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Glucose Sensing Using Glucose Oxidase-Glutaraldehyde-Cysteine Modified Gold Electrode

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The method to develop a stable glucose biosensor with successive attachment of cysteine (Cys), glutaraldehyde (GA) and glucose oxidase (GOx) onto gold electrode is presented. The cyclic voltammetry (CV) suggests the diffusion control of the glucose oxidation. The obtained biosensor shows a fast electron transfer of k^0 = 20.4 s^{-1}, high affinity for glucose with the apparent Michaelis-Menten constant K_M^{app} = 1.15 mM, a low detection limit of 0.94 mM in a linear range 1.5-7 mM. This biosensor exhibits good stability and reproducibility. Good biocompatibility of modified electrode surface, which enhances the covalent bonded enzyme and consequently glucose oxidation, resulted in biosensor with excellent performances. Biosensor was tested in samples containing human serum.

Keywords: Biosensors; Immobilization; Enzyme catalysis; Self-assembly.

1. INTRODUCTION

Diseases such as diabetes mellitus, uremia, heart failure, intestinal diseases, or muscle damages use different enzymes in their metabolic activities [1]. Based on their catalytic activity various types of biosensors are fabricated. Among all types at the overall biosensors market glucose biosensors occupy more than 85 % [2]. World statistic shows that number of people with diabetes is growing rapidly [3], and apart to treatment, the detection of the disease plays a great role, thereby development of an efficient biosensor for monitoring blood glucose levels represents challenge for the scientists. GOx (β-

d-glucose:oxygen 1-oxidoreductase, E.C.1.1.3.4) is the major enzyme used in analytical test kits and biosensors. It is a flavoprotein which catalyzes oxidation of glucose [4].

The first enzyme electrode was reported in 1962 [5]. Since then, tremendous efforts and variety of approaches have been applied to construct reliable device. Since the GOx redox center is hidden and the electron transfer is thereby limited, construction of biosensor has been upgraded constantly from simple, through the first- and second-, towards third-generation glucose biosensors.

There is an increasing interest and need to develop an electrochemical glucose biosensor by immobilizing enzyme on an electrode surface. Nature of the electrode surface as well as immobilization technique is very important due to attached enzyme needs to maintain reasonable activity and, what is most important, high stability. Immobilization techniques include adsorption, encapsulation, entrapment, cross-linking, or covalent binding [6, 7]. However the most common methods are adsorption and covalent linking. In the case of adsorption, although the simplest and the fastest, lack of stability is a fatal drawback. On the contrary, the covalent binding provides a stable complex between the enzyme and support and overcomes these shortcomings.

Gold is frequently used for immobilization of enzymes [8]. The Au surface can be modified by self-assembled monolayers (SAMs), and for more than 20 years SAMs were used as the basis of electrochemical sensors [9]. Variations of gold electrode modifications are multifarious and due to its affinity toward sulfur, the first modification step usually involve compounds with thiol (-SH) group. These groups chemisorb onto the gold surface forming gold-thiol bond [9] giving a highly ordered and densely packed monolayer. Comparing to cystamine, mercaptan compounds and other frequently used reagents, cysteine is cheaper and more stable and it is often recognized as SAMs reagent [10].

There are few papers that describe the usage of cysteine, an essential thiol amino acid, in the first modification step of gold electrode in preparation of biosensors [10-12]. The introduction of reactive amino groups of cystein enables a binding layer for forthcoming steps for electrode modifications.

Glutaraldehyde (GA) is a well-known powerful crosslinker with reactive aldehyde groups on both ends and one of the most widely used reagents for the activation of aminated surface [13]. One terminal aldehyde group interacts with the amino group on the solid surface, while the other reacts with the enzyme amino group. GA surface modification is also important from the standpoint of the immobilized enzyme stability. The increase of the system stability represents a crucial task for the industrial application. GA activation of the surface may have a positive impact on the stabilization since it provides the possibility of the enzyme multipoint covalent attachment. Such phenomena may increase the rigidity of the immobilized enzyme and make it less susceptible to conformational changes during the biocatalytic processes [14, 15]. Au has been already modified by GA combined with Cys for peroxidase immobilization in order to obtain a sensor for dopamine determination [11].

