JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 10, No. 3, March 2008, p. 713 - 716

Nanostructurated biosensors development for environmental measurements

M. FLORESCU*, C. M. A. BRETT[a]
Department of Physics, Transilvania University of Brasov, 500036 Brasov, Romania
[a]Departamento de Quimica, Universidade de Coimbra, 3004-535 Coimbra, Portugal

Environment, molecular biology, medicine and other life sciences require for a new class of devices having a fast sensitive and reliable response for investigating biomolecular interactions. This need has fuelled a revolution of a new class of sensors, nanobiosensors that are the product of the integration of nanotechnology, biology, and advanced materials. The possibility to employ new materials and innovative technologies allows for the realizing of conceptually new devices, potentially competitive to the existing in classical technologies.

(Received September 25, 2007; accepted March 12, 2008)

Keywords: Sol-gel nanostructure, Glucose oxidase enzyme, Electrochemical biosensor

1. Introduction

Environment, molecular biology, medicine and other life sciences require for a new class of devices having a fast sensitive and reliable response for investigating biomolecular interactions at the single cell level. Thus, appear the necessity of replacing the microelectromechanical systems (MEMS), with nanoelectromechanical systems (NEMS) - i.e. machines, sensors, computers and electronics that are working on the nanoscale [1]. This need has fuelled a revolution of a new class of sensors, nanobiosensors that are the product of the integration of nanotechnology, biology and advanced materials.

An alternative to ease the analysis in routine of environment, molecular biology, medicine and other life sciences is the biosensors development, turning them technology in a possible methodology to be applied in real samples. Chemical and biological sensors (or biosensors) represent analytical systems based on a recognizing sensing layer being in an intimately contact with a transducer that convert the physicochemical changes occurred at sensing layer level after interaction with a specific analyte into a measurable signal. Some advantages of biosensors are high selectivity and specificity, relative low cost of construction and storage, potential for miniaturization, facility of automation and simple and portable equipment construction for a fast analysis and monitoring outside of laboratory [2]. In the case of biosensors selectivity and specificity of biological compounds are combined with the sensitivity of the transducer. Analytical performances of these devices are obtained by using the biological compound in a very small quantity, biosensors operating relying on conformational changes that occur at biomolecular level.

Inorganic and organic hybrid nanomaterials are a fascinating research area, due to the highly promising potential for versatile properties provoked by combining the merits of both sources and by the nanometer size effect, which is entirely different from that in a bulk material. Moreover, a coupling of biorecognition elements on nanostructures might allow a creation of functional hybrid systems with molecular-scale proximity between the molecular recognition and transduction element. Such functional hybrid systems (i.e., the "marriage" of biomolecules and nanostructures) are essential not only for a new generation of chemical and biological nano-sensors with unique functional and application possibilities, but also for the fundamental research of biological molecules (DNA, RNA, immunospecies, proteins, etc.) and living cells [3]. Developments involving the use of this type of sensor could be employed for on line control of the fermentation process. The concentration of carbohydrates in beverages may change throughout the process of production. For example, the concentration of sucrose, glucose and fructose in wine are dependent on the time when sugar is added (prior or post fermentation) and on the degree of fermentation.

A serious problem that must be overcome for using such biosensors in natural samples is the presence of metabolites or other compounds that represent positive interference due to fact they are oxidized/reduced at the same potential as the sample. One solution is to utilize an electroactive compound that will act as a redox mediator, decreasing the sample reduction or oxidation potential to close to 0.0 V. Transition metal hexacyanoferrates (MHCF) are becoming widely used redox mediators for biosensors because of their mixed-valence clusters organisation that posse semiconductor characteristics and can transfer electrons during reduction and oxidation processes [4,5]. An example of a metal hexacyanoferrate is cobalt hexacyanoferrate (CoHCF).

The present study deals with the development, evaluation and characterisation of a glucose oxidase (GOx) electrochemical biosensor for glucose detection with a bilayer configuration. Electrodes have been modified with cobalt hexacyanoferrate first with a film of CoHCF by potential cycling from solutions containing

cobalt and hexacyanoferrate ions. The second layer was obtained by deposition of the enzymatic layer on top of the mediator layer. In this case the nanostructuration is given by the second film of the sol-gel that was prepared using combination of oxysilane precursors with different grades of hydrophobicity with the enzyme immobilized in the nano-porous gel network.

