

AFRL-ML-TY-TP-2007-4522

PREPRINT



THREE DIMENSIONAL IMMOBILIZATION OF β -GALACTOSIDASE ON A SILICON SURFACE

**Heather R. Luckarift
Air Force Research Laboratory**

**Lorena Betancor
Department of Biochemistry
University of Cambridge
80 Tennis Court Road
Old Addenbrookes Site
Cambridge CB2 1GA**

**Jim C. Spain
School of Civil and Environmental Engineering
Georgia Institute of Technology
311 Ferst Drive
Atlanta GA 30332**

December 2006

**DISTRIBUTION STATEMENT A: Approved for public release;
distribution unlimited.**

Submitted for publication in Biosensors and Bioelectronics.

**Air Force Research Laboratory
Materials and Manufacturing Directorate
Airbase Technologies Division
139 Barnes Drive, Suite 2
Tyndall AFB, FL 32403-5323**

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation; or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

Qualified requestors may obtain copies of this report from the Defense Technical Information Center (DTIC) (<http://www.dtic.mil>).

REPORT NUMBER AFRL-ML-TY-TP-2007-4522 HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION IN ACCORDANCE WITH THE ASSIGNED DISTRIBUTION STATEMENT.

 //signature//
GLENN R. JOHNSON
Work Unit Manager

 //signature//
DANIEL M. SAUCER, Major, USAF
Acting Chief, Airbase Sciences Branch

 //signature//
WENDELL D. BANKS
Chief, Airbase Technologies Division

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government's approval or disapproval of its ideas or findings.

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY)		2. REPORT TYPE		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (Include area code)

THREE DIMENSIONAL IMMOBILIZATION OF β -GALACTOSIDASE ON A SILICON SURFACE

Lorena Betancor^{1, 2, 3}, Heather R Luckarift¹, Jim C Spain^{2†}

¹ Air Force Research Laboratory, 139 Barnes Drive, Building 1117, Suite # 2, Tyndall AFB, FL 32403 (USA), ² School of Civil and Environmental Engineering, Georgia Institute of Technology, 311 Ferst Drive, Atlanta GA 30332 (USA), ³ Present address: Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Old Addenbrookes Site, Cambridge CB2 1GA

Abstract

Many alternative strategies to immobilize and stabilize enzymes have been investigated in recent years for applications in biosensors. The entrapment of enzymes within silica-based nanospheres formed through silicification reactions provides high loading capacities for enzyme immobilization, resulting in high volumetric activity and enhanced mechanical stability. Here we report a strategy for chemically-associating silica nanospheres containing entrapped enzyme to a silicon support. β -Galactosidase was used as a model enzyme due to its versatility as a biosensor for lactose. The immobilization strategy resulted in a three-dimensional network of silica attached directly at the silicon surface, providing a significant increase in surface area and a corresponding 3.5-fold increase in enzyme loading compared to enzyme attached directly at the surface. The immobilized β -galactosidase prepared by silica deposition was stable and retained more than 80% of its initial activity after 10 days at 24°C. The ability to generate three-dimensional structures with enhanced loading capacity for biosensing molecules offers the potential to substantially amplify biosensor sensitivity.

Introduction

Enzymes are commonly used in biosensors because of their high specificity. Biosensor applications require a highly active immobilized enzyme system that allows the maintenance of an efficient connection between the sensing molecule and the transduction component of the biosensor. Because of their moderate stability, many alternative strategies to immobilize and stabilize enzymes have been explored to improve the feasibility and applicability of a wide range of biosensor applications. Such strategies include covalent immobilization, physical adsorption, cross-linking, encapsulation, or entrapment (Boyukbayram et al. 2006; Létant 2004; Malhotra et al. 2005; Rauf et al. 2006; Subramanian et al. 1999).

Clearly, enzyme loading will be increased in a relatively thick densely-packed layer as opposed to a thin adsorbed or covalently attached monolayer. Diffusion and mass transfer limitations, however, can limit the sensor signal to only a proportion of the enzyme that is active at (or near to) the sensing element (Williams and Blanch 1994). The search for a strategy that allows an increase of the surface area with the concomitant increase in sensitivity of the biosensor, without compromising the activity of the sensing molecule is therefore an important advancement in biosensor design (Charles et al. 2004; Laurell et al. 1995; Luckarift 2006a; Vepari 2006).

The sol-gel encapsulation of enzymes has recently undergone important developments motivated by its wide applications in biosensors (Avnir et al. 2006; Pierre

2004). Nevertheless, one of the primary drawbacks of the sol-gel technique is enzyme leakage (Blandino et al. 2000). The problem has in some instances been addressed by designing protocols for the preparation of matrixes with a pore size adequate to allow the flow of substrates and products but small enough to prevent the elution of the entrapped biocomponent (Blandino et al. 2001; Lu et al. 2006).

