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Glucose biosensors based on dendrimer monolayers

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The peculiarities of glucose biosensors based on different generation of dendrimers (G0, G1 and G4) have been studied by amperometry and QCM techniques. It is shown that stable glucose biosensor can be obtained with low generation of dendrimers. The sensor sensitivity, however considerable, increased with increasing number of generation of dendrimers. This can be due to the increased volume of the dendrimer interior as well as with increased number of binding sites for glucose oxidase (GOX). QCM experiments showed that immobilization of GOX resulted in formation of enzyme multilayers on a dendrimer surface. The enzyme turnover for this system (0.1–0.01 s^{-1}) was lower then that for immobilization of GOX onto a supported lipid films by means of avidin–biotin technology (1.1 s^{-1}). However, dendrimer based biosensors are more stable in comparison with sBLM based sensors and could be stored in a refrigerator in dry conditions over 15 days without substantial loss of sensitivity.

Introduction

Enzyme biosensors based on thin amphiphilic layers have been found as a very perspective for detection glucose, urea or organophosphates [1, 2]. The stability of these biosensors depends on the method of enzyme immobilization as well as on the type of amphiphilic layer onto which the enzymes are attached. Recently the monolayers prepared by direct adsorption of several kind of new compounds – dendrimers onto gold substrate have been reported [3]. These monolayers revealed high mechanical stability and can be functionalized without lost of dendrimers from the surface. The large area of dendrimers allowing to increase the number of immobilized functional units, and thus to increase the sensor sensitivity.

Recently a multiarrayed enzyme films composed of a glucose oxidase (GOX) and of the fourth generation of dendrimers (G4) has been reported [4]. Further improvement of sensitivity of this sensor has been obtained by modification of G4 with ferrocene carboxaldehyde [5]. In the above papers the first GOX layer was immobilized by chemisorption onto a cysteamine layer formed on a gold support.

In this work, analysis using another method of glucose biosensor fabrication using advantage of strong physical adsorption of dendrimers onto a gold support combined with chemisorption of hexylmercaptane (HYM). HYM served as a stabilizing component of mixed dendrimer–HYM layer. Moreover, the sensitivity of glucose sensor formed by chemical immobilization of GOX on dendrimer–HYM layers was compared as a function of dendrimer generation. It is shown that successful stability of the sensor can be achieved already with G0, but its sensitivity increased with increasing the number of dendrimer generation. The properties of biosensor have been compared with earlier reported glucose biosensors based on supported lipid films (sBLM) [6, 7].

Materials and methods

The dendrimer monolayer on a gold support (the disk gold electrode with diameter 1.6 mm, Minerale Corp., Poland) has been prepared as follows: the clean electrode (for cleaning procedure see Snejdarkova *et al*. [8]) has been first immersed into ethanol solution of Starburst (PAMAM dendrimer G0 (3.0 mM), G1 (1.5 mM) or G4 (0.2 mM), Aldrich) for 3–5 h at room temperature and then hexylmercaptan (HYM) (1- hexanethiol, 95%, Aldrich) was added to final concentration of 0.1 or 1 mM for 16 h (combination of dendrimers with HYM was necessary for formation of a stable layer on a gold support. Without HYM the dendrimers were detached from the electrode surface). The electrode was then washed with ethanol and deionized water and then has been immersed into the mixture of glucose oxidase (GOX) (0,144 U, Sigma) and glutaraldehyde (GA) (0.25%) in 0.1 M phosphate buffer (pH 7.5). After 20

