Penicillin detection with nanocrystalline-diamond field-effect sensor

Maryam H. Abouzar[1,2], Arshak Poghossian [*,1,2], Arash Razavi[1,2], Astrid Besmehn[5], Nathalie Bijnens[3], Oliver A. Williams[3,4], Ken Haenen[3,4], Patrick Wagner[3], Michael J. Schöning[1,2]

[1] Institute of Nano- and Biotechnologies (INB), Aachen University of Applied Sciences, Campus Jülich, Ginsterweg 1, D-52428 Jülich, Germany
[2] Institute of Bio- and Nanosystems (IBN-2), Research Centre Jülich GmbH, D-52425 Jülich, Germany
[3] Institute for Materials Research, Hasselt University, Wetenschapspark 1, 3590 Diepenbeek, Belgium
[4] Division IMOMEC, IMEC vzw, Wetenschapspark 1, 3590 Diepenbeek, Belgium
[5] Central Division of Analytical Chemistry (ZCH), Research Centre Jülich GmbH, D-52425 Jülich, Germany

PACS 73.40.-c, 73.63.Bd, 81.05.Uw, 81.07.Bc, 81.15.Gh, 87.14.ej

Corresponding author: a.poghossian@fz-juelich.de, Phone:+49-2461-612605, Fax:+49-241-6009-53235

Nanocrystalline-diamond (NCD) films have been utilised for the detection of penicillin G for the first time. The developed penicillin-sensitive biosensor consists of a field-effect capacitive electrolyte-diamond-insulator-semiconductor (EDIS) structure with an immobilised enzyme layer that covers the gate region of the sensor. Undoped NCD thin films of about 100 nm thickness were grown on a p-Si-SiO_2 (50 nm thermally grown SiO_2) structure by microwave plasma-enhanced chemical vapour deposition. The enzyme penicillinase has been adsorptively immobilised directly onto the O-terminated NCD surface. The EDIS biosensors have been characterised in buffer solutions with different content of penicillin G by means of capacitance-voltage and constant-capacitance method. The developed penicillin biosensor possesses a low detection limit of 5 µM and a high sensitivity of 60-70 mV/decade in a wide linear range of 0.005-2.5 mM penicillin G concentration.

1 Introduction

Artificially grown diamond has been recognised as a promising alternative transducer material for chemical and biological sensing [1-3]. Diamond is especially attractive because, in addition to having a wide bandgap and an excellent chemical as well as mechanical stability, it is widely considered to be biocompatible, can be deposited as a robust thin film onto Si or Si-SiO_2 substrate, and offers a new challenge in creating biosensors via coupling of biological recognition elements directly onto the diamond surface. The possibility of application of diamond for a direct electrical sensing of molecular binding events such as DNA hybridisation and antibody-antigen affinity reaction was demonstrated in [4, 5]. An enzyme-modified diamond-based field-effect transistor (FET) has been realised for the detection of urea and glucose [6]. The pH- and ion-sensitive properties of an electrolyte-gate FET with monocrystalline and polycrystalline diamond surfaces have been investigated in [7-15].

Most of diamond-based field-effect (bio-)chemical sensors have been realised on polycrystalline or monocrystalline diamond using a transistor structure. Recently, we have introduced a field-effect capacitive EDIS (electrolyte-diamond-insulator-semiconductor) structure as a platform for (bio-)chemical sensing [16]. In contrast to transistor structures, EDIS sensors are simple in layout, and easy and cost-effective in fabrication. Usually, no photolithographic process steps or complicated encapsulation procedures are required in case of the capacitive field-effect structure (a simple O-ring is enough for the electrical isolation of the sensor chip from the aqueous solution). In addition, alternating current (AC) measurements with the EDIS structure are often more informative than static direct current (DC) measurements with the transistor structure. A feasibility of this platform has exemplarily been demonstrated by realising a pH-sensitive EDIS sensor with nanocrystalline diamond (NCD) films [16]. In addition, the possibility of label-free electrical detection of charged macromolecules with the EDIS structure has been demonstrated using layer-by-layer adsorbed polyelectrolyte multilayers as model system [17].