The entrapment of enzymes through biomimetic silicification reactions provides an efficient method for the preparation of robust immobilized derivatives of a variety of enzymes (Berne et al. 2006; Luckarift 2006b; Luckarift et al. 2004). Among the advantages of the immobilization technique are an absence of enzyme leaching and good mechanical properties; both key criteria for biosensor purposes. The study of silicification reactions has recently provided chemical mechanisms for biological silica formation (Belton 2005; Kroger et al. 2001; Sumper 2004). Amino groups have proven to be crucial in the reactions, leading us to explore the possibility of directly involving amino groups from a functionalized surface in the precipitation of silica as a simple method to covalently associate silica-immobilized enzymes directly and simultaneously to a planar surface. Glutaraldehyde and APTS are common coupling agents used in binding proteins to silicon surfaces (Longo et al. 2006; Williams and Blanch 1994). APTS is used to functionalize the surface, forming amino groups which are further activated with glutaraldehyde to form a layer of aldehyde groups which react with the amino groups of protein. Here, the strategy was used to immobilize β -galactosidase to a glutaraldehyde-activated silicon surface. β -galactosidase was used as a model enzyme because it has been well characterized, is readily quantified, and has been incorporated in

several types of biosensors for the detection and quantification of lactose (Goktug et al. 2005; Sharma et al. 2004; Tkac et al. 2000; Watanabe 1991).

Materials and Methods

Chemicals

β -Galactosidase from *E. coli* (Grade VIII), monoclonal anti- β -galactosidase antibody produced in mouse (G6282), (3-Aminopropyl)triethoxysilane (APTS) and glutaraldehyde (grade II, 25%) were obtained from Sigma-Aldrich. Secondary Antibody; FITC (Fluorescein Conjugate), Polyclonal Goat Anti-Mouse IgG was purchased from Southern Biotech. The synthetic peptide R5, (SSKKSGSYSGSKGSKRRIL) was obtained from New England Peptides. Potassium phosphate buffer (25 mM, pH 7.0) was used throughout unless otherwise indicated. All other reagents were of analytical grade.

Functionalization of the silicon samples

Single-crystalline lightly-doped silicon wafers (0.5 x 0.5 cm squares) were kindly donated by Jae Hyeong Seo and Prof. Oliver Brand from the School of Electrical and Computer Engineering at Georgia Institute of Technology. A cleaning step was carried out in 10% nitric acid at 80°C for 20 min followed by thorough rinsing with distilled water. The cleaned silicon wafers were activated as described previously (Williams and Blanch 1994). All solutions were applied by placing 20 μ l onto the sample surface. Amino-activated silicon samples were prepared by incubating with 10 % APTS (v/v) in distilled water for 4 hours at 80°C before rinsing thoroughly with distilled water. For

glutaraldehyde-activated silicon samples, the surface was subsequently incubated with 15 % (v/v) glutaraldehyde in buffer at 25°C for a minimum of 8 hours. Excess glutaraldehyde was removed by thoroughly rinsing with distilled water.

Enzyme activity assays

β -galactosidase activity was determined spectrophotometrically by increase in absorbance at 405 nm caused by the hydrolysis of o-nitrophenyl- β -D-galactopyranoside (oNPG). The reaction mixture contained 20 mM oNPG and 1 mM MgCl₂ in potassium phosphate buffer. To determine the activity bound to the surface, the samples were incubated in the reaction mixture with gentle agitation. Periodically, samples of the reaction mixture were withdrawn for determination of A₄₀₅. One enzyme unit (IU) was defined as the amount of enzyme that catalyzes the formation of 1 μ mol of product per minute under the specified conditions.

Enzyme immobilization

Immobilization of the soluble β -Galactosidase: Immobilization of the soluble enzyme on the silicon surface was carried out by either 1) immersion or 2) evaporation. For immobilization by immersion; activated samples were incubated with 0.08 IU/mL (in 250 μ L) at 25°C. Periodically, samples of the supernatant were withdrawn and analyzed for enzyme activity. After 2 hrs of incubation, the silicon samples were thoroughly rinsed and stored in buffer at 4°C.

For immobilization by evaporation; immobilization was performed by applying the enzyme solution (10 μ L volume containing 2 IU/mL) directly on the activated surface at 25°C for 15 min until completely dry. The samples were then rinsed thoroughly and stored in buffer at 4°C.