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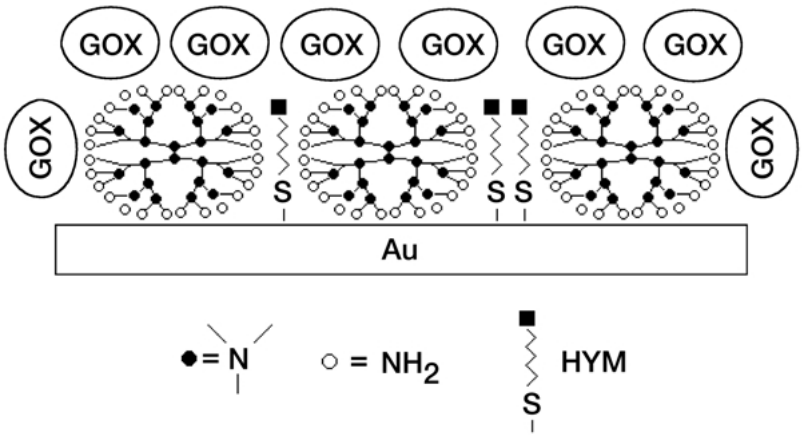

Figure 1 Organization of GOX–(D4 + HYM) layer on the gold electrode surface.

hours the modified electrode was rinsed with deionized water and stored in the refrigerator until the measurements. The sensor design corresponding to GOX immobilized on a dendrimer + HYM monolayer is schematically showed on Fig. 1. In one series of experiments, we prepared also ''sandwich'' type sensor. In contrast with Yoon and Kim [4] that utilized G4 dendrimers, we used G0 and G1 generation of dendrimers for this purpose. The biosensor has been prepared as follows: (1). The gold electrode has been immersed into the ethanol solution of G0 dendrimer (3.0 mM) with HYM (0.1 mM) for 22 h. Then the electrode was rinsed with ethanol, then with deionized water and immersed into the solution of GOX (3.3 μM) in 0.1 M phosphate buffer (pH 7.5) and GA (62 μM) for 20 h. (2). After step (1). the electrode was washed with 0.1 M phosphate buffer and immersed into the solution of 0.1 M phosphate buffer (pH 7.5) contained D1 (1.5 mM) and GA (62 μM). After 3.5 h the first step was repeated. Thus, the biosensor consisted of repeated dendrimer–GOX monolayers (EG0EG1). The integrity of layers and their conductivity have been detected by cyclic voltammetry (CV) and resistivity (see Snejdarkova *et al*. [8] for details).

The measurement of current flow across the supported thin films was performed with a programmable electrometer (Keithley 6512, USA), connected on line with an IBM PC 486 DX through KPC-488.2AT Hi Speed IEEE-Interface board. By analogy with Mirsky *et al*. [9] due to small currents flowing through the cell containing s-BLM $(I \leq 1\mu A)$, no substantial influence of Faraday processes is expected [10], a two electrode configuration was used throughout the experiments. The working electrode was the gold electrode with GOX, while the saturated calomel electrode (SCE) served as reference. The concentration of GOX immobilized on the electrode surface was determined by common QCM technique based on dependence of the quartz crystal on its mass. In this case frequency of oscillation of AT-cut quartz was measured (fundamental frequency 10 MHz) covered by a thin gold layer, onto which the GO + HYM layer deposited, was measured (see Hianik *et al*. [11] for details of experimental apparatus). We studied changes of frequency following immobilization of GOX by glutaraldehyde. All experiments have been performed at $T = 21 \pm 1$ °C in 0.1 M KCl + 0.1 M Tris (pH 7.1).

Results and discussion

Detection of glucose can be performed amperometrically, based on measurement of the current of electrons flowing between GOX and electrode surface due to anodic degradation of hydrogen peroxide, the product of glucose degradation [12]. In our experiments we applied 0.67 V to the working electrode relative to SCE (positive terminal on a gold support). Addition of glucose resulted fast increase of membrane current and its subsequent stabilization on a new steady-state level within approx. 5 min. Fig. 2, shows a typical amperometric response of a G0-based glucose sensor following addition of glucose. Dependence of relative changes of the current $\Delta I/I_0 = (I - I_0)/I_0$ (I_0 is the initial current at absence of glucose in electrolyte) has typical shape characteristics for an enzymatic reaction (Fig. 3). It can be seen that at relatively low concentration of glucose current grows rapidly with subsequent saturation at glucose concentration above 10 mM. The degree of initial rapid growth and, consequently the sensor sensitivity depends on the generation of dendrimer used for fabrication of the sensor as well as on the layer organization. It can be seen from the Fig. 3, that with increasing the number of dendrimer generation also amperometric response of the sensor increases. This is also seen from Table I, when comparison of sensitivity of glucose biosensors based on dendrimers and sBLM is

