Biosensors and Bioelectronics 24 (2009) 1276–1280

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Comparison of a potentiometric and a micromechanical triglyceride biosensor

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ARTICLE INFO

Article history:
Received 14 April 2008
Received in revised form 19 July 2008
Accepted 21 July 2008
Available online 5 August 2008

Keywords:
EISCAP
Biosensor
Microcantilever
Triglyceride

ABSTRACT

Sensitive biosensors for detection of triglyceride concentration are important. In this paper we report on two types of silicon based triglyceride sensors: an electrolyte–insulator–semiconductor capacitor (EISCAP) which is a potentiometric device and a polysilicon microcantilever. The detection principle for both sensors is based on the enzymatic hydrolysis of triglyceride though the sensing mechanisms are different: electronic for the EISCAP and mechanical for the microcantilever. The characteristics and performances of the two sensors are critically compared. The EISCAP sensor necessitates the presence of a buffer for stable measurements which limits the sensitivity of the sensor at low concentrations of the bioanalyte to 1 mM. The cantilever sensor works without a buffer which improves the lower level of sensitivity to 10 μm. Both sensors are found to give reproducible and reliable results.

1. Introduction

Monitoring of triglyceride concentration in the body is necessary in order to avoid many health risks. Established triglyceride estimation techniques such as the amperometric (Feldbrügge et al., 1994) and the chemical assays use bulky pH electrodes (Wang et al., 1999) or multiple enzymes for the analysis (Tkáč et al., 2000). A biosensor for sensing triglycerides based on electrolyte–insulator–semiconductor capacitor (EISCAP) has been reported earlier Basu et al. (2005). The reaction is mediated by the enzyme lipase which hydrolyzes tributyrin, a short chained Triglyceride, to produce Butyric acid causing a shift in the flat-band voltage of the EISCAP. The flat-band voltage varies as the pH of the electrolyte changes and this can be sensed by measuring the capacitance–voltage (*C–V*) characteristics of the EISCAP. Micromechanical cantilever beams have the potential to transduce a variety of chemical and physical phenomena into a mechanical movement on a micrometer scale (Raiteri et al., 2001). The products of tributyrin hydrolysis – butyric acid and glycerol – make the solution more viscous and dense. The cantilever beams vibrating in a denser fluid have a lower resonance frequency (Tamayo et al., 2001) and this property of the cantilever beam can be used to detect the triglyceride concentration. We have measured the resonance frequency of a cantilever beam immersed in the solution to monitor the reaction products. The reactions are carried out by the addition of optimized quantity of free enzymes to the triglyceride solution and can also be performed by immobilizing the enzyme on the sensor. For the covalent immobilization of the enzyme, the sensor surfaces were modified with 3-aminopropyltriethoxysilane (APTES), then the amine group of APTES was reacted with glutaraldehyde to yield an imine linkage with the primary amine group on proteins (Davis et al., 2002). The objective of this study is to compare the performance of the EISCAP and microcantilever sensors with respect to the sensitivity ranges and efficiency of these two sensors for the detection of triglycerides. The enzyme lipase has the highest activity at pH 7. However, using a buffer in the electrolyte is counterproductive since it would neutralize the change in pH due to the enzymatic hydrolysis, especially at low concentrations. We found the *C–V* measurements became unstable at low buffer concentrations and the lowest tributyrin concentration we could measure was 1 mM. The cantilever, on the other hand, functions well even without buffer which immediately allows measurements at much lower concentrations.

2. Materials and methods

Crystalline silicon, p-type, ⟨1 0 0⟩ oriented, wafers of resistivity 1–10 Ω cm were used. All the chemicals (APTES, toluene, acetone, hydrogen peroxide, ethanol, trichloroethylene, nitric acid) were purchased locally. Lipase (*Pseudomonas cepacia*) was bought from Amano, Japan.

