A Novel Technique for Fabricating Plastic Lab-on-a-Chip Devices with an Immobilized Enzyme

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Abstract

In this study we design new fabrication techniques and demonstrate the potential of using dense CO_2 (i.e. high pressure carbon dioxide that possess a liquid-like density) for facilitating crucial steps in the fabrication of plastic lab-on-a-chip (LOC) micro-devices by embedding bio-molecules at temperatures well below the plastic's glass transition temperature (T_g). The polymer polystyrene (PS) is the plastic that was used in this study and has a T_g of ~105°C. The T_g is the temperature at which the plastic becomes rubbery and deformable, below this temperature it is glassy and acts as a brittle solid. These new techniques are environmentally friendly and done without the use of a clean room. Carbon dioxide at 40°C and between 4.48 and 6.89 MPa was used to immobilize the biologically active molecule, β-galactosidase (β-gal), on the surface of PS micro-channels. To our knowledge, this is the first time dense CO_2 has been used to directly immobilize an enzyme in a micro-channel. β-gal activity was maintained, and shown via a fluorescent reaction product, after enzyme immobilization and micro-channel capping by the designed fabrication steps at 40°C and pressures up to 6.89 MPa.

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1. Introduction

1.1 Lab-on-a-Chip Applications

In recent years researchers have decreased the size of diagnostic biomedical devices. One category of these devices is lab-on-a-chip (LOC), which have channels for fluid flow with a diameter on the order of micrometers down to nanometers. LOC micro-devices are gaining popularity due to their wide variety of diagnostic uses, fast sampling time, and ease of use. The miniaturization of these fluidic devices has reduced the required quantity of sample for analysis, the amount of human labor and the time for analysis. The devices are designed to execute sampling, sample pretreatment, mixing, separation, and detection all on a single chip.[1] Combined with portable electronic equipment LOCs have diverse applications such as detecting explosives,[2] monitoring nutrients in agricultural water, controlling quality of food production, controlling processes in the chemical industry,[3] point-of-care analysis of bodily fluids,[4] analyzing forensic evidence,[5] and tracking pollution in environmental or waste waters.[6] The global demand for these types of devices is tremendous, a single hospital can do as many as hundreds of thousands of point-of-care tests on patients each year.[7] A major incentive to improve further upon the current technology is to help global human health, especially in developing countries, by diagnostic testing using chemically and physically robust LOCs.[8]

The first devices were fabricated from polycrystalline silicon which is expensive and requires high processing costs. More recently, low-price polymers have been introduced as the material for fabricating these devices, thus decreasing their cost. Polymers are made with a variety of properties to fit the need of the device being designed, including physical, chemical, and thermal resistance, optical characteristics, and biocompatibilities. Some LOCs contain immobilized enzymes to perform a specific reaction on a substrate to produce a more easily measurable product, or to create an electrical charge.[9] Immobilizing an enzyme can increase its robustness[10-

[14], however the high temperatures or organic solvents used during polymer processing are still lethal for these fragile molecules causing the final product to be useless.

1.2 Immobilization Techniques

A variety of immobilization and impregnation techniques have been reported (e.g. adsorption,[14-16] cross-linking,[17-21] covalent bonding,[12, 22-28] entrapment/encapsulation,[29-32] and dense gas processing),[33-38] each with intrinsic advantages and disadvantages for a variety of applications. Dense CO_2 has been used previously to impregnate thermally labile compounds into polymer matrices; Kazarian *et al*. immobilized ibuprofen into poly(vinylpyrrolidone),[34] Sproule *et al.* immobilized an immunoglobin into poly(methylmethacylate),[37] Powell *et al*. immobilized paclitaxel into polylactic acid,[39] and Kayrak-Talay *et al*. immobilized glucose oxidase into polypyrrole and polyurethane/polypyrrole composite foam.[38] Immobilized enzymes/proteins within a LOC can be located in different places; such as on an electrode, on porous media placed in a micro-channel, or on the walls of the channel itself. Our focus was to design a technique to immobilize enzyme in accordance with the latter of the three.

Acquiring fast results is a commonality for micro-devices due to the low sample volume, high local concentration ratio of enzyme to reactive substrate, and short diffusion path lengths for the substrate and product. Many of the techniques mentioned for immobilizing enzymes in a micro-channel are multistep and require many different reagents, some involving organic solvents. Immobilization using dense CO_2 is a one step technique that requires nothing more than the enzyme solution and the CO_2. In this study we demonstrate the feasibility of developing a one step distinctive physical, rather than chemical, process for immobilization of a biologically active compound into a polymeric micro-channel.