Silica entrapment of β -Galactosidase: Silica-entrapment was performed as described previously (Luckarift et al. 2004). Enzyme solutions (0.5 ml) containing 0.02 IU/mL in potassium phosphate buffer (25 mM, pH 8.0) were mixed with 0.125 mL of R5 peptide (100 mg/mL) and 0.125 mL of hydrolyzed tetramethyl orthosilicate (TMOS) solution. The TMOS was hydrolyzed by dilution in hydrochloric acid (1 mM) to a final concentration of 1 M. The particles were agitated for 2 minutes at 22°C, collected by centrifugation for 10 seconds (14,000 x g) and then washed twice in phosphate buffer before use.

Immobilization of the silica entrapped enzyme on the activated samples: To attach the silica particles to the amino-activated surface, the silica-entrapment was performed directly on the activated surface. Enzyme solutions (10 μ L) containing 2 IU/mL in potassium phosphate buffer (25 mM, pH 8.0) were mixed on the sample surface with 2.5 μ L of R5 (100 mg/mL) and 2.5 μ L of hydrolyzed TMOS solution. After the silica precipitation the samples were washed 4 times by incubation in buffer with gentle agitation.

Loading capacity experiments

The loading capacities of the glutaraldehyde-activated samples with soluble enzyme and the amino-activated samples with the silica-entrapped enzyme were determined by immobilizing increasing concentrations (2 to 20 UI/mL) of β -galactosidase, as described above.

Detection of immobilized β -galactosidase on glutaraldehyde activated samples.

Each silicon sample was incubated with 2 mL of a 1/2000 dilution of monoclonal anti- β -galactosidase antibody in phosphate buffered saline (PBS) for the detection of native or denatured enzyme molecules. The incubation was carried out for 3 hours at 25°C. Samples were then washed thoroughly with PBS and further incubated with a 1/2000 dilution of secondary antibody (fluorescein conjugated) for 3 hours at 25°C. Samples were washed thoroughly with PBS and kept in the same buffer at 4°C for further analysis. Fluorescence at the surface was imaged on a Zeiss Axiovert 200 microscope equipped with a Zeiss filter set #9 (EX: BP 450-490, FT 510, EM: LP 515). Images were recorded using a Zeiss Axiocam MRc digital camera.

Results and Discussion

Immobilization of soluble β -galactosidase to silicon samples

A silicon wafer surface was functionalized with APTS and glutaraldehyde to form a layer of aldehyde groups which react with the amino groups of β -galactosidase. The glutaraldehyde-activated samples were submerged in enzyme solution and immobilization was followed by measuring activity of the remaining free enzyme in the supernatant (Figure 1). Almost 89 % (\pm 4.5) of the initial enzyme activity was immobilized within 2 hours. As an alternate strategy, immobilization of the same amount of enzyme could be achieved by using evaporation to drive immobilization. The evaporation method significantly reduced the incubation time required for complete immobilization to 15 min and yielded substantially higher immobilization efficiencies consistent with previously reported values for the immobilization of β -galactosidase from *E. coli* (Pessela et al. 2007) (Figure 2). Non-specific adsorption of enzyme using either method was 18.5 % (\pm 0.5). Enzyme attached by non-specific binding, however, was readily removed by washing.

The immobilization of β -galactosidase through glutaraldehyde chemistry was confirmed by fluorescence microscopy using antigen-antibody interactions (Figure 3). Fluorescence microscopy showed an evenly distributed fluorescence signal in the functionalized samples and no signal for a negative control containing a non-functionalized silicon wafer incubated with β -galactosidase (Figures 3).

Immobilization efficiencies of approximately 60 % (56.35 % \pm 6.16) were obtained for enzyme loadings lower than 0.06 IU (Figure 4). When the initial β -

galactosidase activity was increased further, a percentage of the activity remained in the supernatant due to saturation of the surface, which decreased the immobilization yield. A maximum loading capacity of the activated silicon samples of 0.045 IU, however, was obtained at high enzyme concentrations despite the lower overall immobilization yield. (Figure 4).

Immobilization of silica-encapsulated β -galactosidase to silicon samples

In order to further enhance the stability and loading capacity of the silicon samples we next investigated the formation of 3-dimensional silica-immobilized enzymes directly at the surface. Because amino groups are critical for biological silica formation, we explored the integration of amino groups from a functionalized surface with the precipitation of silica particles, in order to covalently associate silica-immobilized enzymes directly and simultaneously to a planar surface. Silicon samples were functionalized with amino groups by activation with APTS and used to prepare surface-immobilized β -galactosidase. The biomimetic procedure for silica-entrapment of enzymes (Luckarift et al. 2004) was performed directly on the surface of the functionalized samples. Silica precipitation occurred rapidly for enzyme concentrations of 0.1 to 2 IU. No activity was found in the wash fractions following immobilization. The strategy led to immobilization of 3.5 times more enzyme activity than in the glutaraldehyde-activated samples, where enzyme attachment was most likely limited to a surface-associated monolayer (Figure 5).

