Miniaturized Biocatalysis on Polyacrylate-Based Capillary Monoliths

Christian Elsner,[1] Claudia Ernst,[1] Michael R. Buchmeiser[1,2,3]

[1]*Leibniz-Insitut für Oberflächenmodifizierung e. V., Permoserstrasse 15, D-04318 Leipzig, Germany*
[2]*Institut für Polymerchemie, Universität Stuttgart, Pfaffenwaldring 55, D-70550 Stuttgart, Germany*
[3]*Deutsche Institute für Textil- und Faserforschung Denkendorf (DITF), Körschtalstraβe 26, D-73770 Denkendorf, Germany*

Received 14 January 2010; accepted 19 April 2010
DOI 10.1002/app.32657
Published online 18 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: We developed a synthetic concept for the immobilization of enzymes onto monolithic supports. In addition, we elaborate on a generally applicable method for the rapid screening of the activity of such immobilized enzymes. For these purposes, we prepared monolithic acrylate-based systems by electron-beam (EB)-triggered free-radical polymerization within the confines of 200-μm capillary columns. Aiming for protein immobilization, we subjected the polyacrylate-based monoliths to EB-mediated grafting processes to introduce functional surface-located groups suited for the subsequent generation of functional units that themselves could bind different proteins. For the generation of the functional units, we used ring-opening metathesis polymerization and free-radical polymerization. The produced systems were tested for their ability to bind or repel proteins as exemplified by the use of the serine protease trypsin, which was used to catalyze the hydrolysis of N-α-benzoyl-L-arginine ethyl ester (BAEE). Finally, the monolith-immobilized trypsin systems were used for enzymatic peptide synthesis purposes, such as the acyl transfer of BAEE to amino acid amides. Complementarily, we used an immobilized trypsin variant, which we additionally subjected to on-column chemical modification with succinic anhydride to alter its synthetic properties. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 1450–1458, 2011

Key words: electron beam curing; enzymes; proteins; ROMP

INTRODUCTION

Microsystem technologies enable the miniaturization of (consecutive) chemical processes on microfluidic devices; this combines synthesis, purification, and analysis in a highly integrated manner. The term *lab-on-a-chip* is commonly used for such systems.[1,2] The major advantages of this technology are an accelerated speed combined with a high sensitivity; this allows the use of small amounts of analytes and offers access to high-throughput processes. Miniaturized reaction systems have already found a number of applications in analytical chemistry, catalyst screening, and synthetic chemistry. Within that context, (bio)catalysts immobilized within microfluidic flow reactors offer a number of advantages over conventional batch reactor systems.[3–8] With such systems, the quantities of the enzymes, substrates, and cofactors can be significantly reduced; this is of particular relevance in the study and optimization of biotransformation processes. Additionally, secondary processes, such as substrate and product inhibition or product degradation processes, can be avoided or managed in a favorable way. Furthermore, biocatalyst immobilization allows enzyme reuse and increases the stability of the protein. In the case of miniaturized systems, this is of special importance, particularly when noncommercial enzymes have to be screened, where typically only very minor amounts are available. In addition, the miniaturized immobilization of proteins allows for straightforward chemical modifications and functional transformations. In addition to adsorption on microchannel surfaces, membranes,[9,10] crosslinked enzymes, agarose beads,[11] and controlled pore glass,[12] monolithic media are particularly suitable supports for the immobilization of biocatalysts in a miniaturized way.[13–15] Furthermore, they are well-suited tools for the separation of both high- and low-molecular-weight analytes.[16–18] Finally, the unique structure of porous polymeric monoliths decreases the danger of bleeding and system contamination or blockage and results in low backpressures, even at high linear flow rates. These features and fast mass transport make them highly favorable for miniaturized, high-throughput applications.[19] Several techniques for the preparation of monolithic supports are known, for example, thermally triggered free-radical polymerization;

Correspondence to: M. R. Buchmeiser (michael.buchmeiser@ipoc.uni-stuttgart.de).

Contract grant sponsor: Federal Ministry of Education and Research; contract grant number: 0315333B.
Contract grant sponsor: Federal Government of Germany.
Contract grant sponsor: Freistaat Sachsen.

UV-, γ-, or electron-beam (EB)-induced free-radical polymerization; polycondensation and polyaddition; and quasi-living and living polymerization techniques, such as TEMPO-initiated free-radical polymerization and ring-opening metathesis polymerization (ROMP).[20,21] Among these polymerization techniques, radiation-based ones are particularly suitable for the preparation of monoliths in microdimensional systems, for example, in capillaries or chips, because of the ease of filling with prepolymeric, low-viscous material and the possibility of spatially resolved polymerization and/or functionalization. This was already demonstrated for the UV-light-triggered free-radical polymerization of polyacrylate-derived porous polymeric monoliths used for chip electrochromatography.[22]

Recently, we reported the pulsed EB-triggered, free-radical polymerization-based synthesis of large-diameter monolithic columns.[23] Because any additional preparative scale modification of these supports for bioapplications, that is, the immobilization of proteins, is expensive, the underlying immobilization chemistry was evaluated on a small scale. Consequently, preliminary studies with miniaturized systems followed by scale-up appeared advantageous. In addition, most of the miniaturized disposable microdevices consist of inexpensive polymeric and/or UV-absorbing materials or are fixed on UV-absorbing supports,[24,25] for example, hot-embossed or UV- or injection-molded polymers. Consequently, an EB-based approach is necessary to treat, that is, functionalize, such devices. Here, we describe our attempts to evaluate both the immobilization chemistry and the activity of an immobilized enzyme. For our purposes, the aforementioned EB-based synthetic approach for the generation of monolithic structures appeared favorable because it could be applied to both microscale and macroscale systems. This resulted in comparable monolithic structures and, thus, facilitated upscaling. We, thus, studied the immobilization of the proteolytic enzyme trypsin as a model system on microscaled monolithic EB-derived supports with different surface chemistries. Thereby, the EB-derived monolith acted as a scaffold for further modification processes by radiation-induced grafting or transition-metal-initiated ROMP. The generated systems were tested in terms of their ability to bind or repel the protein. Both are important features for the spatially resolved protein immobilization, as exemplified by Svec and coworkers.[26] In addition, the use of these miniaturized biocatalytic systems in peptide synthesis are delineated.

