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Surface Modification for Microreactor Fabrication

Dorota G. Pijanowska [1,*], Elżbieta Remiszewska [1], Cecilia Pederzolli [2], Lorenzo Lunelli [2], Michele Vendano [2], Roberto Canteri [2], Konrad Dudziński [1], Jerzy Kruk [1] and Wladyslaw Torbicz [1]

[1] Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences, ul. Ks. Trojdena 4, 02-109 Warsaw, Poland

[2] Centro Per La Ricerca Scientifica E Tecnologia, ITCirst, Via Sommarive 18, Povo, 38050 Trento, Italy

[*] Author to whom correspondence should be addressed. E-mail: depijano@ibib.waw.pl

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Abstract: In this paper, methods of surface modification of different supports, i.e. glass and polymeric beads for enzyme immobilisation are described. The developed method of enzyme immobilisation is based on Schiff's base formation between the amino groups on the enzyme surface and the aldehyde groups on the chemically modified surface of the supports. The surface of silicon modified by APTS and GOPS with immobilised enzyme was characterised by atomic force microscopy (AFM), time-of-flight secondary ion mass spectroscopy (ToF-SIMS) and infrared spectroscopy (FTIR). The supports with immobilised enzyme (urease) were also tested in combination with microreactors fabricated in silicon and Perspex, operating in a flow-through system. For microreactors filled with urease immobilised on glass beads (Sigma) and on polymeric beads (PAN), a very high and stable signal (pH change) was obtained. The developed method of urease immobilisation can be stated to be very effective.

Keywords: Microreactors, surface modification, enzyme immobilisation, lab-on-a-chip.

Introduction

Application of silicon technology in fabrication of microfluidics systems offers an opportunity for development of micro total analytical systems (μTAS). The measuring procedure is shared between two blocks of the system: bioreactor for the sample preparation and biosensors – the detection unit.

These micro analytical systems are complex devices composed of functional units: sample transportation (microvalves, micropumps), sample preparation (microreactors, mixers, microdispensers) and detection of the analytes (chemical and biochemical sensors) parts. The design of these microsystems is multidisciplinary; it combines micromechanics, silicon technology and biotechnology. The micro analytical systems are designed for several applications such as biochemical analysis, medical diagnosis, food industry, pollution monitoring, etc. Thanks to microtechnology, the scale of reactors has been considerably lowered down resulting in a decreased consumption of chemicals and pollution production.

The main problems related to manufacture micro analytical systems and biosensors are related to the local immobilisation of bioreceptors (such as enzymes and antibodies, etc.) onto micro structured surfaces of the bioreactors and/or biosensors. The most welcome immobilisation methods should be simple and mild. In many cases, loss of the enzyme activity during the immobilisation procedure was caused by the aggressive reagents used for chemical treatment.

In the paper, the influence of different methods of chemical modification on effectiveness of bioreactors with bioreceptors immobilised on different supports such as silicon, glass and polymeric beads made with application of polyacrylamide modified with GOPS (EUPERGIT®) and polyacrylonitrile (PAN) is discussed.

For the purpose of enzyme immobilisation, the following agents: glycidoxypropyltriethoxysilane (GOPS), aminopropyltriethoxysilane (APTS) and glutaraldehyde (GA) were applied. Depending on the reactive group of the reagents used for surface modification, the immobilisation of the enzyme was obtained in the alkilation reaction between the amino group and the epoxy ring [1] and Schiff's base formation between the amino and aldehyde groups [2,3] for the two silanes: GOPS and APTS, respectively.

The surface of silicon modified with APTS and GOPS with immobilised enzyme was characterised by atomic force microscopy (AFM), time-of-flight secondary ion mass spectroscopy (ToF-SIMS) and infrared spectroscopy (IR).

Two types of microreactors, batch type filled with the enzyme immobilised on the glass and polymeric beads and lamella type with parallel microchannels etched in silicon, were designed.