Glucose oxidase (GOx) is a first choice as a biosensing model molecule in many studies because it is stable and relatively low cost compound [16, 17]. Also, GOx is used in biofuell cells [18] and in clinical praxis [19-21].

In this work, we presented a method for the construction of a stable glucose biosensor by successive attachment of cysteine, glutaraldehyde and glucose oxidase onto gold electrode and tested such biosensor for glucose sensing. The electrooxidation ability of glucose was tested by CV. In

addition, the stability of modified surface was examined. The dependency of anodic currents *vs*. glucose concentration was examined for a set of concentrations used for the glucose measurements in samples.

2. MATERIALS AND METHODS

2.1.1. Chemicals

Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4), Type VII, 149 800 U g^{-1} (~ 150 U mg^{-1} solid), L-cysteine and D-(+)-glucose were purchased from Sigma Aldrich. Glutaraldehyde was purchased from Acros Organics, and salts for the phosphate buffer from Merck Alkaloid. A Milipore Waters Milli-Q purification unit was used in order to obtain deionized water.

2.1.2. Immobilization procedure

The modification of electrode with cysteine (Au-Cys) was performed as it was described previously [22]. Briefly, previously cleaned electrode (in a way described for preparation of electrode surfaces) was immersed into 30 mM cysteine aqueous solution at the room temperature (24 h). Then the electrode was rinsed using deionized water (to remove cysteine - loosely attached).

Secondly, the Au-Cys modified electrode was immersed into 2.5 % solution of glutaraldehyde in phosphate buffer (0.1 M pH 8.0) at the room temperature (1 h). It was then washed with the same phosphate buffer. Finally, Au-Cys-GA modified electrode was immersed into 3 mg cm^{-3} glucose oxidase solution in phosphate buffer (0.1 M pH 7.0) at 4 $^{\circ}$C (3 h). The obtained enzyme electrode Au-Cys-GA-GOx was washed with the same buffer (pH 7.0) and used in further experiments.

2.1.3. The preparation of samples containing human serum

The human serum was collected and clinically prepared from ten healthy volunteers and spiked with glucose as was described previously [23].

2.2. Electrochemical experiment

The electrochemical experiment was performed as previously described [23].

3. RESULTS AND DISCUSSION

3.1. Electrochemical behavior of glucose over the modified electrodes

Immobilization of GOx on modified electrode (Au-Cys-GA) was electrochemically characterized by CV in phosphate buffer. It was already published that the lipase from *C. rugosa*

enzyme is most active in pH 7.0 phosphate buffer [24] and it seems that this medium is the best choice for the electrochemical enzyme studies**.** The performance of this (Au-Cys-GA) electrode was compared to Au electrode containing GOx immobilized by absorption and Au electrode (Fig. 1). The difference in the CV curves for the investigated electrodes in the region of surface oxidation/reduction occurs. Voltammogram of Au-Cys-GA-GOx presented in the Fig. 1 shows the small current increase in the area before oxide formation, while in the oxide formation area a significant current increase can be observed.

