Macromolecules
ARTICLE

Macromolecules **2010**, *43*, 9601–9607 **9601**
DOI: 10.1021/ma101922s

A Continuous Bioreactor Prepared via the Immobilization of Trypsin on Aldehyde-Functionalized, Ring-Opening Metathesis Polymerization-Derived Monoliths

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Received August 23, 2010; Revised Manuscript Received November 3, 2010

ABSTRACT: The ring-opening metathesis polymerization (ROMP) of norborn-2-ene (NBE) and *cis*-cyclooctene (COE) was initiated with well-defined Grubbs-type initiators, i.e., $RuCl_2(CHPh)(PCy_3)_2$ (**1**), $[RuCl_2(PCy_3)(IMesH_2)(CHPh)]$ (**2**), and $[RuCl_2(3\text{-Br-Py})_2(IMesH_2)(CHPh)]$ (**3**) ($MesH_2$ = 1,3-bis(2,4,6-trimethylphenyl)-imidazolin-2-ylidene, PCy_3 = tricyclohexylphosphine, 3-Br-Py = 3-bromopyridine). Reaction of the living polymers with O_2 (air) resulted in the formation of aldehyde−semitelechelic polymers in up to 80% yield, depending on the initiator and monomer used. To proof aldehyde formation, the terminal aldehyde groups were converted into the corresponding 2,4-dinitrophenylhydrazine derivatives, and the structure of the hydrazones was confirmed by NMR and IR spectroscopy. This simple methodology was then used for the functionalization of ROMP-derived monoliths prepared from NBE, 1,4,4a,5,8,8a-hexahydro-1,4,5,8-*exo*-*endo*-dimethanonaphthalene (DMN-H6) and $(NBE\text{-}CH_2O)_3SiCH_3$, to yield aldehyde-funtionalized monoliths. The extent of aldehyde formation was determined by hydrazone formation. Up to 8 μmol of aldehyde groups/g monolith could be generated by this approach. Finally, these aldehyde-functionalized monoliths were used for the immobilization of trypsin. Excellent proteolytic activity of the immobilized enzyme was found both under batch and continuous flow conditions.

Introduction

Ring-opening metathesis polymerization (ROMP) has become a powerful tool for polymer synthesis.[1−4] For this purpose, Ru-based initiators have attracted significant attention. Their ease of use, enhanced stability, high catalytic activity, and their excellent functional group tolerance relative to the other catalysts have broadened their synthetic utility beyond olefin metathesis.[5−7] Interestingly, there exist diverging reports on the stability of Grubbs' type initiators vs oxygen, water, nitriles, amines, etc.[8−13] Gibson et al. were the first to use this reaction for the preparation of semitelechelic ROMP-derived polymers.[14,15] Recent reports show that on exposure to oxygen these initiators can afford Ru−hydride complexes along with organic byproducts.[10,16,17] Thus, reaction of $RuCl_2(CHPh)(PCy_3)_2$ (**1**) with air in benzene has been reported to result in the formation of benzaldehyde as well as *cis*- and *trans*-stilbene along with various Ru-containing byproducts (Scheme 1).[13,18] For the past decades, several approaches toward the synthesis of telechelic or semitelechelic polymers have been reported.[19−41] In view of the synthetic potenial of the metathesis reaction of Ru−alkylidenes with oxygen, we decided to employ this reaction for the preparation of aldehyde-functionalized, ROMP-derived polymeric monoliths. This study describes as to what extent ROMP-derived polymers can be functionalized upon termination with O_2 and in which form this reaction can be useful to material science and biocatalysis.