The amount of immobilized enzyme bound to the silicon surface increased linearly with increasing concentration of enzyme in the reaction solution. The immobilization yield of the resulting samples, however, remained constant at 9.7 % (\pm 1.0) irrespective of the starting concentration. This effect was not observed with the β -galactosidase directly bound to the surface through glutaraldehyde (Figure 5) which reached a plateau concentration as the surface became saturated. In comparison, entrapment of enzyme (even at higher enzyme concentrations) yielded 45.3 % (\pm 0.2) immobilization efficiency when the silica particles were freely suspended in buffer. The low activity on the silicon surface is therefore attributed to mass transfer problems associated with diffusion of substrate and product into the silica particles and access to the enzyme's active site. This limitation has been previously reported in the design of three dimensional structures for biosensors (Charles et al. 2004).

Silicon samples that had been washed but not activated with APTS did not retain any enzyme activity after the silica deposition step indicating that the APTS functionalization was essential to associate the silica particles to the surface. SEM micrographs of the silica-immobilized enzyme attached to the silicon samples revealed an uneven coverage by a three dimensional network of silica particles (Figure 6).

Enzyme stability issues are always of high significance in the production of a stable and reproducible biosensor. Furthermore, the practical application of otherwise interesting putative sensing enzymes is often hindered by their poor stability (Kim et al. 2006). The immobilization of enzymes on unusual surfaces and especially by covalent attachment is sometimes detrimental to the stability of the enzyme (Brena et al. 2003).

The immobilized samples prepared herein by silica deposition retained more than 80% of their initial activity after 10 days at 24°C. Moreover, the activity of the samples remained unchanged when stored at 4°C for more than 4 weeks (data not shown).

Conclusions

In this work we have demonstrated a method for three-dimensional enzyme immobilization by involvement of amino groups of a functionalized silicon surface with the biomimetic reactions used to deposit silica. Unlike other attempts to attached silica particles to silicon substrates, where extremely high temperatures were used (900°C) (Zou and Yang 2006), the system described works at conditions suited to the physiological requirements of biomolecules. In addition, immobilization, attachment and hence stabilization of the enzyme occur simultaneously providing a simple and rapid method for preparation.

The use of silica particles to build a 3-dimensional structure not only provides an increased capacity for the immobilization of β -galactosidase but also an improved stability of the sensor molecule. The loading capacities obtained demonstrate the applicability of the concept for biosensors where high loadings of stable sensing molecules are needed. Analysis of the silica-coatings, however, indicates a non-uniform deposition of silica on the surface. A similar observation was also made for the lysozyme-mediated precipitation of silica at a gold surface (Luckarift 2006b) and for covalent immobilization strategies in general (Subramanian et al. 1999). The preliminary

technique described here with further optimization could, however, provide significant enhancement to increase the sensitivity of biosensors or as a strategy for three dimensional sensors (Charles et al. 2004; Rubina et al. 2004).

Acknowledgements

This work was supported by funding from the Air Force Office of Scientific Research. L.B and H.R.L were supported by postdoctoral fellowships from the Oak Ridge Institute for Science and Education (US Department of Energy). The authors thank Prof. Oliver Brand and Jae Hyeong Seo for the preparation of the silicon wafers and the SEM pictures. They would also like to thank Prof. Nils Kroger and Dr. Nicole Poulsen for help with the fluorescence microscopy.

Legends to Figures

Figure 1. Time course of β -galactosidase immobilization onto glutaraldehyde-activated samples

Enzyme activity in supernatant (\blacklozenge) from enzyme incubated by immersion with silicon wafer; Control (\bullet) from enzyme activity in buffer. Immobilization (%) = activity in the supernatant / initial activity x 100

Figure 2. Immobilization yield obtained by various immobilization methods

Immobilization yield (%) = activity bound to the sample / initial activity x 100

Figure 3. Detection of immobilized β -galactosidase by fluorescence microscopy

Immobilized samples were treated sequentially with a monoclonal antibody and a secondary FITC-conjugated antibody. A: β -galactosidase immobilized to a functionalized silicon sample. B: control (non functionalized sample incubated with β -galactosidase)