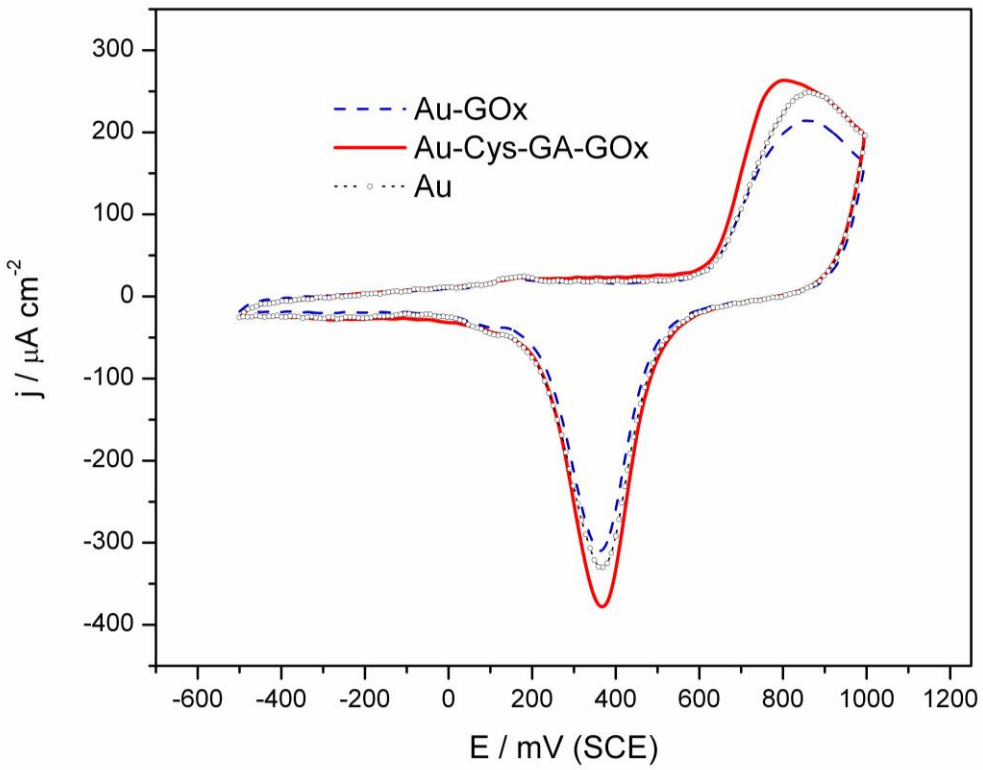

Figure 1. CVs obtained on gold electrode using phosphate buffer (0.1 M, pH 7.0) (dotted line), Au-GOx (dashed line) and Au-Cys-GA-GOx (full line) at scan rate 50 mVs^{-1}.

The peak of the oxide reduction also increases in comparison to the oxide reduction of the Au electrode in phosphate buffer solution. It is obvious that Au covered only with adsorbed GOx exerts current decrease in the region of oxide formation and reduction in comparison to Au electrode. This indicates the existence of different bonds between the enzyme and Au electrode and the Cys-GA-GOx and Au electrode surface.

The adsorption of the GOx molecules on the gold electrode should mainly be governed by hydrophobic interactions, and the hydrophobic regions (colored in orange in Fig. 2) are located in the vicinity of the active site. After the GOx adsorption on the gold electrode, the access of glucose molecule to enzyme active site was restricted as is presented in Fig. 2. Thus, the main reason for the Au electrode surface modification with cysteine and glutaraldehyde was creating different environment and allowing covalent interactions of introduced groups with the enzyme amino groups. Those groups

are located opposite to active site access and after immobilization approach of glucose molecules is easier.

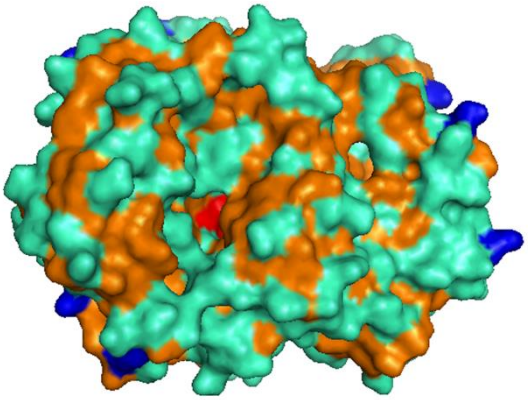

Figure 2. Distribution of hydrophobic amino acid residues (orange), residues with amino group (dark blue) and catalytic site (red) on the surface of glucose oxidase from *Aspergillus niger*. The 3D structure was obtained using Pymol *vs*. 0.99 and data obtained from Protein Data Bank (PDB). PDB code for glucose oxidase from *Aspergillus niger* is 1CF3.