EXPERIMENTAL

Instrumentation

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on an UltiMate 3000 system (Dionex GmbH, Idstein, Germany) equipped with an LPG-3400 M pump, an FLM-3300 flow manager, a VWD-3400 detector (cell volume = 45 nL), a WPS-3000 autosampler module, and a CAP 100 flow-splitter cassette (split ratio = 1 : 100). The mobile phases were as follows: A consisted of 95% H_2O and 5% acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA), and B consisted of 5% H_2O, 95% ACN, and 0.1% TFA, where the gradient was 0′ 4% B, 15′ 30% B, 17′ 4% B, and 30′ 4% B [flow rate = 4 μL/min, Acclaim PepMap C18, 3 μm, 100 Å, inside diameter = 300 μm, 15-cm length (LC Packings), detection: UV (254 nm)]. For the postsynthesis modifications, an UltiMate 3000 high performance liquid chromatography system from Dionex (Idstein, Germany) consisting of an LPG-3400 M pump and a WPS-3000 autosampler module was used. Attenuated total reflection (ATR)–IR spectroscopy was performed on a Bio-Rad FTS 6000 Fourier transform infrared spectrometer (Krefeld, Germany) and a Bruker FTIR-Spectrometer Vector 22 (Leipzig, Germany). Scanning electron microscopy (SEM) was carried out on an Ultra 55 (Carl Zeiss SMT, Oberkochen, Germany). NMR spectra were recorded on a Bruker Avance^{II} 600 and Bruker Spectrospin 250 spectrometer (Rheinstetten, Germany) in the indicated solvent at 25°C and are listed in parts per million downfield from trimethylsilane as an internal standard for protons and carbon.

Chemicals

Trypsin from bovine pancreas (14923 U/mg) was purchased from Fluka (Buchs, Switzerland); the amino acid amides and N-α-benzoyl-L-arginine ethyl ester (BAEE) were from Bachem (Bubendorf, Switzerland). SR-415 [ethoxylated (20) trimethylol propane triacrylate (TMPTA)] and tripropylene glycol diacrylate (TPGDA) were from Sartomer (Paris, France). Glutaric dialdehyde was from ABCR (Karlsruhe, Germany). *N*-acryloxysuccinimide (M II) was from Acros Organics (Nidderau, Germany). TMPTA, ethyl methacrylate (EMA), 3-(trimethoxysilyl)propyl methacrylate, hydroxypropyl acrylate (**M V**; a mixture of isomers), tetraethylene glycol diacrylate (**M VI**), poly(ethylene glycol) methacrylate (**M I**), 1-dodecanol, acetonitrile, water (HPLC grade), and TFA (99.5%) were obtained from Sigma-Aldrich (Seelze, Germany).

Synthesis

Bicyclo[2.2.1]hept-5-en-2-ylmethyl acrylate (**M III**)

5-Norbornene-2-methanol (a mixture of endodiastereomer and exodiastereomer at 1.51 g or 12.1 mmol) was dissolved in 20 mL of $CHCl_3$ followed by the dropwise addition of triethylamine (3.4 mL). The mixture was stirred for 20 min and cooled to 0°C.

Subsequently, acrylic acid chloride (1.088 g, 0.973 mL, 12.09 mmol) was added dropwise. The reaction mixture was kept at 0°C for 2 h. After the mixture was washed with 50 mL of water and an aqueous $NaHCO_3$ solution (5 wt %) and water, the organic layer was dried over Na_2SO_4. Then, the solvent was removed *in vacuo,* and the obtained brown oil was purified by flash column chromatography (silica gel 60, 35–70 μm) in 11 : 1 pentane/ethyl acetate. All volatiles were removed *in vacuo* to yield 1.07 g (50%) of a colorless oil.

^{1}H-NMR ($CDCl_3$, 600 MHz, δ): diastereomer I: 6.42–6.39 (1 H, dd, ^{3}J = 17.4 Hz, ^2J = 1.2 Hz, CH_2=CH—, trans), 6.18–6.16 (1 H, dd, ^{3}J = 2.8/5.8 Hz, —$C_{tert}H$—CH=CH—), 6.15–6.05 (1 H, m, CH_2=CH—), 5.96–5.95 (1 H, dd, ^3J = 2.8/5.6 Hz, —$C_{tert}H$—CH=CH—), 5.83–5.81 (1 H, dd, ^{3}J = 10.5 Hz, ^{2}J = 1.2 Hz, CH_2=CH—, cis), 3.96–3.93 (1 H, dd, ^{2}J = −10.8 Hz, ^{3}J = 6.7 Hz, —O—CH_2—), 3.76–3.73 (1 H, dd, ^{2}J = −10.5 Hz, ^{3}J = 9.7 Hz, —O—CH_2—), 2.90 (1H, s, —CH—$C_{tert}H$), 2.78 (1 H, s, —CH—CH_2—$C_{tert}H$), 2.45–2.41 (1 H, m, —O—CH_2—CH—), 1.88–1.84 (1 H, m, —O—CH_2—CH—CH_2—), 1.46–1.45 (1 H, m, —$C_{tert}H$—CH_2—C_{tert}H—), 1.26–1.23 (1 H, m, —C_{tert}H—CH_2—C_{tert}H—), 0.60–0.57 (1 H, m, —O—CH_2—CH—CH_2—); diastereomer II: 6.44–6.41 (0.3 H, dd, ^3J = 17.4 Hz, ^2J = 1.2 Hz, CH_2=CH—, trans), 6.15–6.05 (3x 0.3 H, m, —C_{tert}H—CH=CH—, CH_2=CH—), 5.84 (0.3 H, dd, ^3J = 10.8 Hz, ^2J = 1.2 Hz, CH_2=CH—, cis), 4.26–4.23 (0.3 H, dd, ^2J = —11.0 Hz, ^3J = 6.4 Hz, —O— CH_2—), 4.07–4.04 (0.3 H, d, ^2J = −10.8 Hz, ^3J = 9.3 Hz, —O—CH_2—), 2.85 (0.3 H, s, —CH—$C_{tert}H$), 2.73 (0.3H, m, —CH— CH_2— $C_{tert}H$), 1.79–1.73 (0.3 H, m, —O— CH_2—CH—), 1.37–1.23 (3x 0.3 H, m, —C_{tert}H—CH_2—C_{tert}H—, —O—CH_2—CH—CH_2—), 1.21–1.17 (0.3 H, m, —O—CH_2—CH—CH_2—). ^{13}C-NMR ($CDCl_3$, 150 MHz, δ): diastereomer I: 29.1, 37.9, 42.4, 44.0, 49.5, 68.1, 128.9, 130.6, 132.2, 137.8, 166.4; diastereomer II: 29.7, 38.1, 41.7, 43.8, 45.1, 68.6, 128.8, 130.7, 136.4, 137.1, 166.5. Gas chromatography–mass spectroscopy: *m/z* Calcd for $C_{11}H_{14}O_2$:178.1. Found: 178 ($M^{+\cdot}$), 66 (C_5H_6). IR (ATR mode, wavenumber $\tilde{\nu}$, cm^{-1}): 3059 (m), 2964 (s), 2868 (m), 1722 (s), 1635 (m), 1620 (m), 1406 (s), 1294 (s), 1271 (s), 1184 (S), 1053 (s), 985 (s), 966 (m), 810 (s), 721, 631.

