CHEMICAL MODIFICATION OF POROUS SILICON MIRROR FOR BIOSENSING APPLICATIONS

G. Palestino Escobedo*[a], R. Legros[a] , B. de la Mora Mojica[b], J.A. del Río Portilla[b], J.E. Pérez López[C], C. Gergely[a]
[a]GES-UMR 5650, CNRS, Université Montpellier II, Montpellier Cedex 5, France
[b]CIE, Universidad Nacional Autónoma de México AP 34, Temixco, Mor. CP 62580, México
[C]Instituto de Física, Universidad Autónoma de San Luis Potosí, SLP, CP 78000, México

ABSTRACT

Porous silicon (PSi) nanostructures have remarkable optical properties that can be used for biosensing applications. In this paper we report first on the fabrication of heavily doped p-type PSi with pore diameters in the range of 400-4000 nm. The nonspecific and specific binding of the Glucose Oxidase protein (GOX) was then studied onto the PSi mirror-like substrate. Adsorption of GOX was tuned by the pH of the protein solution (pI = 4.2) depending of the surface charge. PSi matrixes were first stabilized by thermal oxidation and GOX adsorption was performed once directly on the oxidized PSi surface, and also on previously functionalized PSi surfaces. In the latter case the GOX was coupled to the PSi via the S-H group of the 3-(mercaptopropyl)trimethoxysilane (MPTS). The silane-GOX and GOX interactions on the PSi surface were monitored by the Fourier Transformed Infrared spectra that display characteristic bands of the linked molecules. The interference spectrum shows a large blue shift in the Fabry- Perot interference pattern caused by the change in the refractive index of the medium implying a decrease in the effective optical thickness. Quantitative analysis shows that chemically modified PSi samples admit approximately 24% of GOX. Activity assay proved that the protein preserves its catalyst properties under these adsorption conditions.

Keywords: functionalization, glucose oxidase, protein crystallization, Bragg mirrors

1. INTRODUCTION

There is a great need for the development of easy-to-use, sensitive, robust and inexpensive biosensors for the detection and identification of biological substances. A good example is the class of novel biosensors based on protein-functionalized semiconductor devices, where the protein provides the desired biological answer. Conventional sensors are mainly based on electrochemical detection of enzymatic conversion products of the protein. These sensors, designed for implantation, often showed a severe loss of sensitivity due to surface fouling and enzyme inactivation caused by proteins and immune factors.[1] A modified approach is to use external sensors. Therefore, various label-free biosensors that do not use the traditional methods have been developed to provide quick and simple biomolecular detection by converting the molecular recognition events into easily detectable optical signal.[2] Synthesis of materials using nanostructured templates has emerged as a useful and versatile technique to generate ordered nanostructures. Templates consisting of micropores membranes, zeolites and crystalline colloidal arrays have been used to construct and elaborate electronic, mechanical, or optical structures.[3] The most important advantages of PSi structures are that its sensing parameters can effectively work at room temperature. On the other side, it supplies a handy template to hold chemical and biological species. Target species like biomolecules, enzymes, DNA fragments and antibodies[4] can be immobilized in the porous silicon matrix. Techniques for immobilization range from physical adsorption to replacement of hydride bonds with Si alkyls and to antibody bonding at functionalized porous silicon surface with subsequent antibody-antigen interactions. Species identification can be performed by probing optical, electrical or chemical properties.

* gabriela.palestino@ges.univ-montp2.fr; phone 33 4 67 14 37 11; fax 33 4 67 14 37 60

Bioengineered and Bioinspired Systems III, edited by Paolo Arena, Ángel Rodríguez-Vázquez, Gustavo Liñán-Cembrano,
Proc. of SPIE Vol. 6592, 65920E, (2007) · 0277-786X/07/$18 · doi: 10.1117/12.721912

Porous silicon is an attractive material for use as a template also because its porosity and average pore size can be easily tuned by adjusting the electrochemical preparation conditions, thus allowing the construction of photonic crystals [5], dielectric mirrors [6], microcavities [7] and other optical structures.