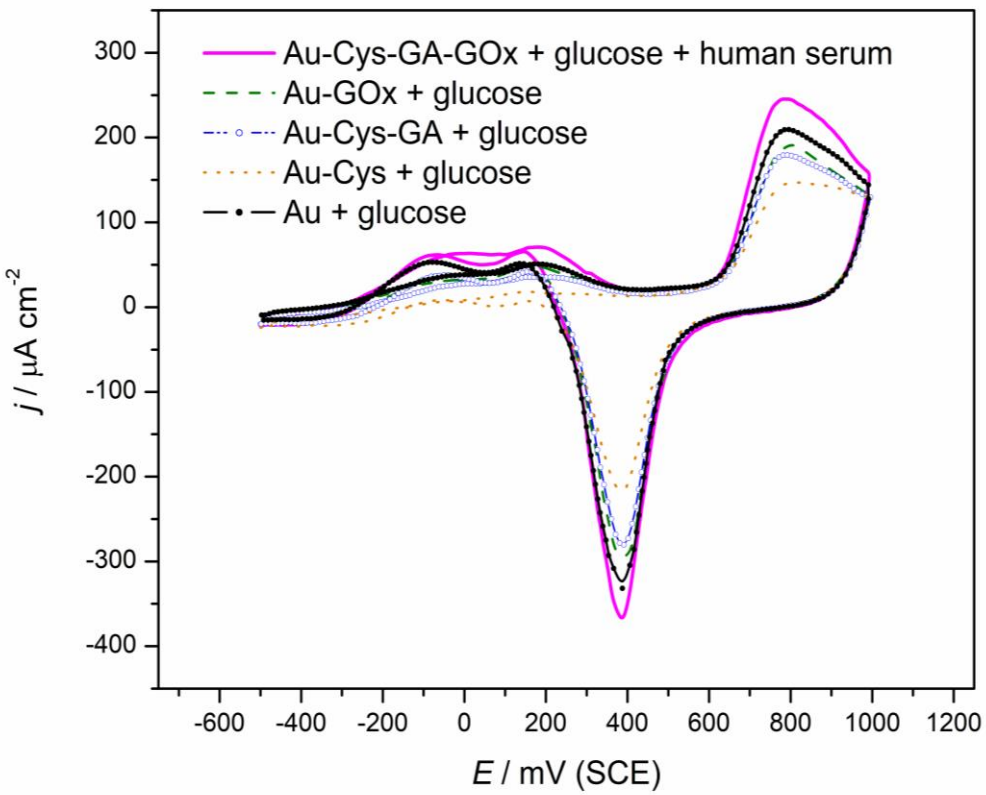

Figure 3. CVs obtained on gold electrode using phosphate buffer (0.1 M, pH 7.0) + 5 mM glucose (- · -), Au-Cys (….), Au-Cys-GA (- - · --), Au-GOx (---), Au-Cys-GA-GOx (⁻) + human serum at scan rate 50 mVs^{-1}.

The immobilized GOx on modified electrode as well as the electrodes with or without cross-linking agents were subsequently tested for the oxidation of glucose (Fig. 3). It is clear from Fig. 3 that

glucose exhibited high electrooxidation ability especially with the immobilized GOx on modified electrode (full violet line in Fig. 3). In Fig. 1 in the range of the potential from -0.2 V to 0.4 V only the currents of gold double layer are observed. In the presence of glucose in the same range of the potential, as is presented in Fig. 3, the high activity of glucose electrooxidation is apparent.

The detailed explanation of the oxidation of glucose presented in Fig. 3 will be discussed separately for the electrodes with or without cross-linking agents and for the immobilized GOx on modified electrode.

Au-Cys electrode exhibits low activity toward glucose oxidation. The further addition of organic layers increases its activity by enlarging the peak intensity and by shifting the beginning of the oxidation to more negative potentials. However, the higher current response (approximately 50 % compared to bare gold electrode) in the oxidation of glucose is observed for the covalently immobilized glucose oxidase (Au-Cys-GA-GOx electrode). While trying to understand the origin of oxidation ability of investigated steps of modified electrode one should take into account that gold surface is able to catalyze the oxidation of different sugars including glucose [25-27]. Namely, the glucose oxidation is correlated with the AuOH formation on the gold surface since in the potential range -0.1 to 0.3 V chemisorption of OH– occurs [28]. This indicates that the Cys-GA-GOx film on Au surface is not compact and that there were some bare regions which remain catalytically active. This finding is in agreement with the investigation of the influence of gold support on the activity of GOx immobilized on SAM modified electrodes [25]. Also, the obtained result indicates the strong interaction as a result of covalent bond formation between GOx and cross-linking agents providing better activity of Au-Cys-GA-GOx electrode. At high potentials, in which a layer of gold oxide is formed onto the electrode surface, an apparent catalytic effect on glucose oxidation [29] is observed as well as for Au-Cys-GA-GOx electrode (Fig. 3).