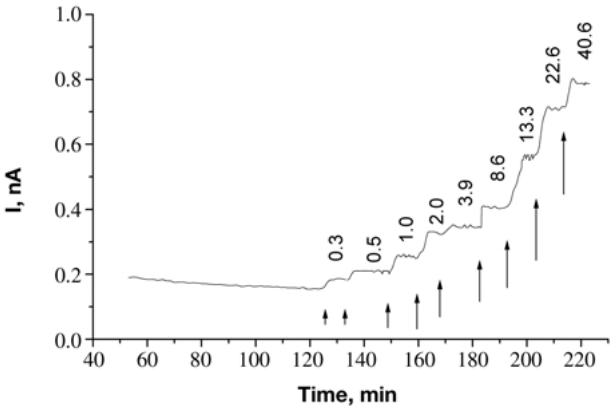

Figure 2 Amperometric response to glucose for GOX–G0 biosensor in 0.1 M KCl + 0.1 M Tris, pH 7.1 at 0.67 V vs. SCE. Arrows indicate the moment of addition of glucose (final concentration in millimoles is showed at upper side of the curve).

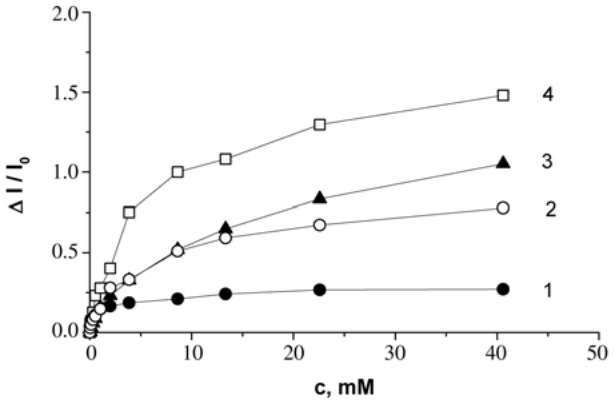

Figure 3 Dependence of relative changes of the current $\Delta I/I_0 = (I - I_0)/I_0$ (I_0 is the initial current at absence of glucose in electrolyte) on concentration of glucose for biosensor based on different generation of dendrimer monolayers and on enzyme–dendrimer sandwich: 1-G0, 2-G1, 3-G4, 4-EG0EG1.

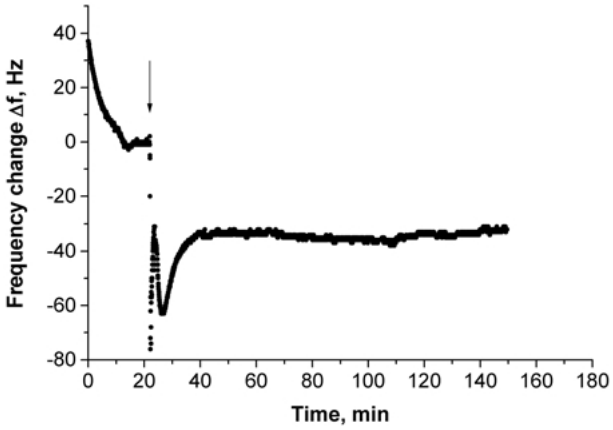

Figure 4 QCM frequency changes versus time of the AT-cut quartz crystal covered by G0 + HYM layer following addition of GOX at concentration of 5.3 μM and glutaraldehyde at concentration of 62 μM. The arrow indicates the time at which GOX was injected into the buffer solution.