1.1 Porous silicon (PSi) state of the art

Devices based on bulk silicon semiconductors have been available for *in vitro* biosensing applications for several years. However, this form of silicon is not biocompatible and so far this has prevented its use *in vivo*. Bulk silicon-based integrated circuits need 'packaging' in a biocompatible material if they are to be used and linked to living tissues. Contrary, nanostructured PSi has properties that make it a very promising biomaterial, in particular for sensing devices that need to be linked to the biological system.

Possible potential applications for PSi concern mainly three areas: i) in vitro biosensors, ii) the development of intelligent implantable medical devices and iii) biologically interfaced networks [7]. Biosensors that use silicon-based devices are currently available for *in vitro* applications. In vitro biosensors could be classified into two types: those that quantitatively detect single compounds in complex biological matrices using immobilized receptors or enzymes [8,9] and those that sense the viability of intact living cells.[10] The former type of sensor could be improved through the use of PSi if the increased surface area of this material over conventional silicon substrates can be harnessed. This would result in an increase in sensitivity through the immobilization of a greater concentration of receptor/enzyme per surface area. If silicon's lack of biocompatibility has prevented its use *in vivo* and the majority of *in vivo* applications rely on the ability of PSi to directly interface with living cells [11]. The absorption of human serum albumin (HSA) and fibrinogen has been measured onto porous silicon. Hydration of the porous surface significantly decreases the adsorption of HSA but increases its quantity deeper in the porous film. Hydration does not affect the adsorption of fibrinogen, a protein essential in blood clotting processes. Another important test is the *in vitro* deposition of hydroxyapatite onto the surface of a biomaterial from a simulated body fluid [12]. This test has become a standard indicator of potential bioactivity of materials for bone implantation.

Nanocrystalline PSi films have been used both like biosensor and as chromatography matrices. Films with pore sizes in the range of a few nanometers are used as a size-exclusion matrices to perform an on-ship determination of macromolecule dimensions.[13] For that porpose porous silicon films are prepared with a controlled distribution of pore sizes in a way that allow the simultaneous separation and detection of a protein. The ability to controllably trap and release proteins may be useful for drug delivery applications, as PSi has been shown to be biocompatible and readily bioresorbable. Otherwise, photonic crystals can also be generated from PSi structures removing the PSi film from the substrate using a current pulse and mechanically agitate or ultrasonicate to create particles. The multilayered photonic crystals generated in this fashion display a very sharp line in the optical reflectivity spectrum by tuning the preparation conditions, this line can appear anywhere in the visible to near-infrared spectral range. The most important feature of these PSi particles are the fluorescence spectrum that can be much narrower that the fluorescence spectrum from a molecule or core-shell quantum dot. [14] The use of encoded silicon nanostructures in medical diagnostic applications has advantages over organic dyes or quantum dots. *In vivo* studies have shown the biocompatibility of PSi, as well as the long-term stability of reflectance data from multilayer structures.[15]

2. MATERIALS AND METHODS

2.1. Porous silicon based Bragg mirrors

Bragg's mirrors are periodic stacks (Fig. 1) of two quarter-wavelength optical thickness layers of different refractive indices. The periodicity gives a photonic band, in which light propagation is forbidden and incident light is reflected. [16] Dielectric mirrors can reflect light for any direction of propagation in specific wavelength region. This specific structure can be used to confine, manipulate, and guide photons allowing the creation of optical integrated circuits from PSi. Dielectric mirror fabricated with Porous silicon (PSi) may be also used to enhance the sensibility to detect molecules and

biomolecules trapped in the porous structure. For this purpose, the sensing principle in PSi structures is based in the effective refractive index of PSi multilayer defined by the porosity and the refractive index of the medium inside the pores. Selective incorporation of an organic analyte in the PSi layer can modify the refractive index in two ways: it should increase the average refractive index (n) of the medium in the pores by replacing air (n=1) with organic matter (n>1), and it can also decrease the n of PSi structures by modifying the carrier concentration in the semiconductor. [17]

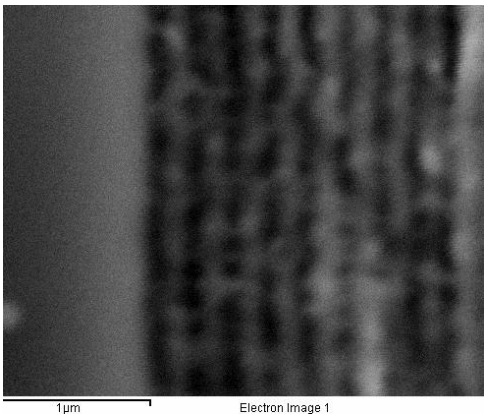

Fig. 1. Cross sectional SEM images of a multilayer structure (Bragg mirror) formed in highly doped p-type silicon (0.01 $\Omega \cdot cm^{-1}$) at current densities of 5 mA/ cm^2 and 25 mA/cm^2.

