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Enzymatic Microreactor-on-a-Chip: Protein Mapping Using Trypsin Immobilized on Porous Polymer Monoliths Molded in Channels of Microfluidic Devices

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Enzymatic microreactors have been prepared in capillaries and on microfluidic chips by immobilizing trypsin on porous polymer monoliths consisting of 2-vinyl-4,4-dimethylazlactone, ethylene dimethacrylate, and acrylamide or 2-hydroxyethyl methacrylate. The azlactone functionalities react readily with amine and thiol groups of the enzyme to form stable covalent bonds. The optimized porous properties of the monoliths lead to very low back pressures enabling the use of simple mechanical pumping to carry out both the immobilization of the enzyme from its solution and the subsequent analyses of substrate solutions. The Michealis−Menten kinetic characteristics of the reactors were probed using a low molecular weight substrate: *N*-α-benzoyl-L-arginine ethyl ester. The effects of immobilization variables such as the concentration of trypsin in solution and percentage of azlactone functionalities in the monolith, as well as the effect of reaction time on the enzymatic activity, and of process variables such as substrate flow velocity and residence time in the reactor, were studied in detail. The proteolytic activity of the enzymatic microreactor on chip was demonstrated at different flow rates with the cleavage of fluorescently labeled casein used as a substrate. The excellent performance of the monolithic microreactor was also demonstrated with the digestion of myoglobin at the fast flow rate of 0.5 μL/min, which affords a residence time of only 11.7 s. The digest was then characterized using MALDI-TOF MS, and 102 out of 153 possible peptide fragments were identified giving a sequence coverage of 67%.

An enormous effort has been directed toward the development of new microfabricated analytical devices and their integration to create micro total analytical systems (μTAS). These systems offer the promise of increased throughput, lower sample and reagent consumption, smaller size, and lower operating costs than full size instrumentation.[1,2] Among the various applications of microfluidic devices, analytical techniques such as electrophoresis,[3−8] electrochromatography,[9−13] assays involving enzymes,[3,14−18] and immunoassays[19−21] have already been demonstrated in this format. Despite the undeniable success of microfluidic chip technologies, some problems persist. For example, the vast majority of microfluidic chips feature open channel architecture. Consequently, these channels exhibit rather small surface-to-volume ratios. This may be a serious problem in applications such as chromatographic separations, solid-phase extraction, and heterogeneous catalysis that rely on interactions with a solid surface. Since only the channel walls can be used to provide the desired interactions, the microdevice can only handle minute amounts of compounds.

The use of immobilized proteins in analytical processes is well established, and a number of routine assays are based on

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(1) van den Berg, A.; Lammerink, T. S. J. *Top. Curr. Chem.* **1998**, *194*, 21−49.
(2) Bruin, G. J. M. *Electrophoresis* **2000**, *21*, 3931−3951.
(3) Gao, J.; Xu, J. D.; Locascio, L. E.; Lee, C. S. *Anal. Chem.* **2001**, *73*, 2648−2655.
(4) Hofmann, O.; Che, D. P.; Cruickshank, K. A.; Muller, U. R. *Anal. Chem.* **1999**, *71*, 678−686.
(5) Kutter, J. P.; Jacobson, S. C.; Matsubara, N.; Ramsey, J. M. *Anal. Chem.* **1998**, *70*, 3291−3297.
(6) Lazar, I. M.; Ramsey, R. S.; Ramsey, J. M. *Anal. Chem.* **2001**, *73*, 1733−1739.
(7) Liu, Y. J.; Foote, R. S.; Jacobson, S. C.; Ramsey, R. S.; Ramsey, J. M. *Anal. Chem.* **2000**, *72*, 4608−4613.
(8) Wang, J.; Chatrathi, M. P.; Mulchandani, A.; Chen, W. *Anal. Chem.* **2001**, *73*, 1804−1808.
(9) Ceriotti, L.; de Rooij, N. F.; Verpoorte, E. *Anal. Chem.* **2002**, *74*, 639−647.
(10) Ericson, C.; Liao, J. L.; Nakazato, K.; Hjertén, S. *J. Chromatogr., A* **1997**, *767*, 33−41.
(11) Ericson, C.; Holm, J.; Ericson, T.; Hjertén, S. *Anal. Chem.* **2000**, *72*, 81−87.
(12) Ngola, S. M.; Fintschenko, Y.; Choi, W. Y.; Shepodd, T. J. *Anal. Chem.* **2001**, *73*, 849−856.
(13) Throckmorton, D. J.; Shepodd, T. J.; Singh, A. K. *Anal. Chem.* **2002**, *74*, 784−789.
(14) Drott, J.; Lindstrom, K.; Rosengren, L.; Laurell, T. *J. Micromech. Microeng.* **1997**, *7*, 14−23.
(15) Laurell, T.; Drott, J.; Rosengren, L.; Lindstrom, K. *Sens. Actuators, B* **1996**, *31*, 161−166.
(16) Mao, H.; Yang, T.; Cremer, P. T. *Anal. Chem.* **2002**, *74*, 379−385.
(17) Wang, J.; Chatrathi, M. P.; Tian, B. M. *Anal. Chem.* **2000**, *72*, 5774−5778.
(18) Wang, J. *Electrophoresis* **2002**, *23*, 713−718.
(19) Sato, K.; Tokeshi, M.; Odake, T.; Kimura, H.; Ooi, T.; Nakao, M.; Kitamori, T. *Anal. Chem.* **2000**, *72*, 1144−1147.
(20) Dodge, A.; Fluri, K.; Verpoorte, E.; de Rooij, N. F. *Anal. Chem.* **2001**, *73*, 3400−3409.
(21) Sato, K.; Tokeshi, M.; Kimura, H.; Kitamori, T. *Anal. Chem.* **2001**, *73*, 1213−1218.

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enzymatic reactions and immunoabsorption.[22] This has led to attempts to extend these techniques to the microfluidic format. For example, Dodge et al.[20] immobilized protein A on the wall of a microfluidic chip and used it for heterogeneous immunoassays. Sato et al.[19] introduced polystyrene beads with immobilized antibodies in the channel of a microchip and demonstrated its use in an assay of antigens. The use of the solid support enabled a dramatic increase in sensitivity, while the microfluidic format decreased the time of analysis from hours to minutes. Richter et al.[23] immobilized xanthine oxidase and horseradish peroxidase on glass beads for the determination of xanthine using chemiluminescent detection.

