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Enzyme immobilisation on planar and porous silicon substrates for biosensor applications

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Abstract

Two methods for the immobilisation of enzymes on silicon-based so-called electrolyte–insulator–semiconductor (EIS) structures are suggested. These EIS structures are used as a basis for potentiometric biosensors. In the first method, heterobifunctional cross-linker molecules are employed to covalently bind enzymes to these capacitive layer structures which possess a planar surface that contains amine groups. Porous EIS sensors which, in comparison to planar sensors, exhibit an enlarged surface area, are used in the second method. For the first time, pH-sensitive Si_3N_4 was deposited on the walls and bottoms of the SiO_2-covered pores. Here, a large amount of enzyme molecules can adsorptively be bound inside the porous structure. Penicillinase is used as a model enzyme. Capacitance–Voltage and Constant Capacitance measurements are performed in order to examine the respective penicillin sensor responses and thus to validate both immobilisation methods. Whereas the sensitivity of the sensors prepared by both methods is nearly identical for low penicillin concentrations up to around 0.25 mM, a difference of the calibration curves in the higher concentration range indicates a larger amount of immobilised enzyme in the case of the porous structures.

Keywords: Silicon; EIS structure; Porous silicon; Biosensor; Cross-linker

1. Introduction

After Caras and Janata [1] introduced the concept of an enzyme field-effect transistor (EnFET) in 1980, considerable effort has been devoted to the development of biosensors based on semiconductor devices. The combination of a biologically sensitive material with silicon technology is very attractive due to the possibility of miniaturisation and integration of signal processing devices [2–4].

One of the most frequently described methods of immobilising the biologically sensitive material, e.g., enzyme molecules, on the surface of a silicon chip is that of homobifunctional cross-linking with glutaraldehyde [5]. However, a shortcoming of sensors prepared in this way is the insufficient adhesion of the sensor membrane and the fast leaching out of the enzyme molecules during the sensor operation. Besides, the activity of the enzyme is decreased if a large number of cross-linker molecules is bound to a single enzyme molecule and thus, hinder its conformational changes during the catalytical

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reaction. In contrast, the pure adsorptive immobilisation of enzyme molecules on semiconductor surfaces is a very mild method which leads to high enzyme activities but also to a rather low stability in the long term. To overcome the drawbacks of the methods mentioned above, we suggest two ways to immobilise enzyme molecules on the pH-sensitive surface of so-called electrolyte–insulator–semiconductor (EIS) structures. These field-effect structures are employed as a basic device for potentiometric biosensors.

In the first method, heterobifunctional cross-linkers which possess two different reactive groups are used to covalently bind enzyme molecules to planar surfaces. The immobilisation procedure is performed in two distinct steps: first, the cross-linker molecules react with certain surface groups of the EIS structure and after adding the enzyme, the remaining second reactive group is linked to the enzyme. Since no enzyme is present in the first step, a polymerisation of the enzyme molecules, which often occurs when homobifunctional cross-linkers are used, is prevented. Thus, the enzyme molecules are stably bound to the surface instead of being mainly cross-linked to each other.

In the second method, the enzyme molecules are deposited into the sponge-like structure of porous EIS sensors. For that purpose, porous silicon is prepared by an anodic etching process in concentrated hydrofluoric acid. Depending on the type of silicon and on the parameters during the etching process, various pore sizes and shapes can be achieved [6]. After coating the enlarged surface of the silicon with the pH-sensitive insulator materials, SiO_2 and Si_3N_4, the enzyme molecules are adsorptively bound to the pore walls. The 3D-structure allows an embedded immobilisation of a large amount of enzyme molecules and protects them against a fast leaching out.

Penicillinase is used as a model enzyme for both immobilisation methods. It converts penicillin in aqueous solutions to penicilloic acid which results in a pH change that can be detected by the EIS sensors [7–9]. The specific sensor characteristics resulting from both approaches are presented and discussed.

2. Experimental

2.1. Materials

The planar sensors were fabricated from p-type silicon wafers with (100)-orientation (18–24 Ω cm). For the porous sensors n-type silicon with (100)-orientation (12–23 Ω cm) was used. The enzyme penicillinase (EC 3.5.2.6.) from *Bacillus cereus* (Sigma) had a reported specific activity of 1650 units mg^{-1} protein. The heterobifunctional cross-linking reagent *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) was purchased from Pierce. The calibration of the penicillin sensors was carried out with penicillin G sodium salt (Sigma) dissolved in Polymix buffer [10]. All other chemicals used were of analytical-reagent grade.

