Change in the surface density of immobilized enzyme molecules induced by photoelectron processes in a silicon substrate

A V Kozlowski, E D Kiseleva, A A Maslennikova and S V Stetsyura
Department of Nano- and Biomedical Technologies, Saratov State University, Saratov, 410012, Russia
kozlowsky@bk.ru

Abstract. We report on a change in the number of adsorbed glucose oxidase molecules depending on both salt concentration and illumination of a silicon substrate during glucose oxidase adsorption on its surface. Glucose oxidase molecules were adsorbed from its aqueous solution onto a silicon substrate either with a polyethyleneimine layer or without it. Based on obtained atomic force microscopy (AFM) data, it was found that addition of salt led to a more prominent effect of photo-assisted adsorption. In addition, illumination led to a monotonic change in adsorbed enzyme coverage (either decrease or increase depending on silicon substrate conductivity type), while variation of salt concentration resulted in the nonmonotonic change. The obtained results can be used for fabrication of biosensors with easily controllable parameters such as sensitivity, lower limit of analytic detection, etc.

1. Introduction
Enzyme adsorption on various surfaces occurs not only in fundamental biological processes, but also in many biotechnological applications, such as biosensors, protein separation, drug delivery, biomaterials, etc. Therefore, enzyme adsorption has attracted the attention of many researchers and has been widely studied by both theoretical [1] and experimental [2] methods to understand, predict, and control it on various surfaces including semiconductors.

The key step for developing biosensor is to maintain the bioactivity of enzyme after its immobilization on a solid surface. When enzyme is adsorbed with the favourable orientation for recognition of the enzymatic reaction by the transducer, the biocatalytic activity of the enzyme is provided. Physical adsorption and chemical attachment are two common methods to immobilize enzymes. Chemical attachment may provide a more stable immobilization of proteins but, frequently, it is accompanied by the loss of their bioactivity. Physical adsorption is a simple method to control adsorbed amount, orientation, and conformation of enzyme molecules. Previous experimental data of glucose oxidase (GOx) adsorption and molecular simulation results of other proteins [1,2] have shown that electrostatic interactions play an important role in controlling the adsorption. Variation of ionic strength and pH of solution is well-known method to adjust the electrostatic interactions between GOx and solid surface. This method is used in Langmuir-Blodgett technique as well as in layer-by-layer adsorption of charged polyelectrolyte molecules onto charged planar or curved surfaces. Atomic force microscopy (AFM) is an effective method for monitoring the surface of hybrid and biosensor structures based on solid substrate and organic components (such as enzyme, DNA, bacteria, etc.).
Semiconductors are widely used as a substrate for enzyme adsorption. Semiconductors are promising materials for biosensor technologies as transducer. Additionally, semiconductors allow remote changing surface charge density (SCD) of a substrate affecting the electrostatic interaction between enzyme molecules and the substrate. Obviously, the remote change of SCD of the substrate can be achieved by either a field effect using external electric field or a super band-gap illumination of the semiconductor substrate. Our previous results demonstrated the effect of photo-assisted adsorption on the processes of immobilization of GOx molecules [3] and branched polyethylenimine (PEI) onto a silicon surface [4].

The mentioned approaches are not state-of-the-art, while the effect of their combinations is not investigated properly. Therefore, in this work, AFM was used to study the GOx adsorption onto differently charged surfaces at different salt concentration and/or SCD through photo-assisted adsorption, with the aim to improve the efficiency of enzyme immobilization method.

2. Experimental

2.1. Materials and methods

Experiments were performed with single-crystal Si wafers of n-type ($\rho \approx 4 \ \Omega \ cm$) and p-type ($\rho \approx 8 \ \Omega \ cm$). Initially, the wafers were boiled in a peroxide-ammonia solution and rinsed in deionized water. This treatment led to “reconstruction” of a native oxide layer to SiO$_2$. It can be noted that isoelectric point (IEP) of the SiO$_2$ surface is ca. pH=2-3 [5], above this pH value the surface is negatively charged. Afterwards, wafers were cut into substrates of $8 \times 8 \ mm^2$.