The rapid acceleration of research in the area of proteomics now requires the development of new technologies. Peptide mapping is one of the methods that appear ideally suited for both the identification of proteins and the determination of posttranslational modifications.[6,24] The protein of interest is digested by a proteolytic enzyme, most often trypsin, and the peptides in the resulting mixture are identified using mass spectrometry. This process affords a peptide map that is unique for each protein and allows identification through a search of existing databases. However, the use of soluble trypsin also results in its autodigestion with the undesired formation of additional peptides, which may complicate the unambiguous assignment of the studied protein.[6] Such unwanted autodigestion can be eliminated by site isolation of the proteolytic enzyme moieties via immobilization on a solid support.[25] Obviously, such immobilization may only occur on surfaces that are fully accessible to the enzyme and will not hinder subsequent access of substrate to the active sites. The larger the surface available on the support, the higher the amount of the accommodated enzyme. Therefore, porous supports are favored and some conjugates of enzymes and porous polymer bead conjugates have already been commercialized (Poroszyme, Applied Biosystems) for use in conjunction with ESI mass spectrometry.

It is generally thought that microscale protein digestion will enable faster and automated protein identification via peptide mapping while using very small quantities of samples.[26] Indeed, several reports supporting this assumption have already been published.[6,27−29] For example, Ekstrom et. al.[28] significantly improved the solution-phase methodology carried out in microfabricated nanovials reported originally by Litborn et al.[27] by covalently immobilizing trypsin on the surface of microvials that had been first anodized to make them porous and therefore to increase their surface area. Wang et al.[29] developed a microfluidic system that enabled the packing of a cavity in a chip with immobilized enzyme beads through a side channel. They found that this "packed bed" reactor enabled better protein processing at a higher speed.

Despite such initial successful demonstrations, beads remain seldom used in microfluidic devices due to the difficulty of packing a bed of beads in the channels of a microchip. In contrast, it is much easier to produce a porous polymer monolith in a chip using an in situ approach. The rigid monolithic materials that were introduced in the early 1990s were first used in a number of macroscopic applications.[30,31] Their preparation, utilizing the recently developed photolithographic-like technique involving photopolymerization through a mask, enables the formation of reactive porous monoliths within a specific part of a microfluidic channel system.[32] This technology has now been demonstrated in the fabrication of microfluidic columns for electrochromatography,[12,33,34] solid-phase extraction devices,[35] and static mixers.[36]

Macroscopic monolithic materials have also been used as supports for immobilization of proteins.[37−39] An overview of these results has recently been presented in a short review.[40] However, the glycidyl methacrylate-based monoliths have epoxide functionalities that react only slowly with proteins, and extended reaction times of up to 5 days are required to achieve the desired activity.[39] Multistep reaction schemes involving the modification of epoxide groups with a diamine followed by activation using a dialdehyde accelerate the overall immobilization process, but they remain impractical for use on a chip.[37] In contrast, the use of 2-vinyl-4,4-dimethylazlactone introduced for beads by Heilmann et. al.[41,42] of the 3M Company more than a decade ago has enabled us to prepare very reactive monolithic materials by copolymerization of this monomer with a cross-linker.[43,44] The azlactone functionality of these monoliths readily reacts with the amino or thiol groups of proteins thereby enabling their rapid and efficient immobilization.[38]

Following our success with the immobilization of proteins using Heilmann's azlactone chemistry[38] as well as the microfabrication of both solid-phase extraction microdevices[35] and micromixers[36] on a chip using photopolymerized polymer monoliths with well-controlled porous properties, we had anticipated that the application of azlactone monolith in microfluidic format would also be well-suited for the fabrication of an enzymatic microreactor on chip. In this paper, we report the preparation of reactive porous

(22) Tischer, W.; Wedekind, F. *Top. Curr. Chem.* **1999**, *200*, 95−126.
(23) Richter, T.; Shultz-Lockyear, L. L.; Oleschuk, R. D.; Bilitewski, U.; Harrison, D. J. *Sens. Actuators, B* **2002**, *81*, 369−376.
(24) Gevaert, K.; Vandekerckhove, J. *Electrophoresis* **2000**, *21*, 1145−1154.
(25) Moulin, C. In *Methods in Enzymology*; Colowick, S. P., Caplan, N. O., Eds.; Academic Press: New York, 1987; Vol. 136.
(26) Sanders, G. H. W.; Manz, A. *Trends Anal. Chem.* **2000**, *19*, 364−378.
(27) Litborn, E.; Emmer, A.; Roeraade, J. *Anal. Chim. Acta* **1999**, *401*, 11−19.
(28) Ekstrom, S.; Onnerfjord, P.; Nilsson, J.; Bengtsson, M.; Laurell, T.; Marko-Varga, G. *Anal. Chem.* **2000**, *72*, 286−293.
(29) Wang, C.; Oleschuk, R.; Ouchen, F.; Li, J. J.; Thibault, P.; Harrison, D. J. *Rapid Commun. Mass. Spectrom.* **2000**, *14*, 1377−1383.
(30) Svec, F.; Fréchet, J. M. J. *Anal. Chem.* **1992**, *64*, 820−822.
(31) Tennikova, T. B.; Bleha, M.; Svec, F.; Almazova, T. V.; Belenkii, B. G. *J. Chromatogr.* **1991**, *555*, 97−107.
(32) Yu, C.; Svec, F.; Fréchet, J. M. J. *Electrophoresis* **2000**, *21*, 120−127.
(33) Fintschenko, Y.; Choi, W. Y.; Ngola, S. M.; Shepodd, T. J. *Fresenius J. Anal. Chem.* **2001**, *371*, 174−181.
(34) Shediac, R.; Ngola, S. M.; Throckmorton, D. J.; Anex, D. S.; Shepodd, T. J.; Singh, A. K. *J. Chromatogr., A* **2001**, *925*, 251−263.
(35) Yu, C.; Davey, M. H.; Svec, F.; Fréchet, J. M. J. *Anal. Chem.* **2001**, *73*, 5088−5096.
(36) Rohr, T.; Yu, C.; Davey, M. H.; Svec, F.; Fréchet, J. M. J. *Electrophoresis* **2001**, *22*, 3959−3967.
(37) Petro, M.; Svec, F.; Fréchet, J. M. J. *Biotechnol. Bioeng.* **1996**, *49*, 355−363.
(38) Xie, S. F.; Svec, F.; Fréchet, J. M. J. *Biotechnol. Bioeng.* **1999**, *62*, 30−35.
(39) Vodopivec, M.; Berovic, M.; Jancar, J.; Podgornik, A.; Strancar, A. *Anal. Chim. Acta* **2000**, *407*, 105−110.
(40) Josic, D.; Buchacher, A.; Jungbauer, A. *J. Chromatogr., B* **2001**, *752*, 191−205.
(41) Rasmussen, J. K.; Heilmann, S. M.; Krepski, L. R.; Jensen, K. M.; Mickelson, J.; Johnson, K. Z.; Coleman, P. L.; Milbrath, D. S.; Walker, M. M. *React. Polym.* **1992**, *16*, 199−212.
(42) Coleman, P. L.; Walker, M. M.; Milbrath, D. S.; Stauffer, D. M.; Rasmussen, J. K.; Krepski, L. R.; Heilmann, S. M. *J. Chromatogr.* **1990**, *512*, 345−363.
(43) Tripp, J. A.; Svec, F.; Fréchet, J. M. J. *J. Comb. Chem.* **2001**, *3*, 216−223.
(44) Tripp, J. A.; Stein, J. A.; Svec, F.; Fréchet, J. M. J. *Org. Lett.* **2000**, *2*, 195−198.

