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Comparison of a potentiometric and a micromechanical triglyceride biosensor

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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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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