Results and Discussion

Reactivity of Ru-Based Initiators vs Air (O_2). In order to obtain a mechanistic understanding, we first reacted initiators **1**−**3** ($IMesH_2$ = 1,3-bis(2,4,6-trimethylphenyl)imidazolin-2-ylidene, PCy_3 = tricyclohexylphosphine, 3-Br-Py = 3-bromopyridine (Figure 1) with air (O_2) in the absence of any monomer in benzene for 24 h. In accordance with the results of Grubbs et al. on the decomposition products of **1**,[13] this reaction afforded a mixture of benzaldehyde and *cis*/*trans*-stilbene (Scheme 1, Figure S1, Supporting Information) along with several Ru byproducts, which were not further characterized. The yields of benzaldehyde and the stilbenes were determined by GC-MS with the aid of an internal standard and reference compounds and are summarized in Table 1.

As can be seen, the benzaldehyde yield increased in the order **1** > **2** ∼ **3** while the yield of the stilbenes decreased in the order **2** > **1** > **3**. Particularly the low stilbene yields obtained with **3** clearly illustrate the reduced propensity of **3** to react with an aldehyde to give the corresponding stilbene.

Reactivity of Living ROMP-Derived Polymers vs O_2. Next, starting with a high-ring strain monomer, i.e., norborn-2-ene, we synthesized aldehyde−telechelic polymers via the reaction of the living Ru−alkylidene of the ROMP-derived poly(NBE) with O_2 as outlined in Scheme 2. With the three different initiators **1**−**3** used in this study, the desired aldehyde telechelic polymers were formed to a different extent. Thus, in case a ROMP-derived poly(NBE) initiated by **1** was reacted with air for 16 h, an aldehyde-semitelechelic polymer with one polymer end bearing an aldehyde group was obtained in 80% yield (Table 2) while the termination of living poly(NBE) prepared by the action of **2** or **3** with O_2 gave only 47% and 29% of aldehyde−semitelechelic polymers. These data clearly reflect the different chemical stabilities of

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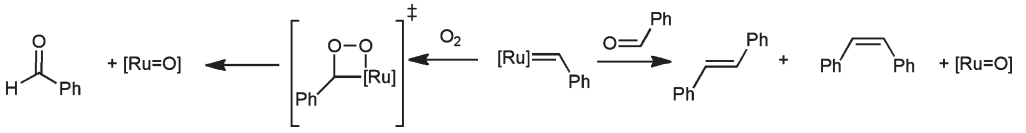
Published on Web 11/16/2010

Scheme 1. Proposed Pathway for the Formation Benzaldehyde and of the Stilbenes



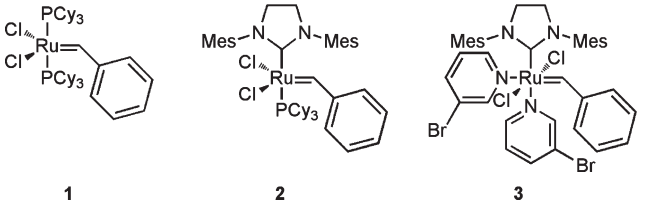


Figure 1. Structures of the first- (**1**), second- (**2**), and third-generation (**3**) Grubbs initiator. PCy_3 = tricyclohexylphosphine, Mes = mesityl.

Table 1. Results of the Reaction of Initiators 1, 2, and 3 with O_2[a]

initiator	benzaldehyde (%)[b]	stilbenes (%)	cis:trans
1	44	7	1:4
2	24	40	0:1
3	29	1	0:1

[a] Benzene, room temperature, 24 h. [b] Determined by GC-MS analysis using *n*-dodecane as internal standard.

the initiators vs O_2. Thus, the stability of the living polymer increases in the order **1** < **2** < **3**.

In the polymers obtained with **1**−**3**, the peaks at $\delta = 9.61$ and 203.6 ppm in the ^{1}H and ^{13}C NMR correspond to the aldehyde proton and carbon, respectively (Figures S2 and S3, Supporting Information). Size exclusion chromatography (SEC) revealed monomodal distributions (1.82 < PDI < 1.98).