Table 1. Polymerization Mixtures Used for the Preparation of Monolithic Supports

polymer	A	B	C	D	E	F	G	H
format[a]	Ca	Ch	Ca	Ca	Ch	Ca	Ca	Ca
ethylene dimethacrylate, wt %	20	20	20	20	20	20	20	20
acrylamide, wt %	12	12	12	12	12			
2-hydroxyethyl methacrylate, wt %						12	16	18
2-vinyl-4,4-dimethylazlactone, wt %	8	8	8	8	8	8	4	2
1-decanol, wt %	60	60	55	40		60	60	60
cyclohexanol, wt %			5	20				
1-dodecanol, wt %					60			
2,2-dimethoxy-2-phenylacetophenone[b]	1	1	1	1	1	1	1	1
polymerization time, min	3	6	3	3	6	6	6	6

[a] Ca , capillary; Ch, microfluidic chip; [b] Percentage of initiator with respect to monomers.

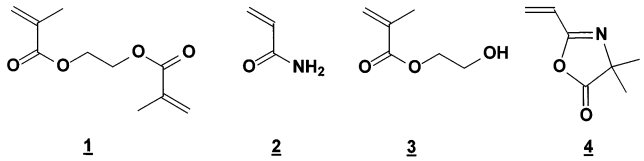
monoliths within microchannels, their use as supports for the immobilization of trypsin, and the application of the resulting conjugates for the rapid digestion of proteins.

EXPERIMENTAL SECTION

Materials. Ethylene dimethacrylate (98%, EDMA), 2-hydroxyethyl methacrylate (97%, HEMA), 2,2-dimethoxy 2-phenylacetophenone (99%), tris(hydroxymethyl)aminomethane (TRIS), acrylamide, sodium sulfate, benzamidine, 1-decanol, sinapinic acid, and 3-(trimethoxysilyl)propyl methacrylate (98%) were purchased form Aldrich (Milwaukee, WI). Trypsin from bovine pancreas, *N*-α-benzoyl-L-arginine ethyl ester (BAEE), equine myoglobin, and ethanolamine (98%) were obtained from Sigma (St. Louis, MO), and BODIPY-labeled casein was from Molecular Probes (Eugene, OR). Fused-silica tubing was purchased from Polymicro Technologies (Phoenix, AZ), and the microfluidic chips were from Micralyne, (Edmonton, Alberta, Canada). 2-Vinyl-4,4-dimethylazlactone (VAL) was a gift from 3M Company (St. Paul, MN) and was distilled prior to use. EDMA and HEMA were passed through basic alumina followed by a distillation under reduced pressure. All other reagents were used as received.

Instrumentation. An Oriel deep-UV illumination series 8700 (Stratford, CT) fitted with a 500-W HgXe lamp was used for UV initiation of the polymerization reactions. The radiation power of this lamp with its uniform beam was measured and reported previously.[36] The unreacted components remaining in the pores of the monoliths within both capillaries and chips were then washed out using a pressurized flow of methanol actuated by an Isco (Lincoln, NE) 100 DM syringe pump. Immobilization and digestion in capillaries were carried out with an Ultra-Plus II Capillary LC equipped with a UV detector (Micro-Tech Scientific, Sunnyvale, CA). Similar experiments on microchips were performed using the Microfluidic Tool Kit (Micralyne). This instrument is equipped with a 5-mW diode laser, which emits light at a wavelength of 525 nm. Porosity data were obtained using an Autopore III 9400 mercury intrusion porosimeter (Micromeritics, Norcross, GA). Scanning electron microscope (SEM) images were collected using an ISI high-resolution analytical scanning electron microscope (Topcon, Tokyo, Japan). MALDI-TOF mass spectra were obtained using a Voyager DE Biospectrometry Workstation (Applied Biosystems, Foster City, CA).

Monolithic Supports. The internal wall surface of UV-transparent fused-silica capillaries (100-μm I.D.) and microfluidic chips (channel width 50 μm, depth 20 μm) was first vinylized to enable covalent attachment of the monolith to the walls.[10] The wall capillaries and channels were rinsed with acetone and water, activated with a 0.2 mol/L sodium hydroxide solution for 30 min, washed with water followed by 0.2 mol/L HCl for 30 min, rinsed with water and acetone, and dried with a stream of nitrogen. The capillary or channel was then filled with a 30% solution of 3-(trimethoxysilyl)propyl methacrylate in acetone. The device was then sealed, allowed to equilibrate for 24 h at room temperature, then rinsed with acetone, and dried. The sections of the capillary or chip that should *not* contain a monolith were covered with an opaque mask. For capillaries, this mask was produced by painting the surface with a flat black fast-dry enamel, while for chips, the masks simply consisted of black electrical tape covering the areas that should remain unexposed. The length of the unmasked area was kept constant at 20 mm. The "molds" were then completely filled with a polymerization mixture consisting of monomers (ethylene dimethacrylate, 2-vinyl-4,4-dimethylazlactone, and acrylamide or 2-hydroxyethyl methacrylate) (Figure 1), porogenic solvent (dodecanol, decanol, or its mixtures with cyclohexanol), and a photoinitiator (2,2-dimethoxy-2-phenylacetophenone). The compositions of the individual polymerization mixtures are summarized in Table 1. Polymerization was initiated by placing the molds under the UV lamp and irradiating for the period of time shown in Table 1. Inlet and outlet 50-μm-i.d. fused-silica capillaries were glued to the holes of the microfluidic device prior to filling using a two-component Five Minute Epoxy Glue (Devcon Inc.). The monoliths were washed with methanol for 12 h using a syringe pump attached via the inlet capillary.