2. Experimental

2.1 Chemicals

Three different trioxysilane solutions were used for enzyme encapsulation: tetraethoxysilane obtained from Fluka, 3-glycidoxypropyltrimethoxysilane, and methyltrimethoxysilane from Aldrich. Glucose oxidase (GOX, EC 1.1.3.4, II-type from Aspergillus niger, 35,600 units/mg), α-D(+)-glucose, ($K_3Fe(CN)_6$) and $CoCl_2x6H_2O$ were obtained from Merck. Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm) was used throughout for the preparation and dilution of solutions. The supporting electrolyte was phosphate buffer saline (PBS) (0.1 M phosphate buffer + 0.05 M NaCl). Glucose standard solutions were prepared by dilution of a 100 mM α-D (+)-glucose stock solution prepared in water. The stock solution was prepared 24 h before use to establish the anomeric equilibrium between α and β forms of D-glucose; it was kept in the refrigerator and used within a week.

2.2 Electrochemical measurements

Measurements were made in a one-compartment cell containing the carbon film electrode, a platinum auxiliary electrode and a saturated calomel electrode (SCE) as reference. Amperometric experiments were carried out using CV-50W Voltammetric Analyzer from Bioanalytical Systems, West Lafayette, Indiana, USA, controlled by BAS CV-2.1 software.

2.3 Electrochemical modification of carbon film with cobalt (II)-hexacyanoferrate

The carbon film electrodes were modified by electrochemical deposition of cobalt(II)-hexacyanoferrate (CoHCF). This was accomplished by cycling the electrodes 15 times between 0.0 and +0.9 V at a scan rate of 50 mV s^{-1} in a freshly prepared solution containing 0.5 mM $CoCl_2$, 0.25 mM $K_3Fe(CN)_6$ and 0.05 M NaCl at pH 3 (pH adjusted with HCl) with solution agitation by slow mechanical stirring. Subsequently, the CoHCF film electrodes were stabilised for 1 hour in 0.05 M NaCl, pH 3. After that they were left to dry at room temperature. Modification of the electrodes was always carried out employing the same, identical solution composition for obtaining reproducible results.

2.4 Enzyme immobilisation

Sol-gel solution was prepared by mixing solution of the oxysilanes and water in next ratios: GOPMOS:TEOS:H2O – 130:70:600 µl; and GOPMOS:MTMOS:H2O – 150:70:620 µl. The mixtures obtained were intensively stirred for a few minutes and then sonicated for 15 min. Following this, the solutions were heated to evaporate the alcohol [40] formed during hydrolysis of the oxysilanes in a hot air stream (~70 ºC) until the solutions lost 40 % of their volume and were left 10 min at room temperature to cool down and neutralized to pH 7.0. Then 50 µl of each solution was carefully mixed with 15 µl of GOX solution in 0.1 M phosphate buffer saline (PBS) solution pH 7.0. Then CoHCF- coated carbon film electrodes were immersed in the sol-gel-enzyme solutions for 5 min, removed and then left for sol-gel formation at 4 ºC for 3 days. Electrodes were stored at 4 ºC when not in use.

3. Results and discussion

The most widely used amperometric biosensors are based on oxidase enzymes (e.g., glucose oxidase, GOx) that generate H_2O_2, the transduction path being the electrochemical oxidation of the hydrogen peroxide formed in an enzyme reaction [6-8]. A serious problem that must be overcome for the use of such biosensors in real samples is the presence of metabolites or other compounds that represent positive interference due to fact they are oxidized/reduced at the same potential as H_2O_2.

$$\text{Glucose} + O_2 \rightarrow \text{GOx} \rightarrow \text{gluconolactone} + H_2O_2$$

One solution is to utilize an electroactive compound that will act as a redox mediator with lower oxidative or reductive potential than hydrogen peroxide [9]. Metal hexacyanoferrate films are interesting types of redox mediator. Attachment of these species to electrode surface can be achieved by controlled-potential electrodeposition, galvanostatic deposition, adsorption or entrapping them into polymeric matrices and mechanically transferring them onto the electrode surface.

The stability and electrochemical characteristics of biosensor are governed by the enzyme immobilization on the transducer surface. The analytical performance of the biosensors depends on both the immobilization process and the matrix used for immobilization of the enzyme (a friendly environment is necessary to immobilize enzymes in hybrid nanomaterials). Enzyme immobilisation onto the electrode surface can be done in different ways, using physical or chemical procedures. Good results were obtained using cross-linking methods with glutaraldehyde [10,11], sol-gel [12], layer-by-layer (LbL) technique [13] as immobilisation methods for glucose-oxidase and recently nanostructured materials [8] .

