NOVEL COMBINED TEMPLATE FOR AMPEROMETRIC BIOSENSORS WITH CHANGEABLE SELECTIVITY

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Abstract: Present paper describes innovative approach in design of amperometric biosensors useful in various applications. Original template of the electrodes has been prepared on a base of carbon nanotube support layer deposited on the polycarbonate membrane. Novel template and changeable enzyme layer give rise to creation of new family of biosensors acceptable for detection of wide range of carbohydrates. The morphology and electric properties of the constituent parts of the template electrode are characterized by scanning probe microscopy. The sensitivity, selectivity and stability are described for typical types of the biosensors.

1 INTRODUCTION

Electrochemical biosensors are the most common class of biosensors in various practical applications (A. Chaubey and B. D. Malhotra, 2002). In these sensors, bio-interaction at specific sites of enzymes results in extra electric charge. The extra charge can be transferred from enzyme to electrode and detected by an external electric circuit. Technology of the electrodes and enzyme immobilization is crucial for conversion of biochemical interaction into the response signal and, still, is under intensive studies. This type of sensors has well known advantages such as acceptability for functioning in turbid media, comparable instrumental sensitivity and amenability to miniaturization, and etc. In this report, we present an original approach in biosensor technology based on immobilization of enzymes within special matrix attachable to carbon nanotube electrode.

2 METHODS AND EXPERIMENTS

We developed and tested an original technology acceptable for production of a family of biosensors. Selectivity of two component biosensors can be changed simply by replacing special matrix containing enzymes attached to the single wall carbon nanotube (SWCNT) based electrode. Therefore the biosensors can be adjusted for detection of monosacharydes and disacharydes such as glucose, lactose, galactose, maltose and et cetera.

Nanotube support layers on the polycarbonate membranes were prepared from the industrial SWCNT (Cheap Tubes Inc., USA). The main parameters of these nanotubes ware as follows: diameter is 2 nm, 5.0 – 30.0 μm length, specific surface area 400 m2/g, electrical conductivity 10-2 S/cm. The SWCNT were functionalized with carboxyl groups (2.73 wt%).

In this study we introduced an original protocol for coating of flexible support by SWCNT layer acceptable for biosensor electrode. The protocol includes special filtration of aqueous suspension

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through the isopore polycarbonate membrane and is crucial for electrochemical properties of biosensors.

The prototype biosensors were based on three types of enzymes, namely glucose oxidase from *Aspergillus niger* and pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenases and aldose sugar dehydrogenase. The soluble glucose dehydrogenase (s-PQQ-GDH) from *Acinetobacter calcoacetics* L.M.D. 79.41 was purified by the method reported in (A. J. A. Olsthoorn and J. J. Duine, 1996). The membrane-bound enzyme (m-PQQ-GDH) was purified from *Erwinia* sp. 34-1 (Marcinkevičienė et al., 1999). The water-soluble aldose sugar dehydrogenase (s-PQQ-ADH) was purified from *Escherichia coli* (Southall et al., 2006). Each of the enzyme types was immobilized on individual flexible support of polivinylalcohol coated terylene. Adsorption and cross linking to the support were the methods for immobilisation of enzymes.

Our prototype amperiometric biosensors consisted of SWCNT based electric charge drain and changeable biosensitive detector. The sensor construction is illustrated by a sketch in Fig. 1.

Surfaces of the sensor components, namely, electrode support, SWNT coatings and matrix without and with immobilized enzymes, were analyzed by scanning probe microscope (SPM) D3100 / Nanoscope IVa (Veeco Instruments Inc.). Standard AFM methods such as contact and tapping mode surface scanning were used for visualization of the surface morphology. The surface electrical characteristics were evaluated from measurements of tunneling current obtained in contact mode. Conductive probe of the SPM was firmly pressed to the surface so that it was not damaged. Special module SPM D3100 TUNA (Veeco Instruments Inc.) was used for these experiments. The maps of the current and local point volt-amperic characteristics (VACh) were obtained for the components of the biosensor electrodes in these experiments. The data and SPM mages were processed by the NanoScope Software 6.14 (Veeco Instruments Inc.).