Experimental

For the purpose of enzyme immobilisation, supports of different materials such as silicon, glass and polymeric beads, polyacrylamide modified with GOPS (EUPERGIT®, Degussa, Germany) and polyacrylonitrile (PAN), were tested. The surface of the support was modified with the following silanes: glycidoxypropyltriethoxysilane (GOPS), aminopropyltriethoxysilane (APTS) and glutaraldehyde (GA). Depending on the reactive group of the reagents used for surface modification, the immobilisation of the enzyme was obtained in the alkilation reaction between the amino group and

the epoxy ring [1] as well as Schiff's base formation between the amino and aldehyde groups [2,3] in the case of silanes: GOPS and APTS, respectively.

Two types of microreactors: the batch type, filled with enzyme immobilised on the glass and polymeric beads, and the lamella type, with parallel microchannels etched in silicon, were designed. As a model system the urea hydrolysis catalysed by urease, resulting in a pH change of the sample solution was used. The microreactors with immobilised urease were tested in a flow-through system, operating in the closed loop mode. The flow was driven with a peristaltic pump. The concentration of urea in the sample solution was changed by the standard addition method. The output signal (pH) was monitored by a pH microelectrode.

Materials

For batch type microreactors, the following supports for enzyme immobilisation: 200 μm diameter polyacrylamide beads modified with GOPS (EUPERGIT® C 250 L, Degussa, Germany) of 100 nm size pores and 80-120 μm diameter porous glass beads of 100 nm size pores (PG 1000-120, Sigma), were used.

Reagents used for the surface modification: glycidoxypropyltriethoxysilane (GOPS) as well as aminopropyltriethoxysilane (APTS) and glutaraldehyde (GA) were purchased from Fluka and Sigma, respectively.

Enzyme: urease from Jack bean (E.C. 3.5.1.5) type IX, activity of 500 kU/g was obtained from Sigma. For measurements as well as for enzyme immobilisation, 5 mM phosphate buffer containing 0.1 M NaCl, pH 6.03 was used.

Other chemicals, polyacrylonitrile fibres and solvents: toluene and ethyl alcohol were purchased from POCH, Poland. Before use, the solvents were freshly distilled. Then, the toluene was stored with sodium.

Microreactors

Three types of micro reactors, the first in Perspex and two in silicon, were realised. The first reactor was in the form of a 100 μl volume flow-through column made of Perspex with an inlet and an outlet protected by Nylon® net (Dupont).

Two types of microreactors were realised in silicon: the lamella and batch type. The lamella type microreactors were fabricated in the form of 31 parallel channels of the total length of 0.496 m. The 90 μm deep channels were anisotropically etched in a silicon wafer of <100> crystal orientation [1]. The third channel of the batch type was in the form of 220 μm deep cells (6 mm by 21 mm) divided into two compartments with a volume ratio 1:2 by a 130 μm high threshold (lock sill).

Then, the silicon wafers with etched structures of microreactors were anodically bonded to the Pyrex plate, 2 mm thick. Prior to the anodic bonding, in the glass plate, inlets and outlets for each microreactor were made. Finally, the silicon-glass sandwich was diced into chips (Fig. 1).

The silicon microreactors were fabricated at the Institute of Electron Technology, Warsaw.