In this work, a capacitive field-effect EDIS structure with an enzyme penicillinase-modified NCD film is investigated for the detection of penicillin for the first time. The determination of different kinds of penicillin is very impor-

tant in medicine, pharmaceutical production, environmental monitoring, process control in fermentation broths and food industry. At present, many Si-based enzyme biosensors have been developed for the detection of penicillin G, mainly for the analysis of fermentation broths (see, e.g. [18, 19] and literature there), where it requires the determination of relatively high concentrations of penicillin. However, for many fields of application (e.g., drug control-analysis of antibiotic tablets, capsules and injectables, food control) (bio-)chemical microsensors for the detection of small amounts of penicillin are needed [20, 21]. For instance, benzyl penicillin or penicillin G is frequently used as antibiotic in veterinary practice for prevention and treatment of bacteria infection [22]. This may lead to a not-desired presence of penicillin residues in food (milk, meat, etc.) obtained from medicated animal, and a small quantity of this compound might be responsible for allergic reactions in human beings.

2 Experimental

2.1 Functioning principle of a penicillin-sensitive EDIS biosensor

The structure and operation principle of the penicillin biosensor is shown in Fig. 1. The developed penicillin biosensor consists of a pH-sensitive field-effect p-Si-SiO_2-NCD structure with immobilised ß-lactamase (penicillinase).

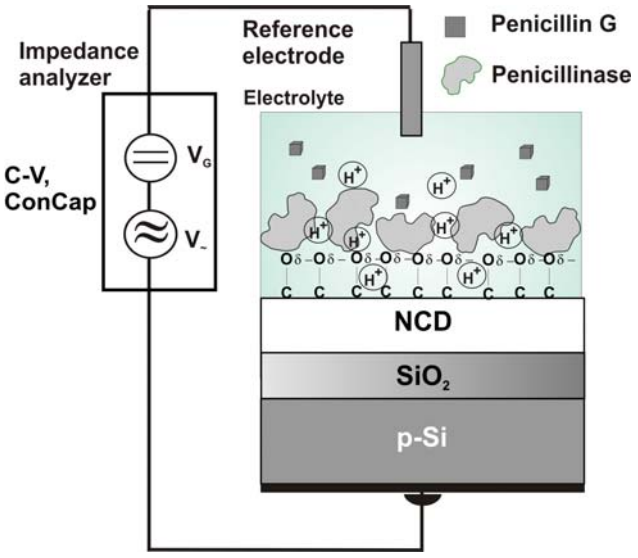

Figure 1 Structure and operation principle of the penicillin biosensor based on a field-effect EDIS structure with penicillinase-modified O-terminated nanocrystalline diamond.

The operation principle of the penicillin biosensor is as follows [18-21]: The pH-sensitive transducer material (here: O-terminated NCD) detects variations in the H^+-ion concentration resulting from the catalysed hydrolysis of penicillin by the enzyme penicillinase according to reaction (1):

$$\text{penicillin} + H_2O \xrightarrow{\text{penicillinase}} \text{penicilloicacid} + H^+ \quad (1)$$

A resulting local pH decrease near the surface of the O-terminated NCD layer leads to a change in the NCD surface charge and thus, modulates the space-charge capacitance in the Si and consequently, the flatband voltage and capacitance of the EDIS structure.

2.2 Preparation and physical characterisation of NCD films

Undoped NCD thin films of about 100 nm thickness were grown on a p-Si-SiO_2 (ρ=1-10 Ωcm, 50 nm thermally grown SiO_2) structure by microwave (2.45 GHz) plasma-enhanced chemical vapour deposition from a mixture of methane (CH_4) and hydrogen (H_2) in an ASTeX 6500 reactor. Prior to growth, the SiO_2 surface was seeded with a monodisperse colloid of nanocrystalline diamond particles in water with an ultrasonic bath. The gas flow rate, gas pressure, microwave power, substrate temperature and growth time were 485 sccm H_2, 15 sccm CH_4, 22 Torr, 3000 W, 490 °C, and 240 min, respectively. An Al film was deposited on the rear side of the Si chip as a contact layer. The chip size of the EDIS sensors has been 10 mm x 10 mm. For the process details of preparation of NCD films, see [23, 24].