1 2 3 4

Figure 1. Structures of monomers used in this study: ethylene dimethacrylate (**1**), acrylamide (**2**), 2-hydroxyethyl methacrylate (**3**), and 2-vinyl-4,4-dimethylazlactone (**4**).

Preparation of Bulk Polymer. A larger volume mold consisting of a circular Teflon base plate and a 10-cm quartz window separated by a 700-μm-thick polysiloxane gasket, held between an aluminum plate and an aluminum ring and held together with screws, was used for bulk polymerization. The polymerization mixtures were purged with nitrogen for 10 min, filled in the mold, and irradiated using the same conditions as those used for the

Table 2. Porous Properties of Monoliths Prepared in Bulk Mold from Polymerization Mixtures Shown in Table 1[a]

polymerization mixture	A	B	C	D	E	F	G	H
pore volume, mL/g	1.01	1.01	0.95	0.85	1.06	1.33	1.34	1.37
median pore diameter, μm	0.95	0.95	0.72	0.69	0.74	1.02	1.08	1.08
porosity, %	41.2	41.2	40.4	39.4	45.9	51.9	49.9	53.4
specific surface area, m^2/g	4.9	4.9	5.9	5.6	6.8	6.4	6.2	6.2

[a] Porosity data calculated for pore sizes between 0.05 and 10 μm.

preparation of the monolith in capillaries and chips. Once the polymerization was completed, the mold was disassembled, and the layer of solid polymer was recovered, broken into smaller pieces, extracted in a Soxhlet apparatus for 12 h with methanol, and dried in a vacuum oven at 60 °C for 12 h. These monoliths were then subjected to mercury intrusion porosimetry.

Immobilization of Trypsin. A modification of our earlier procedure was used to immobilize trypsin on the monoliths.[38] Specifically, trypsin was dissolved in an aqueous solution containing 0.5 mol/L sodium sulfate and 0.1 mol/L sodium carbonate, and 0.05 mol/L benzamidine was added to avoid the undesired autodigestion. The concentrations of trypsin in these solutions were kept in the range of 2−10 mg/mL (Table 2). The immobilization solution was pumped through the monolith for a specific period of time varying from 1 to 8 h. The monolith was then washed for 1 h with 1 mol/L ethanolamine to quench the unreacted azlactone functionalities followed by equilibration with a 50 mmol/L TRIS buffer (pH 8.0) for 12 h.

Assay of Enzymatic Activity. The activity of the free trypsin (32.4 μmol min^{-1} mg^{-1}) was determined using a standard procedure.[45] The activity of immobilized enzyme was assayed by pumping 500 μL of BAEE solutions (15−230 mmol/L) in a 50 mmol/L TRIS buffer (pH 8.0) through the monolith at a flow rate of 3 μL/min with detection of resulting products at 254 nm. The enzyme activity was calculated by multiplying the flow rate by conversion obtained from the difference in absorption between blank and converted solutions. The activity is expressed in nanomoles of converted substrate in 1 min normalized to 1 mL of reactor volume.

To determine both enzymatic activity and extent of digestion, a 240 μg/mL myoglobin solution in 50 mmol/L TRIS buffer was pumped through the microchip reactor and the eluent collected, combined in a ratio of 1:10 with sinapinic acid used as a matrix, and spotted onto a MALDI plate. The resulting mass spectra were then compared against the protein digestion database (protein prospector, http://prospector.ucsf.edu/) to identify the digest.[46]

Since UV detection is not available in our microchip developmental testing unit, a 10 μg/mL solution of BODIPY-labeled casein with intermolecularly quenched labels in 50 mmol/L TRIS buffer was used for the determination of enzymatic activity. An increase in the fluorescent signal positively identifies the presence of proteolytic activity.[47]

(45) Rick, W. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1965; pp 807−818.
(46) Clauser, K. R.; Baker, P.; Burlingame, A. L. *Anal. Chem.* **1999**, *71*, 2871−2882.
(47) Jones, L. J.; Upson, R. H.; Haugland, R. P.; Panchuk-Voloshina, N.; Zhou, M. J. *Anal. Biochem.* **1997**, *251*, 144−152.

RESULTS AND DISCUSSION

Preparation of Monolithic Microreactors. The compositions of the polymerization mixtures used in this study for the in situ preparation of monolithic materials were inspired from those we used previously.[38] However, in contrast to the original thermally initiated process, the UV-initiated polymerization used in this study occurs at room temperature. Since temperature has a significant effect on both the porous properties of the monolith and the solubility of monomers in the polymerization mixture, the porogenic solvent system had to be redesigned. For example, the solubility of acrylamide in tetradecanol and dodecanol at room temperature is not sufficient to achieve the required concentration. After searching through a variety of solvents and their mixtures, 1-decanol and its mixtures with cyclohexanol were found to provide the desired level of solubility for acrylamide.

The kinetics of UV-initiated polymerization constitutes a very important variable that would likely affect throughput should the process be implemented to prepare chips in quantity. Our experiments carried out within the framework of this study demonstrate that complete conversion of monomers to the monolithic polymer is always achieved in less than 10 min. For example, the polymerization time in a 100-μm-i.d. capillary could be reduced to only 3 min using the highest power output of the UV lamp (46 mW/cm^2).