Figure 4. Loading capacity of the glutaraldehyde-activated silicon samples

β -galactosidase was applied to a glutaraldehyde activated sample at a range of concentrations. Immobilization yield (%) (\blacksquare); Immobilized IU (\blacklozenge). Immobilization yield (%) = activity bound to the sample / initial activity x 100

Figure 5. Loading capacity of functionalized silicon samples upon silica-enzyme entrapment

Immobilization of β -galactosidase on glutaraldehyde-activated samples (■);

Immobilization of silica entrapped β -galactosidase on amino-activated samples (◆)

Figure 6. SEM micrographs of silica entrapped β -galactosidase on amino-activated samples. A, B and C show different areas of the immobilized samples. Samples were sputter coated with gold before analysis.

Figure 1

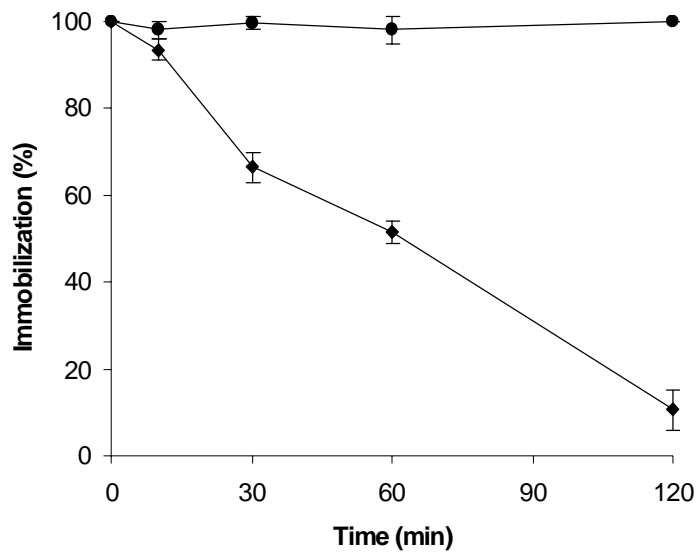


Figure 2

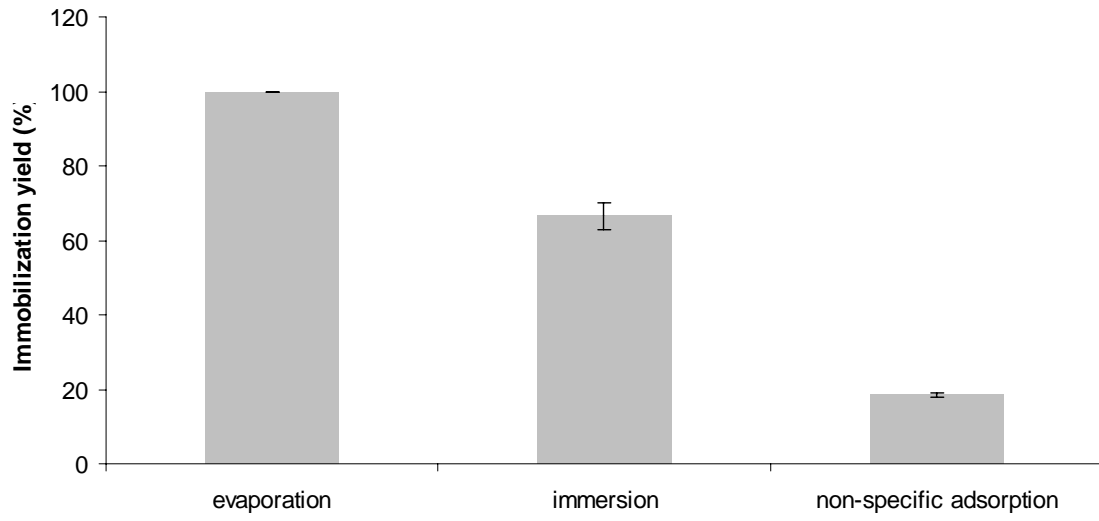


Figure 3

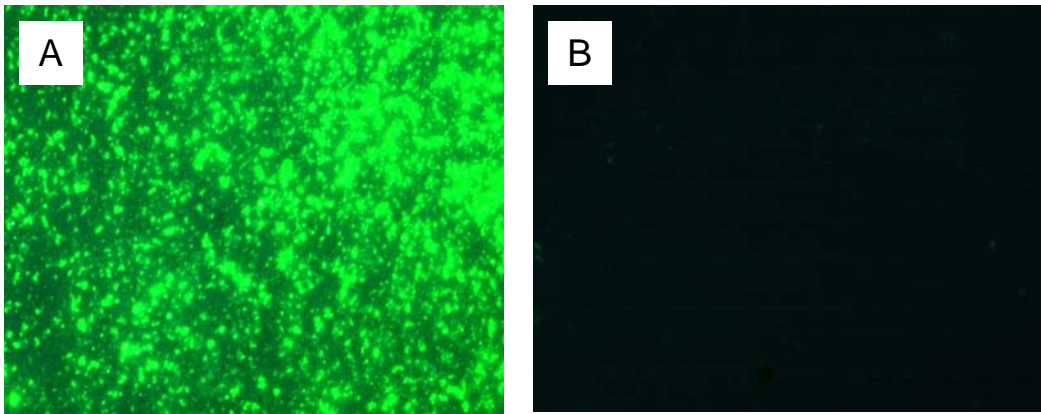


Figure 4

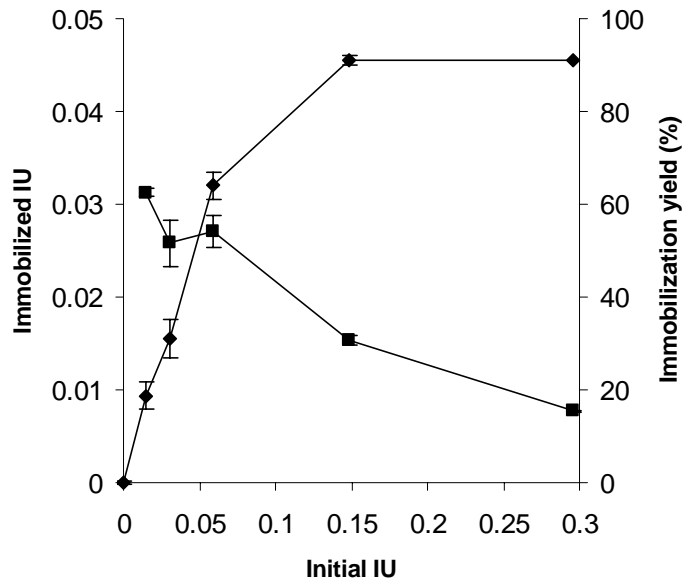


Figure 5

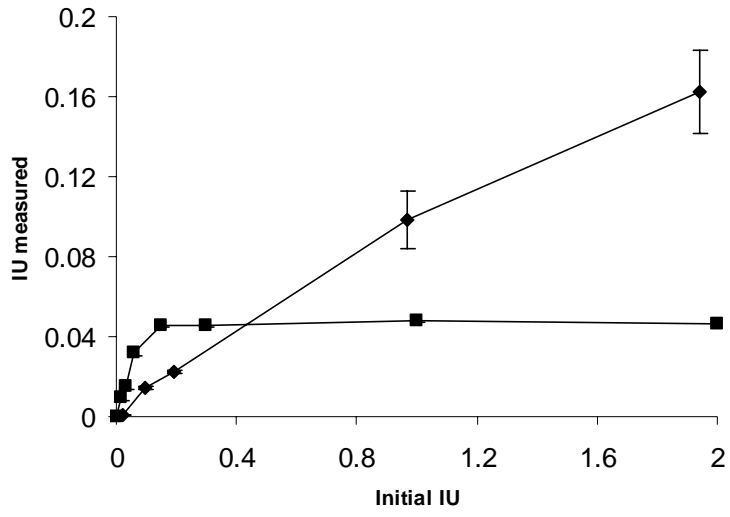
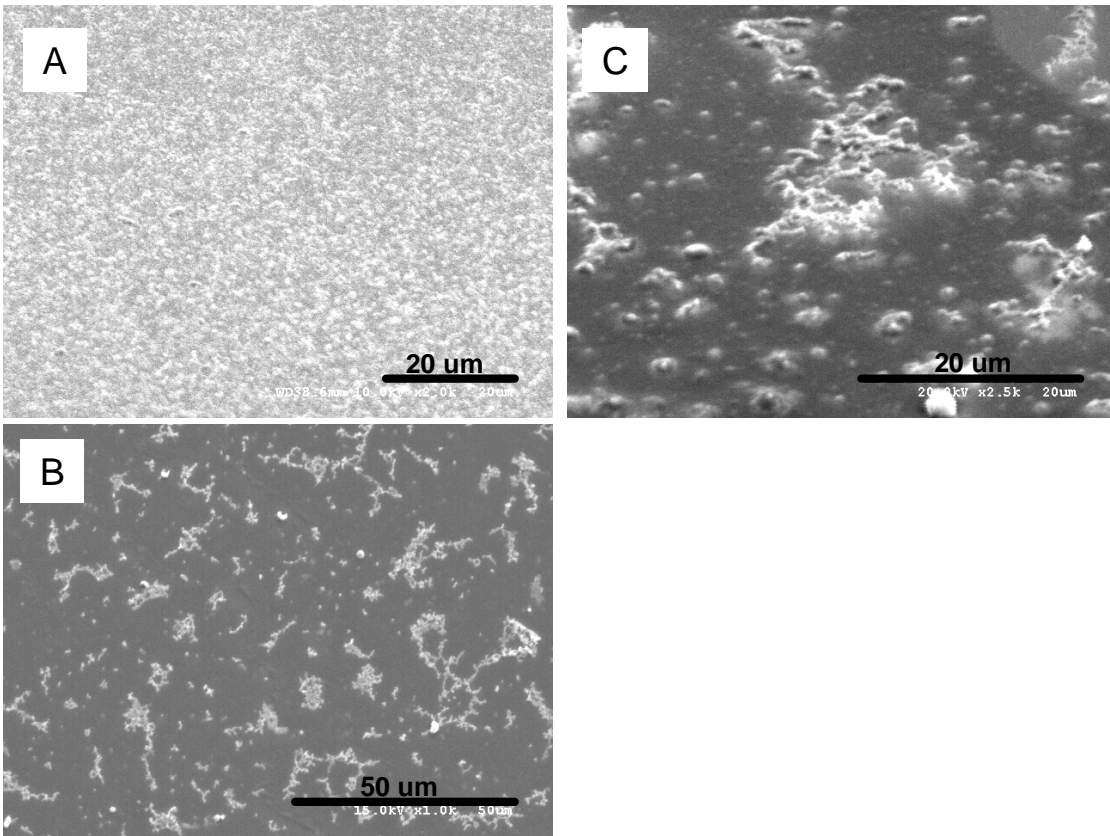