2,5-Dioxopyrrolidin-1-yl-(1*R*,4*R*)-7-oxabicyclo [2.2.1]hept-5-ene-2 carboxylate (**M IV**)

N-Hydroxysuccinimide (NHS; 138 mg, 1.20 mmol) and triethylamine (121 mg, 1.20 mmol) were dissolved in dry CH_2Cl_2 (15 mL). Subsequently, 7-oxanorborn-5-ene carboxylic acid chloride was added dropwise (164 mg, 1.0 mmol). The reaction mixture was stirred for 4 h and was washed twice with ice-cold water. The organic layer was dried over $MgSO_4$, and the organic solvent was removed *in vacuo*. The solid raw product was washed with ice-cold diethyl ether and purified by flash column chromatography (silica gel 60, 35–70 μm) in ethyl acetate. The organic solvent was removed *in vacuo* to yield 130 mg (55%) of a white crystalline product.

^{1}H-NMR (600 MHz, $CDCl_3$, δ): 6.47–6.45 (1H, dd, J = 5.8, 1.5 Hz, —CH=CH—), 6.40–6.39 (1H, dd, J = 5.7, 1.4 Hz, —CH=CH—), 5.34 (1H, s, —CH—CH—CO), 5.16–5.15 (1H, d, J = 4.4 Hz, —CH—CH_2—CH—), 2.84–2.83 (4H, d, J = 9.8 Hz, —CO—CH_2—), 2.72–2.70 (1H, dd, J = 8.5, 4.0 Hz, —CH—CO), 2.27–2.24 (1H, dt, J = 4.3, 11.7 Hz, —CH_2—CH—CO—), 1.70–1.73 (1H, dd, J = 8.5 Hz, 11.7 Hz, —CH_2—CH—CO—). ^{13}C-NMR DEPT (150 MHz, $CDCl_3$, δ): 169.6, 169.3, 137.6, 132.0, 81.1, 78.2, 40.4, 30.0, 25.7. IR (ATR mode, $\tilde{\nu}$, cm^{-1}) 3100–3000 (w), 2949 (m), 1807 (s), 1778 (s), 1730 (S), 1571 (m), 1427 (m), 1361 (s), 1315 (m), 1273 (m), 1200 (S), 1155 (m), 1140, 1105, 1061, 1047, 1020, 995, 947, 895, 872, 850, 804, 737, 706, 642.

Functionalized monoliths in capillaries

Pretreatment of the capillaries

The inner walls of the capillaries were treated by the subsequent passing of 4 mL of ethanol, 4 mL of distilled water, 4 mL of 1*M* NaOH, and 8 mL of distilled water through the device followed by drying at 40°C in a vacuum furnace. Silanization was accomplished by the use of a 50 wt % solution of 3-(trimethoxysilyl)propyl methacrylate in toluene for 12 h at 60°C. Then, the devices were rinsed with 4 mL of toluene and 4 mL of acetone followed by vacuum drying. In all cases, a syringe pump (World Precision Instruments, Sarasota, FL) was used with a flow rate of 0.8 mL/min applied for the 200-μm capillaries.

Typical procedure of the polymerization inside a capillary

The polymethacrylate-based monoliths were prepared inside a capillary according to the procedure described earlier with a 10-MeV linear accelerator ELEKTRONIKA (Tory Co., Moscow, Russia).[23,27] The capillary was filled with the polymerization mixture, which consisted of 15 wt % EMA, 15 wt % TMPTA, 30 wt % 2-propanol, 30 wt % 1-dodecanol, and 10 wt % toluene; sealed at the ends; and irradiated by the application of a dose of 22 kGy. After irradiation, the capillary was connected to the HPLC pump and flushed overnight with acetonitrile or chloroform at a flow rate of 5 μL/min.