Since all liquids must flow through the pores of the monolithic material during its use, it is essential to control the size of the pores that permeate it. Obviously, flow resistance is inversely proportional to pore size. The pore size of monoliths obtained in a series of polymerization reactions is controlled by composition of porogenic system consisting of various mixtures of 1-decanol and cyclohexanol. The pore size increases from about 200 to 950 nm as the amount of decanol in the polymerization mixture is increased from 17 to 55%. At even higher percentages of decanol, the slope of pore size versus percentage decanol becomes steeper as the percentage of decanol is increased beyond 55%. This is likely the result of the poor solubility of acrylamide in these very nonpolar mixtures. In contrast to the effect of decanol on pore size, its percentage in the polymerization mixture only has a minor effect on the pore volume.[36]

All polymerization mixtures had to be heated to ∼40 °C to achieve a complete dissolution of all components and formation of a homogeneous monomer phase. It is likely that the solution becomes oversaturated with acrylamide when the mold is cooled to room temperature for polymerization. However, no change in appearance of the solution is observed during cooling. An added benefit of the increased temperature used to prepare the monomer solutions is its effect in lowering the viscosity, facilitating the filling of the capillaries and small channels.

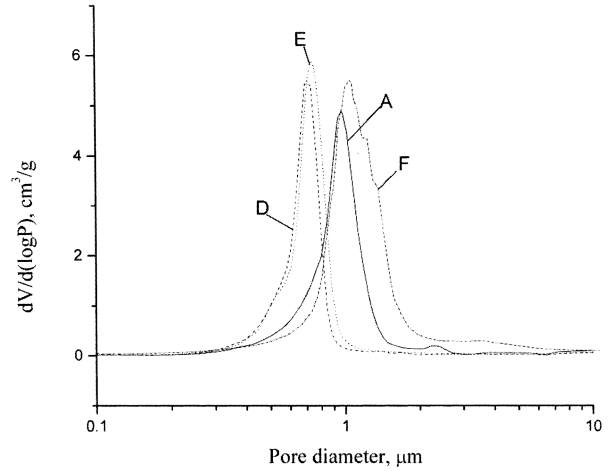


Figure 2. Pore size distribution profiles for monoliths A and D−F determined by mercury intrusion porosimetry.

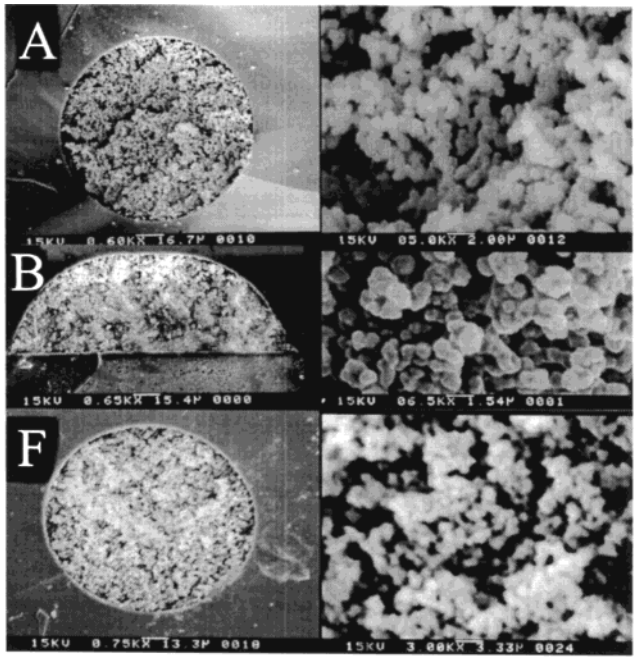


Figure 3. SEM micrographs of internal structure of monoliths A, B, and F.

The differential pore size distribution profiles determined by mercury intrusion porosimetry for some of our monoliths are shown in Figure 2. These materials were prepared from the polymerization mixtures shown in Table 1 using the larger volume mold, and their pore size distributions highlight the very significant effect of both the type of porogenic solvent used and the comonomer since the overall percentages of all components in all of these mixtures were kept constant. Specifically, mixtures A and F involve 1-decanol, while D is a combination of 1-decanol and cyclohexanol, and E has 1-dodecanol as the porogen. Mixtures A, D, and E use acrylamide as the inert hydrophilic comonomer and 2-hydroxyethyl methacrylate is used for mixture F. Interestingly, the effect of the comonomer functionality appears to be very small despite the fact it accounts for a large proportion (30%) of the monomer mixture. The SEM images of Figure 3 show the internal morphologies of monoliths A, B, and F and demonstrate that the porous polymers are homogeneous across the entire

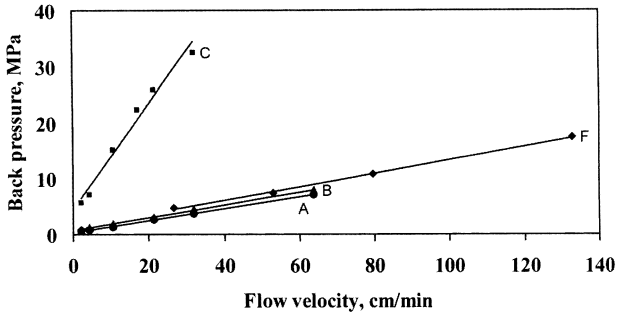


Figure 4. Effect of flow velocity on back pressure for monoliths A, B, C and F with immobilized trypsin. Conditions: mobile phase, 50 mmol/L TRIS buffer solution.

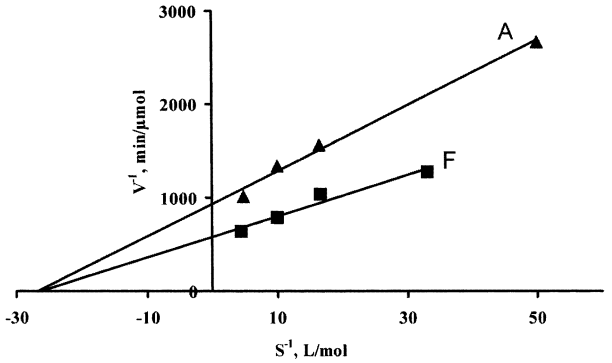


Figure 5. Lineweaver−Burke plots for trypsin immobilized on monoliths A and F. Conditions: immobilization, 2 mg/mL trypsin solution in 0.5 mol/L sodium sulfate, 0.1 mol/L sodium carbonate, and 0.05 mmol/L benzamidine; determination of activity, 15−230 mmol/L BAEE in 50 mmol/L TRIS buffer.