presented. The sensor based on G0 dendrimers is characterized with rather low sensitivity. Considerable improvement of sensitivity take place when G1 is used instead of G0. Further increase of sensitivity is obvious for biosensor based on G4 dendrimer. The increase of sensitivity of biosensors with increasing the number of generation of dendrimers can be connected with at least two reasons: (1) Conductivity of monolayers increases in the order $(g)_{G4} > (g)_{G1} > (g)_{G0}$ (see Table I). This is due to the increased volume of the dendrimer interior. This interior contains large number of voids that facilitate diffusion of small molecules (e.g. H_2O_2) to the electrode surface. (2) With increased generation of dendrimers also number of binding sites for GOX increases. Thus, the concentration of GOX should be higher for G4 based biosensor in comparison with that for lower generation of dendrimers. Further increase of sensitivity has been obtained with ''sandwich'' type biosensor. This biosensor was composed of G0 layer adjacent to the electrode surface onto which GOX has been immobilized. Next layer was composed of G1 dendrimers with immobilized GOX (EG0EG1). As it is seen from the Table I, the sensitivity of this type of biosensor is higher than that for G4 based sensor. The sandwich design obviously allowing to increase the number of active GOX molecules. The sensitivity of biosensor based on G0 layer is even lower than that based on sBLM (see Table I). However, it should be noted that sBLM was formed on the tip of freshly cuted stainless steel wire. The surface of such electrode is rather rough and lipid film contains a huge number of structural defects. Even an uncovered metallic surface could take place in such a sensor design [13]. Further improvement of sensor sensitivity can be achieved by modification of dendrimer layers with electron-transferring mediators, e.g. ferrocene methanol, as has been reported recently [5].

Using the dependence of current vs. glucose concentration we determined the Michaelis constant, K_{ME} for the enzyme degradation of glucose on the electrode surface (see Hianik *et al*. [6] for detail). The Michaelis constants for glucose biosensors based on sBLM and on dendrimer layers are listed in Table I. The K_{ME} value is similar for biosensors composed of different generation of dendrimers. Considerably lower K_{ME} value for sBLM-based biosensor indicates higher affinity of GOX binding site to the glucose.

The immobilization of GOX on a dendrimer layer could influence the conformational freedom of GOX. This phenomenon was discussed earlier when properties of sBLM-based biosensors were compared with that based on free standing BLM and on glassy carbon electrodes [6]. The turnover of GOX immobilized on a surface of lipid film by means of avidin–biotin technology ($1.1\,s^{-1}$) is shown to be close to that measured in homogeneous solution ($5\,s^{-1}$). It would therefore be interesting to compare the above mentioned values with turnover of the GOX immobilized on a dendrimer layer. For this purpose it is necessary to know number of GOX molecules immobilized on the electrode surface. In our previous work [6] this value was

TABLE I Comparison of some parameters of the glucose biosensors based on dendrimer layers and sBLM. GOX is immobilized on dendrimer–hexylmercaptane monolayer composed of different generation of dendrimer (1) G0, (2) G1, (3) G4, (4) biosensor of ''sandwich'' type based on two dendrimer and two enzyme layers EG0EG1 (for explanation see the text). (5) avidin–GOX complex is immobilized on sBLM from biotinylated phospholipids [6]. *gs* is specific conductivity of the biosensor at absence of glucose. The values represent mean ± S.D. If not indicated the experimental errors did not surpass 10%