Electrochemical experiments were performed using a conventional three-electrode system containing a screen-printed carbon electrode as a working electrode, a platinum wire as a counter electrode and an Ag/AgCl in saturated KCl as a reference electrode (all potential values presented in the text are vs. this reference electrode). 0.05 M acetate buffer (pH 6.0) containing 1 mM of Ca^{2+} and 0.2 mM N-methylphenazonium methyl sulphate was used as a default buffer. Steady state currents of the biosensors were recorded at 0.4 V using a polarographic analyzer "PARSTAT 2273" (Princeton Applied Research, USA).

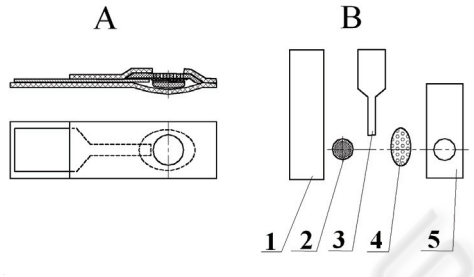

Figure 1: General side and top views (A) and the components (B) of the biosensor: 1 – insulating film, 2 – enzyme immobilized on terylene film, 3 – contact zone, 4 – SWCNT-polycarbonate membrane, 5 – insulating film.

3 RESULTS AND DISCUSSIONS

Characteristics of the biosensor family were obtained only for four types of the biosensors based on the original prototype structure in present study. The SWCNT layer on polycarbonate membrane and changeable enzyme based detector are the most important results of the sensor technology in present study. It was proved by experiments with the prototype biosensors that SWCNT based structure is acceptable for the sensor electrode and immobilization of enzymes. The attachable enzyme detectors were reproducible and stable for comparatively long time.

3.1 Surface Properties of the Electrodes

The morphology and electric properties were described for separate components of the template electrodes by the SPM experiments. The results were obtained for the components at intermediate stages of the technology.

Typical structure of the SWCNT coating is illustrated by a SPM image in Fig. 2. It is seen in Fig. 2 the SWCNT were found in vertical and horizontal positions on the membrane. Since the membrane contained the pores deep valleys were found in the nanotube layer. It was revealed by high aspect ratio SPM tests that SWCNT are in vertical position in the areas corresponding to the pores in the membrane. On the flat surfaces of the membrane there were no preferable orientations of the SWCNT with respect to the membrane surface. The SWCNT layer

was comparatively thick and at least several layers of horizontal nanotubes were detectable.

It was proved by measurements of tunneling current that electric conductivity highly depends on the structure of the SWNT layer. The areas with vertical nanotubes were more conductive that that with horizontal SWCNT. Electrical properties of individual areas of the SWCNT layer are compared in Fig. 3 by typical voltamperic characteristics (VACh) that were measured by special SPM module TUNA in contact mode. The tunneling current was measured by the SPM conductive cantilever tip diameter of which is about 20 nm.

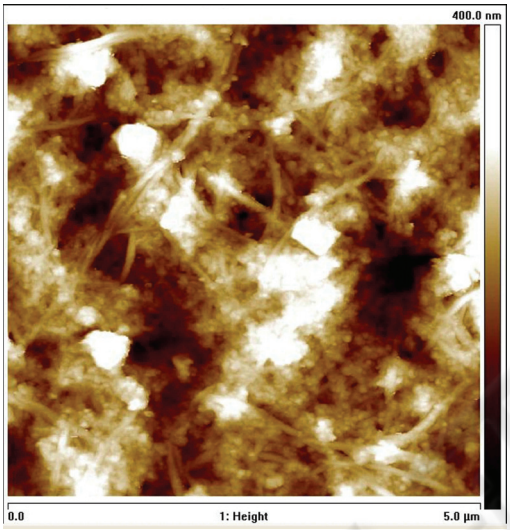

Figure 2: The SPM image of the surface of the SWCNT coating on polycarbonate membrane. The surface area of the image is 5x5 µm^2 and the maximum height is 400 nm.