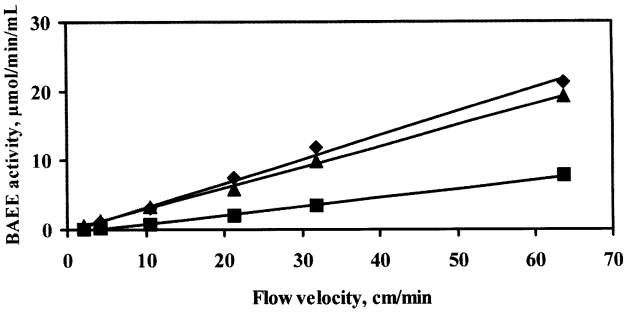


Figure 6. Effect of immobilization time and flow rate of the substrate solution on activity of immobilized trypsin. Conditions: support, monolith A; immobilization, 2 mg/mL trypsin solution in 0.5 mol/L sodium sulfate, 0.1 mol/L sodium carbonate, and 0.05 mmol/L benzamidine; immobilization time 1 (■), 3 (▲), and 8 (◆) h; determination of activity, 20 mmol/L BAEE in 50 mmol/L TRIS buffer.

monolith. The pore sizes estimated from these micrographs closely match those determined by mercury intrusion porosimetry (Table 2). These images also reveal the complete absence of voids between the wall and the monolith.

Flow through the Monoliths. In general, electroosmotic flow (EOF) is used to propel liquids through microfluidic devices.[4,5,7,8,10−12,17] The use of EOF is very convenient since it does not require any mechanical device with moving parts and is simple to implement on a chip. In addition, the profile of electroosmotically driven flow is generally flat, which has a beneficial effect on the separations. However, to create EOF, ionizable functionalities must be present in the system and only a limited number of polar

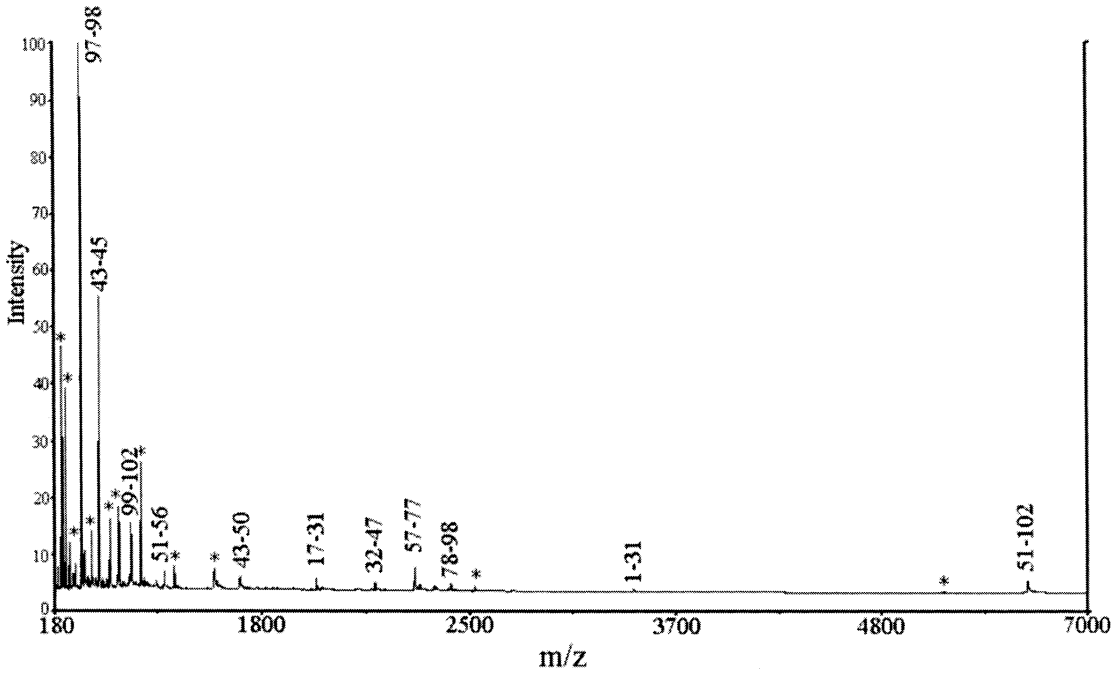


Figure 7. MALDI-TOF MS spectrum of tryptic digest of equine myoglobin. Conditions: trypsin immobilized on monolith F, myoglobin solution, 14.2 pmol/μL in 50 mmol/L TRIS buffer; Flow rate, 0.5 μL/min. Positive identification of myoglobin using protein prospector (Swissprot accession number P02188); 102 out of 153 peptides matched; sequence coverage 67%; asterisks indicate unidentified peaks.

Table 3. Michaelis Constant and Maximal Velocity for Monoliths and Immobilization Mixtures at a Substrate Flow Rate of 3 μL/min

monolith	trypsin concn, mg/mL[a]	V_{max}, nmol/min	K_m, mmol/L
A	2	1.07	32.8
C	2	1.81	7.7
F	2	1.7	33.8
F	5	2.27	41.1
F	10	9.13	285.0
G	2	2.12	54.2
H	2	4.26	77.2

[a] Trypsin concentration in the solution used for immobilization.

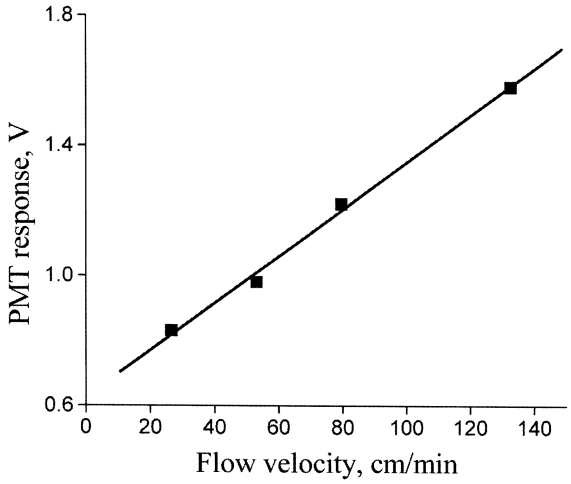


Figure 8. Effect of flow velocity on the extent of on-chip tryptic digestion of BODIPY-labeled casein characterized by fluorescence in the microfluidic chip. Conditions: support, monolith E; casein solution 10 μg/mL in 50 mmol/L TRIS buffer, pH 8.0.