The glucose sensing presented with violet line in Fig. 3 is the same and reproducible in the samples containing human serum. In addition, the surface of modified electrode impersonate more suitable environment allowing the enzyme to take the optimal conformation for catalytic activity expression. It was already suggested that covalent coupling of the enzymes could preserve the enzyme activity, increasing the electron transfer rate in large extent [30].

Very often sensors suffer from interference of O_2 and H_2O_2 endogenously coexisting in biological systems or as an enzymatic reaction products [31]. Under our experimental conditions (Figs. 1-3) there is no possibility for O_2 and H_2O_2 reduction because it proceeds at more negative area of the potential [32-34] and consequently no interferences with molecule of glucose could occur. This suggests high selectivity of modified electrode enabling to proper glucose detection in biological systems.

Fig. 4a shows the cyclic voltammograms of GOx covalently immobilized on modified gold electrode, in phosphate buffer (0.1 M) containing 5 mM glucose, at different scan rates. The linear increase of anodic peak currents with the square root of scan rate between 20 and 140 mV s^{-1} implies diffusion-controlled electron transfer process (Fig. 4b). For such processes difference between peak potential and half-height peak potential is equal to $|Ep - Ep_{1/2}| = 47.7/(\alpha n)$, where α is the charge transfer coefficient, and *n* the number of the electrons in the rate determining step [35]. From the value of slope of logarithm of anodic peak current *vs.* logarithm of scan rate (0.58), it can be concluded that

this is a diffusion-controlled process, since it is very close to the theoretical value (0.5). So the calculate value of n is 1.68 i.e. $n \approx 2$ which is in accordance with previously published results [36-38]. The electron-transfer coefficient (k^0) for the diffusion-controlled process can be calculated by using the Laviron equation [37].