1.3 Microchip Fabrication

Bonding the caps on polymeric lab-on-a-chip micro-channels has been accomplished in a variety of different ways, such as using organic solvents,[40, 41] hot embossing at temperatures above the T_g,[1] low temperature plasma activation,[42] localized heating,[43] and using dense CO_2.[44, 45] All except for the two latter processing techniques are detrimental to biologically active molecules. Another potential problem during bonding is that as the features on the chips are reduced in size, bonding without geometric deformation becomes a more prominent issue, but Yang *et al*. has eliminated this dilemma by bonding at moderate conditions with CO_2, even down to the nanometer scale.[45-47]

One of the objectives of this study was to develop a low temperature, solvent free, non-clean room technique for bonding an enclosing cap on enzyme activated micro-channels fabricated from an inexpensive polymer, while retaining enzymatic activity. We used dense CO_2 at moderate temperatures and pressures for immobilization of the enzyme and capping of the micro-channels. Polystyrene was used as the polymer matrix to immobilize β–gal. We have eliminated foam formation, a major issue in previous dense gas immobilization,[37] and engineered a non-foaming microstructure by meticulously governing the depressurization rate.

2. Experimental

2.1 Immobilization of β-Gal into PS Micro-Channels

The physical set-up for enzyme immobilization, Fig. 1, was assembled by placing a PS substrate complete with micro-channels in a high pressure vessel (Jerguson Site Gauge), then placing a piece of porous glass, with maximum pore diameter of 4-8 μm, on top of it. One hundred μL of 5 mg/mL β–gal in distilled water was dripped onto the porous glass. The β–gal solution percolated through the porous glass and came into contact with the PS substrate. The high pressure vessel was then sealed air tight and submerged in a 40°C water bath. A minimum of 15 minutes was

waited to ensure thermal equilibrium, and then an ISCO syringe pump (500D) was used to pressurize it with CO_2 (4.48-6.89 MPa). The temperature and pressure were held constant for 2.5 hours during which the immobilization of the β–gal into the PS took place.

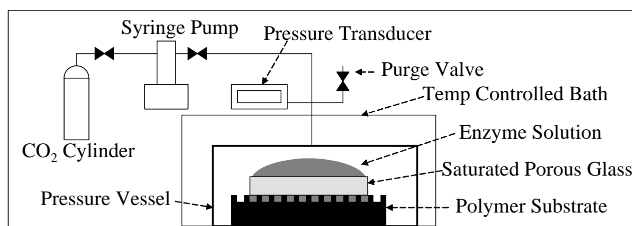

Figure 1. Schematic of enzyme immobilization set-up. The size of the sample and the pressure vessel have been magnified.

The system was then depressurized over nearly 3 hours to avoid foaming of the sample. After depressurization the enzyme immobilized PS sample was sonicated (Unisonics ultrasonic cleaner (FXP8)) in deionized water at room temperature for 10 minutes to remove the free β–gal from the surface. The PS with immobilized enzyme was then sealed in a plastic bag and stored at 5°C until future application and characterization.

2.2 Capping Micro-Channels

In this study we use a benign dense CO_2 at the low temperature of 40°C to bond the cap on the micro-channels to preserve both enzyme activity and micro-channel features. Polystyrene substrates (1.0 x 1.0 x 0.1 cm) with micro-channels were capped by another piece of PS measuring the same dimensions. Prior to bonding a 0.6 mm diameter drill bit was used to bore holes in the PS cap for fluid injection/extraction ports.

During the process the two separate PS substrates were sandwiched by Teflon film and then surrounded by polished stainless steel plates. Two 19mm foldback clips (standard office binder clips) were used to apply the compressive force, which measured 46±3.0 N over the entire bonded surface area (Honeywell load cell model 13). The samples were placed in a Thar

Technologies Reactor (R100W) and heated up to 40°C. An ISCO 500D syringe pump was used to pressurize the system with CO_2 to a desired pressure of 4.48 to 6.89 MPa. The vessel was kept at these conditions for a period of time that varied between 1 and 2.5 hours. The system was then depressurized slowly using the syringe pump.

3. Characterization Techniques

3.1 Immobilized *β*-Gal Confirmation by Fluorescence

The presence of immobilized *β*–gal after dense CO_2 processing was confirmed by confocal fluorescent microscopy on uncapped PS microchannels. *β*–gal was labeled with Oregon Green® 488 dye, via the manufacturer's instructions (Molecular Probes), which binds to the primary amines of the protein and has an absorption/emission light spectrum at 496/524 nm, respectively. Next, the labeled enzyme was immobilized using the previously described dense CO_2 processing technique and again an ultrasonic cleaner was used to remove free enzyme from the micro-channel surfaces.