In Fig. 3, the surface areas with the lowest and the highest conductivity are represented by the VACh measured at the tip-points of individual surface area. The lowest conductivity was obtained for the tip attached to the horizontal SWCNT (2 in Fig. 3). The highest conductivity of the SWCNT layer was found in the areas corresponding to the pores in the membrane (1 in Fig. 3).

Detailed distribution of the electrical conductivity over the surface of the SWCNT layer was visualized by scanning of the surfaces with the SPM TUNA. It was found that the spots of high conductivity are measured over the flat surface of the membrane if vertical SWCNT are detected in this area. Comparatively large areas were characterized by intermediate electric conductivity. It was supposed that only part of the SWCNT are connected so that produce conductive mesh of the electrode. The major part of the vertical SWCNT is only partly connected to this mesh and, therefore, limits electric charge transfer from the enzymes to the measurement circuit. This electric limitation reduces the effectiveness of the biosensor electrode.

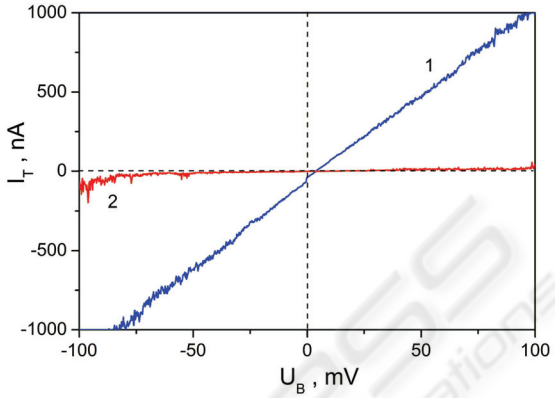

Figure 3: Tunneling current versus dc-potential between the SPM cantilever and the sample.

3.2 Biosensor Characteristics

Since the main advantage of PQQ-dependent dehydrogenases is functional independence of oxygen these enzymes are highly attractive for development of biosensors (Razumiene et al., 2005; Razumiene et al., 2006). All these enzymes were chosen also due to different ability to oxidise a number of carbohydrates. Thus, integration of these biosensors in whole sensing system allows detecting broad range of sugars, encompassing clinically important such as lactose, galactose, maltose and et cetera that is usually not detectable in body fluids although are associated with several diseases. In spite of numerous modifications of these enzymes that can be acceptable for detection of various important compounds we probed only a few types in this study.

Typical calibration curve for glucose obtained using s-PQQ-GDH based biosensor is shown in Fig. 4.

In Fig. 4, the current generated at the electrode during electrocatalytic oxidation of glucose by the enzymes was measured as a function of glucose concentration in the solution. Similar dependences were measured for all types of biosensors manufactures and probed in this work.

Kinetic characteristics, namely the apparent Michaelis constant (K_M^{app}) and maximum current (I_{max}^{app}), calculated for each type of the biosensors are summarized in Table 1.

Table 1: Kinetic characteristics of SWCNT-based biosensors with different enzymes.

Biosensor type	K_M^{app}, mM	I_{max}^{app}, μA	n	r^2
s-ADH	305.4	42	7	0.9913
s-GDH	5	27	12	0.9945
m-GDH	0.11	10	8	0.9986
GOx	5.8	260	7	0.9870

Results in Table 1 shows that enzymes possess different kinetics of action. From the functional standpoint, there are also different, i.e. (s-) soluble types operate in cytoplasma and (m-) membrane-bound is tightly bound to the outer surface of the cytoplasmic membrane (Matsushita et al., 2003). It has been shown that they are different enzymes with different pH-optima, molecular weights and substrate specificity.

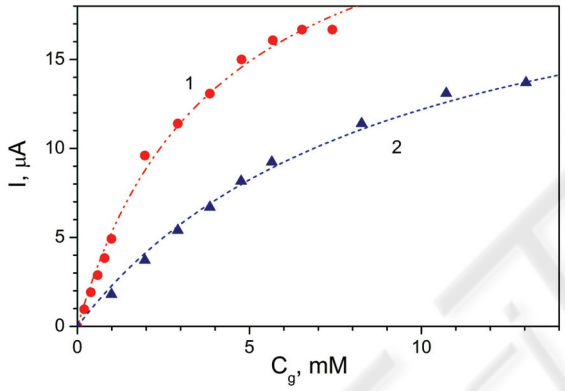

Figure 4: The calibration curves of the biosensors based on direct immobilization of s-PQQ-GDH on SWCNT (1) and with changeable s-PQQ-GDH film attached to SWCNT electrode (2). C_g is glucose concentration in the solution.