The NCD films have been characterised by ellipsometry, scanning (SEM) and transmission (TEM) electron microscopy, and X-ray photoelectron spectroscopy (XPS) methods. The SEM and TEM micrographs in Fig. 2 demonstrate an example of the morphology of a 100 nm thick NCD film and cross-section of the layer structure, respectively. As can be seen, the film comprises randomly oriented fine grains and is totally closed. There are no pinholes visible in the film. The average grain size of the 100 nm thick NCD film, as determined by the SEM image, was around 100 nm.

Typically, as-prepared NCD surfaces are H-terminated. To obtain an O-terminated NCD surface with insulating properties, it was treated in an oxidising boiling mixture of H_2SO_4 and KNO_3 at 300 °C for about 45 min. In order to establish the relative coverage of the surface functional groups generated by the oxidation of NCD surfaces, XPS analysis was carried out. The results of XPS analysis have shown that the O1s/C1s atomic concentration ratio for the O-terminated NCD films is about 12 at. %, as calculated by dividing the relevant peak areas in the recorded spectra by appropriate bulk atomic sensitivity factors. These values are comparable to those that have been reported in [16, 25].

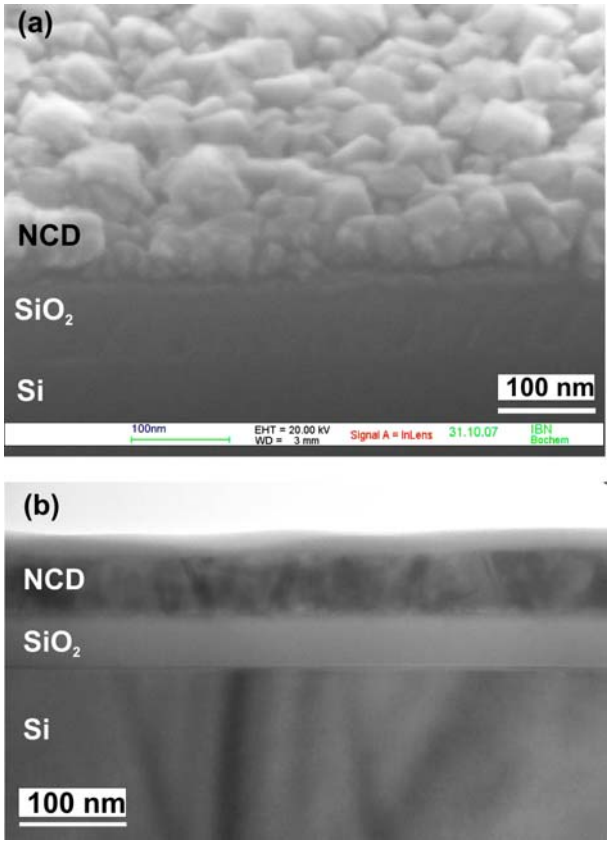

Figure 2 SEM (a) and TEM (b) picture of the surface morphology of a 100 nm thick NCD film and cross-section of layer structure, respectively.

2.3 Enzyme immobilisation

The characteristics of enzyme biosensors based on a pH-sensitive field-effect structure depend on the type of pH-sensitive transducer material used, on the pH and capacity of the buffer solution, on the thickness of the enzyme membrane and finally, on the enzyme immobilisation method. The most frequently used immobilisation techniques are enzyme entrapment and cross-linking methods (see, e.g., recent reviews [18,19] and literature there). In this study, we have examined the performance of the EDIS biosensor with adsorptively immobilised penicillinase (EC 3.5.2.6., *Bacillus cereus* from Sigma, specific activity: 1650 units/mg protein) directly onto the O-terminated NCD film. The main advantages of the adsorptive immobilisation technique are its simplicity and cheapness without any loss of the enzyme activity and the possibility of subsequent enzyme (sensor) regeneration, i.e. the possibility of a repeated enzyme immobilisation in the case of washing out of the loosely attached enzyme molecules from the sensor surface during storage, measurement and rinsing steps [18, 20, 21].