3.2 Activity of Immobilized *β*-Gal by Fluorescent Microscopy

The activity of the *β*–gal after immobilization into the PS was assessed in the capped micro-channels qualitatively using a fluorescent substrate, resorufin *β*–D-galactopyranoside. This molecule is composed of a galactose sugar unit with one of the oxygen atoms bonded to resorufin. The resorufin can be fluoresced via laser excitation after being separated from the sugar. *β*–gal cleaves the aforementioned bond, leaving the resorufin by itself and easily identifiable by fluorescent microscopy. If the *β*–gal is no longer active the bond cleavage will not occur, thus leaving the original molecule intact and non-fluorescable.

A dilute solution ($5x10^{-6}$ M) of resorufin *β*–D-galactopyranoside in dimethylsulfoxide (DMSO) was dripped onto a PS substrate with immobilized *β*–gal. The fluorescent molecule was excited

by a laser at 543 nm and monitored at 580-650 nm by a Leica DMIRE2 inverted stand fluorescent confocal microscope. The transmitted light images were illuminated using a 633 nm laser. This fluorescent enzymatic substrate solution was used to qualitatively verify the activity of the β–gal after the micro-channels had been capped by injecting the solution into the channels.

3.3 Lap Shear Bonding Samples

A study of the lap shear bond force was conducted to quantify how well the cap was bonded on to the micro-channels. An Instron 5567 equipped with a 10 kN load cell and a cross head speed of 1 mm/min was used to extensionally pull three samples at each condition to test them for their lap shear bond strength. Pieces of PS for lap shear bonding samples, Fig. 2, were cut from a compression molded PS sheet and micro-channels were hot embossed into one of the pieces to be bonded. Once the two pieces of PS were bonded together, tabs were glued on. The tabs were needed to make sure the extensional force during breaking was applied at the bonded interface and in a perpendicular geometry.

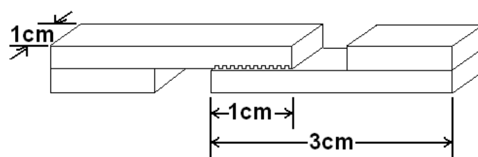

Figure 2. Schematic of lap shear bonding sample

Samples were all bonded at 40°C and with the same compressive force applied, but the CO_2 pressure and bonding time was varied. Two 19mm foldback clips (standard office binder clips) were used to apply the compressive force during bonding, just as the bonding during the capping of the micro-channels.

4. Results and Discussions

4.1 Enzyme Immobilization and Activity

Dense CO_2 was used to immobilize the enzyme, β–gal, on the walls of a PS channel. Carbon dioxide is inexpensive, readily available, environmentally benign, nontoxic, nonflammable, noncorrosive, a tunable plasticizer,[36] and has good sterilization properties.[48] Dense CO_2 is efficient as a polymer processing agent due to its gas-like transport properties and liquid-like densities. See Fig. 3 for a graphical representation of the following process. As a small linear molecule it readily diffuses into polymer matrices causing swelling, increased void space, enhanced polymer chain mobility,[47] and T_g depression.[49] The mechanism for enzyme immobilization in this process is thought to be analogous with that of the micro-void model by Von Schnitzler and Eggers.[50] Their model can be summarized as follows, when a polymer is exposed to dense CO_2, it swells, thus increasing the free volume between polymer chains. At this point the enzyme diffuses into the free volume, as known as micro-voids, of the polymer by a concentration gradient. The size of the micro-voids is unknown, but is thought to increase as the T_g is approached; the size of the folded β–gal is 17.5 x 13.5 x 9.0 nm.[51] The CO_2 pressure is then released, the polymer relaxes back to it original size, and the enzyme is physically trapped within the polymer matrix. A disadvantage to using this technique is that if the enzyme is immobilized too deeply into the polymer matrix, it will not have its active site positioned correctly at the surface of the channel, thus no reaction will occur.

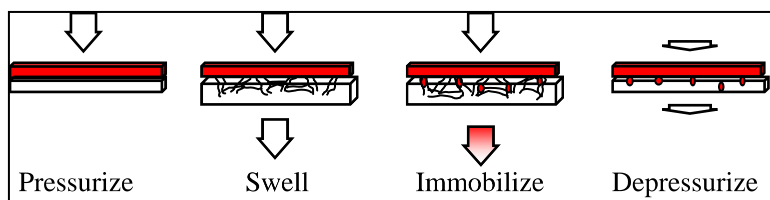

Figure 3. Mechanism for biomolecule immobilization. Red is the biomolecular solution, white is the polymer matrix with some polymer chains shown. Only the effects of CO_2 are shown, the molecule is not.

We demonstrated that the enzyme is immobilized in the PS micro-channels using a fluorescence imaging technique. A reconstructed stack of 2-D confocal images to form one 3-D image of the fluorescently labeled immobilized enzyme in the PS micro-channels is presented in Fig. 3. The control sample, with no immobilized enzyme (not shown), showed no fluorescence. The relative intensity of the imaged fluorophore increased 88% between the two experimental conditions, based on the average of 3 samples at each. Similar florescent intensities were measured on samples that had been processed at 4.48, 5.52 and 6.89 MPa. This significant intensity increase of the processed sample proves that dense CO_2 dramatically increases the quantity of enzyme immobilized into the micro-channels over low pressure (0.1 MPa) CO_2. The conditions used in this work have been shown to successfully immobilize the enzyme without causing the sample to foam.