EB-based functionalization of monoliths

Monomers **M I–M III, M V,** and **M VI** (Fig. 1) were used. The columns were flushed with the monomer/solvent systems listed in Table I at a flow rate

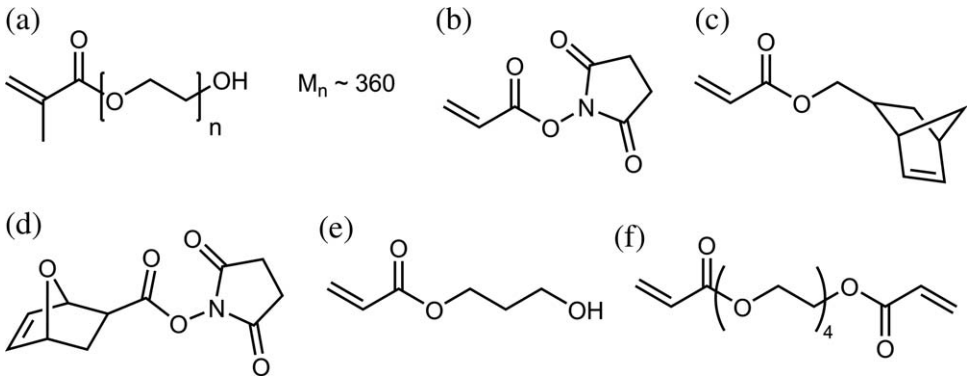


Figure 1 Monomers used for the postsynthesis modification of EB-derived monoliths: (a) **M I** (where M_n = number average molecular weight), (b) **M II,** (c) **M III,** (d) **M IV,** (e) **M V,** and (f) **M VI.**

of 5 μL/min. After complete filling, the columns were exposed to EB irradiation (dose = 22 kGy) and, finally, washed with the corresponding solvent.

ROMP-based functionalization of monoliths

A **M III**-modified monolithic column was prepared by the EB-triggered grafting of **M III** onto the monolith, as described previously, and flushed with ethanol and dry $CHCl_3$ to remove traces of water; this was followed by flushing with a solution of the first-generation Grubbs initiator $RuCl_2(PCy_3)_2(CHPh)$ in dry $CHCl_3$ (4 mg/mL, flow = 5 μL/min, injection volume = 100 μL). After an incubation period of 60 min for immobilization of the initiator, any excess initiator was removed by washing with dry $CHCl_3$. Subsequently, 100 μL of a solution of **M IV** in dry $CHCl_3$ (10 mg/mL) was injected onto the column. After an incubation period of 60 min, the column was flushed with ethyl vinyl ether in $CHCl_3$ (10 mg/mL) and then with $CHCl_3$.

Trypsin immobilization

A solution of trypsin (100 μL) in a 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0, 6 mg/mL) was pumped through the monolithic column by the use of an HPLC–autosampler–pump system. The completeness of filling was tested with the aid of an indicator paper. After incubation for 1 h at room temperature, any unbound trypsin was removed by flushing with water for 1 h. In all cases, the flow rate was 5 μL/min.

TABLE I
Postsynthesis Modification of EB-Derived, Polyacrylate-Based Monolithic Columns

Column	Monomer	Concentration of the monomers during EB grafting
Monolith **M I**	**M I**	10 wt % in H_2O
Monolith **M V/M VI** (5 : 1)	**M V/M VI**	1 wt % in H_2O
Monolith **M II**	**M II**	5 wt % in $CHCl_3$
Monolith **M IV**	**M III**	5 wt % in ethanol

Injection volume = 100 μL; dose = 22 kGy.

Succinylation of immobilized trypsin

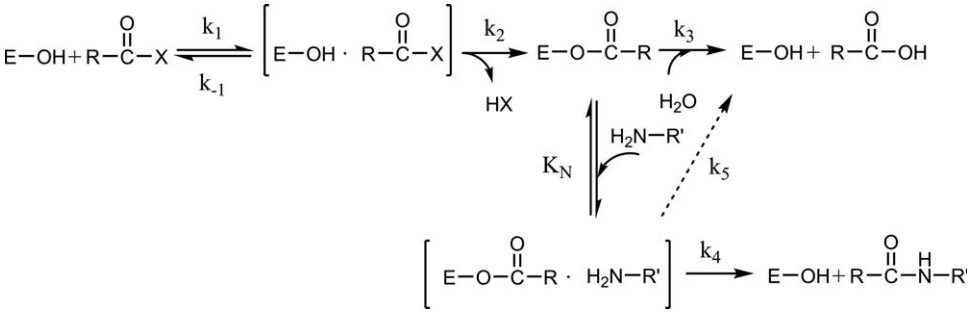
Succinic anhydride (30 mg) was dissolved in 50 μL of dimethyl sulfoxide and 950 μL of water. The filtered solution was flushed through the capillary column (injection volume = 100 μL) at a flow rate of 30 μL/min for 30 min followed by flushing with water.

Acyl-transfer reactions

The reaction mixtures, which contained 2 m*M* BAEE and 25 m*M* N_α-unprotected amino acid amides ($[NH_2—R']$) in 50 m*M* HEPES buffer (pH 8.0, 100 m*M* NaCl, 10 m*M* $CaCl_2$) were prepared. On the basis of their pK_a values, the concentrations were corrected according to eq. (1), where $[NH_2—R']^0$ corresponds to the total amino acid amide concentration and $[NH_2—R']$ corresponds to the unprotonated species at a given pH:

$$[NH_2—R'] = [NH_2—R']^0/(1 + 10^{pK-pH}) \quad (1)$$

The reaction was started by the loading of 100 μL of the reaction mixture onto a trypsin-modified monolithic column with the aid of an Ultimate 3000 autosampler module (Dionex); this was followed by flushing with a 50 m*M* HEPES buffer (pH 8.0) and water. The fractions were collected in a mixture of water and methanol (50 : 50 wt % containing 1 wt % TFA) with a fraction collector and were analyzed by RP-HPLC to determine the ratio (p) of the hydrolysis



Scheme 1 Mechanism for serine-protease-catalyzed acyl-transfer reactions, where E—OH is the free enzyme, R–COX is the acyl donor, X is the leaving group, and NH_2—R′ is the nucleophile. k_x = rate constants, K_N = equilibrium constant.

product ([R—CO_2H]) to the aminolysis product {[R—CONH—R′]; eq. (2), Scheme 1}:

$$p = [NH_2—R'][R—CO_2H]/[R—CONH—R'] \quad (2)$$

RESULTS AND DISCUSSION

Strategies for the functionalization of the monolithic scaffolds

In the first step, monoliths were prepared by EB-triggered, free-radical polymerization with EMA as a monomer, TMPTA as a crosslinker, 2-propanol as a macroporogen, and toluene as a microporogen.[23,27,28] A representative scanning electron microscopy picture is shown in Figure 2. To contribute to a potential upscaling, no further functional monomer was added. Such an approach offers two advantages. First, the total amount of functional monomer used in the following grafting step is low, which reduces costs. Second, the monolithic support can be prepared and designed in terms of porosity independently from the functional monomer to be used in the grafting process. This prevents any unwanted swelling of the support and changes in backpressure, particularly in case gradient elution is applied later on.