No.	System	Conductivity, gs $10^{-8}\,\Omega^{-1}\,cm^{-2}$	Sensitivity, nA $mM^{-1}\,cm^{-2}$	K_{ME}, mM	Enzyme turnover s^{-1}
1	D0	1.2 ± 0.4	2.0	5.1 ± 1.2	0.1-0.01
2	D1	46.8 ± 3.0	8.6	6.0 ± 0.4	—
3	D4	63 ± 4.0	28	5.2	—
4	ED0ED1	—	95.1	4.5	—
5	sBLM	—	14.1	0,7 ± 0.2	1.1

estimated using known dimensions of GOX molecule (cross-sectional area: $5.6 \times 10^{-13}\,\mathrm{cm}^2$) and known area of electrode surface [6]. Using QCM technique, it is possible to determine the number of molecules of GOX from the decrease in oscillation frequency of quartz crystal using a modified Sauerbrey equation $\Delta f = 2.26 \times 10^{-6} f_0^2 \Delta \mathrm{m}/A$, where Δf is the frequency shift due to the increased mass of the layer, f_0 is the quartz crystal resonance frequency ($\approx 10\,\mathrm{MHz}$ in our case) and $\Delta \mathrm{m}/A$ is the surface mass loading [14]. The change of frequency following immobilization of GOX onto a gold layer of AT-cut quartz crystal is shown on Fig. 4. The GOX immobilization resulted in decrease of frequency due to increase of mass loading of the crystal. The steady state value $\Delta f \approx 37\,\mathrm{Hz}$ is reached after approx. 12 min. The frequency of the crystal did not change substantially. Using frequency change and known molecular weight of GOX ($M = 154\,000$ Da) from the Sauerbrey equation the surface concentration of GOX molecules can be estimated: $N/A = \Delta f N_A/(2.26 \times 10^{-6} f_0^2\, M))$, where $N_A = 6.02 \times 10^{23}/\mathrm{mol}$ is the Avogadro number and A is the area of active electrode surface (in our case $A = 2.8 \times 10^{-3}\,\mathrm{cm}^2$). It should be noted that rather high variations in frequency changes (30–340 Hz) following immobilization of GOX have been observed in different experiments. These variations could be connected with variations in homogeneity of dendrimer + HYM layer on AT-cut quartz surface. This is particularly connected with most soft procedure of cleaning the quartz as well as with the fact that we did not use specially polished AT-cut quartz crystals, i.e. the surface could contain some micro-inhomogeneities or can be roughed. Thus using the above frequencies and Sauerbrey equation, $N = 6.4 \times 10^{13}$ to 7.1×10^{14}, of GOX molecules per cm^2, respectively. Using known cross-sectional area of GOX molecule, i.e. $5.6 \times 10^{-13}\,\mathrm{cm}^2$, the maximal density of GOX monolayer can be 1.8×10^{12} of GOX molecules per cm^2. It can be seen that this maximal density is considerably lower than that obtained in QCM experiments. It is assumed that immobilization procedures with glutaraldehyde resulted in the appearance of enzyme multilayers on dendrimer surface. Using the procedure reported earlier [6], from estimated surface density of GOX and experimentally determined total number of glucose molecules converted into lacton molecules on the electrode per second (i.e. $(I - I_0)/2e$, where $I - I_0$ corresponding to the maximal changes of the current, i.e. at maximal velocity of enzymatic reaction, $e = 1.602 \times 10^{-19}\,\mathrm{C}$ is the elementary charge) we estimated enzyme turnover, which varied in the interval 0.1–$0.01\,\mathrm{s}^{-1}$ for lower and higher GOX concentration, respectively. The obtained values are, however lower than that for sBLM based biosensor (see Table I). This means that chemical immobilization of GOX on a dendrimer layer resulted in higher restriction of the conformational changes of GOX in comparison with more soft immobilization of GOX on a lipid film surface using avidin–biotin technology. It is also possible that in enzyme multilayers some GOX molecules are inactive, or glucose molecules have restricted access to the enzyme active sites.

However, considerable advantage of glucose biosensor, even fabricated from low generation of dendrimers is its high stability. The sensitivity of G0 based glucose biosensor was almost unchanged during 15 days. In these experiments the sensor was kept in refrigerator at dry conditions at 4 °C overnight and then, prior calibration it has been activated during 10–30 min in 0.01 M glucose solution.

Thus, stable glucose biosensor can be obtained with low generation of dendrimers. The sensor sensitivity, however considerable increased with increasing number of generation of dendrimers.

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