture of hydrofluoric acid (48%) and ethanol at the ratio 1:1 (v/v) at the constant current of 50 mA/cm^2. Such preparation mode allows obtaining porous layers of 60% porosity (Bisi et al, 2000) with emission in visible region (maximum is situated at 590–620 nm) and high quantum yield (Gelloz et. al., 2005; Skryshevsky, 2000).

The photoluminescence measurements were performed at room temperature using a pulse nitrogen laser (λ=337.1 nm, τ = 8 ns, 20 kW in each pulse) as a photo-excitation source. The samples of porous silicon on silicon wafer were placed in a quartz cuvette filled with the buffer solution and irradiated with a laser beam. The sample emission directed via an optical fiber toward a monochromator (MS2004, Solar T2) was synchronically detected by a photomultiplier (Hamamatsu C6270), converted by an analog-to-digital device and finally, processed by PC.

3. Results and discussion

It was shown (Fig. 1) that the photoluminescence intensity of porous silicon on Si wafer increased by 1.7 times when increasing glucose concentration in the GOD-containing reaction medium from 0 to 3.0 mM (Fig. 1a), and, in contrast, decreased by 1.45 times at the same increase in the urea concentration in the urease-containing reaction medium (Fig. 1b).

Direct analysis of substrates by the proposed biosensor systems using the PL effect of porous silicon is based on the following enzymatic reactions (Dzyadevych et. al., 2006; Dzyadevych et. al., 2003):

GOD

$$\beta\text{-D-glucose} + O_2 \rightarrow \text{D-gluconolactone} + H_2O_2 \Rightarrow$$
$$\text{D-gluconic acid} + H_2O \leftrightarrows \text{acid residue} + H^+ \qquad (1)$$

Urease

$$\text{Urea} + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^- \qquad (2)$$

In the course of these enzymatic reactions the concentration of protons changes and correspondingly varies the solution pH. As can be seen from equations (1), (2) the enzymatic action of GOD and urease shifts the pH value of reaction medium in different ways: GOD - to acidic, urease – to alkaline range.

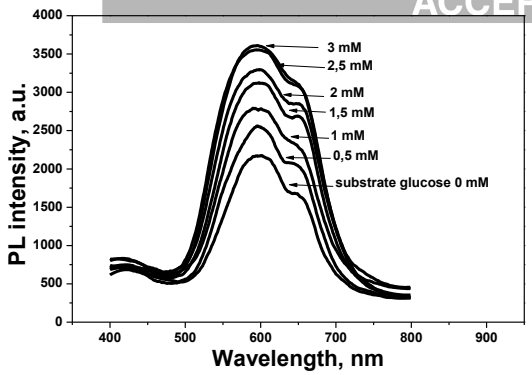

Fig. 1(a). Dependence of PL spectra of porous silicon in solutions containing GOD (130 U) on glucose concentration. Measurements were conducted in 5 mM phosphate buffer with pH 6.5.

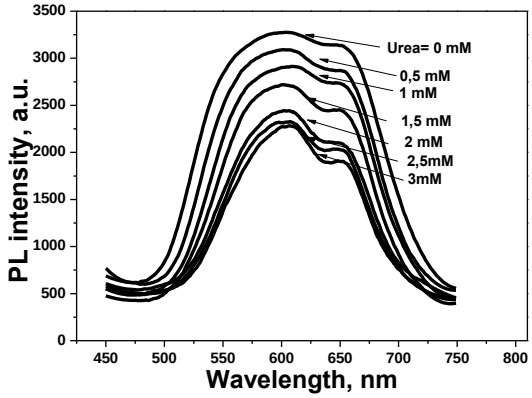

Fig. 1(b). Dependence of PL spectra of porous silicon in solutions containing urease (99.4 U) on urea concentration. Measurements were conducted in 5 mM phosphate buffer with pH 6.5.

When the porous silicon layer in phosphate buffer is exposed to UV irradiation, several competing processes take place simultaneously, namely: 1) the partial rupture of Si-H bonds and hydrogen effusion, which results in an increase in the concentration of surface traps acting as nonradiative recombination channels; 2) the renewal of the Si-H bonds due to the hydrogen adsorption from the buffer solution, 3) molecular water oxidation activity under the conditions of intense UV irradiation, which results in the formation of a silicon oxide layer on the porous silicon surface, affecting the PL intensity (Benilov et.al., 2007) .