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doi:10.1016/j.bios.2008.07.054

2.1. EISCAP sensor

EISCAP sensor device is similar to a MOS (metal oxide semiconductor) capacitor, except that metal is replaced by an electrolyte. EISCAP theory was first developed by Bergveld (1970, 1972) and Siu and Cobbold (1979). An EISCAP structure contains a stack of pH sensitive dielectric layers deposited on silicon. The electrochemical relation between flat-band shift of the CV curve and the pH of the electrolyte is given by the "Nernst response" (Schoning et al., 2005). SiO_2, Si_3N_4, Al_2O_3 and Ta_2O_5 are the commonly used dielectrics. Among these, Ta_2O_5 has the highest sensitivity of about 58 mV/pH (Schoning et al., 2005). However, SiO_2 and Si_3N_4 are interesting too, as they are compatible with CMOS technology (Reddy et al., 2001). SiO_2 suffers from low sensitivity (35 mV/pH) and hydration problems. This is the reason why Si_3N_4 (55 mV/pH) is commonly used as a pH sensitive layer. The fabrication, characterization and applications of an EISCAP cell was reported by Basu et al. (2005) where a stack of oxide followed by nitride was used as dielectric to exploit the superiority of the Si–SiO_2 interface on one hand and enhanced pH sensitivity on the other.

The silicon wafers, were cleaned using standard cleaning procedure and cut into small pieces and oxidized for 2 h at 1000 °C. Silicon nitride was deposited by plasma enhanced chemical vapor deposition technique (PECVD) on thermally oxidized silicon sample in order to obtain a good interface between the insulator layer and semiconductor since the interface state density is negligible at the Si–SiO_2 interface. PECVD nitride deposition was done for 10 min to obtain a thickness of 80 nm. The samples were annealed at 800 °C for 20 min in nitrogen ambient. The samples were cut into 2.5 cm × 2.5 cm size pieces and were loaded on to the custom made teflon cell which contained 1.6 ml of 5 mM tributyrin solution in phosphate buffer (1 mM, pH 7), to which 1 M KCl was added as an ionic strength adjuster. The contact was taken from a platinum-wire dipped in the electrolyte and the semiconductor contact was taken by providing an aluminium base at the bottom of the cell. *C–V* measurements were done with an HP 4274A LCR meter at 4 kHz with 15 mV signal amplitude by sweeping the voltage from −7 to 0.5 V in steps of 100 mV.

2.2. Microcantilever sensor

The oxide anchored polysilicon cantilever beams, of length 200 μm, width 20 μm and thickness 2 μm with 1.6 μm gap between the beam and the substrate were fabricated in house by surface micromachining (Bhat and Bhattacharya, 2007). A Doppler vibrometer, with suitable modifications, was used to detect the resonance frequency of the cantilever beams. It is a non-contact, non-destructive technique and ensures high measurement accuracy with reduced testing time. The whole system is integrated with a computer *via* a software program, written in LabVIEW so as to minimize any measurement errors. The cantilever beams were immersed in the tributyrin–lipase solution and were excited with a sine wave of 10 V_{p-p} coupled with a dc-offset voltage of 100 mV. The frequency of the signal was varied from 5 to 100 kHz. The frequency at which the amplitude of vibration was found to be maximum was taken as the resonance frequency of the beam. A cantilever beam immersed in water has a smaller resonance frequency since water, being denser than air, contributes an extra mass (Δm) to the cantilever mass. The resonance frequencies of the cantilever vibrating in air and water were measured as 76.4 and 48.3 kHz respectively.

2.3. Enzyme immobilization

The samples were silanized as reported earlier (Davis et al., 2002; Li et al., 2002). A solution of lipase (0.8 mg/ml) in phosphate buffer (10 mM, pH 7) was used for immobilization. The silanized and glutaraldehyde activated samples were immersed in 0.8 mg/ml lipase solution for 24 h (Fernandez et al., 2008). The samples were washed thoroughly with DI (deionized) water to remove any unreacted enzyme.