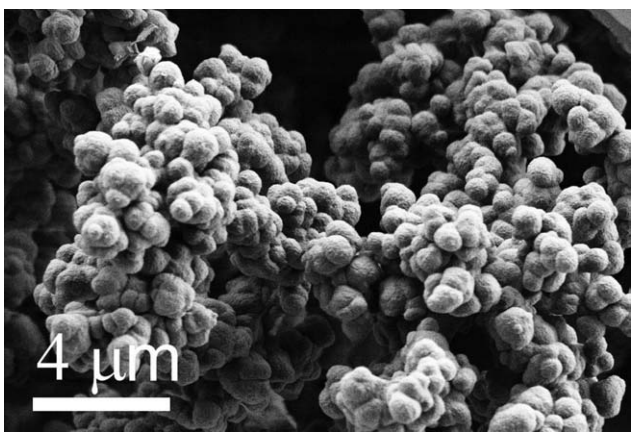


Figure 2 Scanning electron micrograph of the monolithic structure inside a capillary.

In a second step, the monolithic scaffold was subjected to further modification via an EB-assisted grafting process (*vide infra*) with different functional monomers to selectively modify the surface of the monolithic support and preserve the structural integrity of the monolithic three-dimensional structure.[29] With monomers **M I-M III**, **M V**, and **M VI**, either protein-repellent surfaces (**M I, M V, M VI**) or functional ones (**M II, M III**), which were finally suited for the immobilization of proteins via covalent attachment, were obtained.

To investigate the protein-repellent character of monoliths grafted with **M I, M V,** and **M VI,** monoliths modified with one of the aforementioned monomers were characterized for any nonspecific protein adsorption by treatment with trypsin. Then, the capability to hydrolyze a 20 m*M* BAEE solution under flow conditions after repeated injections was measured. Generally, BAEE is a very sensitive substrate for trypsin ($k_{cat}/K_M \approx 2 \times 10^6$ M^{-1} s^{-1}), k_{cat} = turnover number, K_M = equilibrium constant according Michaelis–Menten kinetics and therefore, even small amounts of any nonspecifically adsorbed protein can be detected and quantified by this approach.[30] Practically, 100 μL of a 20 m*M* BAEE solution was pumped through the monolithic column. Because of the nature of the column and the experimental conditions, the obtained hydrolysis product, benzoyl-L-arginine, was not separated inline from the remaining BAEE during the run; instead, the reaction mixture was analyzed separately by HPLC on a commercial HPLC column after collection of the eluate. Typical graphs from selected hydrolysis experiments are shown in Figure 3. As we deduced from these results, an equilibrium was obtained after about five injections. The hydrolysis results after 20 injections are summarized in Table II.

As we deduced from these results, the parent acrylate-based monolithic scaffolds showed significant nonspecific protein adsorption. It could, however, be reduced by a factor of four via the modification of the monolith's surface with glycol units, for example, by poly[poly(ethylene glycol) methacrylate] [poly(**M I**)] or

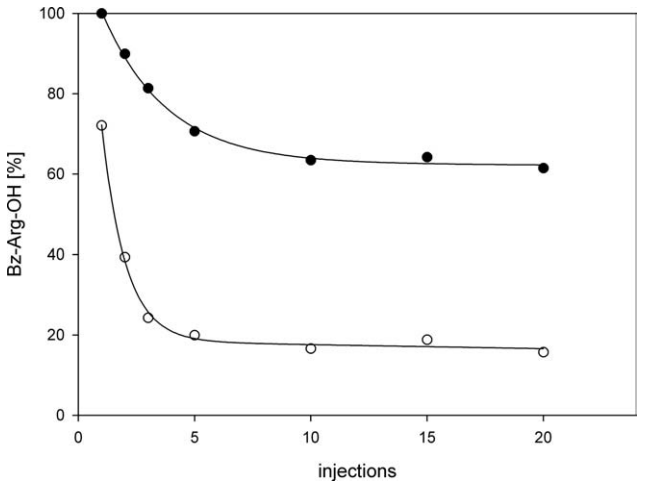


Figure 3 BAEE hydrolysis by trypsin adsorbed onto monolithic columns: (●) parent monolith and (○) monolith–poly(**M V**-*co*-**M VI**)-modified monolith.

poly(hydroxypropyl acrylate-*co*-tetraethylene glycol diacrylate) [poly(**M V**-*co*-**M VI**)], a finding that was in accordance with those reported by other groups.[26,31,32]