2.2 Etching procedure

PSi multilayers have been fabricated by wet electrochemical etching of highly boron-doped silicon wafer (SQI Inc: P+ type, boron-doped, 0.01-0.02 $\Omega \cdot cm^{-1}$, <100> oriented) in a teflon two electrode electrochemical cell. In order to have better interfaces, an aqueous HF/ethanol/glycerol electrolyte with 15% HF, 75% ethanol and 10% glycerol concentration is used to anodize the silicon substrate. During the etching process, in order to maintain a constant HF concentration over the interface between Si and PSi under chemical attack, a peristatical pump is used to circulate the electrolyte within the teflon cell. Anodization begins when a constant current is applied to the silicon wafer and the electrolyte by means of an electronic circuit controlling the anodization process (fig. 2). To stabilize the PSi multilayer structure, all the samples are thermally oxidized in an oxygen atmosphere at 300 ˚C for 15 min. In order to fabricate a stable mirror the first layer has to have a low porosity layer. Unfortunately, this prevents a good penetration of the biomolecule into the porous structure. To overcome this problem, we prepared a basic mirror consisting of a first layer produced with a current of 70 mA/cm^2. A second layer was then fabricated with a current of 5 mA/cm^2, followed by a third layer produced with a current of 45 mA/cm^2. The procedure involving the production of the last two layers was repeated 22 times. In these conditions, the alternating porosities of these layers are of the order of 35%-55%, 60%-85% and 90%-95% respectively.

2.3 Functionalization of oxidized porous silicon

The silanization of PSi surfaces was performed at room temperature by placing the surfaces in a solution of 10 % v/v 3-(Mercaptopropyl)trimethoxysilane in 2-propanol for 1 hr. After silanization, samples were rinsed with clean solvent 5 times (5 min each), and dried under a stream of N_2. Glucose oxidase (GOX) from Aspergillus Niger (VII-S type; Sigma, MW= 16 x 10^4 Da) at a concentration of 0.00022 g/ml was prepared in two ways: negatively charged in 8.3 mM phosphate buffer pH 7.2 and positively charged in 50 mM acetate buffer pH 4.0. The oxidized and functionalized pSi mirror were incubeted for 2 hr in the positive respectively negative charged protein solutions. Afterward the samples were rinsed 3 times (5 min each) with buffer, and dried under a stream of N_2.