Figure 6



References

- Avnir D, Coradin T, Lev O, Livage J. 2006. Recent bio-applications of sol-gel materials. *Journal of Materials Chemistry* 16(11):1013-1030.
- Belton DJ, Patwardhan, S V, Perry C 2005. Spermine, spermidine and their analogues generate tailored silicas. *Journal of Materials Chemistry* 15(3):4629-4638.
- Berne C, Betancor L, Luckarift HR, Spain JC. 2006. Application of a Microfluidic Reactor for Screening Cancer Prodrug Activation Using Silica-Immobilized Nitrobenzene Nitroreductase. *Biomacromolecules* 7(9):2631-2636.
- Blandino A, Macias M, Cantero D. 2000. Glucose oxidase release from calcium alginate gel capsules. *Enzyme and Microbial Technology* 27(3-5):319.
- Blandino A, Macias M, Cantero D. 2001. Immobilization of glucose oxidase within calcium alginate gel capsules. *Process Biochemistry* 36(7):601.
- Boyukbayram AE, Kiralp S, Toppare L, Yagci Y. 2006. Preparation of biosensors by immobilization of polyphenol oxidase in conducting copolymers and their use in determination of phenolic compounds in red wine. *Bioelectrochemistry* 69(2):164-171.
- Brena BM, Irazoqui G, Giacomini C, Batista-Viera F. 2003. Effect of increasing co-solvent concentration on the stability of soluble and immobilized [beta]-galactosidase. *Journal of Molecular Catalysis B: Enzymatic* 21(1-2):25-29.
- Charles PT, Goldman ER, Rangasammy JG, Schauer CL, Chen M-S, Taitt CR. 2004. Fabrication and characterization of 3D hydrogel microarrays to measure antigenicity and antibody functionality for biosensor applications. *Biosensors and Bioelectronics* 20(4):753-764.
- Goktug T, Sezginurk MK, Dinckaya E. 2005. Glucose oxidase-[beta]-galactosidase hybrid biosensor based on glassy carbon electrode modified with mercury for lactose determination. *Analytica Chimica Acta* 551(1-2):51-56.
- Kim J, Grate JW, Wang P. 2006. Nanostructures for enzyme stabilization. *Chemical Engineering Science* 61(3):1017-1026.
- Kroger N, Deutzmann R, Sumper M. 2001. Silica-precipitating Peptides from Diatoms. THE CHEMICAL STRUCTURE OF SILAFFIN-1A FROM CYLINDROTHECA FUSIFORMIS. *J. Biol. Chem.* 276(28):26066-26070.
- Laurell T, Drott J, Rosengren L. 1995. Silicon wafer integrated enzyme reactors. *Biosensors and Bioelectronics* 10(3-4):289-299.
- Létant SE, Hart B. R, Kane S. R, Hadi M. Z, Shields S. J, Reynolds J. G. 2004. Enzyme Immobilization on Porous Silicon Surfaces. *Advanced Materials* 16(8):689-693.
- Longo L, Vasapollo G, Guascito M, Malitesta C. 2006. New insights from X-ray photoelectron spectroscopy into the chemistry of covalent enzyme