Obviously, the nature of changes in the PL quantum yield is defined by both the solution pH and the intensity of the laser radiation used for the PL excitation. The hydrogen is preferably adsorbed on the porous silicon surface when the sample is in contact with low-pH liquids. Since Si-H bonds are known to passivate the porous silicon surface (Zho et. al., 2003), the hydrogen adsorption leads to the healing of nonradiative recombination channels, and quantum yield of exciton recombination increases. Use of alkaline solutions causes a decrease in the volume of adsorbed hydrogen as well as an additional etching of the porous silicon layer, i.e. its degradation and reduction of the sample PL intensity.

Considering a possible impact of UV irradiation on the samples PL in the solutions without enzymatic reactions, the time dependence of PL intensity of porous silicon was tested in the buffer solution in the absence of any enzyme and in the presence of each separate enzyme (without substrates).

The PL intensity decreases by less than 3% during 15 min (the spectra were recorded during 5 min). Thus, the PL changes due to the oxidation and aging of porous Si film are much smaller than those due to the enzymatic reactions, i.e. porous silicon oxidation and aging slightly influence the results of biosensor measurements.

At a fixed enzyme concentration, the enzymatic reaction rate increases as the substrate concentration rises tending to the saturation. When changing the enzyme amount in biosensor systems, the sensitivity to the substrate and a linear detection range may vary. Therefore, the biosensor systems were investigated relative to their dependence on the enzyme (GOD or urease) volume in the reaction medium. Accordingly, a number of calibration curves of the biosensor systems for glucose and urea determination were obtained at different amount of the enzymes (Fig. 2).

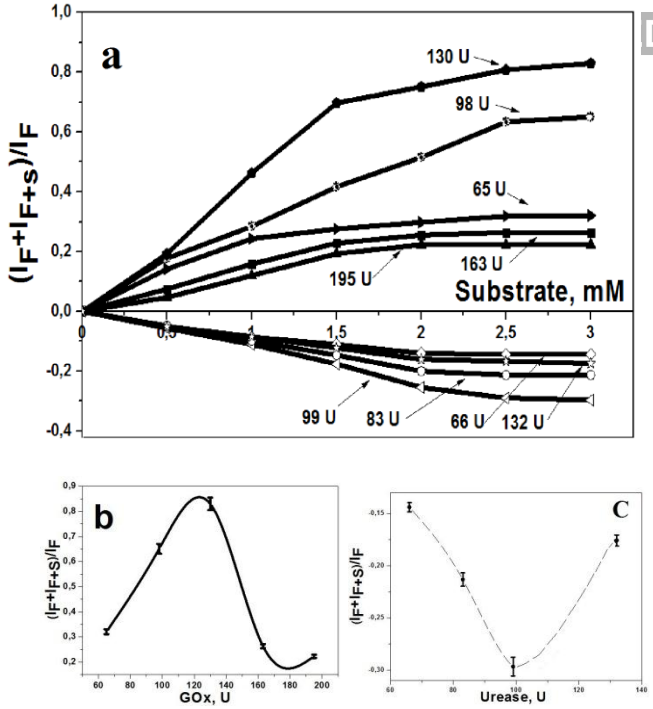

Fig. 2. Calibration curves (**a**) of two biosensor systems for determination of substrate (glucose or urea) at different concentrations of enzymes. Positive values on y-axis correspond to GOD, negative - to urease. Dependence of PL intensity of porous silicon in biosensor systems based on GOD (**b**) and urease (**c**) on enzymes concentration. Measurements were conducted in 5 mM phosphate buffer with pH 6.5. I_{F+S} is maximum intensity in the presence of enzyme and substrate; I_F is maximum intensity in the presence of enzyme only (initial point).

As seen (Fig. 2), at small substrate concentrations the enzymatic reaction rate is proportional to their values, which is typical and confirmed by the results of previous studies (Zhylyak et. al., 1995; Volotovsky et. al., 1998). However, at further increase in the substrate concentration up to saturation, all enzyme molecules are involved in the substrate molecules conversion at maximum rate. As shown in Fig. 2, both biosensor systems (with different concentrations of the enzymes) were characterized by almost similar linear ranges of determination of the relevant substrates. The linear ranges of the GOD - and urease-based biosensor systems remained virtually unchanged at varying concentration of the enzymes.