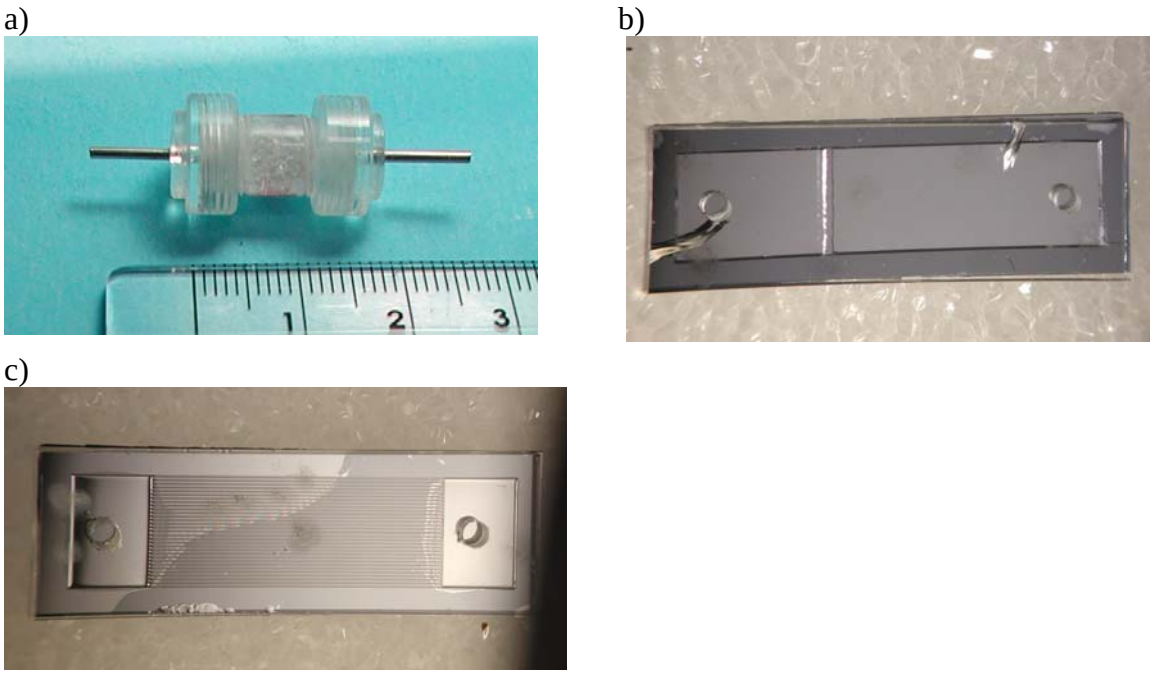

Figure 1. Microreactors: the batch type made in Perspex (a), the batch type made in silicon (b) and the lamella type made in silicon (c).

Silicon samples

For surface investigations, 1 cm^2 silicon samples with a 50 Å thick native silicon oxide layer were prepared. The samples were modified according to the procedure used for the silicon microreactors modification.

Polyacrylonitrile beads

Polymer beads were made from reduced polyacrylonitrile. Polyacrylonitrile fibres were broken up mechanically to form powder. The polymer was dried at 100 °C for 16 hours. Then, it was stored in a desiccator with a drying agent.

The reduction of polyacrylonitrile was performed in a 2 dm^3 glass reactor poured with 500 cm^3 of dry toluene containing 18.4 g of sodium. After the boiling point was reached, small portions of polyacrylonitrile and ethyl alcohol were added with a dropper filled with 76 cm^3 of anhydrous ethyl alcohol. The complete set-up consisting of a mechanical stirrer, a reflux condenser and a heater is shown in Fig. 2.

The toluene was kept boiling during the reaction. After all the portions of polyacrylonitrile and anhydrous ethyl alcohol were added, the reactor was poured with 300 cm^3 of 95% ethyl alcohol, in order to remove the sodium residue. Then, the reactor was cooled down and the reduced polyacrylonitrile was washed with 95% ethyl alcohol, which contained 3% of ether. The product was washed until ethyl alcohol became colourless. The reduced polyacrylonitrile was dried at 80 °C for 8 hours.

To form polymeric beads, the reduced polyacrylonitrile was dissolved in N-methylpyrrolidone of the concentration of 12%. Then, the solution was dropped into deionised water with an automatic

measuring pipette. The spherical shape was formed during the gelation of the polymer droplets in deionised water. In order to complete the gelation, the polymer beads remained in water for 24 hours. The deionised water was changed a period required after 12 hours. Then, the beads were dried at 80 °C for 8 hours.

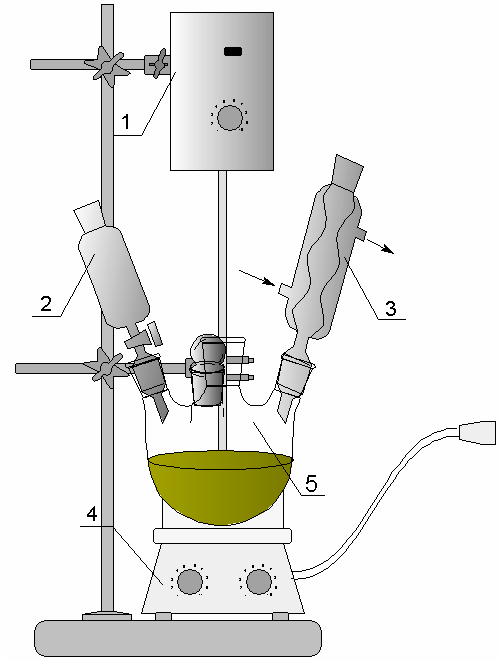

Figure 2. The scheme of the reaction set-up. 1 - mechanical stirrer; 2 - dropper with dry ethyl alcohol; 3 - reflux condenser; 4 - electric heater; 5 – reactor.