solvents can be used. A high electric field is also required to generate adequate flow, and the combination of solvent and electric field can generate heat via the Joule effect. Therefore, only eluents with a low ionic strength may be used to prevent excessive generation of Joule heat. Pressurized flow through microchips have also been reported by using mechanical and electrokinetic pumps.[48−54] Since pressurized pumping technology is becoming more readily available in microfluidics, we mimicked this method through the use of an external micro-LC pump to generate flow through both capillaries and microchip channels. The latter were attached to the pump via a short piece of fused-silica capillary glued to the chip. This implementation enables variations in flow rates over a wide range from 0.1 to 3 μL/min for capillaries and from 0.1 to 0.5 μL/min for microchips. It is worth noting that the cross sections for the 100-μm-i.d. capillary and the microchip channels are 7.9×10^{-5} and 6.3×10^{-6} cm^2, respectively. Thus, the top flow rates translate into very high flow velocities of 10.6 mm/s for the capillaries and 22.1 mm/s for the microchips.

In contrast to EOF, the pressure created by the pump is required to achieve flow through the monoliths, and the back pressure observed is indirectly proportional to the pore size with smaller pores exerting higher pressures.[55] Figure 4 shows the back pressures generated at different flow rates and detected by

(48) Kar, S.; McWhorter, S.; Ford, S. M.; Soper, S. A. *Analyst* **1998**, *123*, 1435−1441.
(49) Hosokawa, K.; Fujii, T.; Endo, I. *Anal. Chem.* **1999**, *71*, 4781−4785.
(50) Fan, Z. H.; Mangru, S.; Granzow, R.; Heaney, P.; Ho, W.; Dong, Q. P.; Kumar, R. *Anal. Chem.* **1999**, *71*, 4851−4859.
(51) Culbertson, C. T.; Ramsey, R. S.; Ramsey, J. M. *Anal. Chem.* **2000**, *72*, 2285−2291.
(52) Tokeshi, M.; Minagawa, T.; Kitamori, T. *Anal. Chem.* **2000**, *72*, 1711−1714.
(53) Dutta, D.; Leighton, D. T. *Anal. Chem.* **2001**, *73*, 504−513.
(54) Ni, J.; Zhong, C. J.; Coldiron, S. J.; Porter, M. D. *Anal. Chem.* **2001**, *73*, 103−110.

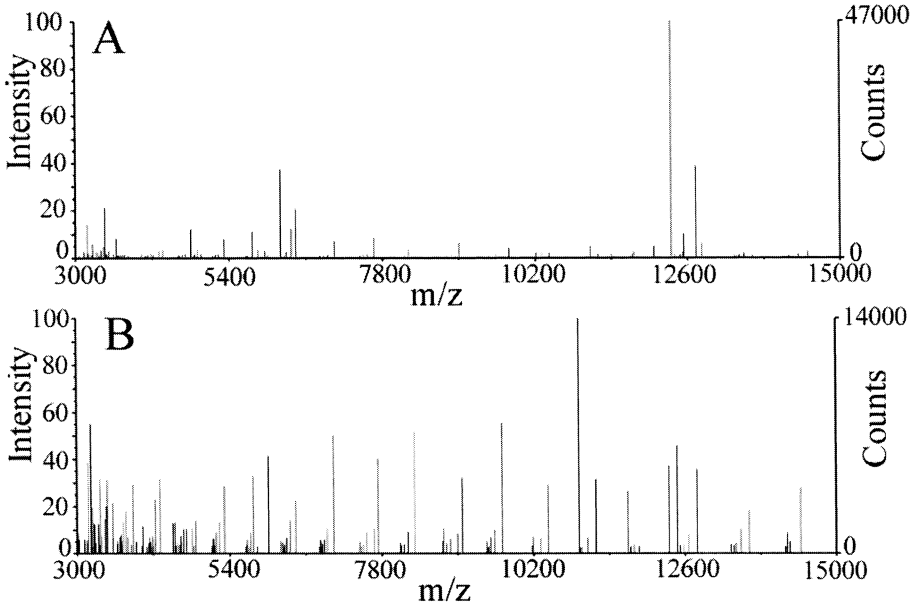


Figure 9. MALDI-TOF MS spectrum of fluorescently labeled casein (A) and its tryptic digest (B). Casein solution: 10 μg/mL in 50 mmol/L TRIS buffer, pH 8.0; Flow rate for digestion 26.5 cm/min; Reactor prepared from monolith E.

the pressure sensor of the micropump for a series of monoliths. As expected, the back pressure monitored for monoliths A, B, and F with a pore size of ~1 μm is much lower at all flow velocities than is the case for monolith C with its smaller pore size of 0.75 μm. The 2-hydroxyethyl methacrylate-based monolith generates a lower back pressure than the acrylamide-based monolith with a similar pore size. It should be noted that the back pressures observed for both the microchip and the capillary are about the same at a given flow velocity. This confirms again that monoliths with essentially the same characteristics are formed by polymerization of the same mixture regardless of the "mold" used.

Activity of Enzymatic Microreactors. The activity of an enzyme, and consequently of the microreactor, is characterized by the values of the Michaelis constant (K_m) and maximum velocity (V_{max}). These values are most often derived from a linearized form of the Michaelis−Menten equation using a Lineweaver−Burke plot.[28,56] Figure 5 shows two of these plots for the conversion of BAEE in enzymatic microreactors prepared by immobilization of trypsin from a 2 mg/mL solution on monoliths A and F that differ only in the type of hydrophilic comonomer used. The kinetic characteristics for several microreactors prepared within the framework of this study are shown in Table 3.

The data of Table 3 enable an evaluation of various effects. For example, the effect of pore size can be discerned by comparing reactors prepared from monoliths A and C. The K_m value is much lower and the V_{max} is higher for monolith C featuring smaller pores. This means that a higher reaction rate can be obtained at a lower concentration of BAEE in the substrate solution. Since monolith C has a larger surface area, it is likely that it can accommodate more enzyme in readily accessible pores. Unfortunately, our current instrumentation does not allow the direct determination of the amount of enzyme immobilized in the microreactor to confirm this hypothesis. The microreactor prepared from monolith F containing HEMA as the comonomer exhibits a higher maximal velocity than the reactor based on monolith A while the Michaelis constants are almost equal for both, indicating that the immobilized enzyme is more accessible in the former.