3. Results and discussion

3.1. EISCAP sensor

The silicon nitride deposited EISCAP was prepared as discussed in Section 2.1. In order to examine the potentiometric response of the sensor to tributyrin, millimolar solutions were prepared in phosphate buffer (1 mM, pH 7.0) and sonicated to get a homogenous stock solution from which different concentrations of tributyrin were derived. 1 mg of lipase was used per 10 ml of tributyrin solution in phosphate buffer and was incubated at room temperature for 20 min. The *C–V* characteristics were taken for various concentrations of hydrolyzed tributyrin solutions varying from 0.5 to 10 mM as shown in Fig. 1. To analyze the *C–V* characteristics U_{bias} was defined as the voltage applied across the device to get 60% of maximum capacitance and ΔU_{bias} is the difference in U_{bias} at a particular concentration from that of the buffer U_{bias} value. From Fig. 2 it can be noted that the sensor responded linearly in the 1–7 mM range but showed a smaller change at 0.5 mM. Below 0.5 mM concentration of tributyrin, the pH changes were not measurable and this could be because of the presence of the phosphate buffer which nullifies small changes in pH of the solution. The presence of the buffer is required for optimum functioning of the enzyme. A lower buffer concentration would improve the response of the sensor at lower tributyrin concentrations but the *C–V* measurements tend to be unstable at low buffer concentrations. Hence there is a trade-off between the sensitivity of the sensor and the stability of measurement.

3.2. Enzyme immobilized EISCAP sensor

Lipase was immobilized on the silicon nitride layer of the EISCAP as given in Section 2.3 and the amount of immobilized enzyme as quantified using the pNPP assay (Pimentel et al., 1994; Fernandez et al., 2008) was found to be 90 μg. The EISCAP sensor with covalently immobilized enzymes was loaded into the custom made teflon cell to which 5 mM tributyrin solution in phosphate buffer was added and the sensor response was tested by measuring the CV characteristics at different times. The tributyrin hydrolysis was mediated by the enzyme immobilized on the surface of the sensor thus changing the pH of the solution. Fig. 3 shows the ΔU_{bias} vs time graph of an enzyme immobilized EISCAP for 5 mM solution of tributyrin over a period of time. The measurements were taken at regular intervals until there was no more shift in the *C–V* characteristics. After a period of 45 min the *C–V* characteristics did not show much shift. The reduction in the shift in the *C–V* was due to the reduction in the hydrolysis reaction rate. The EISCAPs showed a shift of 29.9 mV in the *C–V* plots for a pH change from 6.97 to 5.54 in 45 min.

3.3. Triglyceride detection using microcantilever sensors

Tributyrin solutions of varying concentrations were prepared in deionized water to which 1 mg lipase (1 mg/10 ml of the solution) was added and the resulting solution was incubated for 20 min for the hydrolysis. The surface micromachined polysilicon cantilever beams were immersed in the hydrolyzed tributyrin solution. A molecule of tributyrin hydrolyzes to one molecule of glycerol and

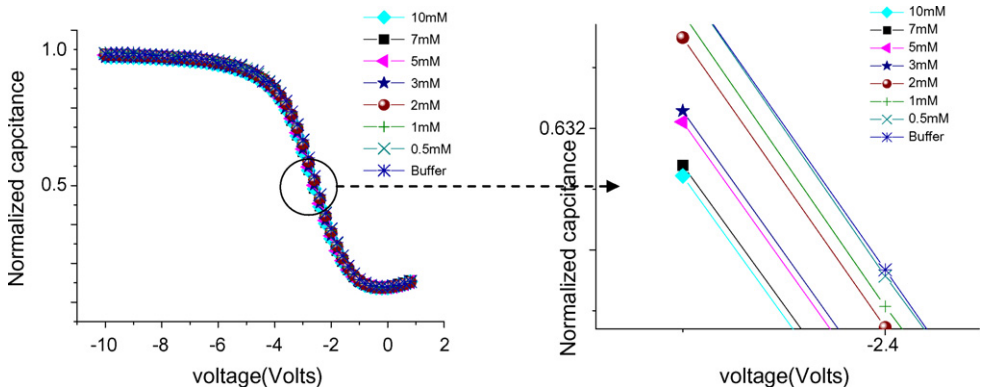

Fig. 1. Normalized *C–V* curves on EISCAP for various concentrations of tributyrin.