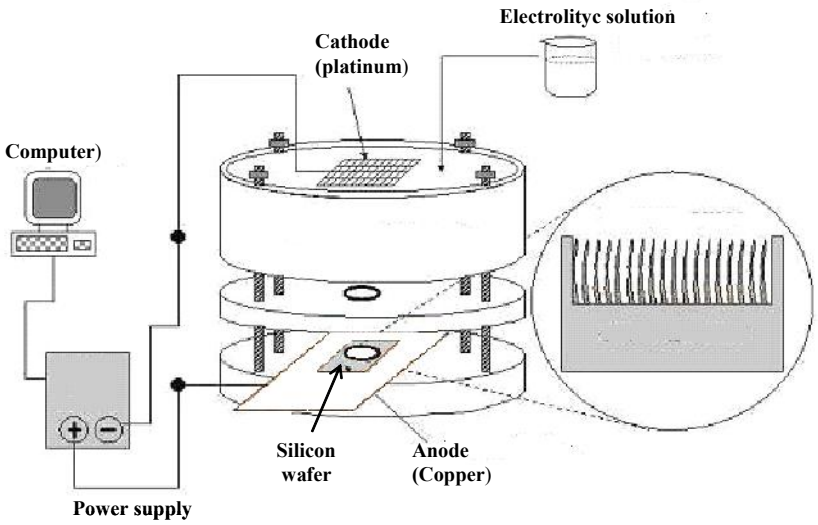

Fig.2. Electrochemical anodization system

3. RESULTS

3.1 Atomic Force Microscopy (AFM)

AFM images (obtained by a Nanoscope IIIa in tapping mode at 250 KHz) were acquired to monitor topology of pSi mirrors such as external pore diameter size and roughness. The porous silicon surface was imaged before (Fig.3) and after (Fig. 4) silane and protein binding. The morphological analyses of these two samples reveal a structure composed by pores and channels. The analysis show that pSi samples have external pore diameters ranging from 0.5 to 4 μm and 1 to 7 μm, respectively. Furthermore, the porous silicon surface roughness quantified in terms of rms (root-mean-square) was 105 and 62 nm respectively, suggesting that the surface silanization and protein binding smoothens the sample. The pore size increased tremendously after silane and protein binding revealing pores and channels with lateral dimensions larger than 5 μm. The increase in the size of the pores and channels can be interpreted also in terms of fused material [18] due to loss of some part of the walls of the pSi pores. However, the AFM images confirm that the thermal oxidation of pSi followed by silanization of the surface, turns the substrate stable enough toward corrosion in aqueous environment.

3.2 Fourier Transform Infrared Spectroscopy (FTIR)

The correct silanization and GOX binding on pSi samples was verified by HATR (Horizontal Attenuated Total Reflectance) Infrared Spectra (Perkin-Elmer GX). The HATR spectra are shown in Fig. (5), from top to bottom for the thermal oxidized pSi, then GOX-binded to pSi, finally silanized and GOX-binded samples. In the fist case the binding mechanism between the negatively charged pSi oxidized surface and the positively charged protein (by tuning the pH of protein, pI_{GOX}=4.2) was driven by electrostatics forces. In the second case the binding mechanism between MPTS groups and the GOX protein were the very stable disulphure bridge (S-S), formed by the interaction of sulfhydril groups and cysteine residues. The characteristic bands for the thermally oxidized sample is shown in Fig. 5(A): it displays n absorption band at 1076 cm^{-1} corresponding to the pSi-oxide υ(Si-O-Si). The characteristic bands of protein are appearing in Fig. 5(B) and 5(C), when GOX is adsorbed onto the nacked, respectively previously silanized pSi surface. For both the cases the FTIR spectrum displays characteristics bands of amide I υ (C=O) at 1644 cm^{-1} and amide II δ (N-H) at 1545 cm^{-1}. The methylene bands at 2920 $\upsilon_{AS}(CH_2)$, 2842 $\upsilon_{A}(CH_2)$, and 1467 cm^{-1} $\delta(CH_2)$ are also observed.

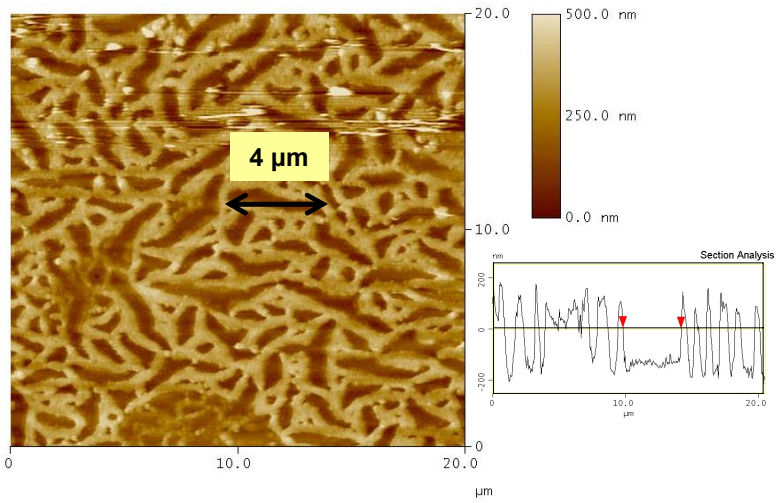

Figure 3. AFM images (tapping mode) 20X20 µm. PSi mirror thermally oxidized at 300˚C.