The sol-gel encapsulation of biomaterials and organic complexes has recently seen important developments, sol–gel being an interesting and versatile way to prepare modified electrodes and biosensors [14, 15]. This inorganic material is particularly attractive for the fabrication of biosensors since it possesses physical rigidity, chemical inertness, high photochemical and thermal stability, excellent optical transparency, and

experiences negligible swelling in aqueous and organic solvents. Most of the useful sol–gel solutions are organic orthosilicates having a solid and a gel phase, which is formed by gelation of a colloidal suspension and can be dried to form a xerogel, a dry porous silicate with controlled porous surface area, network structures, surface functionalities and processing conditions. The gels are mostly interesting because an enzyme can be immobilized by building the porous gel network around each enzyme macromolecule by encapsulation that not involve any covalent bound between the support and the enzyme, allowing the preservation of enzyme activity [16]. In this way the gels make possible to achieve the nano-encapsulation of each individual enzyme molecule, provided these macromolecules did not agglomerate during the encapsulation process [17]. Additionally, external substrates as well as transformed products must remain free to diffuse in and out of the nano-capsule walls. In this way the hydrophobic-hydrophilic balance of the support present a great importance. Hence, it becomes necessary to analyze in more details the hydrophilic-hydrophobic nature of the silica gels made in conditions compatible with biosensors application [18].

The CoHCF layer was first deposited on the electrode surface and a layer of a sol–gel with entrapped enzyme was then deposited on top of the mediator layer. Many parameters in the synthetic protocol of a sol-gel can be adjusted. The hydrophobic or hydrophilic properties of a dry gel were related to their textural and surface structural characteristics [18]. The changes in proportions of hydrophilic and hydrophobic surface groups of sol-gel precursors can influence the capillary contraction of the whole structure and then the contraction of the nano-cages where the enzyme is immobilised [17].

From literatures was observed the hydrophobic character of GOPMOS compared with the less hydrophobic of MTMOS and hydrophilic of TEOS [17]. Since the highest sensitivity was observed with the biosensor with encapsulated GOx using GOPMOS as sol–gel precursor and the highest limit of detection with the longer linear range were observed at the widely used TEOS based sol–gel [12], the combination of GOPMOS and TEOS precursors was studied and compared with GOPMOS plus MTMOS in the development of glucose-oxidase biosensors. Different proportions of each precursor have been tested [19] and the optimum composition was found to be GOPMOS : TEOS : H_2O – 130 : 70 : 600 µl and GOPMOS : MTMOS : H_2O – 150 : 70 : 620 µl. These compositions will be abbreviated to GT and GM, respectively, in the text that follows.

The concentration of the entrapped enzyme has a big influence on biosensor response, since it has been observed that an increase in the concentration of some entrapped enzymes can result in a marked decrease in specific activity that can form aggregates at higher concentrations [20]. For this reason different concentrations of enzyme in PBS solution were used: 4%, 10% and 15%, that correspond to concentrations of approximately 1.0%, 2.3% and 3.5% in the resulting dry gel on the electrode surface

Glucose detection was done for both type of dry gel CoHCF mediated sensors and data from analysis of the curves are given in Table 1.

Table 1. Data from calibration plots of CoHCF-mediated glucose biosensors with GM and GT sol-gel precursor mixtures.

Sol-gel	[Enzyme] / %	Sensitivity / nA mM^{-1}	Intercept / nA	LOD / µM	Linear range / mM
GM	1.0	47.6 ± 0.3	0.64 ± 0.70	59	0 – 0.4
	2.3	39.7 ± 0.19	0.51 ± 0.30	34	0 – 0.4
	3.5	138.8 ± 0.1	1.09 ± 0.90	24	0 – 0.2
GT	1.0	19.7 ± 0.1	0.56 ± 0.30	71	0 – 1.0
	2.3	29.9 ± 0.1	0.33 ± 0.20	30	0 – 0.25
	3.5	57.4 ± 0.1	0.53 ± 0.50	46	0 – 0.5

The best sensitivity of the biosensors of 138.8 nA mM^{-1} and a limit of detection of 24 µM was obtained when GM sol-gel have been used, but for a short linear range 0-0.2 mM and using a big concentration of immobilised enzyme (3.46%). The longer linear range (0-1 mM) was observed for the biosensor prepared with GT sol-gel, with lower sensitivity and a bigger limit of detection (71.4 µM) for a concentration of immobilised enzyme of 1% (Fig. 1).

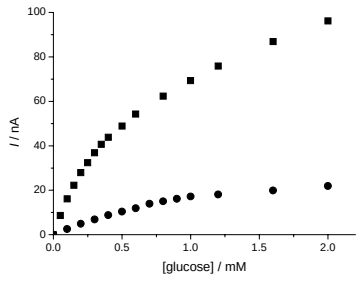

Fig. 1. Calibration curves at (■) GM-based biosensor and (●) GT-based biosensor at applied potential 0.0 V vs SCE for successive additions of glucose to 0.1 M PBS pH 6.94.

Higher concentrations than 3.46% of immobilised enzyme were used for sol-gel-based biosensors development but results were not different (results not shown).

The results can be explaining taking into account that the concentration of the entrapped enzyme has a big influence on his function and the accessibility of analyte to the entrapped enzyme is determined largely by the pore size and the electrostatics of the material, which can be tuned by various methods including variation of precursors, sol–gel synthesis conditions, silane:solvent ratio, aging conditions and so on.

