

Novel Dense CO₂ Technique for β -galactosidase Immobilization in Polystyrene Microchannels

In this study we design new fabrication techniques and demonstrate the potential of using dense CO₂ for facilitating crucial steps in the fabrication of polymeric lab-on-a-chip (LOC) microdevices by embedding biomolecules at temperatures well below the polymer's glass transition temperature (T_g). These new techniques are environmentally friendly and done without the use of a cleanroom. 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 polystyrene (PS) microchannels. To our knowledge, this is the first time dense CO₂ has been used to directly immobilize an enzyme in a microchannel. β -gal activity was maintained and shown via a fluorescent reaction product after enzyme immobilization and microchannel capping by the designed fabrication steps at 40°C and pressures up to 6.89 MPa.

Enzyme Immobilization and Activity

Dense CO₂ 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, and has good sterilization properties. Dense CO₂ is an efficient polymer processing agent because of its gas-like transport properties and liquid-like densities. As a small linear molecule, it readily diffuses into polymer matrices causing swelling, increased void space, enhanced polymer chain mobility, and glass transition temperature depression. 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. Their model can be summarized as follows: when a polymer is exposed to dense CO₂, 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. The CO₂ pressure is then released, the polymer relaxes back to its 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 available for reacting.

We demonstrate that the enzyme is immobilized in the PS microchannels using a fluorescence imaging technique. A reconstructed stack of 2-D confocal images that forms one 3-D image of the fluorescently labeled immobilized enzyme in the PS microchannels is presented in Figure 1. 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 fluorescent 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 CO₂ dramatically increases the quantity of enzyme immobilized into the microchannels over low pressure (0.1 MPa) CO₂. The conditions used in this work have been shown to successfully immobilize the enzyme without causing the sample to foam, but they have not been optimized for enzyme loading or processing time.

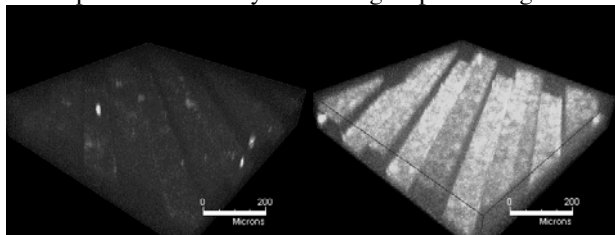


Figure 1. β -gal immobilization at 40°C, 2.5 hours, and CO₂ pressure of 0.1 MPa (left) and 6.89 MPa (right)

The results of the Leica fluorescent confocal microscopy analysis demonstrate that β -gal maintained activity after immobilization and capping of the microchannel. Both the control test (a and b) and also the validation of active β -gal after CO₂ processing (c and d) are depicted in Figure 2. In Figure 2a we present an optical image of a microfluidic chip with a cap bonded on and no immobilized enzyme. The bonding conditions for capping the channels were $T = 40^\circ\text{C}$, $P_{\text{CO}_2} = 6.89 \text{ MPa}$, and $t = 1.0 \text{ hour}$. The channels could not be imaged through the cap because this particular piece of PS was slightly opaque, so the image was taken through part of a fluid injection/extraction port. Resorufin β -D-galactopyranoside in DMSO ($5 \times 10^{-6} \text{ M}$) was injected through the port and incubated for 30 minutes at room temperature. Figure 2b shows limited auto-fluorescence after being excited at 543 nm.

An optical image of a microfluidic chip with immobilized enzyme is shown in Figure 2c. The immobilization conditions were $T = 40^\circ\text{C}$, $P_{\text{CO}_2} = 6.89 \text{ MPa}$, and $t = 2.5 \text{ hours}$, and the bonding conditions were $T = 40^\circ\text{C}$, $P_{\text{CO}_2} = 6.89 \text{ MPa}$, and $t = 1.0 \text{ hour}$. This image was taken through the cap because it was transparent and the microchannels can be seen horizontally. Resorufin β -D-galactopyranoside in DMSO ($5 \times 10^{-6} \text{ M}$) 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 Figure 2d, corroborating that the β -gal is active after CO₂ processing of enzyme immobilization and capping of the channels.

Bonding a Cap on Microchannels

It is critical in the fabrication of LOCs to retain the original microstructure dimensions and geometry during processing. The ideal case would be high bond strength