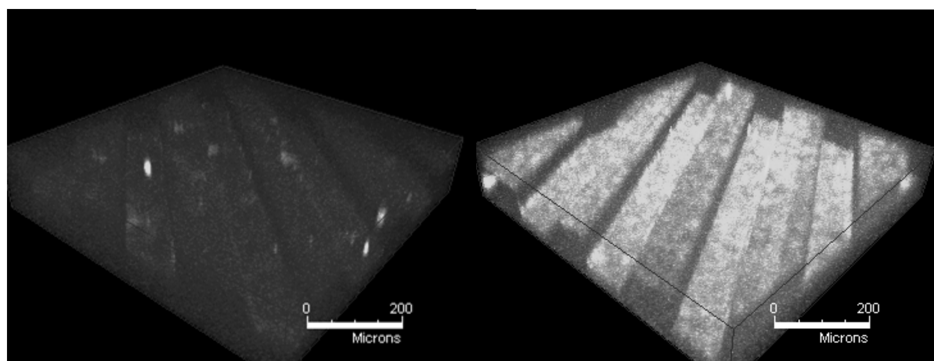

Figure 3. β–gal immobilization at 40°C, 2.5 hours, and CO_2 pressure of 0.1 MPa (left) and 6.89 MPa (right). The light areas are fluoresced molecules.

Fluorescent microscopy with high resolution and a low limit of detection was used with a reactive substrate to qualify that the enzyme was still active after processing. The results of the Leica fluorescent confocal microscopy analysis demonstrate that β–gal maintained activity after immobilization and capping of the micro-channel. In Fig. 4 both the control test (a and b) and also the validation of active β–gal after CO_2 processing (c and d) are depicted. In Fig 4a we

present an optical image of a micro-fluidic chip with a cap bonded on and no immobilized enzyme. The bonding conditions for capping the channels were T = 40°C, P_{CO_2}= 6.89 MPa, and t = 1.0 hour. The channels could not be imaged through the cap due to this particular piece of PS being slightly opaque, so the image was taken through part of a fluid injection/extraction port. Resorufin β–D-galactopyranoside in DMSO (5x10^{-6} M) was injected through the port and incubated for 30 minutes at room temperature. Fig. 4b shows limited auto-fluorescence after being excited at 543 nm.

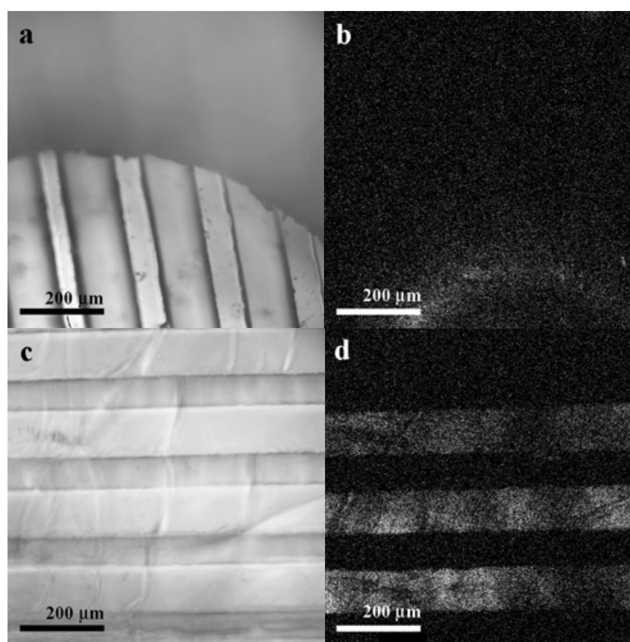

Figure 4. (a)Optical image through hole in cap of chip without immobilized enzyme (b)Fluorescent image of sample shows mild auto-fluorescence when resorufin β–D-galactopyranoside is added (c)Optical image through cap of chip with immobilized enzyme (d)Fluorescent image shows intense fluorescence in wells filled with resorufin confirming β–gal is active

An optical image of a micro-fluidic chip with immobilized enzyme is shown in Fig. 4c. The immobilization conditions were T = 40°C, P_{CO_2}= 6.89 MPa, and t = 2.5 hours and the bonding conditions were T = 40°C, P_{CO_2} = 6.89 MPa, and t = 1.0 hour. This image was taken through the cap because it was transparent and the micro-channels can be seen horizontally. Resorufin β–D-

galactopyranoside in DMSO ($5x10^{-6}$ M) was injected through the ports in the cap and was incubated for 30 minutes at room temperature. The laser was set to the same conditions as the control sample and the fluid in the micro-channels intensely fluoresced as can be observed in Fig. 4d; showing that the β–gal is active after CO_2 processing of enzyme immobilization and capping of the channels.