In our previous paper (Laurinavicius et al., 2004) has been demonstrated that due to the immobilization the active center of enzyme can be distorted that leads to different affinity to substrates as in the case of the native enzyme. Aiming to evaluate the affinity of enzymes operating in heterogeneous biosensing systems, the selectivity to clinically important metabolites such as glucose, lactose, galactose and maltose were investigated for all types of the proposed biosensors. The responses to individual metabolites were represented by the signal ratio with respect to the detection of 100 % glucose. The results are summarized in Table 2.

In order to understand an influence of the enzyme immobilization method of the of s-PQQ-GDH on the the selectivity and main kinetic parameters of the biosensor we investigated the electrodes based on SWCNT and carbon paste electrodes (CE) and manufactured by the method previously described in (Razumiene et al., 2006). The K_M^{app} and I_{max}^{app} parameters for three types of the electrodes are summarized in Table 3.

The I_{max}^{app} for all substrates can be explained by the s-PQQ-GDH catalyzed oxidation of main substrates such as glucose, lactose, and galactose with almost the same rate ratio for all probed types of biosensors (Table 3). However, the kinetic parameters are individual for the biosensors with differently immobilized enzymes (Table 3). The increase in K_M^{app} results in extension of the interval of the linear calibration curve. We associate it with diffusion limited access of the substrate.

The stability of the s-PQQ-GDH and GOx based biosensors was investigated during couple of weeks. The responses to the standard glucose solution (5 mM) were periodically recorded at room temperature equal to about 25 °C during these experiments. The residual response of the probed biosensors was not less than about 80 % of initial magnitude over the period of the tests.

4 CONCLUSIONS

Original technology was developed and probed for manufacturing of prototype biosensors with changeable selectivity. The template of the electrodes has been prepared on the basis of SWCNT conductive layer deposited on the polycarbonate membrane. The attachable flexible matrix with immobilized enzymes was proved functionally acceptable for catalysis of biochemical reactions and detection of these reactions. Vertical arrangement of the SWCNT in the electrodes was related to the areas of high electric conductivity of the electrodes that was assumed essential for functioning of the biosensors.

Using glucose oxidase, two types of pyrroloquinoline quinone dependent glucose dehydrogenases (namely s-PQQ-GDH, m-PQQ-GDH) and water-soluble aldose sugar dehydrogenase s-PQQ-ADH four versions of the prototype biosensors were manufactured and investigated in this work. In the tests, the responses to clinically important metabolites such as glucose, lactose, galactose, arabinose, manose and glucose-6-phosphate were measured. It was

Table 2: Responses to different substrates of SWCNT-based biosensors.

Biosensor type	Glucose	Lactose	Galactose	Manose	Arabinose	Glucose-6-phosphate
s-ADH	100	61	120	7	102	60
s-GDH	100	95	99	87	68	15
m-GDH	100	0	76	57	72	91
GOx	100	0	3	1	0	0

Table 3: Kinetic parameters of SWCNT-based and CE-based biosensors with differently immobilised s-PQQ-GDH.

Substrate	CE, enzymes on terylene		SWNT, enzymes on terylene		enzymes adsorbed on CE	
	I_{max}^{app}, μA	K_M^{app}, mM	I_{max}^{app}, μA	K_M^{app}, mM	I_{max}^{app}, μA	K_M^{app}, mM
Glucose	5.2	4.8	23.4	9	0.37	5.7
Lactose	4.7	8.1	21.7	7.2	0.3	8
Galactose	3.6	10.1	10.4	4.2	0.24	4

proved that the prototype biosensors are sufficiently stable so that can be acceptable for practical use.

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