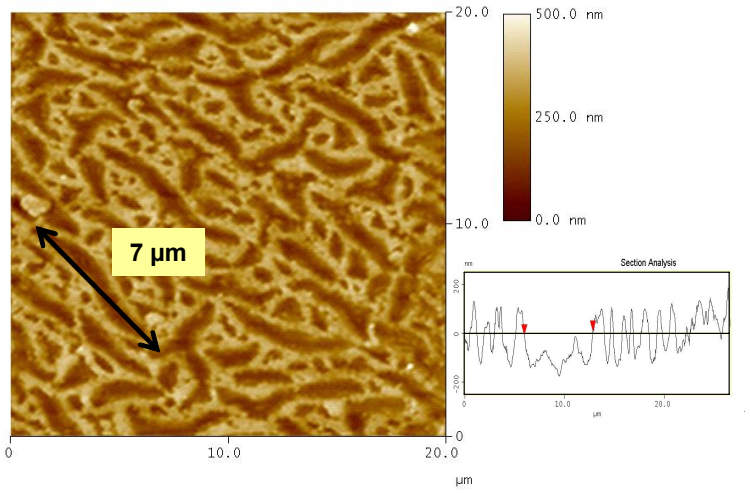

Figure 4. AFM images (tapping mode) 20X20 µm. PSi mirror thermally oxidized at 300˚C and chemically modified with 3-mercaptopropyl-trymethoxisilane and covered with glucose oxidase protein (0.22 mg/ml, in phosphate solution at 8.3 mM, pH 7.2).

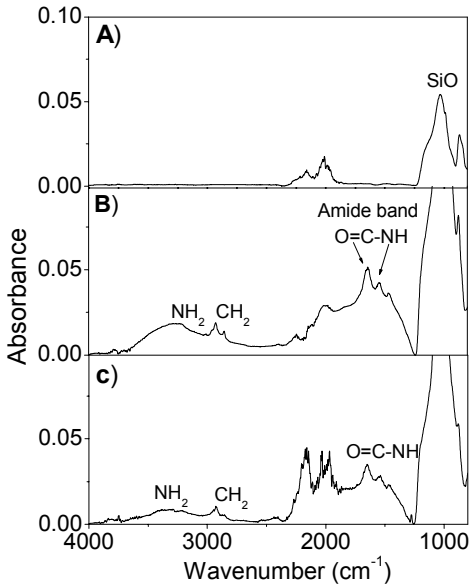

Fig 5. FTIR-HATR infrared absorbance spectrum of pSi oxidized mirror (A), with Glucose oxidase (0.22 mg/ml in acetate buffer pH 4.0) directly adsorbed on PSi (B) and with GOX binding on previously silanized pSi (C).

3.3 Interferometric reflectance spectra

Reflectance UV spectra (Shimadzu UV3010 UV-vis-NIR spectrophotometer, at 5° incidence angle) were recorded to monitor the presence of molecules in the pSi structures (Fig.6). pSi-GOX (Fig. 6B) and pSi-silane-GOX (Fig 6C) mirrors produce a blue-shift compared to the naked oxidized pSi spectra. The control experiments revealed no blue shift in presence of solely the buffer solution Fig 6(A). The observed blue-shifts in the pattern of dielectric mirror fringes suggest a decrease in the effective refractive index of the protein-film onto the pSi surface. This behavior might be the consequence of oxidation process of the highly dopped [p+boron, (0.01-0.02 $\Omega \cdot cm^{-1}$)] silicon wafer due to an incomplete biomolecule coverage. This oxidation reaction implies a decrease of average diameter of the crystalline cylinder forming the PSi. Moreover it can produce an accumulation of the carrier charge in the pores tips, as it has been already observed for certain semiconductor surfaces. This is confirmed by the increasing of the oxide specific bands (Si-O) of the IR spectra of these samples when silanization and respectively biofunctionalization is practiced.

3.4 Scanning Electron Microscopy (SEM)

The macrostructure of pSi Bragg mirrors was studied by scanning electron microscopy. In agreement with our AFM results the SEM images (Fig. 7) for thermal oxidized mirror of top (A) and cross-sectional (B) view shows a surface morphology like channels with high percentage of avoid space. These results are confirmed by the porosities obtained from gravimetric measurements that provided also 90-95 % of avoid space for this specific structure. The Figure (C) shows the cross-sectional view of pSi mirror after its functionalization with positively charged glucose oxidase. The presence of crystals into the nanostructure following GOX deposition is clear, that might be the result of protein crystallization. It's well known that supersaturation conditions are necessary to promote the nucleation. The local supersaturation is possible near any surface if attractive forces are still strong enough. These conditions are realized in the case of neutral-particle capillary condensation and adsorption of charged particles onto an oppositely charged surface. According to the traditional approach, the attractive force between opposite charges of a molecule and a surface can produce a sufficiently large difference in the surface free energy to produce a local supersaturation of charged particles resulting in heterogeneous nucleation.[19] Therefore, the net sign of the molecule charge and the surface charge is crucial for crystallization of charged molecules in general. In our case the nucleation mechanism might be enhanced