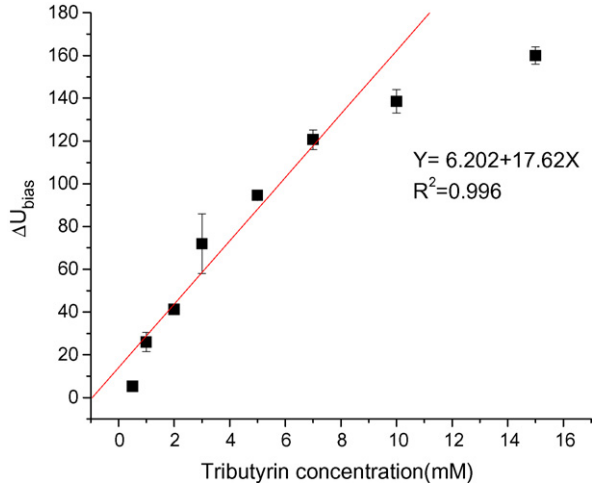

Fig. 2. ΔU_{bias} vs tributyrin concentration response of three different EISCAPs with hydrolyzed tributyrin concentrations (relative standard deviations included).

three molecules of butyric acid.

$$\underset{\text{(Tributyrin)}}{C_{15}H_{26}O_6} + \underset{\text{(Water)}}{3H_2O} \overset{\text{Lipase}}{\rightleftharpoons} \underset{\text{(Glycerol)}}{C_3H_8O_3} + \underset{\text{(Butyric acid)}}{3C_4H_8O_2}$$

The cantilever sensor was first calibrated using different concentrations of butyric acid + glycerol solutions, stoichiometrically equivalent to fully hydrolyzed tributyrin solutions. The cantilevers were vibrated in equivalent solutions of glycerol and butyric acid mixtures in which concentrations of glycerol varied from 24 to 480 μM and the butyric acid concentrations were thrice that of glycerol. It was noted that the response of the sensor was linear at lower concentrations and with increasing concentrations it showed saturation in its response. As in the case of the cantilever beam sensor, the values after 100 μM were not very much repeatable. The experiments were repeated using enzymatically hydrolyzed tributyrin solutions and the results were found to be similar to the calibrated readings. Measurements could not be carried out for concentrations above 100 μM as bubbles started forming which disturbed the laser beam spot. We can see that the points at 150 and 250 μM have a wide range of readings which was because of the interference of the bubble formation. The resonance frequency of the sensor was seen to reduce from ~48 to 28 kHz for 10–100 μM of tributyrin concentration. Fig. 4 compares the response of the cantilever with butyric acid + glycerol solutions and tributyrin hydrolyzed solutions.

3.4. Enzyme immobilization on a microcantilever sensor

Enzyme immobilization was done on cantilever beams as a prelude to a miniaturized and user friendly microcantilever based triglyceride sensor. Contrary to the method described in Section 2.3, prior to silanization the cantilever beams were treated with 5%

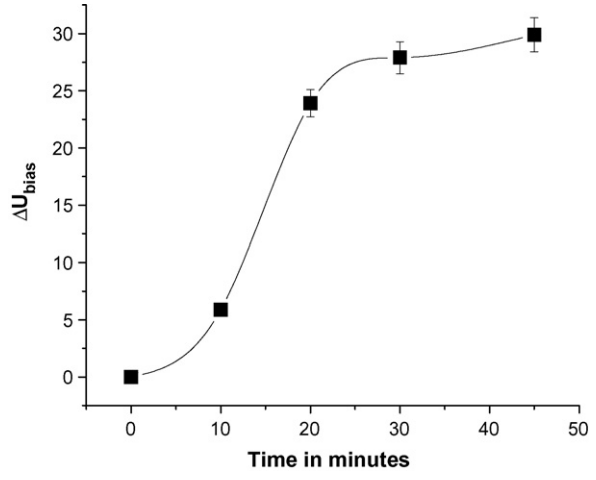

Fig. 3. ΔU_{bias} vs time response of an enzyme immobilized EISCAP for 5 mM solution of tributyrin as a function of time.