2.2. Heterobifunctional cross-linking to planar EIS structures

To prepare the planar EIS structures, SiO_2 was thermally grown on top of the p-type silicon substrates with a thickness of 30 nm. Afterwards 70 nm Si_3N_4 was deposited by PECVD (Plasma Enhanced Chemical Vapour Deposition). As an ohmic contact 200 nm Al was evaporated on the rear side of the wafers. Finally, the wafers consisting of Al/p-Si/SiO_2/Si_3N_4 were cut into single chips of 10 mm × 10 mm [11]. Fig. 1a shows a schematic of the resulting pH-sensitive layer structure.

The immobilisation with the cross-linker ANB-NOS had to be carried out in the dark since the cross-linker molecule possesses a phenyl azide group that can be photoactivated by UV light. First, the Si_3N_4 surface of the sensor chip was incubated with 5 mM ANB-NOS in 200 mM triethanolamine buffer (TEA), pH 8, to let the succinimide ester group of the cross-linker react with the surface amine groups

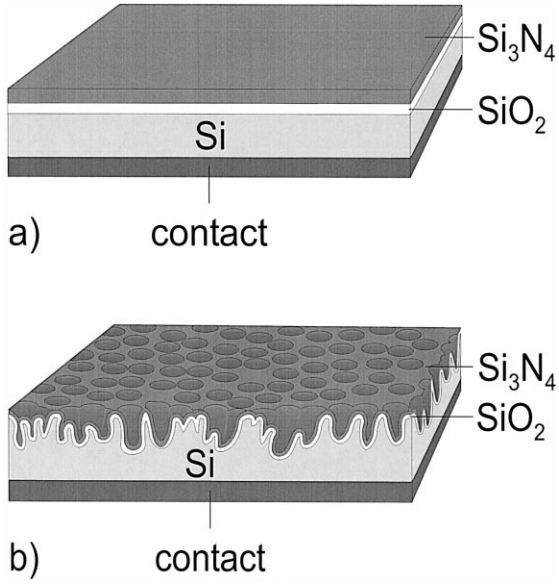

Fig. 1. Schematic of the planar (a) and the porous (b) EIS structures.

of Si_3N_4. Thereafter, the chip was rinsed with TEA buffer to remove surplus cross-linker molecules. Then, the enzyme solution consisting of 0.34 mg protein per ml TEA buffer, pH 8, was incubated with the sensor surface. The activation of the phenyl azide ring and thus, the binding of the enzyme to the cross-linker was achieved by UV radiation. Detailed immobilisation conditions are given elsewhere [9]. The dry sensors were stored at 4°C. Before use, they were incubated with 0.2 mM Polymix buffer, pH 8, for at least 12 h to let the enzyme membrane equilibrate.

2.3. Embedded immobilisation in the porous EIS structures

On the rear side of the n-type silicon wafers, a layer sequence of 5 nm Cr, 150 nm AuSb and 20 nm Au was deposited by means of vacuum evaporation. By anodic etching for 10 min in a 1:1 mixture of 50% HF solution and pure ethanol, a macroporous layer was formed [12]. As a dielectric and pH-sensitive transducer material 30 nm SiO_2 and 70 nm Si_3N_4 were deposited by PECVD technique onto the porous surface [13]. Finally, the samples were cut into single chips of 10 mm × 10 mm. The resulting porous EIS structure is schematically shown in Fig. 1b.

In order to immobilise the enzyme into the porous EIS structures, the enzyme solution was prepared by dissolving the protein in a 1:1 mixture of TEA buffer, pH 8, and pure ethanol to a concentration of 0.34 mg ml^{-1}. The solution was pipetted onto the samples and incubated at room temperature for 20 h. Then, the sensors were rinsed for 1 min in bi-distilled water and dried under N_2 atmosphere. The storage and equilibration of the porous EIS sensors followed as described for the planar sensors.