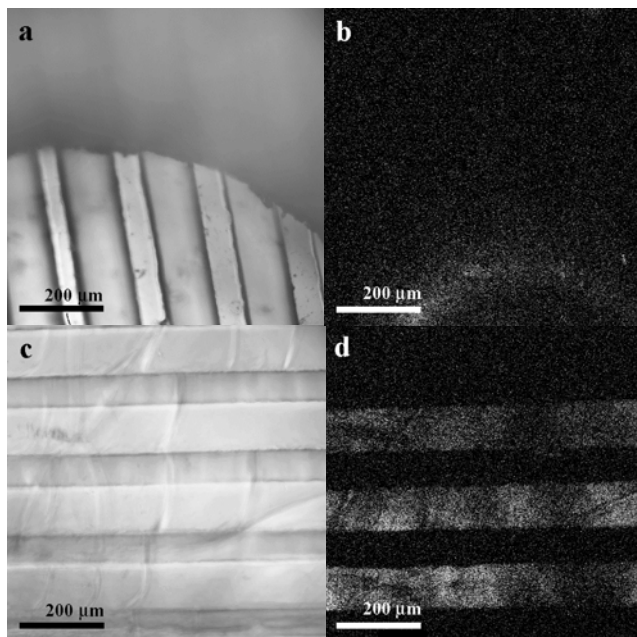


Figure 2. (a) Optical image through hole in cap of chip without immobilized enzyme; (b) Fluorescent image of sample in a shows mild auto-fluorescence when resorufin β -D-galactopyranoside is added; (c) Optical image through cap of chip with immobilized enzyme; (d) Fluorescent image of sample in c shows intense fluorescence in wells filled with resorufin confirming β -gal is active.

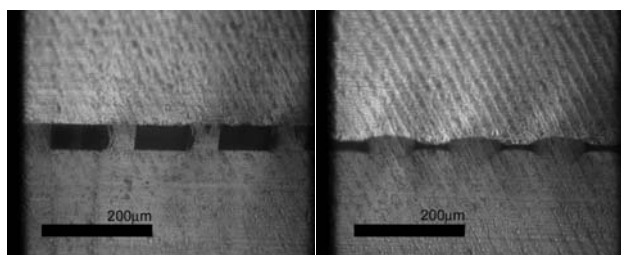


Figure 3. Optical images of the micro-channels hot embossed in to PS after being capped at $T = 40^{\circ}\text{C}$, $P_{\text{CO}_2} = 6.89 \text{ MPa}$, and $t = 2.5$ hours with no deformation (left) and substantial deformation (right)

with no deformation of the microstructures. For all samples fabricated in our study, the microchannel structure was transferred to the bonded cap to some extent, but none of the samples deformed enough to block the channels from fluid flow, hence all samples were still usable as microfluidic devices. Optical images, taken by an Olympus BX60 microscope equipped with an Olympus DP10 digital camera and a 20x lens, of the cross section of a bonded microchip sample fabricated from PS by our dense gas process at bonding conditions of $T = 40^{\circ}\text{C}$, $P_{\text{CO}_2} = 6.89 \text{ MPa}$, and $t = 2.5$ hours are shown in Figure 3, the cap is on top of the PS containing the microchannels. Both images are from the same cross section at different locations. The sample was first scored on both the top and bottom using a stainless steel surgical knife and then broken at the score line, all at room temperature. The fracture left a rough edge, so it was milled until smooth. As shown, some places bonded with

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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.

Conclusions

We were successful in both immobilizing an enzyme in microchannels and bonding a cap on the channels using only environmentally benign dense CO_2 . β -galactosidase was immobilized in polystyrene microchannels at 40°C using CO_2 at pressures from 4.48 to 6.89 MPa in a process that took 5 hours to complete. We have shown by confocal fluorescence 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 microchannels with a second piece of PS by using CO_2 at 40°C and pressures of either 5.52 or 6.89 MPa in a process that took up to 5 hours. 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 microchannels. The results of this study and other previous impregnation/immobilization studies of proteins demonstrate the potential of dense CO_2 for immobilizing various robust biomolecules into polymeric matrices at moderate temperatures. The depressurization profiles designed in this study can eliminate the issue of foam formation in polymers with large CO_2 solubility that may occur in dense gas systems. The process we have developed in this paper can be considered “green” because it uses only non-toxic, non-corrosive, environmentally benign chemicals and it can be applied for the non-cleanroom fabrication of lab-on-a-chip devices.

Polymer Solvent Welding via CO_2

A polymer welding apparatus has been fabricated. It is a stainless steel high pressure vessel capable of handling pressures up to 15 MPa at 150°C . It has windows on opposing sides in order to visualize the sample during welding. Also, it has a sample stage that is translatable in its vertical position and a load cell located directly above it, in order to measure the compressive force applied to the sample during the welding process. It also contains an RTD to monitor temperature *in situ*.

Polymers, shaped like a “T”, will be welded together as butt end samples at conditions varying temperature, CO_2 pressure, compressive force, and time. They will then be pulled by an Instron to obtain a stress vs. strain plot and, ultimately, their bond strength. This data will be used to further understand the polymer chain welding mechanism and to create a thermodynamic model that can predict bond strength based on processing conditions.

Publications

1. Ellis, J., Tomasko, D., Dehghani, F., “A Novel Integrated Technique for Fabricating Polymeric Lab-on-a-Chip Devices with an Immobilized Enzyme,” accepted in *Biomacromolecules* (Jan 2008)

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