Surface modification

Modification of the glass beads and silicon samples

The deposition of the enzymatic layer is a multi-stage process, consisting of hydration, silanisation and enzyme immobilisation.

In order to increase the number of Si-OH surface groups, the substrates: glass beads, the silicon wafers and the lamella type microreactors, were hydrated by dipping in 3% hydrogen peroxide solution for 72 hours. After washing with deionised water, the substrates were silanised.

For the silanisation step, two types of silanes: glycidoxypropyltriethoxysilane (GOPS) and aminopropyltriethoxysilane (APTS), were used.

Silanisation with APTS was performed in 9% aqueous solution of APTS in a round-bottomed flanged reactor of 250 ml capacity, with five-necked cover, reflux condenser, thermometer and Teflon sleeves for ground joints (Bibby Quickfit, Great Britain). The temperature was kept constant at 40-42 °C for 4 hours. After cooling the mixture to about 30-35 °C, the supports were taken out from the reactor and washed four-times with deionised water. The fourth stage washing was done in an ultrasonic bath. The wafers were left for drying overnight. Prior to enzymatic modification, the silicon samples, lamella type silicon microreactors and glass beads modified with APTS were treated with

glutaraldehyde (10% aqueous solution) for 3 hours. Afterwards, the substrates were thoroughly washed with deionised water.

Silanisation with 20% GOPS in anhydrous toluene was performed using reflux at a temperature of 120 ^{o}C for 6 h. Afterwards, the substrates were carefully cleaned in the following sequence: (1) 3-step ultrasonic cleaning with 2-butanone for at least 10 min, (2) rinsing with ethyl alcohol and (3) rinsing with deionised water. In between consecutive washing steps the substrates were dried. The silanisation procedure resulted in a substrate surface functionalisation with epoxy rings.

Modification of PAN beads with GA

Prior to enzymatic modification, the polyacrylonitrile beads were treated with glutaraldehyde (10% aqueous solution) for 3 hours. Afterwards, the beads were thoroughly washed with deionised water.

Deposition of the enzymatic layer

In the last stage, the enzymatic layer was deposited onto the chemically modified supports. In all the cases, apart from the EUPERGIT beads, for enzyme deposition an enzyme solution containing 25 mg of the urease in 1 ml of phosphate buffer was used. To obtain a covalent bound of the enzyme to the substrate, the supports were exposed to the enzyme solution at room temperature overnight.

The excess of the non-attached molecules of the enzyme was removed by washing with the phosphate buffer solution.

The EUPERGIT beads were enzymatically modified according to the manufacturer's recommendations [4]. The enzyme solution was prepared as follows: 25 mg of the enzyme was dissolved in 2 ml of MQ water and then, 4 ml of 1 M potassium phosphate buffer pH 7.5 was added. The enzyme solution and 1 g of the EUPERGIT beads were mixed. The immobilisation was performed at room temperature for 24 hours. Then, the beads were placed on a glass frit and washed using deionised water. The liquid was sucked off and the washing was repeated several times using a copious amount of deionised water.

Measurements

Characterisation of the silicon samples

The surface of silicon modified by APTS and GOPS with immobilised enzyme was characterised by atomic force microscopy (AFM), time-of-flight secondary ion mass spectroscopy (ToF-SIMS) and infrared spectroscopy (FTIR).

Characterisation of microreactors

The batch type microreactors were packed with glass and polymeric beads, with urease immobilised on the surface. Prior to the measurements, the reactors were washed with phosphate buffer at a higher flow rate of 10 ml/min for 30 min. Due to the diameters of the PAN beads, they were packed into a Perspex type microreactor, while the glass and EUPERGIT beads were loaded into silicon onces.