2.4. Measurements

To validate the immobilisation procedures, the respective penicillin sensor response of both EIS structures was examined. For that purpose, the prepared samples were mounted in a measuring cell sealed by an O-ring. The sensors were contacted on the front side by an electrolyte and a Ag/AgCl reference electrode and on the rear side by a gold-plated pin. To study the sensor characteristics, Capacitance–Voltage (C–V) and CONstant CAPacitance (CONCAP) measurements were performed with an AC voltage frequency of 120 Hz and a signal amplitude of 20 mV [9]. Since the maximum capacitance measured in the C–V mode increases proportionally with the surface area of the sensor, this value is suitable to estimate the surface ratio of the planar and porous samples. The CONCAP measuring mode allows a direct investigation of the dynamic behaviour of the sensor signal [14].

All measurements were performed in a dark Faraday cage at room temperature. Electrolyte solutions consisting of 0.2 mM Polymix buffer, pH 8, with 100 mM KCl as an ionic strength adjuster and penicillin G in the concentration range of 0.01 to 1 mM were used as calibration standards. When not in use, the sensors were stored in Polymix buffer at 4°C.

3. Results and discussion

3.1. Planar EIS structures

A typical CONCAP measurement of a planar penicillin sensor prepared by heterobifunctional cross-linking is shown in Fig. 2a. In order to correct the measurements for a possibly occurring drift of the sensor signal, the penicillin samples (indicated by the respective concentration values) and penicillin-free Polymix buffer were alternately measured. The shape of the measuring curve can be explained as follows: the more penicillin is present in the solution, the more molecules are converted to penicilloic acid and the more H^+-ions are released. As a consequence, the decrease of the pH value near the sensor surface and thus the decrease of the measured voltage is dependent on the penicillin concentration.

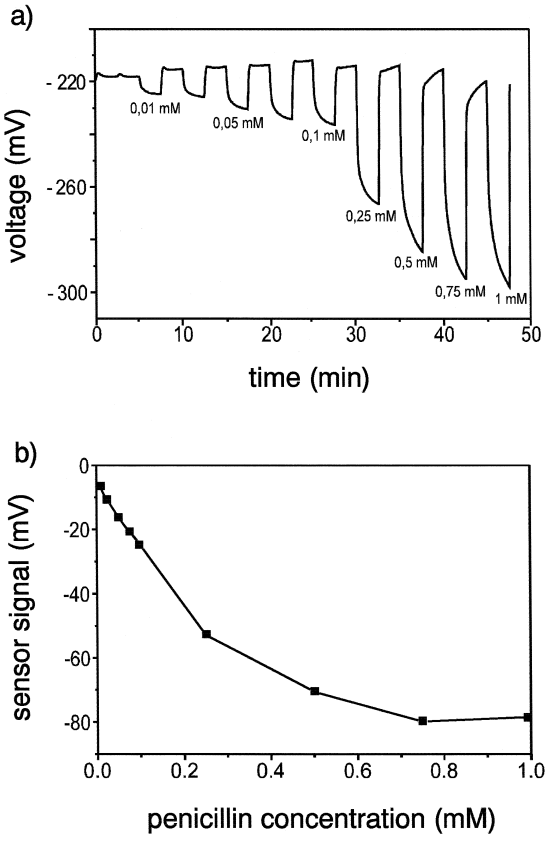

Fig. 2. Typical CONCAP measurement (a) and the corresponding calibration curve (b) of a penicillin sensor prepared by heterobifunctional cross-linking of enzyme molecules on a planar EIS structure.

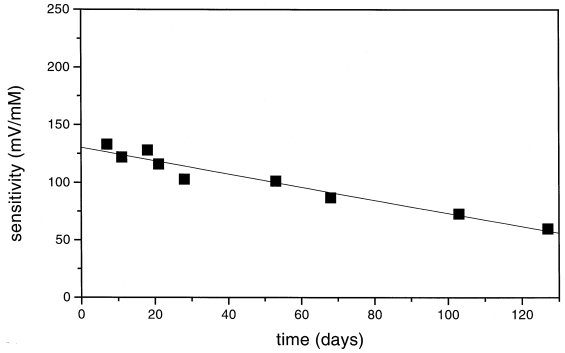

Fig. 3. Long-term behaviour of the planar penicillin sensors. The sensitivity in the linear region of the calibration curves (0.01–0.25 mM) is given vs. the operation time.