Since the immobilization is a bimolecular reaction of the enzyme in solution with the azlactone functionalities at the surface of the solid support, an increase in the concentration of each of these variables, or an increase in reaction time, should lead to microreactors with a higher loading of the catalytically active moieties. Indeed, Table 3 documents that an increase in the concentration of trypsin in the solution used for immobilization from 2 to 10 mg/mL affects the kinetic parameters of the microreactors prepared using monolithic support F. Specifically, the increased concentration leads to an increase in V_{max}. However, this is accompanied by a concomitant increase in the value of K_m. The value of K_m reflects the concentration of substrate required so that the reaction velocity is half of its maximum value. Thus, although the maximum velocity is higher, a higher concentration of substrate must be used to achieve this velocity.

Increasing the percentage of azlactone incorporated in the monolith should have the same effect on the activity of the immobilized enzyme as the use of a higher concentration of enzyme in the solution shown above. Indeed, an increase in both V_{max} and the Michaelis constant can be observed for enzymatic microreactors prepared from monoliths incorporating 2 (H), 4 (G), and 8% (F) azlactone monomer (Table 3).

Figure 6 shows the catalytic activities at different flow velocities of the BAEE substrate solution for monolithic microreactors containing enzyme immobilized using 1-, 3-, and 8-h reaction times. As expected, the performance of the immobilized enzyme improves with an increase in the reaction time from 1 to 3 h. However, a further increase to 8 h contributes only a small increment to the overall enzymatic activity. This suggests that

(55) Viklund, C.; Svec, F.; Fréchet, J. M. J.; Irgum, K. *Chem. Mater.* **1996**, *8*, 744−750.
(56) Hadd, A. G.; Raymond, D. E.; Halliwell, J. W.; Jacobson, S. C.; Ramsey, J. M. *Anal. Chem.* **1997**, *69*, 3407−3412.

the most accessible azlactone functionalities located at the surface of larger pores quickly react in the early stages of immobilization, and only the less accessible reactive groups remain after the initial period. The activity versus flow velocity plots presented in Figure 6 are linear. Since the Michaelis constants found for the immobilized trypsin reactors are rather high, this suggests that the active sites of the immobilized enzyme are not fully saturated under these experimental conditions.

Digestion of Myoglobin. One of the applications of our microreactors is in protein mapping. This procedure involves the proteolytic digestion of a protein followed by the determination of the masses of the digested fragments using mass spectrometry. A subsequent comparison of the mass distribution profile within an appropriate database is then used to identify the original protein. Myoglobin is a model protein often used for the testing of systems including proteolytic digestion.[6,27,28] To demonstrate the performance of trypsin immobilized on the monolithic support, we pumped a 14.2 pmol/μL myoglobin solution through a microreactor prepared from monolith F at a flow rate of 0.5 μL/min, which affords a residence time of only 11.7 s. The effluent from the microreactor was collected, combined with a matrix (sinapinic acid), and analyzed by MALDI-TOF MS. The mass spectrum shown in Figure 7 indicates that only ~5% of the myoglobin remained undigested. This rough estimate is based on the reduction in the intensity of the myoglobin peak (m/z = 16 700) compared to its peak obtained from the original protein solution. However, 102 of 153 peptide fragments in the digest could be positively identified. This results in a sequence coverage of 67% with an average of 3.1 missed cleavages. Repeated experiments demonstrated very good reproducibility of this procedure. Our results can be compared to a standard "in-reservoir" digestion method that requires 5−15 min of digestion time to obtain a sequence coverage between 70 and 95%[13,29] and to 3-min time required for "on-chip" digestion of cytochrome *c* in a large-volume channel (1800 nL) completely packed with trypsin immobilized on agarose beads.[29]

Enzymatic Microreactor-on-a-Chip. The ultimate goal of this study was to fabricate and test a microchip-based enzymatic reactor. Given the very small size of the reactor, this is a difficult task, as the volume of the monolith containing the immobilized enzyme is only 7.5 nL. Although the maximum flow rate we used was only 500 nL/min, this represents a rather high flow velocity of 130 cm/min or 22 mm/s. For evaluation of this microdevice, a 10 μg/mL solution of BODIPY-labeled casein was pumped at different flow rates through the microchip and the fluorescence of the eluted material was monitored. Since the original solution had a very low level of fluorescence, the response shown in Figure 8 clearly demonstrates that the microreactor is rapidly cleaving the high molecular weight protein. Once again, the plot is linear. The quantity of products that are generated by the reactor is directly proportional to the amount of substrate fed in the chip. This indicates that the maximum reaction rate has not been reached under these conditions since the response would otherwise level off at high flow rates. To further demonstrate that the reactor is digesting the substrate, the fluorescently labeled casein was passed through the microreactor, collected, subjected to MALDI-MS analysis, and compared to the original protein. Indeed, results of this experiment shown in Figure 9 confirm that the protein has been digested in our enzymatic microreactor, as demonstrated by the decrease in the number of counts for the labeled casein (m/z = 12 370) and in appearance of more fragments in the spectrum. Sequence coverage could not be determined because the fluorescent tags present on the labeled casein altered the masses of the generated peptides.

CONCLUSIONS

Reactive porous polymer monoliths can be prepared in situ at precise locations within a microfluidic device by using UV photopolymerization through a mask for the preparation of the monolith. Subsequent immobilization of an enzyme such as trypsin converts the monolith into a enzymatic microreactor. Although the performance of our monolithic reactor has only been demonstrated with three substrates so far, low molecular weight BAEE (MW = 343) and two proteins, myoglobin (MW = 16 700) and labeled casein (MW = 12 370), it is likely that this approach can be used quite broadly. The very short digestion time achieved with the microreactor contrasts the longer reaction times required to achieve similar sequence coverage using alternative approaches.[6,28,29] The preliminary results we have obtained are very encouraging since they open new avenues for the design and construction of rapid, high-throughput, protein mapping systems. Our current research is now focused on the study of the digestion of other proteins and the achievement of even higher reactor activities through digestion at higher temperatures. We envision that this technology may rapidly find an application in the area of proteomic research using microfluidic enzyme reactors coupled directly to ESI-MS or used in combination with MALDI-TOF MS with automatic sample preparation.

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