Therefore, the main criterion when selecting the concentration of enzyme as a part of the biosensor system was an appropriate biosensor sensitivity to a specific substrate. As shown in Fig. 2, the greatest sensitivity to glucose and urea had the biosensor systems based on GOD (130 U) and urease (99 U), respectively. At these amounts of relevant enzymes, the sensitivity of the GOD-based biosensor system is determined by the maximum increase, whereas for urease-based biosensor – by maximum decrease in PL intensity of porous silicon.

As can be seen from Figure 2, at large amount of urease and glucose oxidase in the buffer solution the sensor response for glucose and urea drops. A possible explanation of this fact is the following. The luminescence intensity changes due to urea- urease and glucose - glucose oxidase enzymatic reactions only in the area of porous silicon in which photoluminescence is excited. In investigated samples of the porous silicon with thickness of 1 μm the penetration of the solution into porous material is considerably greater than the depth of the photoluminescence generation (absorption coefficient in the porous silicon (60% porosity) at a wavelength of 337 nm is 10^5 cm^{-1} (Behren et al, 1997)). In addition, the photoluminescence quantum efficiency is heterogeneous with depth of porous silicon layer (Skryshevsky, 2000; Serdiuk et. al., 2011). When the concentration of enzyme in the buffer solution growths, enzymatic reactions occur mainly in the non-luminescent region of porous silicon sample.

The main characteristic of enzymes is their high selectivity to substrates, i.e. a capability of selective conversion of the relevant substrate. This distinguishes enzymes from chemical catalysts (platinum, nickel, iron, etc.). Therefore, it was necessary to test the developed biosensor system with regard to the selectivity to glucose as compared to other commonly used carbohydrates fructose and sucrose, which are structurally similar to glucose. The experiments were conducted, the results are shown in Fig. 3. The responses obtained to glucose, fructose and sucrose indicate rather high specificity of the proposed biosensor system.

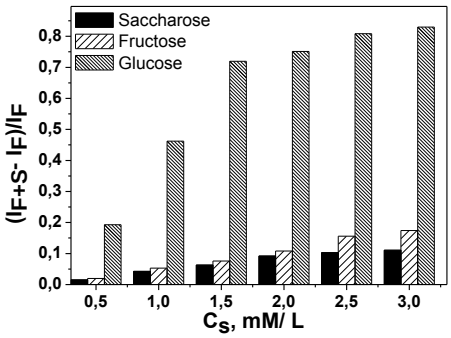

Fig. 3. Values of responses (changes in PL intensity of porous silicon) of GOD-based biosensor system to relevant substrate glucose and two interfering substrates, fructose and sucrose. Measurements were conducted in 5 mM phosphate buffer with pH 6.5. I_{F+S} is PL intensity when adding the enzyme and substrate of certain concentration; I_F is intensity when adding only enzyme (initial point), C_s is concentration of respective carbohydrates.

The same results were obtained by other researchers for the glucose sensor based on photoluminescence of colloidal solutions of Si nanoparticles with GOD (Yi et. al., 2013). The authors studied specificity of developed biosensors for glucose via testing several glucose analogues, including sucrose, fructose, mannose, and galactose. The mentioned substances invoked the photoluminescence intensity change, however, it was an order of magnitude less than that due to glucose.

The results of our experiments clearly demonstrate how the effect of changes in PL intensity of porous silicon at varying pH due to enzymatic reactions can be used for the development of novel optical biosensors or biosensor systems, in particular, for the monitoring of glucose and urea. The proposed biosensor systems were characterized by high reproducibility. The relative standard deviation was in the range 3-4% for both types biosensor system.

Since both proposed biosensor systems have high reproducibility, we considered a possibility of their application for inhibitory analysis of heavy metal ions. It is well known that some enzymes, e.g. urease, are sensitive to heavy metal ions as inhibitors (Zhylyak et. al., 1995; Dzyadevych et. al., 2003) due to the reaction of the latter with sulfhydryl groups of enzymes (Dzyadevych et. al., 2003). A decrease in the enzyme activity results either in lesser number of the protons formed in case of using GOD, or in higher concentration of the protons absorbed when using urease.

An inhibitory effect of various toxic metal ions (Cu^{2+}, Pb^{2+}, Cd^{2+}) on the performance of both biosensor systems was studied. The impact of copper ions of various concentrations on porous silicon PL for glucose and urea biosensor systems is shown in Fig. 4.