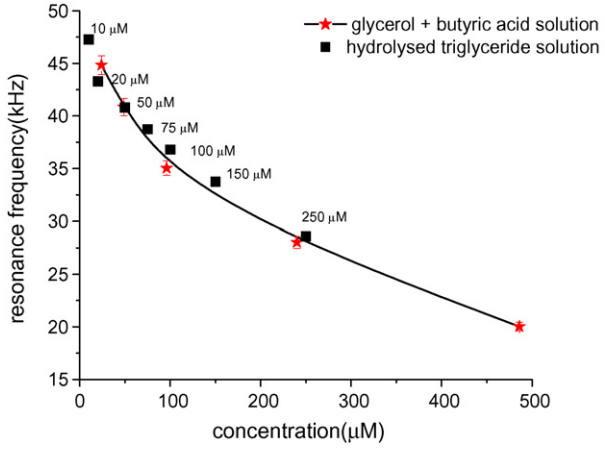

Fig. 4. Variation of resonance frequency with the concentration of enzymatically hydrolyzed tributyrin (■). Also given is the calibration plot (⋆) of frequency vs concentration generated with stoichiometric mixtures of butyric acid and glycerol as discussed in the text.

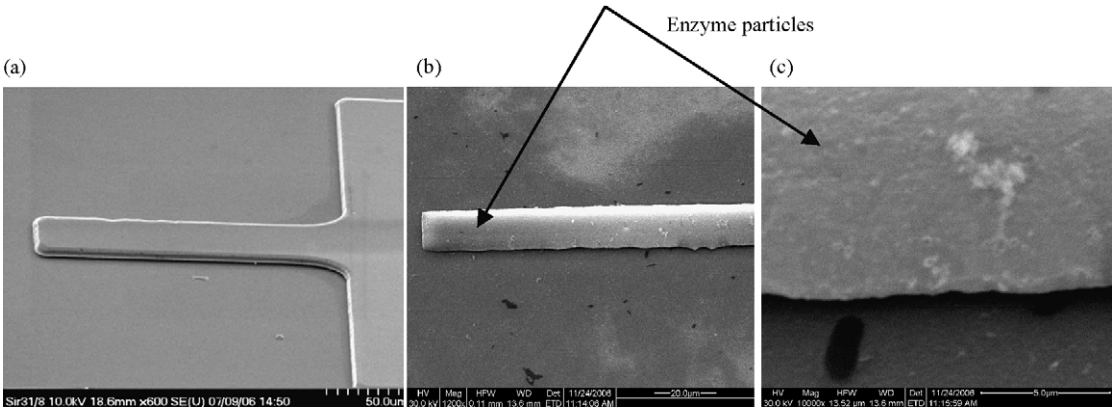

Fig. 5. Cantilever beam (a) before immobilization and with lipase immobilized: magnification (b) 1200× and (c) 10,000×.