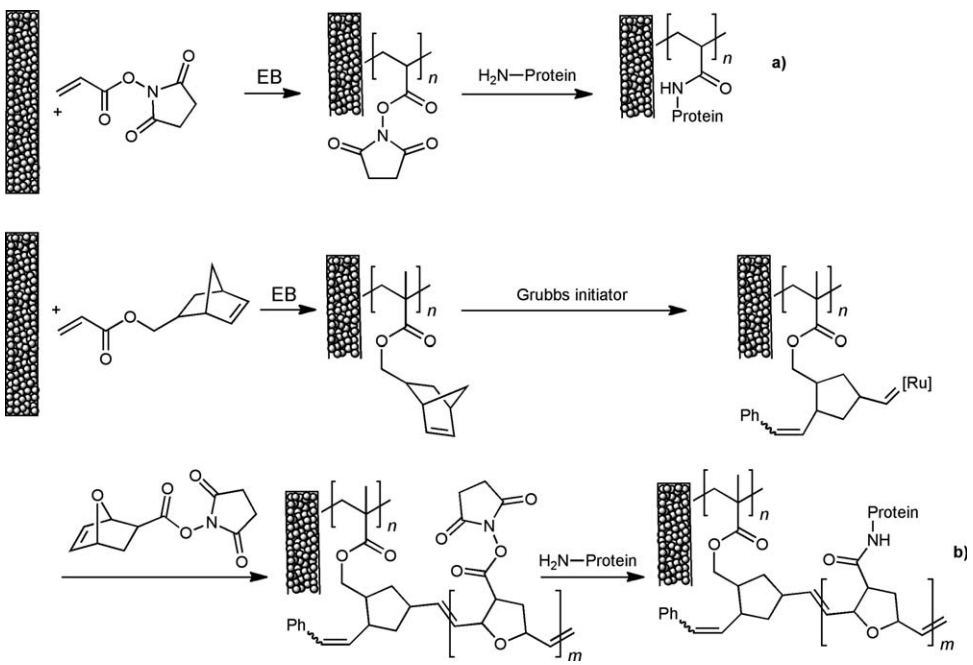
Covalent immobilization of trypsin

Trypsin is the most popular enzyme for the fragmentation of proteins in miniaturized reaction systems and allows their identification and characterization by mass spectroscopy techniques combined with database screening of the obtained peptide fragments. Although it has already been bound to various supports, the use of porous polymeric monoliths that allow for a high rate of substrate perfusion between the fixed enzyme and the substrate has been investigated less.[6,9–11,33–36] However, the immobilization of proteases for peptide synthesis purposes has been investigated, for example, for carboxypeptidase Y and chymotrypsin.[37–39] In addition to the hydrolysis of proteins and peptides, serine proteases can catalyze acyl-transfer reactions from acyl donors to various acceptors.[40] As a result of the reaction mechanism, the acyl enzyme intermediate formed during catalysis can be deacylated competitively by these acceptors, in particular by water and other nucleophiles (e.g., amino acid amides). When carbonic acid esters are used as the carboxyl

TABLE II
BAEE Hydrolysis by Trypsin Immobilized on the Monolithic Columns (170 × 0.2 mm^2)

Column	Yield (benzoyl-L-arginine, %)
Monolith, unmodified	61
Poly(**M I**)-grafted monolith	18
Poly(**M V**-*co*-**M VI**)-grafted monolith	16

Hydrolysis conditions: injection volume = 100 μL, flow = 5 μL/min, BAEE = 20 m*M* in 50 m*M* HEPES (pH 8.0, 100 m*M* NaCl, 10 m*M* $CaCl_2$). Eluents were collected for 1 h in 100 μL of stop solution and analyzed by RP-HPLC. The results correspond to the 20th injection.



Scheme 2 Routes for the functionalization of EB-derived polyacrylate-based monoliths: (a) EB-assisted grafting of an active ester (**M II**) followed by the aminolysis of a protein amino group, (b) EB-assisted grafting of a norborn-2-ene-containing acrylate (**M III**) followed by ROMP-based surface grafting of an active ester (**M IV**) and aminolysis of a protein amino group.

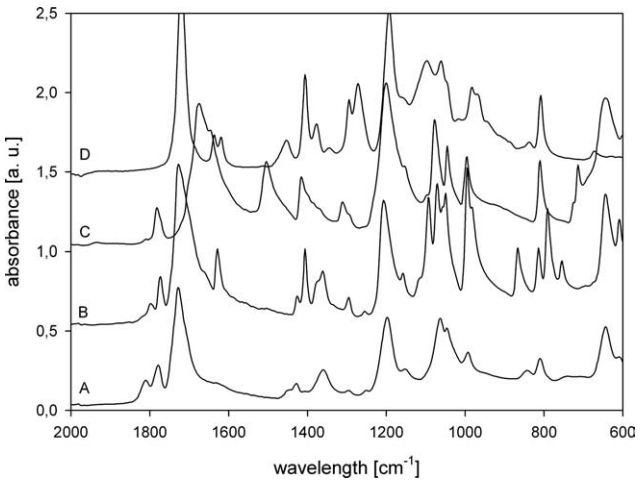


Figure 4 ATR–Fourier transform infrared spectra of (A) poly(acryl–NHS), (B) acryl–NHS, (C) NHS, and (D) TPGDA.

component that fits the primary specificity of trypsin, for example, N_α-protected arginine or lysine esters, and substrate mimetics,[41,42] it is possible to achieve product accumulation above the thermodynamically determined equilibrium concentration. The product accumulation can be improved when chemically or genetically modified trypsin variants are used[43] or when the reaction is performed in frozen aqueous media.[44] A general mechanism for a serine-protease-catalyzed acyl-transfer reaction is given in Scheme 1.

To covalently bind the protein, that is, trypsin, the monolithic scaffold was modified by two different methods, which are summarized in Scheme 2. The general strategy was the generation of different functional units for protein immobilization and their evaluation in terms of protein-binding capacity. For these purposes, we introduced *N*-hydroxysuccinimidyl esters.[45]

A poly(*N*-acryloxysuccinimide) modification of the monolithic scaffolds was obtained via the EB-triggered grafting of **M II** [Scheme 2(a)]. ATR–IR spectroscopy gave no indication for the degradation of the activated NHS–ester groups in the polymer during EB irradiation under the described conditions (Fig. 4). The signal around 1770 cm^{-1} was attributed to the cyclic five-membered ring-based imides and was still present in the monomeric and polymeric form and in NHS itself but was absent in other substituted acrylates, such as TPGDA. Typical signals from the acrylic double bond at 1640 and 810 cm^{-1} were found in the monomeric but not in the polymeric form; this indicated complete polymerization. The reaction of trypsin with the activated ester finally resulted in the immobilization of the protein.

Finally, surface-grafted **M III** was used as an anchor and initiation point for the ROMP-based surface grafting[46] of **M IV** with application of the first-generation Grubbs initiator $RuCl_2(PCy_3)_2(CHPh)$ [Scheme 2(b)]. As evidenced by former studies, the norborn-2-ene unit was retained during the process of EB curing.[47] The reaction of trypsin with the activated ester again resulted in the immobilization of the protein. Because poly{bicyclo[2.2.1]hept-5-en-2-ylmethyl acrylate} possesses a polyether structure, it is highly hydrophilic, and one can expect similar protein-repellent properties, as observed with poly (ethylene glycol)-type structures. Consequently, the measured protein activity was mainly attributed to the covalently bound trypsin.