In principle, this functionalization procedure may be expected to produce a mixture of the aldehyde−semitelechelic polymer via metathesis of the Ru−alkylidene with O_2 as well as a diphenyl-terminated polymer, e.g., via a coupling reaction between a living polymer and an aldehyde−telechelic one (Scheme 2). In such case, the latter polymer should have a molecular weight twice as high as the parent polymer chain. Using **3** as initiator, a comparison of the size exclusion chromatography (SEC) results obtained for an ethyl vinyl ether-terminated poly(NBE) (M_n = 6800 g/mol, PDI = 1.69) and an O_2-terminated poly(NBE) (M_n = 7100 g/mol, PDI = 1.92) revealed that there was no significant bimolecular coupling among the Ru−alkylidenes of the polymer. An overlay over the SEC traces for these two polymers is shown in Figure 2.

Obviously, the by far less oxophilic Ru−alkylidenes are less prone to O_2-triggered bimolecular coupling than the Mo-based Schrock initiators,[42] where almost quantitative bimolecular coupling can be observed in the presence of O_2. In contrast to the initiators, where such a coupling occurs to a significant extent producing substantial amounts of stilbenes, this reaction is less favored (probable) with entangled polymers, where the chain ends have a lower probability to react.

We then extended our investigation toward less strained monomers such as *cis*-cyclooctene (COE). Again, reaction with O_2 (air) resulted in the formation of aldehyde−telechelic polymers. A maximum aldehyde formation of 61% was obtained with **2**-derived poly(COE). The peaks at $\delta = 9.77$ and 203.0 ppm in the ^{1}H and ^{13}C NMR support the formation of the aldehyde semitelechelic polymer (Figures S4 and S5, Supporting Information). In contrast, end-group analysis of **1**- and **3**-derived poly(COE) prepared under the same conditions showed only 15% and 55% of the aldehyde functionality (Table 2). As observed for the poly(NBE)s, SEC revealed a monomodal yet broad molecular weight distribution (1.46 < PDI < 1.82).

To further proof the formation of the aldehyde termini for both poly(NBE) and poly(COE), we converted the aldehyde termini into the corresponding 2,4-dinitrophenylhydrazine derivatives.[43,44] For that purpose, both aldehyde−semitelechelic poly(NBE) and poly(COE) were treated with a freshly prepared acidic solution of 2,4-dinitrophenylhydrazine to afford yellow precipitates of the corresponding hydrazones (Scheme 3). The ^{1}H NMR, ^{13}C NMR, and IR data strongly support the aldehyde functionality in the polymer chain. In the ^{1}H and ^{13}C NMR, the peaks at $\delta = 9.61$ and 203.6 ppm corresponding to the aldehyde group disappeared, and new signals corresponding to the −HC=N− moiety at δ = 7.49 and 155.9 ppm appeared in the ^{1}H and ^{13}C NMR, respectively.

Application: Surface Functionalization of ROMP-Derived Monolithic Supports. The above-described methodology was applied to the functionalization of ROMP-derived monoliths by taking advantage of the truly living character of ROMP.[45,46] Monoliths were prepared from NBE, 1,4,4a,5,8,8a-hexahydro-1,4,5,8-*exo*-*endo*-dimethanonaphthalene (DMN-H6), 2-propanol, toluene, and **1** within stainless steel columns according to published procedures.[45,47] Then the initiator was subjected to a metathesis reaction with O_2 (Scheme 3) by flushing the monolith consecutively with toluene and air. Since a rigid polymer is formed, side reactions as described in Scheme 2 may well be expected to occur to a very minor extent, if at all. In fact, as evidenced by FT-IR, aldehyde groups formed at the surface of the structure-forming microgobules. To further proof aldehyde formation and to quantify the amount of aldehyde groups, these were again converted into the corresponding hydrazones via reaction with 2,4-dinitrophenylhydrazine. Both the signal at 1725 cm^{-1} in the IR spectrum and the nitrogen content (up to 30 μmol/g) as determined by elemental analysis confirmed the successful functionalization sequence (up to 8 mmol/g).