nitric acid in water for 5 min at 80 °C as the longer nitric acid treatment may damage the polysilicon cantilever beams. The beams were dried in nitrogen ambient at room temperature and were tested for their resonance frequencies before and after immobilization. Fig. 5 shows the SEM pictures of sections of an enzyme immobilized cantilever beam. The cantilever beam prior to immobilization showed a resonance frequency of 77.8 kHz and it reduced to 67.3 kHz after enzyme immobilization. The change in resonance frequency of a beam can be correlated to the added mass on the beam due to the immobilized enzyme and this can be calculated using Eq. (1) (Yi et al., 2003)

$$\Delta m = \frac{k}{4\pi^2}\left(\frac{1}{f_0^2} - \frac{1}{f_m^2}\right) \quad (1)$$

where Δm is the change in mass (g), k is the spring constant of the beam (N/m) and f_0, f_m are the resonance frequencies (Hz) of the beam before and after immobilization. E is the Young's modulus of polysilicon which is taken as 160 GPa, t is thickness (2 μm), w is the width (20 μm) and L is the length (200 μm) of the microcantilever.

$$k = \frac{Et^3w}{4L^3} \quad (2)$$

Using Eq. (2), k was calculated as 8 N/m and the amount of immobilized enzyme was calculated by Eq. (1) as 11.2×10^{-9} g. A sample of wafer contains many cantilevers. Immobilization of a sample with enzyme would result in the covalent binding of lipase on the entire wafer including the cantilever under study. Hence it should be noted that the hydrolysis reaction was mediated by the enzymes immobilized on the entire wafer and not only on the cantilever under study. The tributyrin hydrolysis, mediated by the immobilized lipase, was monitored *in situ* continuously over a period of time. 100 μM tributyrin in DI water was used for the study. The production of the byproducts increases the viscosity and density of the solution in turn decreasing the resonance frequency of the cantilever vibrating in the solution. The resonance frequency of the cantilever was noted down over a period of time. The first reading of 46.2 kHz was taken within a minute of the addition of the triglyceride solution as seen in Fig. 6. The resonance frequency was found to be 44.4 kHz after 5 min and decreased further to 38.8 kHz in 30 min but did not show much change after that. The change in the resonance frequency of the cantilever beam is related to the kinetics of the tributyrin hydrolysis reaction. The initial rapid drop in the resonance frequency can be attributed to the high reaction rate and the slower change after 20 min is due to the completion of the hydrolysis reaction.

3.5. EISCAP vs cantilever beam sensor

Although the EISCAP and the cantilever beam sensor have different working principles, both can be pitted against each other to get an overall picture. The sensors were found to be reusable until they are chemically corroded or mechanically damaged. The sensors were cleaned in deionized water after use and stored in vacuum. The typical measurement times for the CV measurement (EISCAP) and resonance frequency determination (cantilevers) were about 15 and 20 s respectively. Hence the response times of the sensors were well within the measurement time limits.

Cantilever beam sensor was able to detect tributyrin concentrations as low as 10 μM while EISCAPs did not respond to concentrations lower than 500 μM. The cantilever sensor on the other hand needs complex fabricating steps while EISCAP sensor is comparably easier to fabricate.

The online monitoring of the tributyrin hydrolysis with enzyme immobilized sensors was essentially done to study the reaction kinetics. From Fig. 3, it can be seen that the reaction rate is highest for the first 20 min, after which it decreases. By comparing the ΔU_{bias} values, it was calculated that 79.9% of the reaction was complete in the first 20 min. The decrease in the reaction rate after that was due to the completion of the hydrolysis reaction. The prediction of the reaction rates were found to be true even when the reaction rate was monitored using an enzyme immobilized cantilever beam. Fig. 6 shows the decrease in resonance frequency as

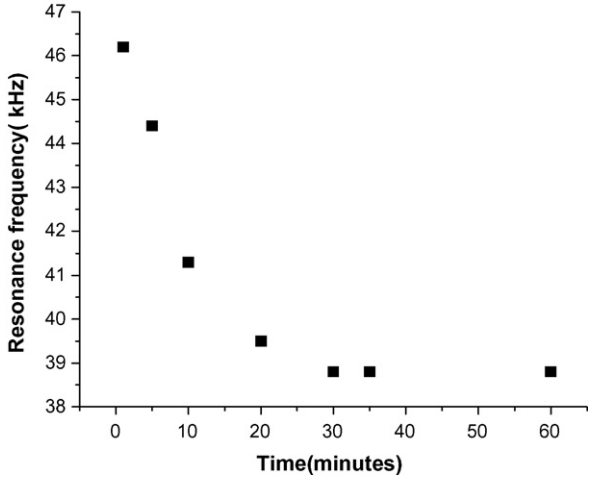

Fig. 6. Variation of resonance frequency with time of a lipase immobilized cantilever beam during hydrolysis.