by the interaction between the negatively charged pSi surface and positively charged protein. However, the penetration and attaching of protein into the pSi multilayer is strongly dependent of the structure and require further research.

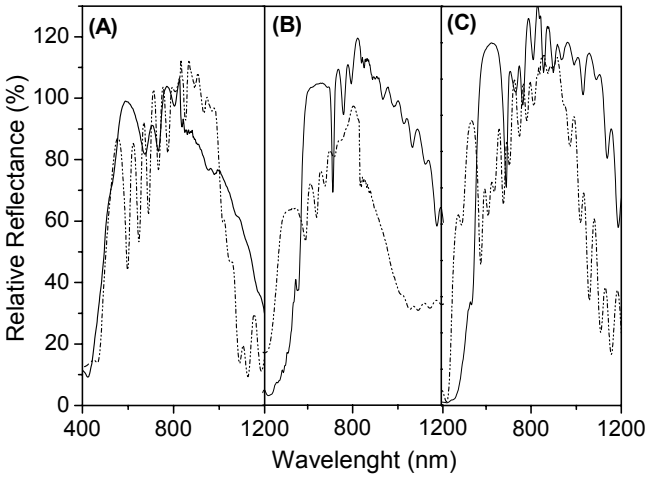

Fig. 6. Interference spectra (Fabry-Perot fringes) of the pSi oxidized mirror (—) in the 400-1200 nm wavelength region; following the GOX (B) and silane-GOX (C) modification (¨–¨). A blue shift in the interference fringes is observed. Contrary, no shift is occurring in presence of only the buffer solution (A).

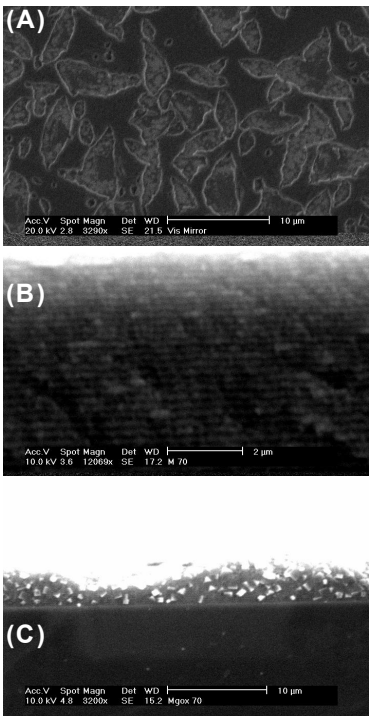

Fig.7 Top (A) and cross-sectional (B) scanning electron microcopy images of thermally oxidized porous silicon Bragg mirror before and after (C) bio-functionalization of pSi surface with glucose oxidase (conc. 0.22 mg/ml, in acetate solution at 50 mM, pH of 4.0)

4. CONCLUSIONS

In summary we can conclude that glucose oxidase protein can be successfully immobilized by physico-chemical adsorption either by electrostatic forces or/and covalent bonding on porous silicon mirror. The presence of molecules could be monitored by both Fourier Transformed Infrared Spectroscopy and Interference reflectance measurements. The AFM and SEM images reveal certain heterogeneity in the morphology of pSi Braggs mirrors resulting from etching process (structure like pores and channels). The apparition of crystalline structures within pores after adsorption of positively charged GOX was also observed. This could be the first observation of a pSi induced protein crystallization.

ACKNOWLEDGEMENTS

This work was partially supported by the CONACyT- 45951 project and the Ecos-North French-Mexican M06P01 program. We thank J. Luis Menchaca (UAZ-Mexico) for the AFM images and Héctor Flores (UASLP-México) for the SEM images. AGP thanks PROMEP support.