4.2 Bonding a Cap on Micro-Channels

Different bonding conditions were studied via measuring the lap shear bond strength using an Instron. The results are presented in Fig 5. The experimental conditions for the lap shear bond samples were chosen by using a 2x2 factorial design with a center point and with 3 replicates at each point. The two sample conditions at the lowest CO_2 pressure yielded bonds to weak to test. An analysis of variance test with α=0.05 was used to analyze the bond strength data for the effects of both CO_2 pressure and time on the bond strength. The analysis of variance test showed that CO_2 pressure (p=0.0073) had a significant effect and that time (p=0.3278) did not have a significant effect on bond strength.

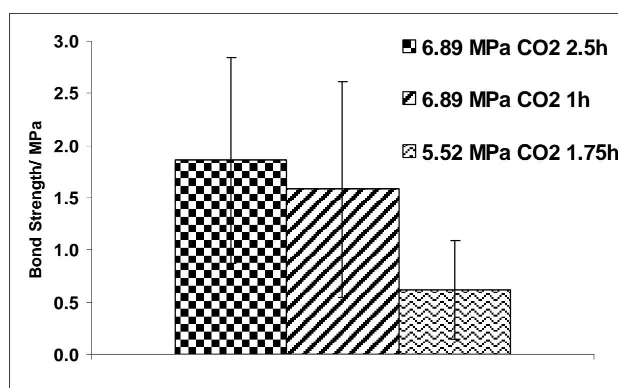

Figure 5. Lap shear bond test results (40°C). Error bars are 3σ from average.

It is critical in the fabrication of LOCs to retain the original microstructure dimensions and geometry during processing. For all samples fabricated in our study, none deformed enough to block the channels from fluid flow, hence all samples were still usable as LOCs. Optical images

of the cross section of a bonded micro-chip sample fabricated from PS by our dense gas process at bonding conditions of T = 40°C, P_{CO_2}= 6.89 MPa, and t = 2.5 hours are shown in Fig. 6, the cap is on the top and the dark rectangular sections are the micro-channels. Some places bonded with no deformation (left) and others bonded with substantial deformation (right), but all channels are still usable, thus proving the validity of this bonding technique for LOC devices.

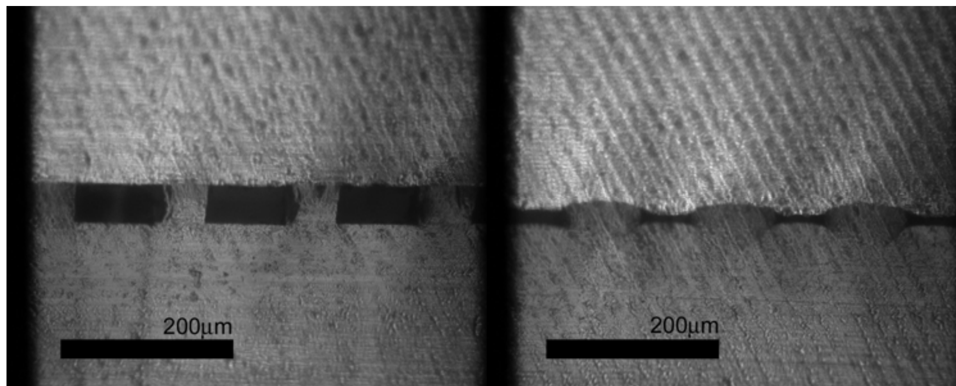

Figure 6. Optical images of the micro-channels hot embossed into PS after being capped at T = 40°C, P_{CO_2}= 6.89 MPa, and t = 2.5 hours with no deformation (left) and substantial deformation (right)

5. Conclusions

The new LOC fabrication technique developed in this study possesses distinct advantages over its predecessors. The primary advantage is the simplicity of the technique, as both enzyme immobilization and bonding of the cap on the micro-channels were performed by using dense CO_2 near biological temperature, without using multistep chemical reaction techniques. In addition, no volatile organic compounds (VOCs) were used in this environmentally friendly process and PS is a recyclable thermoplastic. The novel process opens an avenue for engineering LOCs from other specialty or biomedical polymers with a reasonably low T_g that can be plasticized by CO_2.

