

# Immobilization of Enzymes onto Porous Monolithic Polymer Supports to Facilitate Fabrication of Micro- and Nanofactories

Immobilization of enzymes has been studied for several decades. The very early motivation was to replace as many industrial processes as possible that were catalyzed with enzymes in solution with their counterparts immobilized on a support. The advantages are obvious. The catalyst can be easily removed from the reaction mixture, thus facilitating separation of product, and can be recycled while its activity is preserved. Also, a continuous processes with the biocatalyst immobilized on a solid support placed in a reactor is feasible. These implementations enable a significant decrease in the cost of the biocatalyst used per unit of product, which represents significant savings in operations requiring expensive enzymes. Enzyme catalyzed reactions re-appeared in headlines with the advent of proteomics. In contrast to industrial applications of immobilized enzymes, the sample size in proteomics is very small. Therefore, miniature immobilized enzyme reactors placed in capillaries or microfluidic devices are highly suitable for proteomic studies. An additional benefit is the option to directly link the microreactor to a mass spectrometer.

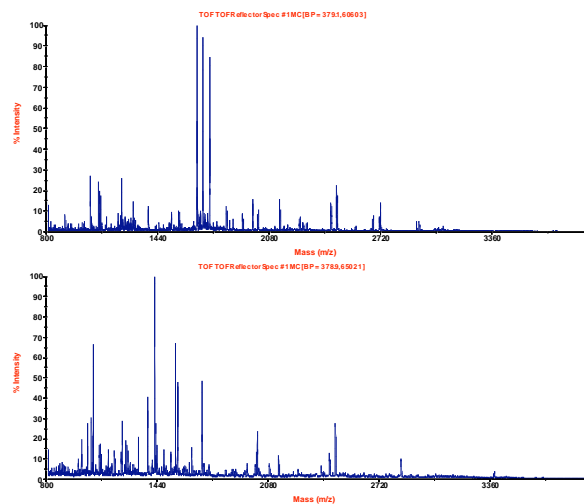
Traditionally, organic polymers have been very popular supports for enzymes. Porous polymer monoliths emerged first as a new class of stationary phases for HPLC in the early 1990s. Soon thereafter, they also were used as supports for the immobilization of proteins and the preparation of both stationary phases for bioaffinity chromatography and enzymatic reactors. These monoliths are typically prepared from a mixture comprising monomers, an initiator, and a porogenic solvent using a simple molding process carried out within the confines of a microfluidic “mold,” such as a capillary or microchip. The major advantage of monolithic supports is their outstanding mass transfer via the convective flow through the pores. Using the novel photografting techniques described in our 2007 report, polymer monoliths can be surface-modified so that they effectively resist non-specific protein adsorption. After hydrophilic photografting, protein-resistant polymer monoliths are well-suited for use as inert, high surface area supports for patterned enzyme immobilization, allowing for the formation of high activity flow-through enzymatic microreactors.

## Immobilization of Trypsin for Rapid Protein Digestion

In contrast to reactive monolithic structures prepared via direct copolymerization of vinylazlactone with ethylene dimethacrylate demonstrated previously, we have now developed a multistep process consisting of (i) polymerization of a monolith from glycidyl methacrylate

and ethylene dimethacrylate, (ii) hydrolysis of epoxide groups to increase hydrophilicity of the monolith, (iii) enhancing hydrophilization via UV initiated grafting of a hydrophilic monomer poly(ethylene glycol) methacrylate and (iv) re-activation using vinylazlactone. This approach enables better control of the surface coverage of the monolith with reactive functionalities and avoids undesired adsorption of proteins and peptides in the reactor.

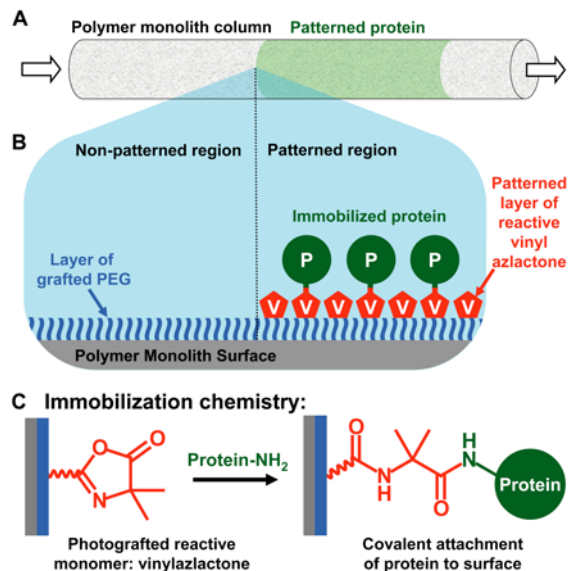
Trypsin was immobilized on these monolithic microcarriers. After polymerization and immobilization conditions were optimized, highly active enzymatic microreactors were obtained and their ability to digest proteins of higher molecular masses tested. Both bovine serum albumin and polyclonal IgG were digested to a high extent in about 4 min, and the peptides were monitored using MALDI TOF MS. The identification of BSA based on peptide mass fingerprinting resulted in the sequence coverage of 44%. Despite the slightly lower sequence coverage compared to digest soluble with soluble enzyme (44% vs. 64%), digestion with the immobilized enzyme was obtained in a much shorter period of time (4 min vs. 24 h) and at a lower temperature of 22 °C. Figure 1 compares the digestion of even larger protein IgG using both soluble and immobilized trypsin and demonstrates again the more efficient digestion using the immobilized trypsin reactor.