As a model system, the hydrolysis of urea catalysed by urease was used. At regular 10-minute time intervals, the concentration of urea in the sample solution was changed by the standard addition method. The microreactors were tested in a flow-through system operating in the closed-loop mode so that the sample should be recirculated in the system and the changes of pH resulting from the enzymatic reaction were recorded by a miniaturised pH sensor. The flow was driven by a peristaltic pump with a flow rate of 0.2 ml/min.

Results and discussion

Silicon samples

Several techniques were used to investigate the chemically modified surface of the silicon samples. Although the samples had different chemical properties in comparison with the unmodified surfaces, the surface of the silicon samples after hydration and the silanisation process were found to look almost identical to the chemically unmodified samples, when observed under AFM (Fig. 3). A distinction between the surface of the two samples modified with APTS and GA and non-modified - that was found with AFM - is limited by a small difference in the sample roughness, i.e. 8 nm and 3 nm for these samples, respectively. However, the differences became appreciable for the samples with the deposited enzymatic layer.

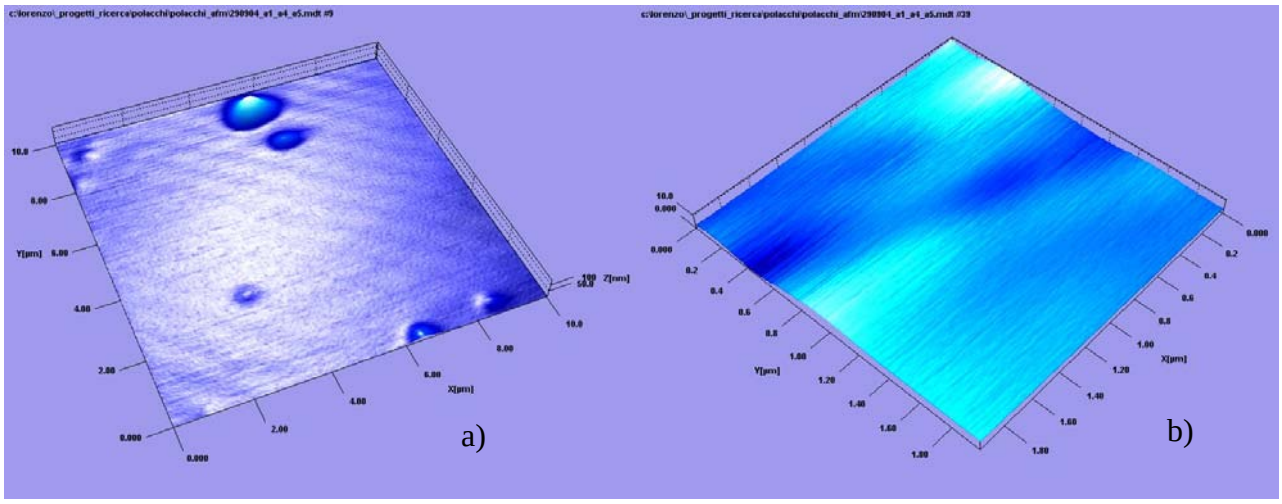

Figure 3. 3D AFM-images for the surface of non-modified silicon samples (a) and modified with APTS and GA (b).

Typical 3D AFM images for the silicon surface with immobilised enzyme are shown in Fig. 4. As it can be seen, the surface of the silicon is tightly packed with large molecules. The ToF-SIMS and IR spectroscopy investigations confirmed the presence of the enzyme on the silicon surface.

Based on the ToF-SIMS results, it was found out that a greater amount of protein (enzyme) was deposited on the surface of the samples modified with APTS than on the surface modified with GOPS.

The difference between the IR spectra for the samples with the enzymatic layer and non-modified samples is shown by the peaks for the wave numbers 1649 cm^{-1} and 1538.8 cm^{-1}. This proves the presence of the peptide coupling (Amide I and Amide II), which is specific for proteins.