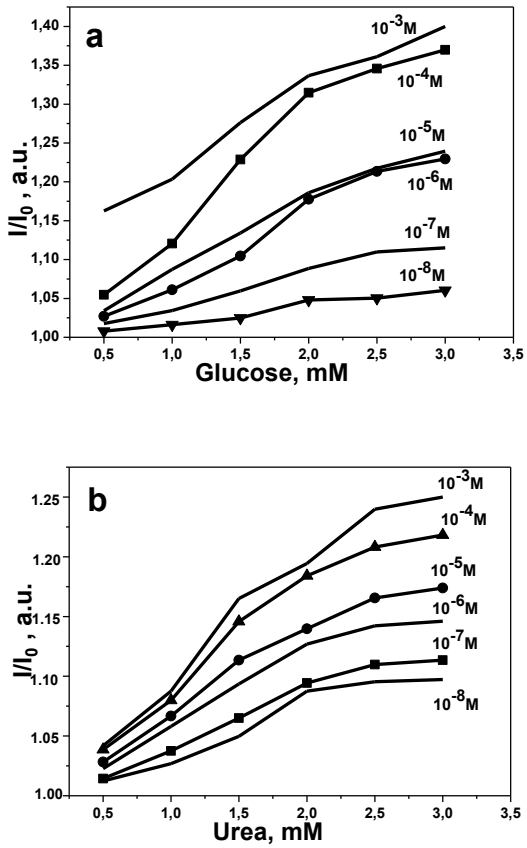

Fig. 4. Dependence of responses of developed biosensor systems based on GOD (130 U) (**a**) and urease (99 U) (**b**) on glucose or urea concentration in the presence of various concentrations of ions Cu^{2+}. Measurements were conducted in 5 mM phosphate buffer with pH 6.5. I is PL intensity after inhibition of biosensor system, I_0 is PL intensity prior to inhibition.

The calibration curves of developed biosensor systems were obtained for the determination of Cu, Pb and Cd (Fig. 5). It should be noted that for the tested solutions of heavy metal ions, the metal salts of 10 nM - 1 mM concentration were used. Since the measurements were conducted in 5 mM phosphate buffer, pH 6.5 (the maximum buffer capacity), even maximum concentrations of metal solutions could not shift the pH of the sample solution. Moreover, the appropriate experiments were carried out to test the non-specific signals, which proved that heavy metal ions act as inhibitors for the enzymes and recover PL intensity of porous silicon samples.

The detection limit (sensitivity) of the developed urease- and GOD-based biosensor systems to heavy metal ions is approximately 10 nM and the linear range is 10 nM to 1 mM, The biosensor systems with such sensitivity are quite competitive compared with the known electrochemical biosensors since the sensitivity of electrochemical biosensors based on the same enzymes is 50-1000 nM (Yin, 2013). Specificity of both proposed biosensor systems and electrochemical biosensors mainly depends on the specificity of the enzyme used.