We were successful in both immobilizing an enzyme in micro-channels and bonding a cap on the channels using only environmentally benign dense CO_2. *β*–gal was immobilized in PS micro-

channels at 40°C using CO_2 at pressures from 4.48 to 6.89 MPa. We have shown by confocal fluorescent microscopy that the immobilization of the enzyme occurs to an appreciable extent only when dense CO_2 is used and not when low pressure is applied. The next step in this fabrication technique was to cap the biologically activated micro-channels with a second piece of PS by using CO_2 at 40°C and pressures of either 5.52 or 6.89 MPa. Both immobilization and capping processes were gentle enough to keep the fragile β–gal active; as shown by identifying the fluorescent product, resorufin, of a specific enzymatic reaction in the sealed micro-channels. The results of this study and other previous impregnation/immobilization studies of proteins demonstrate the potential of dense CO_2 for immobilization of various robust biomolecules into polymeric matrices at moderate temperatures. The depressurization profiles designed in this study eliminated the issue of foam formation in polymers with large CO_2 solubility. The process we have developed in this paper can be considered "green" due to the use of only non-toxic, non-corrosive, environmentally benign chemicals and it can be applied for the non-clean room fabrication of LOCs.

6. References

1. Lee, G. B.; Chen, S. H.; Huang, G. R.; Sung, W. C.; Lin, Y. H., Microfabricated plastic chips by hot embossing methods and their applications for DNA separation and detection. *Sensors and Actuators, B: Chemical* **2001,** B75, (1-2), 142-148.
2. Wang, J.; Tian, B.; Sahlin, E., Micromachined electrophoresis chips with thick-film electrochemical detectors. *Anal. Chem.* **1999,** 71, (23), 5436-5440.
3. Gardeniers, H.; Van Den Berg, A., Micro- and nanofluidic devices for environmental and biomedical applications. *Int. J. Environ. Anal. Chem.* **2004,** 84, (11), 809-819.
4. Vrouwe, E. X.; Luttge, R.; Vermes, I.; van den Berg, A., Microchip capillary electrophoresis for point-of-care analysis of lithium. *Clinical Chemistry (Washington, DC, United States)* **2007,** 53, (1), 117-123.
5. Verpoorte, E., Microfluidic chips for clinical and forensic analysis. *Electrophoresis* **2002,** 23, (5), 677-712.
6. Bowden, M.; Diamond, D., The determination of phosphorus in a microfluidic manifold demonstrating long-term reagent lifetime and chemical stability utilizing a colorimetric method. *Sensors and Actuators, B: Chemical* **2003,** B90, (1-3), 170-174.
7. Park, J. Y.; Kricka, L. J., Prospects for nano- and microtechnologies in clinical point-of-care testing. *Lab on a Chip* **2007,** 7, (5), 547-549.

8. Chin, C. D.; Linder, V.; Sia, S. K., Lab-on-a-chip devices for global health: Past studies and future opportunities. *Lab on a Chip* **2007,** 7, (1), 41-57.
9. Kok, F. N.; Bozoglu, F.; Hasirci, V., Construction of an acetylcholinesterase-choline oxidase biosensor for aldicarb determination. *Biosensors & Bioelectronics* **2002,** 17, (6-7), 531-539.
10. Fortier, G.; Belanger, D., Characterization of the biochemical behavior of glucose oxidase entrapped in a polypyrrole film. *Biotechnol. Bioeng.* **1991,** 37, (9), 854-8.
11. Quinn, C. P.; Pathak, C. P.; Heller, A.; Hubbell, J. A., Photo-crosslinked copolymers of 2-hydroxyethyl methacrylate, poly(ethylene glycol) tetra-acrylate and ethylene dimethacrylate for improving biocompatibility of biosensors. *Biomaterials* **1995,** 16, (5), 389-96.
12. Rejikumar, S.; Devi, S., Immobilization of b-galactosidase onto polymeric supports. *J. Appl. Polym. Sci.* **1995,** 55, (6), 871-8.
13. Sonawat, H. M.; Phadke, R. S.; Govil, G., Covalent immobilization of FAD and glucose oxidase on carbon electrodes. *Biotechnol. Bioeng.* **1984,** 26, (9), 1066-70.
14. Yavuz, H.; Bayramoglu, G.; Kacar, Y.; Denizli, A.; Yakup Arica, M., Congo Red attached monosize poly(HEMA-co-MMA) microspheres for use in reversible enzyme immobilization. *Biochemical Engineering Journal* **2002,** 10, (1), 1-8.
15. Ding, H.-M.; Shao, L.; Liu, R.-J.; Xiao, Q.-G.; Chen, J.-F., Silica nanotubes for lysozyme immobilization. *J. Colloid Interface Sci.* **2005,** 290, (1), 102-106.
16. Park, S.-S.; Cho, S. I.; Kim, M.-S.; Kim, Y.-K.; Kim, B.-G., Integration of on-column immobilized enzyme reactor in microchip electrophoresis. *Electrophoresis* **2003,** 24, (1-2), 200-206.
17. Delvaux, M.; Demoustier-Champagne, S., Immobilization of glucose oxidase within metallic nanotubes arrays for application to enzyme biosensors. *Biosensors & Bioelectronics* **2003,** 18, (7), 943-951.
18. Li, X.; Zhao, K.; Men, Y.; Tu, W., Immobilization and characteristics of b-galactosidase on polymer resin. *Bull. Soc. Chim. Belg.* **1996,** 105, (5), 217-222.
19. Zhou, Q. Z. K.; Chen, X. D., Effects of temperature and pH on the catalytic activity of the immobilized b-galactosidase from Kluyveromyces lactis. *Biochemical Engineering Journal* **2001,** 9, (1), 33-40.
20. Hashimoto, M.; Upadhyay, S.; Suzuki, H., Precise enzyme immobilization at the bottom of a micro flow channel and its application to a sensing system. *Chemical Sensors* **2004,** 20, (Suppl. A), 85-87.
21. Koh, W.-G.; Pishko, M., Immobilization of multi-enzyme microreactors inside microfluidic devices. *Sensors and Actuators, B: Chemical* **2005,** B106, (1), 335-342.
22. Bautista, F. M.; Campelo, J. M.; Garcia, A.; Jurado, A.; Luna, D.; Marinas, J. M.; Romero, A. A., Properties of a glucose oxidase covalently immobilized on amorphous AlPO4 support. *Journal of Molecular Catalysis B: Enzymatic* **2001,** 11, (4-6), 567-577.
23. Holden, M. A.; Jung, S.-Y.; Cremer, P. S., Patterning Enzymes Inside Microfluidic Channels via Photoattachment Chemistry. *Anal. Chem.* **2004,** 76, (7), 1838-1843.
24. Markov, D. A.; Swinney, K.; Bornhop, D. J., Label-Free Molecular Interaction Determinations with Nanoscale Interferometry. *J. Am. Chem. Soc.* **2004,** 126, (50), 16659-16664.
25. Jang, Y.; Oh, S. Y.; Park, J.-K., In situ electrochemical enzyme immunoassay on a microchip with surface-functionalized poly(dimethylsiloxane) channel. *Enzyme Microb. Technol.* **2006,** 39, (5), 1122-1127.