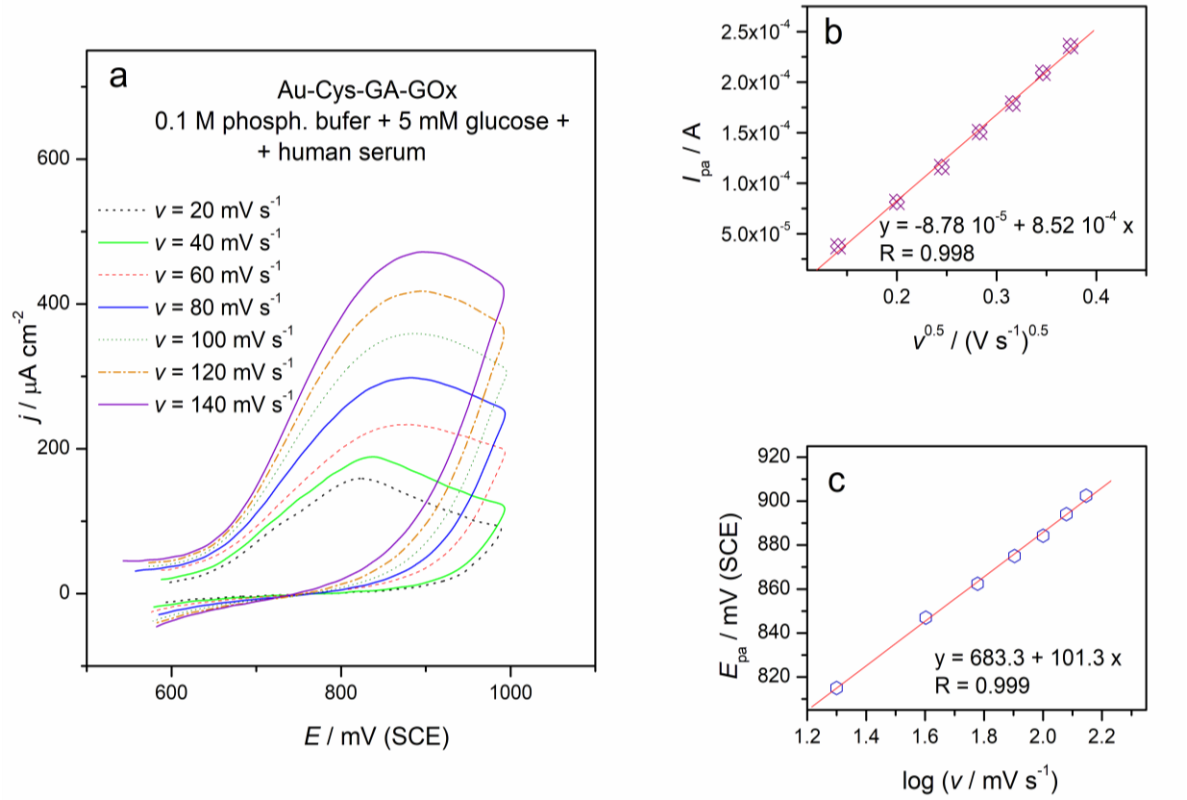

Figure 4. CVs obtained at Au-Cys-GA-GOx electrode using phosphate buffer (0.1 M, pH 7.0) and 5 mM glucose and human serum at different scan rates (a), the dependence of anodic peak currents on square root of scan rate (b), the dependence of anodic peak potentials on logarithm of scan rate (c).

The k^0 value can be determined, when the E^0 value is known, from the intercept of the plot Ep *vs.* log v (Fig. 4c). The E^0 value can be obtained from the intercept of the plot Ep *vs.* v (at v = 0) [39]. In our system for Au-Cys-GA-GOx electrode, E_0 was obtained as 853 mV and the k^0 as 20.4 s^{-1}. The electron-transfer coefficient for the glucose oxidation on electrode containing GOx immobilized by adsorption on Au was calculated to be 1.02 s^{-1}. The small value of k^0 indicates the slow electrochemical process, signifying the importance of cross-linking agents and their influence in better activity for glucose oxidation. The structure of modified electrode surface provides strong interaction due to the formation of covalent bond and more suitable environment for GOx by taking the optimal orientation contributing its catalytic activity toward glucose.

The obtained value for k^0 toward glucose with GOx immobilized on Au-Cys-GA is higher than the value obtained on cobalt hydroxide nanoparticles electrodeposited on the surface of glassy carbon electrode [36] and on graphene oxide modified pencil graphite electrode [39].

Further analysis of the voltammograms is based on linear relationship Ip *vs.* $v^{0.5}$ enabling the determination of diffusion coefficient (D), by using the Randles-Sevcik equation (1) [31]:

$$I_p = 2.99\times10^5 \alpha^{0.5} n^{1.5} A C^* D^{0.5} v^{0.5} \quad (1)$$

where I_p is the peak current, A is the electrode surface area, D is the diffusion coefficient, and C^* is the bulk concentration of glucose. The obtained value of the diffusion coefficient for glucose in this work (2.1×10^{-7} cm^2 s^{-1}) is higher than the one obtained with GC/NiOOH [40], but smaller than those obtained with GC/MWCNT/NiOOH electrode [40] and cobalt hydroxide nanoparticles electrodeposited on the surface of glassy carbon electrode [36].