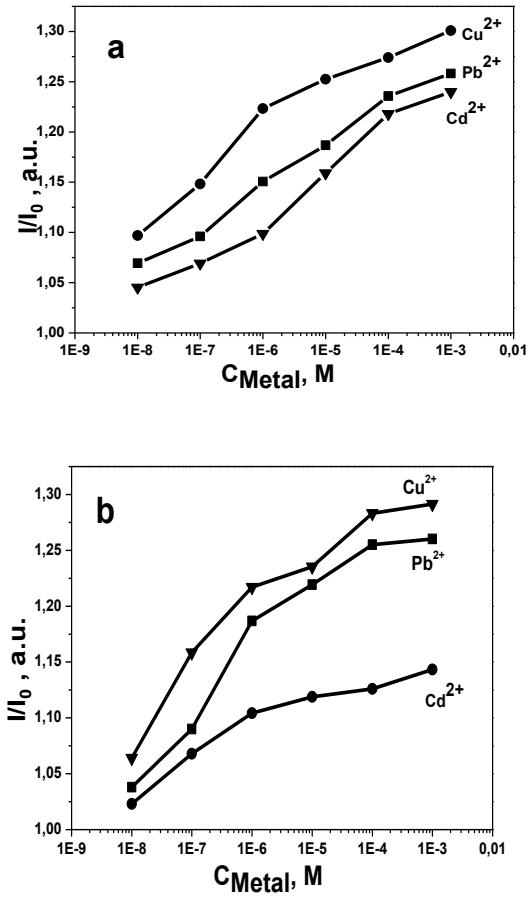

Fig.5. Calibration curves of developed biosensor systems based on GOD (130 U) (**a**) and urease (99 U) (**b**) for determination of concentration of heavy metal ions: Cu^{2+}, $Pb^{2+}$and Cd^{2+}. Measurements were conducted in 5 mM phosphate buffer with pH 6.5, at a concentration of relevant substrate 3mM. C_{Metal} is concentration of heavy metal ions, I is PL intensity after inhibition of biosensor system, I_0 is PL intensity prior to inhibition.

For both biosensor systems developed, ions Cu^{2+} appeared to be the most toxic. In any case, each of these biosensor systems can be used to analyze aqueous samples for the presence of all mentioned metals.

4. Conclusions

Novel biosensor systems were developed for direct determination of substrates glucose and urea and inhibitory analysis of heavy metal ions. Porous silicon was used as highly effective transducer, based on the effect of changing its photoluminescence at varying pH in solution, which caused by the enzymatic reactions. Highly selective enzymes urease and glucose oxidase, which change the medium pH as a result of enzymatic reaction of the substrate conversion served as selective elements of the biosensor system.

The proposed method of conversion of a biochemical signal into photoluminescent response has many advantages such as simple operation, high PL quantum yield, photostability, sensitivity and selectivity, non-toxicity and biocompatibility, which is especially important for in vivo use.

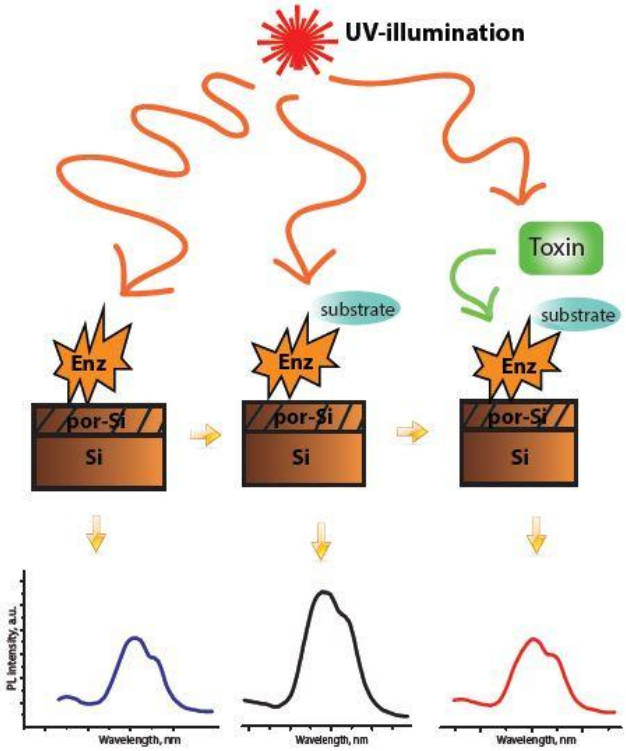

Fig.6. Conception of luminescent biosensors based on GOD or urease for glucose, urea and heavy metals detection.

An approach based on inhibition of enzymatic reactions is suggested in the further development of novel biological nanosensors for the detection of heavy metal ions.

A conception of luminescent biosensors for glucose, urea and heavy metals detection is shown in Fig. 6. The PL spectrum of the porous silicon layer, treated by GOD or urease in buffer solution, is registered. Addition of the substrates to the tested medium causes a decrease or increase in pH and, consequently, an increase or decrease in PL intensity. At the same time, heavy metal ions in the tested solution inhibit the enzymatic reaction, which results in the restoration of PL quantum yield of porous silicon layer.

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Highlights:

1) New biosensor systems are developed for direct determination of glucose and urea by using of enzymatic reactions urea-urease and glucose -glucose oxidase;

2) The enzymatic reaction in buffer solutions changes the intensity of photoluminescence of nanoporous silicon layer;

3) The proposed biosensor systems are characterized by high reproducibility and selectivity, the range of measured glucose and urea concentration lies in range of 0- 3,0 mM;

4) An inhibitory effect of various toxic and low toxic metals (Cu^{2+}, Pb^{2+}, Cd^{2+}) on the performance of both biosensor systems is observed that can be used for detection of heavy metal ions.