REFERENCES

1. G. Piechotta, J.Albers, R. Hintsche, "Novel micromachined silicon sensor for continuous glucose monitoring", Biosensor & Biolectronics, 21, 802-808 (2005).
2. Y. Yang Li, F. Cunin, J. R. Link, T. Gao, R.E. Betts, S. H. Reiver, V. Chin, S. N. Bhatia, M.J. Sailor, "Polymer replicas of photonic porous silicon for sensing and drug delivery applications", Science, 299, 2045-2047 (2003).
3. H. Ouyang, M. Christophersen, R. Viard. B.L. Miller, P. Fauchet, "Macroporous silicon microcavities for macromolecule detection", Adv. Funct. Mater., 15, 1851-1859(2005).
4. M.G. Martemyanov, D.G. Gusev, I.V. Soboleva, T.V. Dolgova, A.A. Fedyanin, O.A. Aktsipetrov, G. Marowsky, "Nonlinear optics in porous silicon photonic crystals and microcavities", Laser Physics, 14(5), 677-684 (2003).
5. V.Agarwal, J.A. del Río, "Tailoring the photonic band gap of a porous silicon dielectric mirrror", applied physics letters, 82(10), 1512-1514(2003).
6. V. Agarwal, J.A. del Río, " Filtres, mirrors and microcavities" Internacional journal of Modern Physics B, 20(1), 99-109(2006).
7. A.H. Mayne, S.C. Bayliss, P.barr, M.Tobin, L.D. Buckberry, "Biologically interfaced porous silicon devices", phys. stat. sol., 182, 505-513(2000).
8. E.J.Anglin, M.P. Schwartz, V. P. Ng, L.A. Perelman, M.J. Sailor, "Engineering the chemistry and nanostructure of porous silicon Fabry-Pérot films for loading and release of a steroid", Langmuir, 20, 11264-11269 (2004).
9. L.De Stefano, L. Rotiroti, I. rendina, L.Moretti,V. Scognamiglio, M.Rossi, S. D´Auria, "Porous silicon-based optical microsensor for the detection of L-glutamine", Biosensor & Biolectronics, 21, 1664-1667(2006).
10. V. Chin, B.E.Collins, M.J. Sailor, S.N. Bhatia, "Compatibility of primary hepatocytes with oxidized nanoporous silicon", Adv. Mater., 13(24), 1877-1880(2001).
11. L.T.Canham, C.L. Reeves, J.P. Newey, M.R. Houlton, T.I.Cox, J.M. Buriak, M.P. Stewart, "Derivatized mesoporous silicon with dramatically improved satability in simulated human blood plasma", Adv. Mater., 11(18) 1505-1507(1999).
12. Buckberry, S Bayliss, "Porous Silicon as a Biomaterial", Materials World, 7(4), 213-215 (1999).

13. EB.E. Collins, K-P S. Dancil, G. Abbi, M. Sailor, "Determinig Protein size using an electrochemically Machined pore gradient in silicon", Adv. Funct. Mater., 12(3), 187-191(2002).
14. F. Cunin, T.A. Schmedake.J.R. Link, Y.Y. Li, J. Koh, S.N. Bhatia, M.J. Sailor, "Biomolecular screening with encoded porous-silicon photonic crystals", Nature materials, 1, 39-41(2002).
15. L.T. Canham, "Derivatized porous silicon mirrors: implantable optical components with slow resorbability, phys. Status Solidi A, 182, 521-525(2000).
16. L. Canham, "properties of porous silicon", INSPEC London, UK,1997
17. V.S.-Y. Lin, K. Motesharei, K-P S. Dancil, M. Sailor, M.R. Ghadiri, "A porous silicon-based optical interferometric biosensor", Science, 278, 840-842(1997).
18. C. Steinem, A. Janhoff, V.S.-Y. Lin, N.H. Vǒlcker, M.R. Ghadiri, "DNA hybridization-enhanced porous silicon corrosion:mechanistic investigations and prospect for optical interferometric biosensing", *Tetrahedron*, 60, 11259-11267(2004).
19. S.Stolyarova, E. baskin, N.E. chayen, Y. Nemirovsky, "Possible model of protein nucleation and crystallization on porous silicon", phys.stat.sol., 202(8), 1462-1466(2005).