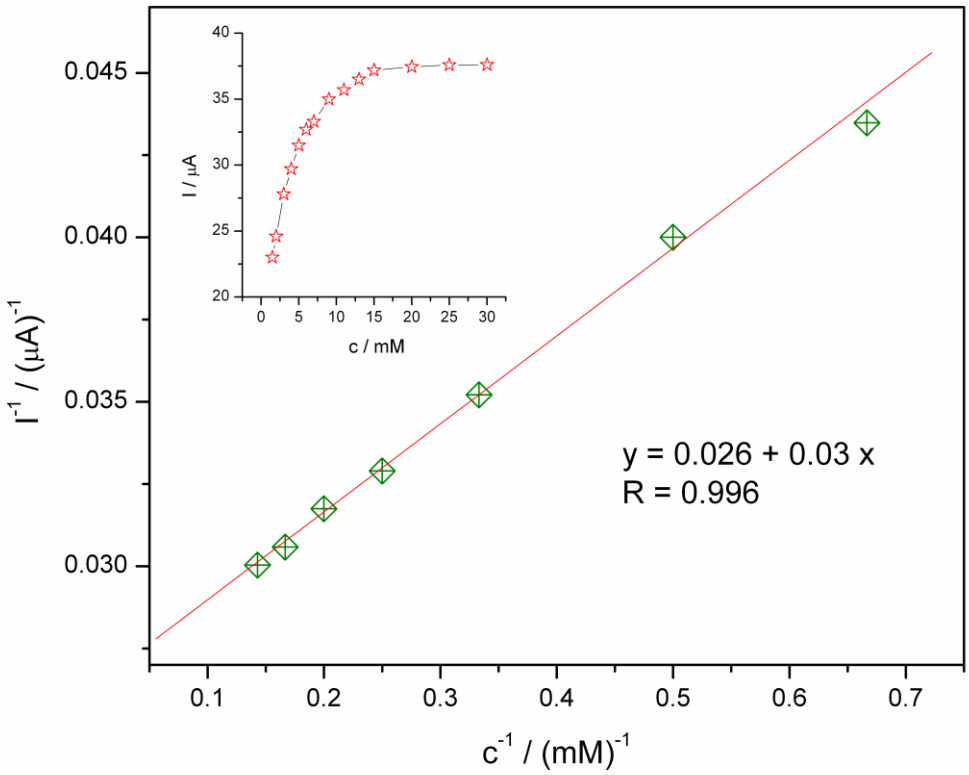

Figure 5. Currents values collected at 170 mV of the Au-Cys-GA-GOx electrode in the presence of various concentrations of glucose in the phosphate buffer solution (pH 7.0). Inset: The Lineweaver-Burk plot.

The dependency of anodic currents of glucose oxidation electrode on different glucose concentrations shows a trend typical for Michaelis-Menten kinetic as is presented in Fig. 5 (the data obtained from results already given in [41]). In inset of Fig. 5, the plot of oxidation currents *vs.* glucose concentration at a potential of 170 mV is displayed. There is a linear relationship between current *vs.* glucose concentration in the range from 1.5 to 7 mM. Detection limit was found to be 0.94 mM while the signal to noise ratio was 3. The sensitivity value, obtained from the slope of the plot, was 2.65 μA mM^{-1} cm^{-2}.

The apparent Michaelis-Menten constant [42] is obtained by using the Lineweaver-Burk equation [43]:

$$\frac{1}{I} = \frac{\frac{1}{I_{\max} + \left(K_M^{app}\right)}}{I_{\max} \cdot c} \quad (2)$$

were I is the steady-state current observed after the addition of glucose, I_{max} is the maximum current measured under saturated glucose conditions and c is the glucose concentration.

The K_M^{app} value (1.15 mM) was calculated from the slope and intercept of the equation 2 (Fig. 5). The additional comparison of analytical parameters of some reported biosensor containing gold with the presented results is shown in Table 1. The investigated Au-Cys-GA-GOx electrode exhibited comparable sensitivity to [44, 45] while the apparent Michaelis-Menten constant is lower regardless to [42, 45-47].

Table 1. Analytical parameters of glucose biosensors on gold electrode modified with different layers

Biosensor	Sensitivity ($\mu AmM^{-1}cm^{-2}$)	Michaelis-Menten constant (mM)	Linear range (mM)	Accuracy - R.S.D (%)	Reference
Au-Cys-GA-GOx	2.65	1.15	1.5-7	2.5 (n = 8)	present work
TEOS/AuNPs/GOx/C	2.43	/	0.5-55	0.5 (n=8)	[44]
Chitosan-gold NP	/	3.5	0.05-1.3	3.3 (n=10)	[42]
AuNWAs-GLA-BSA-GOx-HRP	25.3	2.36	0.005-1	1.33	[46]
GOx-hpAu	22.7	6.3	0.005-10	/	[47]
Au-g-PANI-c-(CS-CNTs)	21	5.35	1-10	5	[48]

The increased affinity of the GOx towards glucose after immobilization indicates changes in the enzyme conformation that enables the access of the glucose molecule to the enzyme active center. GOx redox center contains three amino acid residues (His 516, Glu 412 hydrogen bonded to His 559) [49] buried inside the enzyme structure (colored red in the Fig. 6a). As it can be seen in the Fig. 6b the majority of the enzyme amino groups are located opposite to the active center (dark blue regions). Therefore, the covalent bond enzyme is fixed in the conformation that the active center is positioned to the solution. Also, formed covalent bonds can partially unfold secondary structure and enable more efficient electron transfer by revealing the entrance of the active center. This is in accordance with the obtained voltammetric results presented in Figs. 3 and 5, promoting the Au-Cys-GA-GOx surface

suitable for the further biosensor development. Physical insight in the surface morphology and microstructure of layers of glucose biosensor was performed by Fourier transformed infra red spectroscopy (FTIR), atomic force microscopy (AFM) and optical microscopy (OM) and already presented in [41].