Immobilization of Trypsin. The use of monoliths for the immobilization of transition-metal-based catalysts or enzymes has been reported by our group[48−51] as well as by Svec and Fréchet.[52−54] Monoliths were prepared from NBE and from $(NBE\text{-}CH_2\text{-}O)_3SiCH_3$ according to a published protocol.[55] The reason for the use of $(NBE\text{-}CH_2\text{-}O)_3SiCH_3$ instead of DMN-H6 is that it provides monoliths with better flow-through characteristics.[55] Introduction of the aldehyde groups was carried out as described above (Scheme 3). The aldehyde-functionalized monolithic support was then used for the immobilization of trypsin.[56,57] Because of the steric demands of trypsin, we introduced tetraethylenepentamine (TEPA, Figure 3) as a spacer between the aldehyde-functionalized surface and the protein (Scheme 4).

The primary as well as secondary amino groups were then reacted with glutaraldehyde (GA, Figure 3) to produce a large number of aldehyde functional groups, which were then further used for the reaction with trypsin. The amount of trypsin bound to the monolithic support was quantified by elemental analysis. There, a nitrogen content of 86 μmol/g of

Scheme 2. Synthetic Route to Aldehyde−Semitelechelic ROMP-Derived Polymers and Possible Side Reactions (Exemplified for Poly(NBE))

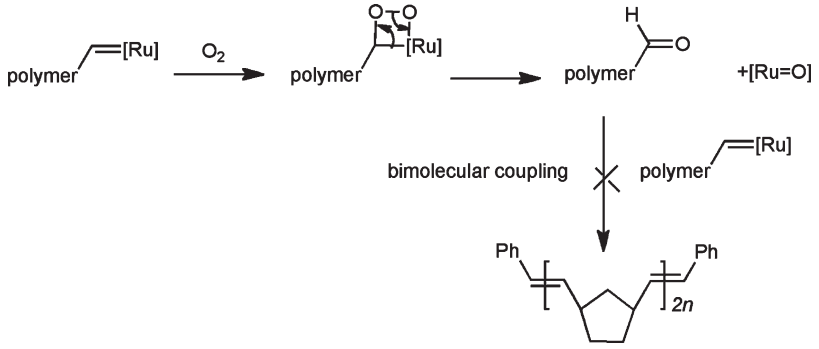


Table 2. Summary of ROMP-Derived Poly(NBE) Prepared by the Action of 1, 2, and 3[a]

initiator	$M_{n,found}$ (g/mol)	PDI	yield (%)[b]	CHO-terminated (%)[c]
1	7500	1.98	91	80
2	8600	1.93	86	47
3	8900	1.82	62	29

[a] Polymerizations conditions: benzene, $[monomer]_0 = 1$ M, monomer: initiator = 30:1; T = 23 °C, t = 1 h. [b] Isolated yield. [c] Determined by ^{1}H NMR spectroscopy.

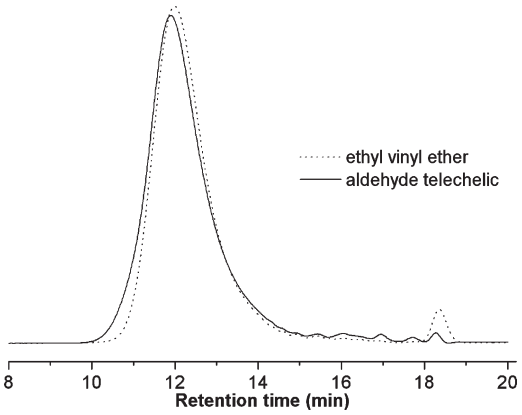


Figure 2. Comparison of the SEC curves between EVE- and O_2-terminated poly(NBE) prepared by the action of **3**.

monolith was found. Since the 8 μmol of aldehyde typically found in such monoliths translates upon reaction with TEPA into 40 μmol of N, the additional 46 μmol of N found by elemental analysis is indicative of the successful immobilization of trypsin on the ROMP-derived monolith.

After its immobilization, the proteolytic activity of the immobilized protein was quantified by using *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) as substrate, which upon hydrolysis produces *N*-α-benzoyl-DL-arginine (BA) and *p*-nitroaniline (PNA). The product formation and the rate of hydrolysis were monitored by RP-HPLC (Figure 4).