26. Yang, T.; Jung, S.-y.; Mao, H.; Cremer, P. S., Fabrication of Phospholipid Bilayer-Coated Microchannels for On-Chip Immunoassays. *Anal. Chem.* **2001,** 73, (2), 165-169.
27. Xiong, L.; Regnier, F. E., Channel-specific coatings on microfabricated chips. *Journal of Chromatography, A* **2001,** 924, (1-2), 165-176.
28. Wu, H.; Zhai, J.; Tian, Y.; Lu, H.; Wang, X.; Jia, W.; Liu, B.; Yang, P.; Xu, Y.; Wang, H., Microfluidic enzymatic-reactors for peptide mapping: strategy, characterization, and performance. *Lab on a Chip* **2004,** 4, (6), 588-597.
29. Lin, T.-Y.; Wu, C.-H.; Brennan, J. D., Entrapment of horseradish peroxidase in sugar-modified silica monoliths: Toward the development of a biocatalytic sensor. *Biosensors & Bioelectronics* **2007,** 22, (9-10), 1861-1867.
30. Podual, K.; Doyle, F. J.; Peppas, N. A., Glucose-sensitivity of glucose oxidase-containing cationic copolymer hydrogels having poly(ethylene glycol) grafts. *J. Controlled Release* **2000,** 67, (1), 9-17.
31. Retama, J. R.; Lopez-Ruiz, B.; Lopez-Cabarcos, E., Microstructural modifications induced by the entrapped glucose oxidase in crosslinked polyacrylamide microgels used as glucose sensors. *Biomaterials* **2003,** 24, (17), 2965-2973.
32. Sahney, R.; Anand, S.; Puri, B. K.; Srivastava, A. K., A comparative study of immobilization techniques for urease on glass-pH-electrode and its application in urea detection in blood serum. *Anal. Chim. Acta* **2006,** 578, (2), 156-161.
33. Kazarian, S. G.; Brantley, N. H.; West, B. L.; Vincent, M. F.; Eckert, C. A., In situ spectroscopy of polymers subjected to supercritical CO2: plasticization and dye impregnation. *Appl. Spectrosc.* **1997,** 51, (4), 491-494.
34. Kazarian, S. G.; Martirosyan, G. G., Spectroscopy of polymer/drug formulations processed with supercritical fluids: in situ ATR-IR and Raman study of impregnation of ibuprofen into PVP. *Int. J. Pharm.* **2002,** 232, (1-2), 81-90.
35. Muth, O.; Hirth, T.; Vogel, H., Polymer modification by supercritical impregnation. *Journal of Supercritical Fluids* **2000,** 17, (1), 65-72.
36. Nikitin, L. N.; Gallyamov, M. O.; Vinokur, R. A.; Nikolaec, A. Y.; Said-Galiyev, E. E.; Khokhlov, A. R.; Jespersen, H. T.; Schaumburg, K., Swelling and impregnation of polystyrene using supercritical carbon dioxide. *Journal of Supercritical Fluids* **2003,** 26, (3), 263-273.
37. Sproule, T. L.; Lee, J. A.; Li, H.; Lannutti, J. J.; Tomasko, D. L., Bioactive polymer surfaces via supercritical fluids. *Journal of Supercritical Fluids* **2004,** 28, (2-3), 241-248.
38. Kayrak-Talay, D.; Akman, U.; Hortacsu, O., Glucose oxidase immobilization on conducting polymers in supercritical CO2 environment: An exploratory study. *Journal of Supercritical Fluids* **2007,** 42, (2), 273-281.
39. Powell, H. M.; Ayodeji, O.; Summerfield, T. L.; Powell, D. M.; Kniss, D. A.; Tomasko, D. L.; Lannutti, J. J., Chemotherapeutic implants via subcritical CO2 modification. *Biomaterials* **2007,** 28, (36), 5562-5569.
40. Ryu, W.; Min, S. W.; Hammerick, K. E.; Vyakarnam, M.; Greco, R. S.; Prinz, F. B.; Fasching, R. J., The construction of three-dimensional micro-fluidic scaffolds of biodegradable polymers by solvent vapor based bonding of micro-molded layers. *Biomaterials* **2006,** 28, (6), 1174-1184.
41. Brown, L.; Koerner, T.; Horton, J. H.; Oleschuk, R. D., Fabrication and characterization of poly(methyl methacrylate) micro fluidic devices bonded using surface modifications and solvents. *Lab Chip* **2006,** 6, (1), 66-73.