The enzyme solution was prepared by dissolving the enzyme penicillinase in a 200 mM TEA buffer, pH 8. 10 µl enzyme solution per sensor was pipetted onto the samples, incubated at room temperature for about 1 h and dried in N_2 atmosphere. Finally, the sensors were rinsed in Titrisol buffer pH 7. Before their first use, the penicillin sensors were incubated in working buffer for a least 1 h to let the enzyme membrane equilibrate. When not in use, the sensors were stored in Titrisol buffer, pH 7, at 4 °C.

2.4 Measurement set-up

The EDIS biosensors have been characterised in buffer solutions with different content of penicillin G by means of capacitance-voltage (*C*-*V*) and constant-capacitance (ConCap) method using an impedance analyzer (Zahner Elektrik) (see measurement set-up in Fig. 1). For operation, a DC polarisation voltage is applied via the reference electrode (conventional Ag/AgCl electrode, Metrohm) to set the working point of the EDIS sensor, and a small AC voltage (20 mV) is applied to the system in order to measure the capacitance of the sensor. All potential values are referred to the Ag/AgCl electrode.

For the measurements, the EDIS sensor was mounted into a home-made measuring cell, sealed by an O-ring and contacted on its front side by the electrolyte and a reference electrode, and on its rear side by a gold-plated pin. The side walls and backside contact of the EDIS sensor chip were protected from the electrolyte solution by means of an O-ring, thereby circumventing the need for a complex encapsulation processes. The contact area of the EDIS sensor with the solution was about 0.5 cm^2. The measurements have been performed in a dark Faraday cage at room temperature. For the measuring procedure, about 1 ml of the working buffer or particular penicillin solution was applied to the EDIS gate surface, and the sensor output signal was recorded for about 3-5 min. After each measurement, the EDIS gate region was rinsed with distilled water or buffer solution.

The penicillin solutions were prepared by dissolving penicillin G (benzyl penicillin, 1695 units/mg, Sigma) in the working buffer. As a working buffer, a 0.5 mM polymix multi-component buffer solution containing 100 mM KCl as an ionic strength adjuster or 0.5 mM phosphate buffer (pH 8), respectively, was used. The pH of the polymix buffer was adjusted to pH 8 by titration with NaOH solution.

3 Results and discussion

3.3 pH-sensitivity of O-terminated NCD films

Before characterisating the penicillin biosensor, the pH sensitivity of an O-terminated NCD film has been proven. Figure 3 exemplarily demonstrates a typical dynamic ConCap response of an EDIS structure with a 100 nm thick O-terminated NCD film recorded in Titrisol buffer with different pH values. The EDIS sensors show an average pH sensitivity of 40-45 mV/pH in the range of pH 4 to pH 10. A mechanism of the pH sensitivity of O-terminated NCD surfaces has been discussed in [9, 16] by using the well-known site-binding model (see, e.g. [18]). Similar to the oxide/electrolyte interface, the presence of hydroxyl

groups at the oxidised NCD surface can result in pH-dependent changes of the surface charge, thus, modulating the space-charge capacitance in the Si and the recorded EDIS sensor signal.

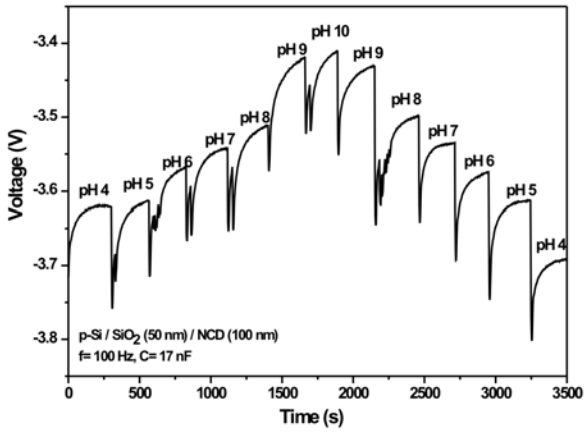

Figure 3 Typical ConCap response of an EDIS structure with O-terminated NCD layer recorded in Titrisol buffers of different pH values from pH 4 to pH 10.