**Figure 1.** MALDI/TOF-MS spectrum of polyclonal IgG digest obtained using soluble trypsin at 37 °C and 24 h reaction time (top) and trypsin immobilized on monolithic support digesting at 22 °C for 4.3 min (bottom).

## Patterned, Spatially Localized Immobilization of Proteins and Fabrication of a Nanofactory

In continuation of our efforts reported in 2007, we have developed a method for photo-patterning multiple enzymes on porous polymer monoliths within microfluidic devices and used it to perform spatially-separated multi-enzymatic reactions. To reduce non-specific adsorption of enzymes on the monolith, its pore surface was modified by grafting poly(ethylene glycol) (PEG), followed by surface photoactivation and enzyme immobilization in the presence of a non-ionic surfactant. In the next step, we employed azlactone attachment chemistry for covalent immobilization of the enzyme to the polymer monolith supports, whereby reactive azlactone groups are introduced onto the monolith surface by photografting the surface with vinyl azlactone. The resulting azlactone functionalities react with amines on proteins, as illustrated in Figure 2. The coupling reaction is relatively rapid and not overly sensitive to hydrolysis. No leaving groups are produced during the immobilization reaction, since azlactone reacts via a ring-

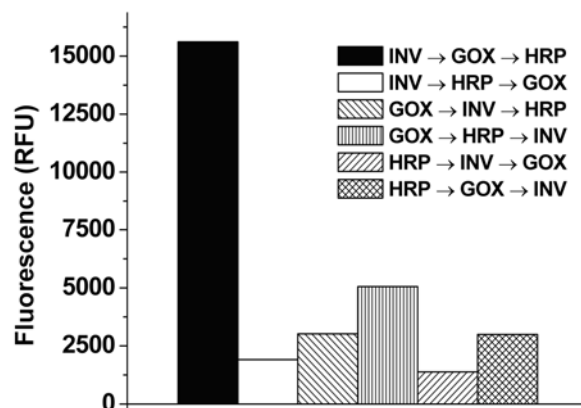


**Figure 2.** Schematic diagram of the photo-patterning process. (A) Protein is immobilized to the surface of a polymer monolith in patterned regions within a microfluidic channel. (B) PEG is grafted to the surface of the polymer monolith to prevent non-specific protein adsorption. Vinyl azlactone is photo-patterned onto the PEG surface and activates the surface for protein immobilization. (C) Azlactone functionality reacts with amines of proteins to form a covalent amide bond between the protein and the polymer monolith surface.

opening, nucleophilic addition. In addition, the azlactone attachment chemistry does not require any modifications of the enzyme prior to immobilization.

Sequential multi-enzymatic reactions were demonstrated using the patternwise assembly of two- and three-enzyme systems. Glucose oxidase (GOX) and HRP

were patterned in separate regions of a single channel and product formation was analyzed as a function of flow direction. Significant product formation occurred only in the GOX to HRP direction.



**Figure 3.** Directional synthesis with a three-enzyme system: INV, GOX, and HRP. Conditions: monolithic support, 9 cm long; INV, GOX, and HRP patterned to a length of 4, 4, and 1 cm, respectively; Product fluorescence was measured from columns prepared with each possible arrangement of the three enzymes, as indicated in the insert; The substrate solution consisted of 10 mg/mL sucrose, 100 μmol/L Amplex Red, and 1.0 % (v/v) DMSO in 50 mmol/L phosphate buffer, pH 7.50; pure oxygen was bubbled through this solution for 15 minutes prior to use. The flow rate was 0.10 μL/min.

A three-enzyme sequential reaction was then performed using invertase (INV), GOX, and HRP. All possible arrangements of the three enzymes were tested, but significant product formation was only observed when the enzymes were in the correct sequential order, as demonstrated in Figure 3. Photo-patterning enzymes on polymer monoliths provide a simple technique for preparing spatially localized multiple-enzyme micro-reactors capable of directional synthesis.

## Publications

1. T.B. Stachowiak, D.A. Mair, T.G. Holden, F. Svec, J.M.J. Fréchet, J. Lee, "Hydrophilic surface modification of cyclic olefin copolymer microfluidic chips using sequential photografting," *Journal of Separation Science* 30, 1088-1093, 2007.