42. Kettner, P.; Pelzer, R. L.; Glinsner, T.; Farrens, S., New results on plasma activated bonding of imprinted polymer features for Bio MEMS applications. *Journal of Physics: Conference Series* **2006,** 34, 65-71.
43. Su, Y.-C.; Lin, L., Localized bonding processes for assembly and packaging of polymeric MEMS. *IEEE Transactions on Advanced Packaging* **2005,** 28, (4), 635-642.
44. Lu, C.; Xie, Y.; Yang, Y.; Cheng, M. M. C.; Koh, C.-G.; Bai, Y.; Lee, L. J.; Juang, Y.-J., New Valve and Bonding Designs for Microfluidic Biochips Containing Proteins. *Anal. Chem.* **2007,** 79, (3), 994-1001.
45. Yang, Y.; Lee, L. J.; Lu, W., Subcritical carbon dioxide assisted polymer nanofabrication at low temperatures. *Journal of Vacuum Science & Technology, B: Microelectronics and Nanometer Structures--Processing, Measurement, and Phenomena* **2005,** 23, (6), 3202-3204.
46. Yang, Y.; Basu, S.; Tomasko, D. L.; Lee, L. J.; Yang, S.-T., Fabrication of well-defined PLGA scaffolds using novel micro-embossing and carbon dioxide bonding. *Biomaterials* **2005,** 26, (15), 2585-2594.
47. Yang, Y.; Zeng, C.; Lee, L. J., Three-dimensional assembly of polymer microstructures at low temperatures. *Advanced Materials (Weinheim, Germany)* **2004,** 16, (6), 560-564.
48. Dillow, A. K.; Dehghani, F.; Hrkach, J. S.; Foster, N. R.; Langer, R., Bacterial inactivation by using near- and supercritical carbon dioxide. *Proc. Natl. Acad. Sci. U. S. A.* **1999,** 96, (18), 10344-10348.
49. Keddie, J. L.; Jones, R. A. L.; Cory, R. A., Size-dependent depression of the glass transition temperature in polymer films. *Europhys. Lett.* **1994,** 27, (1), 59-64.
50. Von Schnitzler, J.; Eggers, R., Mass transfer in polymers in a supercritical CO2 atmosphere. *J. Supercrit. Fluids* **1999,** 16, (1), 81-92.
51. Jacobson, R. H.; Zhang, X. J.; DuBose, R. F.; Matthews, B. W., Three-dimensional structure of beta -galactosidase from E. coli. *Nature (London)* **1994,** 369, (6483), 761-6.
52. Hofland, G. W.; de Rijke, A.; Thiering, R.; van der Wielen, L. A. M.; Witkamp, G. J., Isoelectric precipitation of soybean protein using carbon dioxide as a volatile acid. *Journal of Chromatography, B: Biomedical Sciences and Applications* **2000,** 743, (1 + 2), 357-368.