The immobilization efficiency of the trypsin-modified columns was again tested by the hydrolysis of 20 m*M* BAEE, as described previously. In all cases, the covalent attachment significantly enhanced the amount of bound trypsin (Table III). The best

TABLE III
BAEE Hydrolysis by Trypsin Immobilized on the Monolithic Columns

Column	Column dimensions (mm^2)	Yield (benzoyl-L-arginine, %)
Monolith, unmodified	170 × 0.2	61
Poly(**M II**)-grafted monolith	170 × 0.2	95
Poly(**M IV**)-grafted monolith	170 × 0.2	100
Poly(**M IV**)-grafted monolith	50 × 0.2	57

Poly(**M II**) = poly(*N*-acryloxysuccinimide). The results correspond to the 20th injection for the 170 × 0.2-mm^2 columns and the 21st injection for the 50 × 0.2 mm^2 column.

TABLE IV
Acyl-Transfer Studies from BAEE to a Series of Amino Acid Amides H—Xaa—NH_2 Forming Bz—Arg—Xaa—NH_2 Catalyzed by Immobilized Trypsin Exposed to On-Column Modification with Succinic Anhydride

		p [eq. (2)] of H—Xaa—NH_2		
	Immobilized enzyme	Met	Lys	Gly
Poly(**M IV**)-grafted monolith	Succinyl–trypsin	15	9	87
Trypsin in solution according to ref. 49		17	32	284

Conditions: injection volume = 100 μL, flow = 5 μL/min, BAEE = 2 m*M*, and H—Xaa—NH_2 = 25 m*M* [which corresponds to NH_2—R′ in eq. (1)] in 50 m*M* HEPES at pH 8.0 (100 m*M* NaCl, 10 m*M* $CaCl_2$). Eluents were collected for 1 h in 100 μL of stop solution and analyzed by RP-HPLC.

performance was observed for the monolith grafted with **M IV** via ROMP, which allowed for a 100% conversion of BAEE into the desired hydrolysis product benzoyl-L-arginine.

Acyl-transfer and on-column protein modification

To demonstrate that trypsin immobilized onto monolithic columns by any of the previously described procedures could be used for peptide synthesis, acyl-transfer studies were accomplished with 25 m*M* N_α-unprotected methionine amide, lysine amide, and glycine amide as nucleophiles and 2 m*M* BAEE as the acyl-donor component. For the **M IV**-derivatized monolithic column, the immobilized trypsin was subjected to chemical modification with succinic anhydride. Such a succinylation of the lysine residues has two major effects. It prevents autodigestion and any digestion of Lys-specific proteases, and therefore, it may enhance the stability of immobilized trypsin, especially when unpurified samples are analyzed. In addition, succinylation influences trypsin's secondary specificity via the modification of trypsin–^{60}lysine. Thus, modification results in a pronounced S1′–P1′ interaction if the P1′ position of the substrate is occupied with a basic amino acid.[48] Compared to unmodified trypsin, this is reflected by the reduction of the *p* value for the acyl transfer to P1′-basic amino acids, for example, in H—Lys—NH_2, because the partition of the hydrolysis product and aminolysis product is shifted toward the latter one [eq. (2)]. An enhancement of protease specificities to strictly limited cleavage sites may be of interest in proteomics because few but highly characteristic protein fragments are generated during digestion with so-called restriction proteases; this allows a better assignment of the fragments in complex mixtures. Furthermore, an improved synthetic efficiency may be of interest when proteases are used as biocatalysts for peptide bond formation. As anticipated, an improved acceptance of H—Lys—NH_2 was found for succinylated trypsin by means of the reduction of the *p* value for the acyl-transfer reaction (Table IV). This was indicative of a successful on-column modification of trypsin by succinic anhydride. However, this system has to be further improved for restricted proteolytic approaches.

CONCLUSIONS

The postsynthesis functionalization of polyacrylate-based monoliths via the EB-triggered grafting of functional acrylates followed by different modification steps, including ROMP and free-radical polymerization, were carried out. The obtained materials were characterized in terms of their protein binding and repellent characteristics. Among the systems investigated, the *N*-hydroxysuccinimidyl ester substituted, ROMP-derived monolith showed the best performance in terms of the covalent attachment of trypsin, whereas poly(ethylene glycol)-functionalized polyacrylate-based monoliths had a high tendency to inhibit protein binding. Monolithic capillary columns with surface-immobilized trypsin and succinyl–trypsin were tested for their ability to synthesize peptides via the acyl transfer of BAEE to amino acid amides in a biocatalytic approach. The obtained specificity data were in accordance with literature results. In fact, the results presented here are a suitable base for a future combination, that is, the integration of both biocatalysis and separation of the generated low- or high-molecular-weight analytes within one single microfluidic chip that consists of sections of monolithic phases with different morphologies and surface chemistries. In addition, the methodology developed here will allow the rapid evaluation of the activity of an enzyme (whichever) immobilized by one of the techniques presented here. Moreover, a comparison of the immobilization efficiency of monolithic microsystems due to different surface chemistries is suitable by the presented approach. Our current work focuses on this combination of catalysis and separation with new monolithic stationary phases capable of separating both low- and high-molecular-weight analytes.