GOx molecules from Aspergillus niger were used as enzyme molecules. In a wide pH range of the solution, the GOX molecules have an effective negative charge and the size of $6.0 \times 5.2 \times 7.7 \ nm^3$ [6]. A cationic polyelectrolyte polyethylenimine (PEI) with a molecular weight of 25 kDa was used to increase the adsorption of negatively charged GOX onto the silicon substrates. The PEI molecules were adsorbed onto the silicon substrates from the 1 mg/ml aqueous solution during 10 min followed by rinsing in deionized water ($\rho \approx 18.2 \ \Omega \ cm$) during 10 min and drying in nitrogen flow.

The photo-assisted layer-by-layer adsorption technique suggested in Ref. [7] was used to adsorb GOX from the 0.5 mg/ml aqueous solution onto bare silicon substrates and ones covered with PEI. Additionally, NaCl concentration was varied from 0 to 0.1 M.

2.2. AFM measurements

The topography of the surface was measured using AFM by NTEGRA Spectra (NT-MDT Spectrum Instruments, Russia) in tapping mode with scan frequencies of 0.2-0.5 Hz using HA_NC/W$_2$C cantilevers of ETALON series with resonance frequency of $140 \pm 10\%$ kHz. The Gwyddion software was used for statistical analysis of AFM data. The measurements have not been corrected for the convolution with the tip shape. Therefore, the lateral size of enzyme molecules observed by AFM does not correspond to the real size of the enzyme molecule due to the effect of the instrument on the object resulting in the artefacts appearance ("profile broadening" effect due to the tip-sample convolution). According to Ref. [8], AFM-images of molecules of the GOX with four different tip radii were simulated. As the tip radius increases, the observed lateral dimensions exceed the real size of the molecule significantly and it is impossible to resolve either the structural details or the individual GOX monomers. However, using results from Ref. [8], we can recognize individual molecules of GOX on the scan.

3. Results and discussion

3.1. The effect of salt on the GOX adsorption

Figure 1 shows AFM-images of adsorbed GOX molecules at different NaCl concentrations. According to the Figure 1, adsorption of GOX molecules onto the surface of Si/SiO$_2$ structure increases as NaCl concentration grows from 0 to 0.1 M. The GOX adsorption efficiency can be estimated using a mean surface coverage calculated as the percentage of area covered with GOX molecules from the total substrate area. Table 1 displays the mean surface coverage ($\pm$standard deviation) versus salt concentration. It is obvious that changes of NaCl concentration from 0 to 0.1 M result in corresponding
growth of mean surface coverage from 0.37 to 12.63 %. However, according to Ref. [9], at high salt concentrations (e.g., 0.5 M), the number of adsorbed enzyme molecules can decrease.

The results correlate with the double layer theory [10]. Growth of salt concentration in enzyme solution results in screening of electrostatic repulsion between the GOx molecules and the Si surface, which are both negatively charged. Therefore, it enhances GOx adsorption. With a further increase of salt concentration, a decrease in adsorption can occur due to the screening of intermolecular repulsion, which results in protein aggregate formation in solution instead of their adsorption onto a substrate. Since aggregates and the SiO₂ surface are both hydrophilic, the aggregates do not remain on the surface after rinsing, which results in a decrease of the surface coverage.

Additionally, we observed an increase of adsorbed GOx molecules from salt-free solution onto surface of a Si/SiO₂/PEI structure compared to Si/SiO₂ structure.

At low salt concentration in GOx solution, a number of adsorbed molecules reduces sufficiently in comparison with a salt-free solution. It is similar to the result of studying the adsorption of positively charged lysozyme on the negatively charged surface of SiO₂ [11]. High salt concentration in GOx solution leads to aggregates formation, as described above. However, the aggregates do not disappear after rinsing due to strong electrostatic interaction with a positively charged PEI layer (Fig. 1f).