3.4 Penicillin detection with O-terminated NCD film

Figure 4 shows typical *C-V* curves of a penicillinase-modified EDIS sensor (total number of tested sensors n=3) in a working buffer and in penicillin G solutions with concentrations of 0.1, 0.5 and 2.5 mM. With increasing the concentration of penicillin in the solution from 0.1 to 2.5 mM, the *C-V* curves are shifted in the direction of a more negative flat-band voltage V_{fb}, which results from the increase of the H^+-ion concentration at the sensor surface due to the enzymatic reaction. The flat-band voltage shifts ΔV_{fb} are proportional to the change of the H^+-ion concentration (ΔpH) at the O-terminated NCD surface. At a penicillin concentration of 0.5 mM, ΔV_{fb} was about 70 mV. Considering an average pH sensitivity for O-terminated NCD of 40 mV/pH (see Fig. 3), this voltage shift corresponds to a pH value at the NCD surface of 6.25 (by the pH value of the bulk buffer solution of pH 8), that is a ΔpH of 1.75.

A typical ConCap response and calibration curve of the freshly prepared penicillin biosensor measured in phosphate buffer solution with different penicillin concentrations is shown in Fig. 5. In contrast to the *C-V* method, the ConCap mode allows to monitor the dynamic characteristics of the EDIS biosensor. In this experiment, the capacitance of the EDIS sensor has been kept at a fixed value within the depletion region of the *C-V* curve using a feedback-control circuit, and the penicillin concentration-dependent potential changes at the electrolyte/NCD interface, which result from the increased H^+-ion concentration at the O-terminated NCD surface due to the enzymatic reaction were directly recorded for about 3-5 min. With increasing penicillin concentration from 0.025 to 10 mM, the concentration of the H^+ ions resulting from the penicillin hydrolysis is increased, too. As a result, the voltage that is necessary in order to adjust the constant capacitance raises. The recorded sensor output voltage correlates directly with the respective penicillin concentration in the solution.

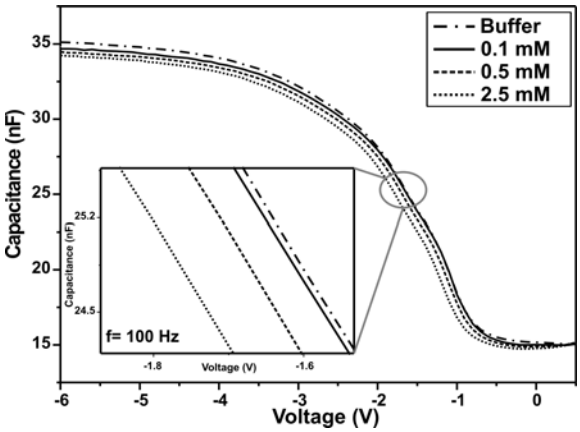

Figure 4 Typical *C-V* curves of a penicillinase-modified EDIS sensor in 0.5 mM polymix working buffer, 100 mM KCl, (pH8) and in penicillin G solutions with concentrations of 0.1, 0.5 and 2.5 mM.