For GM-based biosensors the combination of GOPMOS with MTMOS determines a nano-pore structure of a dry gel that is favourable for the higher concentration of immobilised enzyme. These biosensors present a high sensitivity but the saturation of enzyme is obtained in a short time caused by the big accessibility of the glucose to the entrapped enzyme through the pores of the gel obtained by the combination of two hydrophobic precursors with different grades of hydrophobicity. The pore dimensions of the dry gel seem to be big enough to permit an easy access of the glucose to the enzyme and also to the active sites of immobilised enzyme. Using a high concentration of the enzyme solution assure also the presence of an enough quantity of immobilised enzyme on the biosensor surface, even the big dimensions of the pores allow the leakage of the enzyme.

For the GT-based biosensors the glucose reaches the immobilised enzyme in a longer time that can be caused by the small dimensions of the dry gel nano-pores realised by combination of high hydrophobic GOPMOS with the hydrophilic TEOS. In this case the passing of the substrate through the pores to the enzyme can become more difficult and in the same time the active site of the immobilised enzyme can be buried due to its thronging into the nano-cages where the enzyme is immobilised.

4. Conclusions

Carbon film electrodes have been modified with cobalt hexacyanoferrate redox mediator films followed by enzyme immobilisation in a sol-gel layer from a sol-gel precursor mixture and successfully used as biosensors at low applied potential. The sol-gel was prepared using a mixture of different oxysilane precursors with different grades of hydrophobicity. The changes in proportions of hydrophilic and hydrophobic surface groups of sol-gel precursors influence the performance of gel-based biosensors due to the internal structure of dry gel that can affect the nano-cages dimensions where the enzyme is immobilised and thus the concentration and the activity of immobilised enzyme on the biosensor surface. Such biosensors were used to determine glucose concentrations down to the micromolar level. The most suitable gel film was obtained using GT-based biosensors operated at +0.0 V versus SCE. The linear range was 0–0.5 mM, with a limit of detection of 46 μM. The sensor was used to determine the glucose concentration in synthetic solution and in wines.

Acknowledgements

Financial support from European Project HPRN-CT-2002-00186 (Novtech) and Fundação para a Ciência e Tecnologia (FCT), ICEMS (Research Unit 103), Portugal are gratefully acknowledged. Prof. H. D. Liess is thanked for the gift of the electrical resistors.

References

[1] M. Roukes, Physics World **14**, 2 (2001).
[2] J. Platen, A. Poghossian , M. J. Schöning, Sensors **6**, 361 (2006).
[3] H. T. Luong, C. A. Groom, K. B. Male, Biosensors and Bioelectronics **6**, 547 (1991).
[4] N. R. de Tacconi, K. Rajeshwar, R. O. Lezna, Chem. Mater. **15**, 3046 (2003).
[5] S. M Chen, Electrochim. Acta **43**, 3359 (1998).
[6] F. Xu, L. Wang, M. Gao, L. Jin, J. Jin, Talanta **57**, 365 (2002).
[7] N. Dimcheva, E. Horozova, Z. Jordanova, Z. Naturforsch. **57**, 705 (2002).
[8] S. H. Lima, J. Weia, J. Lina, Q. Lib, J. K. Youc, Biosensors and Bioelectronics **20**, 2341(2005).
[9] A. Chaubey, B. D. Malhotra, Biosens. Bioelectron. **17**, 441 (2002).
[10] M. Florescu, C. M. A. Brett, Talanta **65**, 306 (2005).
[11] M. Florescu, C. M. A. Brett, Anal. Lett. **37**, 871 (2004).
[12] R. Pauliukaite, C. M. A. Brett. Electrochim. Acta **50**, 4973 (2005).
[13] K. Hyde, M. Rusa, J. Hinestroza1, Nanotechnology **16**, S422 (2005).
[14] O. Lev, M. Tsionsky, L. Rabinovich, V. Glezer, S. Samapath, I. Pankratov, J. Gun, Anal. Chem. **67**, 22A (1995).
[15] L. Rabinovich, O. Lev, Electroanalysis **13**, 265 (2001).
[16] A. Kumara, R. Malhotra, B. D. Malhotra, S. K. Grover, Anal. Chim. Acta **414**, 43 (2000).
[17] A. C. Pierre, Biocatal. Biotransform. **22**, 145 (2004).
[18] H. El Rassy, P. Buisson, B. Bouali, A. Perrard, A. C. Pierre, Langmuir **19**, 358 (2003).
[19] M. M. Barsan, J. Klinčar, M. Batič, C. M. A. Brett, submitted.
[20] W. Jin, J. D. Brennan, Anal. Chim. Acta, **461**, 1 (2002).

* Corresponding author: florescum@unitbv.ro