Table 1. The relative change in surface roughness (ΔSa) and average height of irregularities (ΔH), as well as the mean surface coverage (±standard deviation) after GOx adsorption from a salt-free solution and with NaCl.

| Concentration of NaCl, M | p-Si/SiO₂/GOx | p-Si/SiO₂/PEI/GOx |
|-------------------------|--------------|------------------|
|                         | ΔSa, %  | ΔH, % | Surface coverage, % | ΔSa, % | ΔH, % | Surface coverage, % |
| 0                       | 62.13   | 33.78 | 0.37±0.03          | 69.64  | 62.08 | 9.32±1.12          |
| 0.01                    | 72.75   | 53.48 | 4.41±0.45          | 68.52  | 77.10 | 2.17±0.24          |
| 0.1                     | 86.88   | 80.53 | 12.63±1.38         | 78.14  | 69.15 | 18.86±1.65         |

Figure 1. AFM-image: (a-c) Si/SiO₂/GOx and (d-f) Si/SiO₂/PEI/GOx structures. GOx were adsorbed from solution with NaCl concentration: (a, d) 0 M, (b, e) 0.01 M, and (c, f) 0.1 M.
3.2. Illumination and type conductivity of substrate effect

Analysis of AFM-images of adsorbed GOx molecules on p-Si/PEI and n-Si/PEI substrates has shown that amount of adsorbed molecules is larger on the p-Si than on the n-Si type (Fig. 2a,c). However, illumination of the silicon substrate during the adsorption process alters the adsorption significantly. It decreases the number of adsorbed GOx molecules for p-Si/PEI and increases it for n-Si/PEI.

The influence of illumination on the adsorption of GOx molecules can be explained as follows. At equilibrium conditions (i.e., without illumination and external electric fields), the charge of the space-charge region (SCR) of semiconductor substrate is equal in magnitude and opposite in sign to the charge of surface electronic states (SES). The sign of SES charge usually corresponds to main charge carriers in semiconductor. The deposition of a cationic polyelectrolyte molecules (e.g., PEI) changes the structure surface charge to positive. It increases the band bending of p-Si structures and decreases that of n-Si ones. Thus, the total charge of SES, traps in SiO₂, and PEI in structures based on p-Si is greater than zero, while the one of n-Si is close to zero. When a Si/SiO₂/PEI structure is immersed into an aqueous solution of negatively charged enzyme molecules (e.g., GOx), the enzyme molecules are adsorbed onto the PEI surface predominately due to electrostatic interactions. This factor explains a significant difference of mean surface coverage between GOx adsorption on p-Si and n-Si.

Illumination of Si/SiO₂/PEI structures leads to rectification of their energy bands due to the generation of electron-hole pairs and their separation by SCR field. As a result, the charge of SES at the Si/SiO₂ interface decreases. In n-Si/SiO₂/PEI structures, the SES charge may become neutral or even positive (at high illuminance level). It results in a growth of the effective positive charge near the n-Si/SiO₂/PEI structure surface, an enhancement of the electrostatic interactions with GOx molecules, and, as a result, an increase in the mean surface coverage as compared to the case of adsorption in the dark. SCR field facilitates the drift of photogenerated electrons toward the p-Si/SiO₂ interface decreasing the effective positive charge at the PEI/GOx interface and the mean surface coverage as compared to adsorption in the dark. Table 2 displays the mean surface coverage (±standard deviation) versus salt concentration in case of adsorption in the dark and under illumination.