the hydrolysis reaction proceeded. It was calculated that 83.04% of the reaction was completed in first 20 min. Hence online monitoring can be effectively used to study the reaction kinetics of any enzymatic hydrolysis.

4. Conclusion

The EISCAP and microcantilever were tested with various concentrations of hydrolyzed tributyrin solutions. It was found that the sensitivity of the EISCAP was limited to less than 500 μM whereas a microcantilever sensor was able to detect tributyrin levels as low as 10 μM. The micromolar detection capability of a cantilever based sensor makes it a suitable candidate for an intracellular triglyceride monitoring system. Although the microcantilever showed higher sensitivity, EISCAP characterization was easier to perform and the sensitivity of the EISCAP can be improved by having a better choice of the buffering solution. Performance of an EISCAP with immobilized enzyme on the surface was demonstrated using 5 mM tributyrin solutions. The enzyme immobilized cantilever beam was also used to monitor the kinetics of the hydrolysis reaction. It was found that the reaction kinetics predicted by the EISCAP and cantilever beam sensor were in agreement with each other.

References

Basu, I., Subramanian, R.V., Mathew, A., Kayastha, A.M., Chadha, A., Bhattacharya, E., 2005. Sens. Actuators B 107, 418–423.
Bergveld, P., 1970. IEEE Trans. Biomed. Eng. BME-17, 70–71.
Bergveld, P., 1972. IEEE Trans. Biomed. Eng. BME-19, 342–351.
Bhat, S., Bhattacharya, E., 2007. J. Micro/Nanolith. MEMS MOEMS 6, 043013–43017.
Davis, D.H., Giannoulisa, C.S., Johnson, R.W., Desai, T.A., 2002. Biomaterials 23, 4019–4027.
Feldbrügge, R., Renneberg, R., Spener, F., 1994. Sens. Actuators B 19, 365–367.
Fernandez, R.E., Bhattacharya, E., Chadha, A., 2008. Appl. Surf. Sci. 254, 4512–4519.
Li, H.L., Fu, A.P., Xu, D.S., Guo, G.L., Gui, L.L., Tang, Y.Q., 2002. Langmuir 18, 3198–3202.
Pimentel, M.C.B., Krieger, N., Coelho, L.C.C.B., Fontana, J.D., Melo, E.H.M., Ledingham, W.M., Lima-Filho, J.L., 1994. Appl. Biochem. Biotechnol. 49, 59–69.
Raiteri, R., Grattarola, M., Butt, H.J., 2001. Sens. Actuators B 79, 115–126.
Reddy, R.R.K., Bhattacharya, E., Chadha, A., 2001. Biosens. Bioelectron. 16 (4/5), 313–317.
Schoning, M.J., Brinkman, D., Rolka, D., Demuth, C., Poghossian, A., 2005. Sens. Actuators B 111, 423–429.
Siu, W.M., Cobbold, 1979. IEEE Trans. Electron. Dev. ED-26, 1805–1815.
Tamayo, J., Humphris, A.D.L., Malloy, A.M., Miles, M.J., 2001. Ultramicroscopy 86, 167–173.
Tkáč, J., Švitel, J., Novák, R., Šturdik, E., 2000. Anal. Lett. 33 (12), 2441–2452.
Wang, Y.S., Li, G.R., Lu, C.Y., 1999. Prog. Biochem. Biophys. 26 (2), 144–146.
Yi, J.W., Shih, W.Y., Mutharasan, R., Shih, W.H., 2003. J. Appl. Phys. 93, 619–625.