References

1. Belder, D.; Ludwig, M.; Wang, L. W.; Reetz, M. T. Angew Chem Int Ed 2006, 45, 2463.
2. Jähnisch, K.; Hessel, V.; Löwe, H.; Baerns, M. Angew Chem Int Ed 2004, 43, 406.
3. Yu, C.; Xu, M.; Švec, F.; Fréchet, J. M. J. J Polym Sci Part A: Polym Chem 2002, 40, 755.
4. Pierre, S. J.; Thies, J. C.; Dureault, A.; Cameron, N. R.; van Hest, J. C. M.; Carette, N.; Michon, T.; Weberskirch, R. Adv Mater 2007, 18, 1822.
5. Hickey, A. M.; Marle, L.; McCreedy, T.; Watts, P.; Greenway, G. M.; Littlechild, J. A. Biochem Soc Trans 2007, 35, 1621.
6. Krenkova, J.; Lacher, N. A.; Svec, F. Anal Chem 2009, 81, 2004.
7. Nicoli, R.; Bartolini, M.; Rudaza, S.; Andrisano, V.; Veuthey, J.-L. J Chromatogr A 2008, 1206, 2.
8. Wirth, M. J. Anal Chem 2007, 79, 800.
9. Cooper, J. W.; Chen, J.; Li, Y.; Lee, C. S. Anal Chem 2003, 75, 1067.
10. Gao, J.; Xu, J.; Locascio, L. E.; Lee, C. S. Anal Chem 2001, 73, 2648.
11. Wang, C.; Oleschuk, R.; Ouchen, F.; Li, J.; Thibault, P.; Harrison, D. J. Rapid Commun Mass Spectrom 2000, 14, 1377.
12. Banu, S.; Greenway, G. M.; McCreedy, T.; Shaddick, R. Anal Chim Acta 2003, 486, 149.
13. Peterson, D. S. Lab Chip 2005, 5, 132.
14. Peterson, D. S.; Rohr, T.; Svec, F.; Fréchet, J. M. J. Anal Chem 2002, 74, 4081.
15. Ponomareva, E. A.; Kartuzova, V. E.; Vlakh, E. G.; Tennikova, T. B. J Chromatogr B 2010, 878, 567.
16. Minakuchi, H.; Nakanishi, K.; Soga, N.; Ishizuka, N.; Tanaka, N. Anal Chem 1996, 68, 3498.

17. Lubda, D.; Cabrera, K.; Nakanishi, K.; Minakuchi, H. J Sol Gel Sci Technol 2002, 23, 185.
18. Lubbad, S. H.; Buchmeiser, M. R. J Sep Sci 2009, 32, 2521.
19. Svec, F.; Peters, E. C.; Sykora, D.; Yu, C.; Frechet, J. M. J. J High Resolut Chromatogr 2000, 23, 3.
20. Buchmeiser, M. R. Polymer 2007, 48, 2187.
21. Buchmeiser, M. R. J Sep Sci 2008, 31, 1907.
22. Fintschenko, Y. Anal Bioanal Chem 2001, 371, 174.
23. Bandari, R.; Knolle, W.; Prager-Duschke, A.; Gläsel, H. J.; Buchmeiser, M. R. Macromol Chem Phys 2007, 208, 1428.
24. Truckenmüller, R.; Rummler, Z.; Schaller, T.; Schomburg, W. K. J Micromech Microeng 2002, 12, 375.
25. Heckele, M.; Guber, A. E.; Truckenmüller, R. Microsyst Tech 2006, 12, 1031.
26. Logan, T. C.; Clark, D. S.; Stachowiak, T. B.; Svec, F.; Fréchet, J. M. J. Anal Chem 2007, 79, 6592.
27. Bandari, R.; Eisner, C.; Knolle, W.; Kuhnel, C.; Decker, U.; Buchmeiser, M. R. J Sep Sci 2007, 30, 2821.
28. Bandari, R.; Knolle, W.; Buchmeiser, M. R. J Chromatogr A 2008, 1191, 268.
29. Beier, M. J.; Knolle, W.; Prager-Duschke, A.; Buchmeiser, M. R. Macromol Rapid Commun 2008, 29, 904.
30. Maurel, P.; Huibonhoa, G.; Douzou, P. J Biol Chem 1975, 250, 1376.
31. Ostuni, E.; Chapman, R. G.; Holmlin, R. E.; Takayama, S.; Whitesides, G. M. Langmuir 2001, 17, 5605.
32. Krenkova, J.; Lacher, N. A.; Svec, F. J Chromatogr A 2009, 1216, 3252.
33. Li, Y.; Xu, X.; Deng, C.; Yang, P.; Zhang, X. J Proteom Res 2007, 6, 3849.
34. Palm, A. K.; Novotny, M. V. Rapid Commun Mass Spectrom 2004, 18, 1374.
35. Ye, M.; Hu, S.; Schoenherr, R. M.; Dovichi, N. J. Electrophoresis 2004, 25, 1319.
36. Al-Lawati, H.; Welham, K. J. Analyst 2006, 131, 656.
37. Cramer, S. M.; Horvath, C. Biotech Bioeng 1989, 33, 344.
38. Kuhl, P.; Konnecke, A.; Doring, G.; Daumer, H.; Jakubke, H. D. Tetrahedron Lett 1980, 21, 893.
39. Kato, N.; Oishi, A.; Takahashi, F. Mater Sci Eng C 2000, 13, 109.
40. Schellenberger, V.; Jakubke, H. D. Angew Chem Int Ed 1991, 30, 1437.
41. Bordusa, F.; Ullmann, D.; Elsner, C.; Jakubke, H. D. Angew Chem Int Ed 1997, 36, 2473.
42. Günther, R.; Elsner, C.; Schmidt, S.; Hofmann, H. J.; Bordusa, F. Org Biomol Chem 2004, 2, 1442.
43. Elsner, C.; Grahn, S.; Bauer, S.; Ullmann, D.; Kurth, T.; Jakubke, H. D. J Mol Catal B 2000, 8, 193.
44. Hänsler, M.; Jakubke, H. D. Amino Acids 1996, 11, 379.
45. Theato, P. J Polym Sci Part A: Polym Chem 2008, 46, 6677.
46. Buchmeiser, M. R. Adv Polym Sci 2006, 197, 137.
47. Bandari, R.; Knolle, W.; Prager-Duschke, A.; Buchmeiser, M. R. Macromol Rapid Commun 2007, 28, 2090.
48. Schechter, I.; Berger, A. Biochem Biophys Res Commun 1967, 27, 157.
49. Salchert, K.; Ullmann, D.; Jakubke, H.-D. Pharmazie 1996, 51, 710.