Table 2
Table 2. The mean surface coverage (±standard deviation) values and their relative change after photo-assistant GOx adsorption from a salt-free solution and with NaCl.

| Concentration of NaCl, M | p-Si/SiO$_2$/PEI | n-Si/SiO$_2$/PEI |
|-------------------------|------------------|------------------|
|                          | Without illumination | Under illumination | Δ, %            | Without illumination | Under illumination | Δ, %            |
| 0                       | 11.03±0.99        | 5.86±0.45        | -46.9           | 2.32±0.30           | 4.23±0.38        | +82.3           |
| 0.01                    | 10.59±1.06        | 2.96±0.34        | -72.1           | 1.99±0.18           | 4.45±0.42        | +123.6          |

Obviously, addition of salt increases the effect of photo-assisted adsorption. At low salt concentrations, the required surface charge density of the substrate for the adsorption of the enzyme increases [12]. Illumination allows changing the surface charge density of the substrate, thus making possible to control adsorption when the ionic strength changes. Moreover, illumination gives a monotonic change in adsorption (decrease or increase, depending on the type of silicon conductivity), while a change in the salt concentration or pH leads to a nonmonotonic one.

4. Conclusion
In the present study, we have investigated the effects of salt concentration, illumination, and silicon substrate type on the GOx adsorption onto a planar Si substrate. We have shown that both salt concentration and illumination during adsorption affect the enzyme-semiconductor surface interaction. It should be noted that the former leads to a non-monotonous change in protein adsorption. When signs of effective charges of the substrate and molecules of the enzyme are coincident, the changes in salt concentration result in either an increase of protein adsorption (at salt concentration of about 0.1 M) or a reduce due to the formation of massive hydrophilic aggregates (at salt concentration of about 0.5 M). When signs of effective charges of the substrate and molecules of the enzyme are different, the enzyme adsorption decreases at low salt concentration, but the massive aggregates are not removed from the surface after rinsing at high salt concentration.

Depending on the silicon substrate conductivity type, the illumination during adsorption leads either to an increase (for n-Si) or to a decrease (for p-Si) in the number of adsorbed molecules. Moreover, the effect from illumination is enhanced by the addition of salt into the enzyme solution.

As a result of this study, we state that a photo-assisted adsorption on the Si substrate allows controlling over the density of adsorbed enzyme molecules and, as a consequence, different characteristics of biosensor structures (sensitivity, lower limit of analytic detection, etc.). We assume that the effect of photo-assisted adsorption in the dynamics is monotonically decayed. Additionally, we assume that photo-assisted adsorption affects the orientation of the adsorbed enzyme molecule, which also affects the parameters of the biosensor. Further experiments using the scanning probe microscopy methods such as AFM and Kelvin-probe force microscopy might provide additional data to support our observations and hypothesis.

Acknowledgments
The work was supported by the Russian Foundation for Basic Research, projects no. 16-08-00524.

References
[1] Ratner B D, Castner D G, Horbett T A, Lenk T J, Lewis K B and Rapoza R J 1990 J. Vac. Sci. Technol. A 8 2306-17
[2] Xie Y, Li Z and Zhou J 2018 Phys. Chem. Chem. Phys. 20 14587-96
[3] Stetsyura S V and Kozłowski A V 2017 Tech. Phys. Lett. 43 285-8
[4] Malyar I V, Santer S and Stetsyura S V 2013 Tech. Phys. Lett. 39 656-9
[5] Cloarec J-P, Chevalier C, Genest J, Beauvais J, Chamas H, Chevolot Y, Baron T and Souif A 2016 Nanotechnology 27 295602
[6] Hecht H J, Kalisz H M, Hendle J, Schmid R D and Schomburg D 1993 J. Mol. Biol. 229 153-72
[7] Decher G 1997 Science 277 1232
[8] Makky A et al. 2013 J. Mol. Recognit. 26 521-31
[9] Tsapikouni T S and Missirlis Y F 2007 Colloids Surf. B Biointerf. 57 89-96
[10] Bockris J O'M, Devanathan M A V and Muller K 1963 Proc. R. Soc. Lond. A 274 55-79
[11] Su T J, Lu J R, Thomas R K, Cui Z F and Penfold J 1998 J. Colloid Interf. Sci. 203 419-29
[12] Carvalho S J, Metzler R and Cherstvy A G 2016 New J. Phys. 18 083037