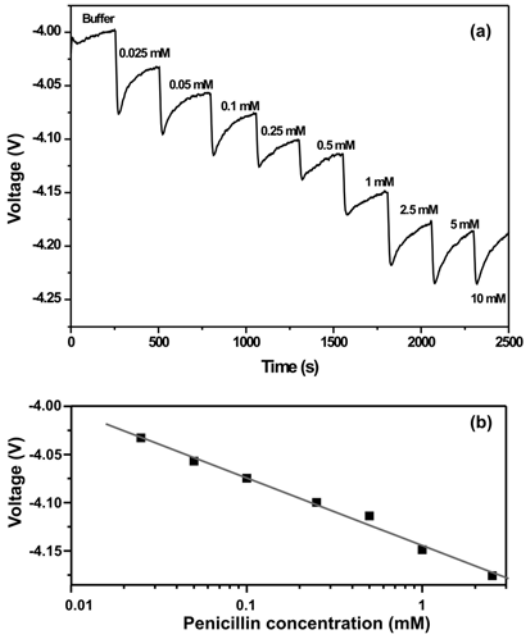

Figure 5 Typical ConCap response (a) and calibration curve (b) of the developed NCD-based penicillin biosensor (buffer: 0.5 mM phosphate buffer, 100 mM KCl, pH 8).

Even after three weeks, the same biosensor shows a clear dependence on the penicillin concentration (see Fig. 6). The developed NCD-based penicillin biosensor possesses a high sensitivity of 60-70 mV/decade in the linear

concentration range from 0.005 to 2.5 mM penicillin G. The upper detection limit is about 5 mM. The response time was between 0.5 and 5 min depending on the particular penicillin concentration.

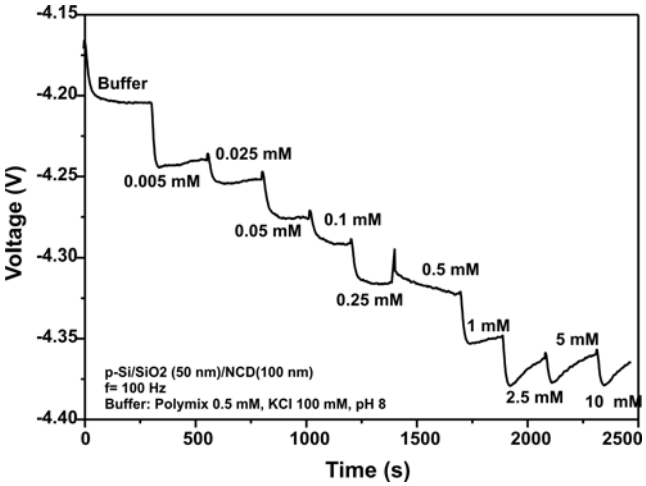

Figure 6 ConCap response of the NCD-based penicillin biosensor after three weeks (buffer: 0.5 mM polymix buffer, 100 mM KCl, pH 8).

Conclusions

A high-sensitive (70 mV/decade) and low detection limit (5 µM) penicillin biosensor has been realised using a capacitive field-effect EDIS structure with an enzyme-modified NCD film. The enzyme penicillinase was adsorptively immobilised directly onto the O-terminated NCD surface, which allows repeatable sensor regeneration. Future experiments will focus on an optimisation of the buffer solution as well as long-term stability investigations of the NCD-based penicillin biosensor.

Acknowledgements The authors thank H. Spelthahn, H.P. Bochem and S. Lenk for technical supports. Part of this work was supported by the Ministerium für Innovation, Wissenschaft, Forschung und Technologie des Landes NRW (Germany) and the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) via the IWT-SBO project # 030219 (Belgium).

References

[1] J. Rubio-Retama, J. Hernando, B. Lopez-Ruiz, A. Härtl, D. Steinmüller, M. Stutzmann, E. Lopez-Cabarcos and J.A. Garrido, Langmuir **22**, 5837 (2006).

[2] V. Vermeeren, N. Bijnens, S. Wenmackers, M. Daenen, K. Haenen, O.A. Williams, M. Ameloot, M. vandeVen, P. Wagner and M. Michiels, Langmuir **23**, 13193 (2007).

[3] R.J. Hamers, J.E. Butler, T. Lasseter, B.M. Nichols, J.N. Russell Jr., K.-Y. Tse and W. Yang, Diamond Relat. Mater. **14**, 661 (2005).