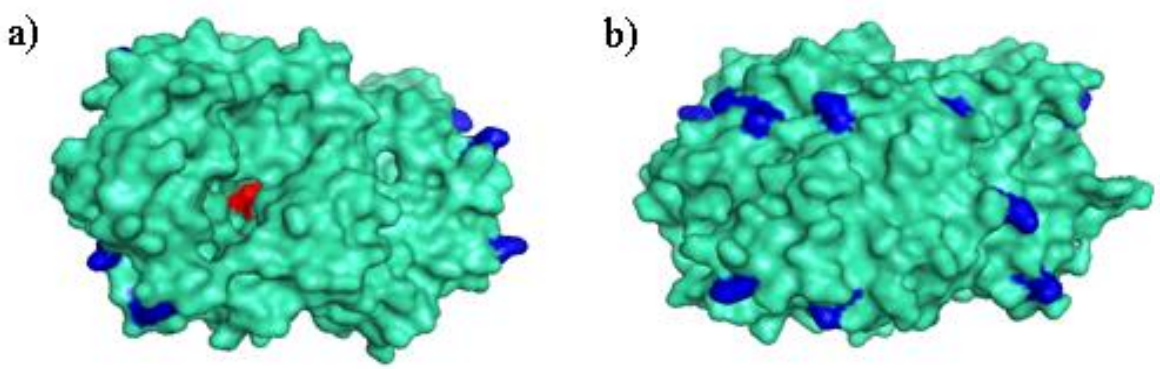

Figure 6. Distribution of amino acid residues with amino group (dark blue) and catalytic site (red) on the surface of glucose oxidase from *Aspergillus niger* (a) Front view on catalytic site. (b) 180^{o} rotation of the front view in x–y plane. The 3D structure was obtained using Pymol *vs*. 0.99 and data obtained from Protein Data Bank (PDB). PDB code for glucose oxidase from *Aspergillus niger* is 1CF3.

3.3. Selectivity and reproducibility of the Au-Cys-GA-GOx

The stability of the Au-Cys-GA-GOx biosensor was investigated after 7 days by storing it at 4 $^{\circ}$C. CV technique was used and after 7 days 95

% of electrode initial current response was retained. This high stability of Au-Cys-GA-GOx is due to the chemical stability of Au-Cys-GA modified electrode and the covalent bond between GOx and Au-Cys-GA.

Six Au-Cys-GA-GOx surfaces were prepared separately and tested by cyclic voltammetry. In this way, the reproducibility of the system as well as the reliability of preparation procedure was checked. The relative standard deviation (R.S.D.) value was found to be 5 %. For Au-Cys-GA-GOx electrode, R.S.D. determined by six successive assays of a 5 mM glucose sample was 2.5 %. The good properties of the Au-Cys-GA-GOx biosensor are the result of the natural features of Au-Cys-GA and the covalent bond with GOx. It can be pointed out that the used procedure for the preparation of immobilized enzyme is efficient in order to retain the enzyme electrocatalytic activity. In addition, the immobilization prevents the loss of enzyme from the electrode surface.

4. CONCLUSION

The electrochemical behavior of glucose oxidase-glutaraldehyde-cysteine modified gold electrode toward glucose oxidation was studied and high activity and affinity for glucose was demonstrated. The obtained results show that Cys-GA can afford a beneficial microenvironment for

the GOx and promote the glucose oxidation. The important advantage of the investigated system enables proper glucose detection in biological systems because of the lack of interferences with O_2 or H_2O_2. The glucose oxidase-glutaraldehyde-cysteine modified gold electrode as a biosensor exhibits excellent sensitivity, selectivity and stability for glucose monitoring. The Au-Cys-GA-GOx presented here may offer an additional approach for developing sensitive and stable system for glucose sensing in samples containing human serum.

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