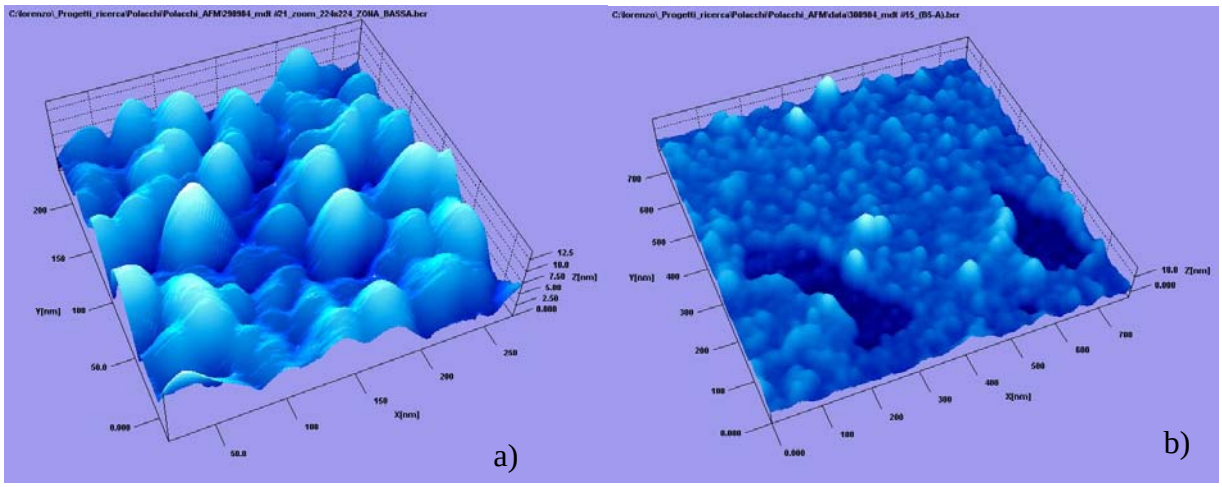

Figure 4. 3D AFM-images for the surface of silicon samples with immobilised enzyme, modified with APTS (a) and GOPS (b).

Microreactors

The output signal (pH) was monitored by a pH microelectrode. To compare effectiveness of different methods of urease immobilisation, the maximal pH difference (ΔpH_{max}) between the initial pH of the sample solution (pH_{ini}) and that observed in the final concentration of urea, i.e. for C_{urea} = 0.385 M (pH_{fin}), for measurements taken in consecutive days, was used.

The comparison of the changes of the output signal in time for the lamella type silicon microreactors modified with APTS and GOPS microreactors is presented in Fig. 5.

For the two lamella type microreactors modified with APTS and GOPS, the output signal decreased very fast during the first days of their operation. After the initial time, the signal became more stable; however, it dropped to a very low level, namely a 0.3 and 0.2 pH unit for the microreactors modified with GOPS and APTS, respectively.

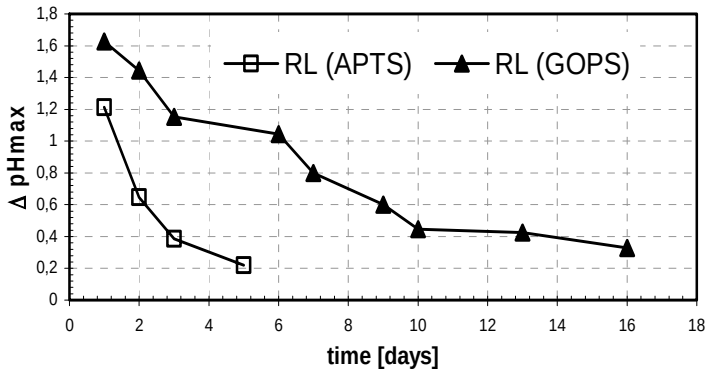

Figure 5. Changes of the output signal (pH_{max}) in time for two lamella type silicon microreactors modified with GOPS and APTS.

The comparison of changes of the output signal in time for the batch type microreactors filled with EUPERGIT, PAN and glass-APTS modified beads is shown in Fig. 6.