[4] K.S. Song, G.J. Zhang, Y. Nakamura, K. Furukawa, T. Hiraki, J.H. Yang, T. Funatsu, I. Ohdomari and H. Kawarada, Phys. Rev. E **74**, 041919 (2006).

[5] W. Yang and R.J. Hamers, Appl. Phys. Lett. **85**, 3626 (2004).

[6] K.-S. Song, M. Degawa, Y. Nakamura, H. Kanazawa, H. Umezawa and H. Kawarada, Jap. J. Appl. Phys. **43**, L814 (2004).

[7] H. Kawarada, Y. Araki, T. Sakai and H. Umezawa, Phys. Stat. Sol. (a) **185**, 79 (2001).

[8] H. Kanazawa, K.-S. Song, T. Sakai, Y. Nakamura, H. Umezawa, M. Tachiki and H. Kawarada, Diamond Relat. Mater. **12**, 618 (2003).

[9] K.-S. Song, Y. Nakamura, Y. Sasaki, M. Degawa, J.-H. Yang and H. Kawarada, Anal. Chim. Acta **3**, 573 (2006).

[10] K.-S. Song, T. Sakai, H. Kanazawa, Y. Araki, H. Umezawa, M. Tachiki and H. Kawarada, Biosens. Bioelectron. **19,** 137 (2003).

[11] B. Rezek, H. Watanabe, D. Shin, T. Yamamoto and C.E. Nebel, Diamond Relat. Mater. **15**, 673 (2006).

[12] B. Rezek, D. Shin, H. Watanabe and C.E. Nebel, Sens. Actuators B **122**, 596 (2007).

[13] J.A. Garrido, A. Härtl, S. Kuch, M. Stutzman, O.A. Williams and R.B. Jackmann, Appl. Phys. Lett. **86**, 073504 (2005).

[14] M. Dankerl, A. Reitinger, M. Stutzmann and A. Garrido, Phys. Stat. Sol. (RRL) **2**, 31 (2008).

[15] A. Denisenko, G. Jamornmarn, H. El-Hajj and E. Kohn, Diamond Relat. Mater. **16**, 905 (2007).

[16] P. Christiaens, M.H. Abouzar, A. Poghossian, T. Wagner, N. Bijnens, W. De Ceuninck, O.A. Williams, M. Daenen, K. Haenen, M.J. Schöning and P. Wagner, Phys. Stat. Sol. (a) **204**, 2925 (2007).

[17] A. Poghossian,, M.H. Abouzar, P. Christiaens, O.A. Williams, K, Haenen, P. Wagner and M.J. Schöning, in: Proceeding *8. Dresdner Sensor-Symposium*, Dresden, Germany, 2007, pp. 191-194.

[18] A. Poghossian and M.J. Schöning, Silicon-based chemical and biological field-effect sensors, Encyclopedia of Sensors (Eds. C.A. Grimes, E.C. Dickey, and M.V. Pishko), Vol. 9 (American Scientific Publishers, Stevenson Ranch, USA, 2006), pp. 463-733.

[19] M.J. Schöning and A. Poghossian, Electroanal. **18**, 1893 (2006).

[20] A. Poghossian, M.J. Schöning, P. Schroth, A. Simonis and H. Lüth, Sens. Actuators B **76**, 519 (2001).

[21] A. Poghossian, M. Thust, P. Schroth, A. Steffen, H. Lüth and M.J. Schöning, Sens. Materials **13**, 207 (2001).

[22] P. Thavarungkul, S. Davan, P. Kanatharana and P. Asawatreratanakul, Biosens. Bioelectron. **23**, 688 (2007).

[23] O.A. Williams, O. Douheret, M. Daenen, K. Haenen, E. Osawa and M. Takahashi, Chem. Phys. Lett. **445**, 255 (2007).

[24] M. Daenen, O.A. Williams, J. D'Haen, K. Haenen and M. Nesladek, Phys. Stat. Sol. (a) **203**, 3005 (2006).

[25] F.B. Liu, J.D. Wang, B. Liu, X.M. Li and D.R. Chen, Diamond Relat. Mater. **16**, 454 (2007).