Each sample was measured for 150 s and afterwards, the buffer signals were subtracted from the following penicillin signals to obtain the net measuring values. These are given in the calibration curve (Fig. 2b). For low penicillin concentrations up to about 0.1 mM steady-state conditions are attained within the measuring time of 150 s. Here, the enzyme reaction is diffusion-limited, i.e., every penicillin molecule that reaches the enzyme layer by diffusion is catalytically converted. In contrast, in the range of higher concentrations the enzyme acts reaction-limited and as a consequence, the sensor signal is dependent on the amount of enzyme immobilised. The mean sensitivity of the sensors prepared by means of the cross-linking method was evaluated in the linear region of the calibration curves between 0.01 and 0.25 mM penicillin. It amounts to 125 ± 4 mV mM^{-1} for the first 20 days of operation. The mean maximum capacitance is around 28 nF which is close to the theoretical value of 27 nF expected for the realized layer structure and a surface area of 0.5 cm^2 given by the size of the O-ring. The long-term behaviour of the sensitivity is given in Fig. 3. During a measuring period of about 120 days, only a slight decrease of 0.6 mV mM^{-1} per day was observed.

3.2. Porous EIS structures

Fig. 4 depicts two scanning electron micrographs (SEM) of a porous silicon layer. The top view (a) shows the homogeneous distribution of the pores which have a mean diameter of about 1 μm. The pore depth was evaluated from cross-section SEMs (b). It amounts up to 2 μm. Cross-sectional transmission electron micrographs verified the complete covering of the pore walls with SiO_2 and Si_3N_4 [13].

The mean maximum capacitance of the porous sensors is 610 nF which is about 20 times higher than the value measured for the planar structures. Assuming that the thickness of the insulating layers of the two types of sensors is exactly the same, a 20-fold increase of the surface area is achieved for the porous sensors. An example of a CONCAP measurement with a porous penicillin sensor is given in Fig. 5a. The penicillin concentration is varied between 0.01 and 1 mM as in the case of the

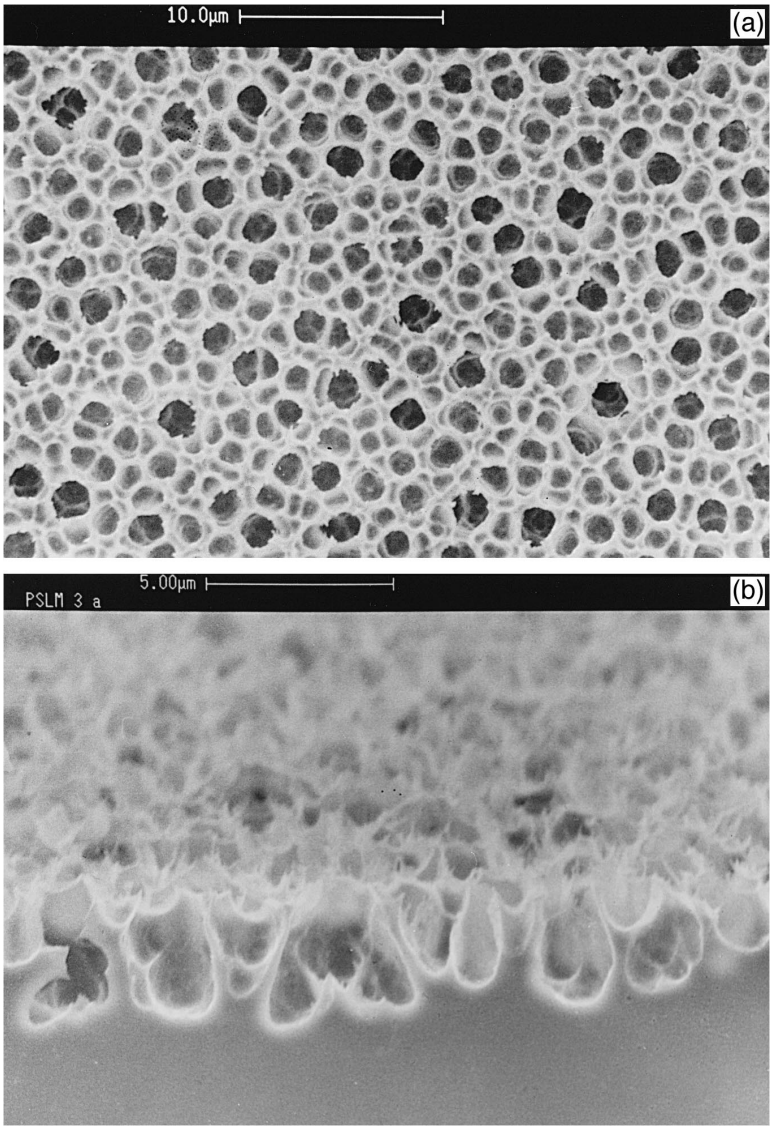

Fig. 4. Top view (a) and cross-section (b) scanning electron micrographs of a porous EIS sensor.