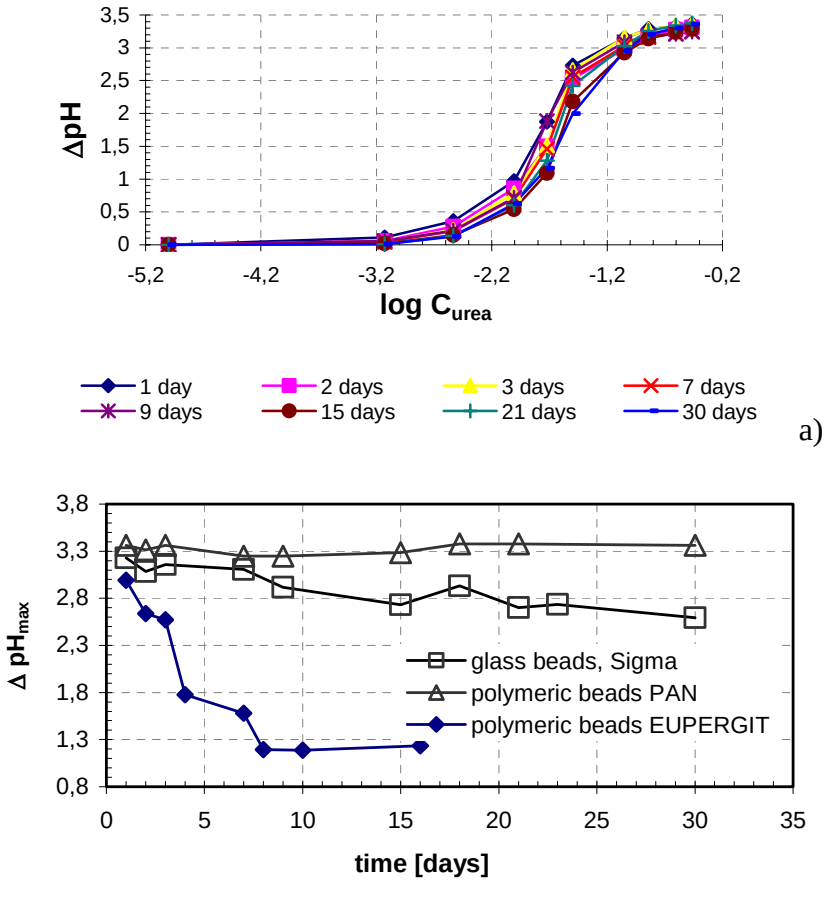

Figure 6. Time dependence of the calibration curves for the batch type microreactor filled with urease immobilised onto PAN-beads (a) and changes of the output signal (pH_{max}) in time for three micro-reactors with immobilised urease: the batch type microreactors filled with EUPERGIT, PAN and glass-APTS-modified beads.

The output signals obtained for the batch type microreactors decreased in time, in particular, during the first days of their operation. This can be explained by the slow washing out of the non-attached molecules from the microreactors. After the initial time, the signals become stable at a certain level, namely 1.2, 2.5 and 3.3 pH units for the microreactors based on the EUPERGIT, the glass beads modified with APTS, the PAN beads and the lamella type microreactor modified with GOPS, respectively. The most stable and the highest output signal was obtained in the batch type microcreactors filled with urease immobilised onto the PAN beads.

Conclusions

A distinction between the surface of the two silicon samples modified with APTS and GA and non-modified, found with AFM, consists in a small difference in the sample roughness, i.e.: 8 nm and 3 nm of these samples, respectively.

The difference between the IR spectrum in sample with the enzymatic layer and the IR spectrum of the non-modified sample is shown by peaks for the wave numbers 1649 cm^{-1} and 1538.8 cm^{-1}. This proves the presence of the peptide coupling (Amide I and Amide II), which is specific for proteins.

For the microreactors, the output signal decreased in time, in particular, during the first days of their operation. This can be explained by the slow washing out of the non-attached molecules from the microreactors. After the initial time, the signal become stable at a certain level, namely 1.2, 2.5 and 3.3 pH unit for microreactors based on the EUPERGIT, the glass beads modified with APTS, and the PAN beads, respectively. For the lamella type, microreactors modified with GOPS and APTS, the output signal stabilises at a very low level, below 0.3 pH unit.

The most stable and the highest output signal was obtained for the batch type microcreactors filled with urease immobilised onto the PAN beads.

Acknowledgements

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