- immobilization, with glutamate dehydrogenase (GDH) on silicon dioxide as an example. *Analytical and Bioanalytical Chemistry* 385(1):146-152.
- Lu Y, Jiang Z-y, Xu S-w, Wu H. 2006. Efficient conversion of CO₂ to formic acid by formate dehydrogenase immobilized in a novel alginate-silica hybrid gel. *Catalysis Today* 115(1-4):263-268.
- Luckarift HR, Balasubramanian, S., Paliwal, S., Johnson, G. R, Simonian, A L. 2006a. Enzyme-encapsulated silica monolayers for rapid functionalization of a gold surface. *Colloids and Surfaces B. Biointerfaces*. Doi: 10.1016/j.colsurfb.2006.08.013
- Luckarift HR, Dickerson, Matthew B., Sandhage, Kenneth H., Spain, Jim C. . 2006b. Rapid, Room-Temperature Synthesis of Antibacterial Bionanocomposites of Lysozyme with Amorphous Silica or Titania. *Small* 2(5):640-643.
- Luckarift HR, Spain JC, Naik RR, Stone MO. 2004. Enzyme immobilization in a biomimetic silica support. *Nat Biotech* 22(2):211.
- Malhotra BD, Singhal R, Chaubey A, Sharma SK, Kumar A. 2005. Recent trends in biosensors. *Current Applied Physics* 5(2):92.
- Pessela BCC, Dellamora-Ortiz G, Betancor L, Fuentes M, Guisan JM, Fernandez-Lafuente R. 2007. Modulation of the catalytic properties of multimeric [beta]-galactosidase from *E. coli* by using different immobilization protocols. *Enzyme and Microbial Technology* 40(2):310-315.
- Pierre AC. 2004. The sol-gel encapsulation of enzymes. *Biocatalysis and Biotransformation* 22(3):145-170.
- Rauf S, Ihsan A, Akhtar K, Ghauri MA, Rahman M, Anwar MA, Khalid AM. 2006. Glucose oxidase immobilization on a novel cellulose acetate-polymethylmethacrylate membrane. *Journal of Biotechnology* 121(3):351-360.
- Rubina AY, Pan'kov SV, Dementieva EI, Pen'kov DN, Butygin AV, Vasiliskov VA, Chudinov AV, Mikheikin AL, Mikhailovich VM, Mirzabekov AD. 2004. Hydrogel drop microchips with immobilized DNA: properties and methods for large-scale production. *Anal Biochem* 325(1):92-106.
- Sharma SK, Singhal R, Malhotra BD, Sehgal N, Kumar A. 2004. Lactose biosensor based on Langmuir-Blodgett films of poly(3-hexyl thiophene). *Biosensors and Bioelectronics* 20(3):651-657.
- Subramanian A, Kennel SJ, Oden PI, Jacobson KB, Woodward J, Doktycz MJ. 1999. Comparison of techniques for enzyme immobilization on silicon supports. *Enzyme and Microbial Technology* 24(1-2):26-34.
- Sumper M, Kröger, N. 2004. Silica formation in diatoms: the function of long-chain polyamines and silaffins. *Journal of Materials Chemistry* 14(14):2059-2065.

- Tkac J, Sturdik E, Gemeiner P. 2000. Novel glucose non-interference biosensor for lactose detection based on galactose oxidase-peroxidase with and without co-immobilised beta-galactosidase. *The Analyst* 125(7):1285-1289.
- Vepari CP, Kaplan, D L. 2006. Covalently immobilized enzyme gradients within three-dimensional porous scaffolds. *Biotechnology and Bioengineering* 93(6):1130-1137.
- Watanabe E, Takagi, M, Takei, S, Hoshi M, Shu-gui, C. 1991. Development of biosensors for the simultaneous determination of sucrose and glucose, lactose and glucose, and starch and glucose. *Biotechnology and Bioengineering* 38(1):99-103.
- Williams RA, Blanch HW. 1994. Covalent immobilization of protein monolayers for biosensor applications. *Biosensors and Bioelectronics* 9(2):159.
- Zou M, Yang D. 2006. Nanoindentation of silica nanoparticles attached to a silicon substrate. *Tribology Letters* 22(2):189-196.