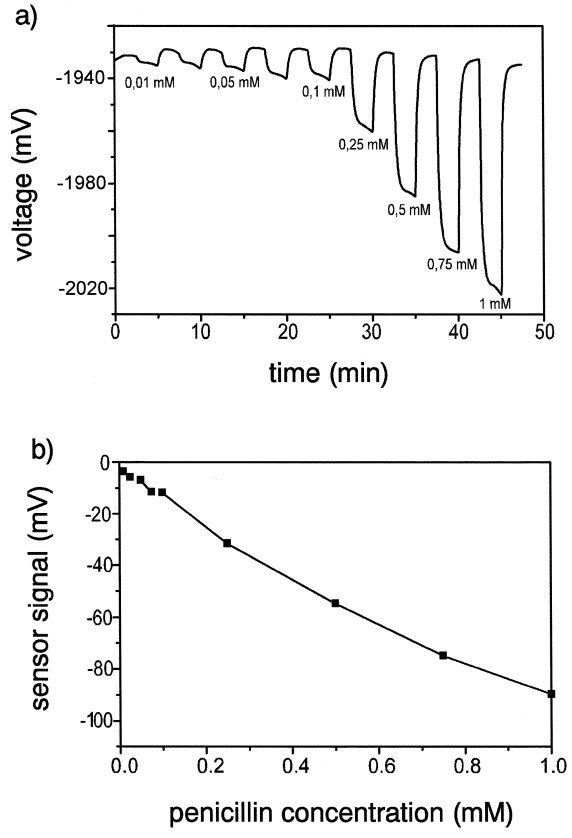

Fig. 5. Example of a CONCAP measurement (a) and the corresponding calibration curve (b) of a porous penicillin sensor.

planar EIS structures. Fig. 5b shows the corresponding calibration curve where the evaluated sensor signal is given vs. the penicillin concentration. The mean sensitivity amounts to 138 ± 10 mV mM^{-1} in the concentration range between 0.025 and 0.25 mM penicillin for the first 20 days of operation. As compared to the planar sensors (see example in Fig. 2b) the calibration curves of the porous structures exhibit a wider linear range from 0.01 up to 0.75 mM penicillin and thus, a stronger decrease for higher concentrations. The mean sensor signal for the highest penicillin concentration (1 mM) amounts to around 100 mV whereas 85 mV is obtained for the planar ones. Since the enzyme acts reaction-limited in this concentration range, the enlarged linear range of the calibration curve and thus the higher signals, indicate a larger amount of enzyme immobilised on the porous sensors as compared to the planar structures. The long-term stability of the EIS sensors prepared on the basis of porous silicon is still under investigation.

4. Conclusions

Two methods for the immobilisation of enzymes on silicon-based EIS structures are presented. The first one uses heterobifunctional cross-linker molecules for a covalent binding of enzyme molecules that is stable in the long term. It is suitable for planar surfaces of amine-group containing dielectrics like Si_3N_4. No activation of the surface such as a silanisation is needed. Due to the two-step character of the procedure, a cross-linking between the enzyme molecules is avoided. Further investigations have to prove the possibility of transferring the method on other enzymes than penicillinase.

In the second method, porous silicon-based EIS sensors are used to adsorptively bind the enzyme molecules in the sponge-like structure. The wider linear range of the calibration curve and the higher sensor signals of the porous sensors as compared to the planar sensors indicate a larger amount of immobilised enzyme. Since the size of the pores is dependent on the experimental conditions during the etching process, tailor-made structures, e.g., with smaller pores and an even larger surface, can be obtained. These could also be suitable for other applications like ‘bioreactors’ presented in literature [15]. Besides, a deposition of different enzymes in single pores or spatially separated porous areas resulting in a sensor array on the basis of porous silicon should be possible [13].

Acknowledgements

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