The BA that formed was analyzed at $\lambda = 214$ nm, whereas the remaining amount of BAPNA was quantified at λ = 310 nm. The hydrolysis yield of BAPNA was determined by comparing the area of BAPNA after hydrolysis to the area of a standard BAPNA solution (0.25 mM). The plot of conversion of BAPNA vs time is shown in Figure 5. As can be seen, a conversion of 65% was accomplished after 60 min. We then switched to continuous flow conditions and monitored the hydrolysis rate of BAPNA for 3 h using an on column (residence) time of 10 min. As can be seen (Figure 6), a slow decrease in the conversion of BAPNA with a total loss in activity of < 20% was observed. Finally, the influence of the flow rate on BAPNA conversion was examined at 37 °C. For this purpose, aliquots were collected at different flow rates and analyzed by RP-HPLC at 310 nm. As expected, lower hydrolysis yields were obtained at higher flow rates, i.e., shorter on column times due to a reduced contact time between substrate and trypsin. A plot for the rate of hydrolysis against the flow rate is shown in Figure 7.

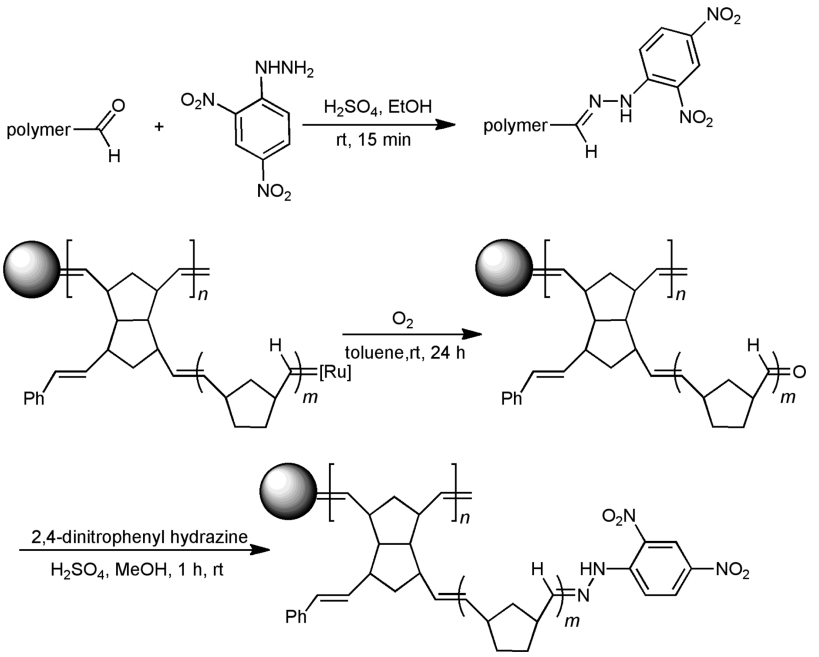
Conclusion

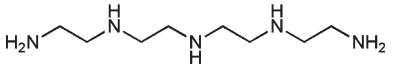
A novel and convenient method for the synthesis of ROMP-derived aldehyde−semitelechelic polymers via the metathesis of the living Ru−alkylidenes of ROMP-derived polymers with O_2 has been developed. The method was checked for all three Grubbs-type initiators. Up to 80% of aldehyde−semitelechelic poly(NBE) were observed when NBE was polymerized by **1** and terminated with O_2. Though this simple reaction does not quantitatively yield aldehyde−semitelechelic polymers and therefore appears less suitable for the selective functionalization of linear and soluble polymers, it is of significant synthetic utility for the surface functionalization of insoluble (cross-linked) polymeric materials, e.g., polymeric monoliths, where a significant degree of surface functionalization up to 8 μmol can be achieved. This amount of aldehyde groups allows for the generation of surface-bound (branched) oligoaldehydes which finally offer access to the permanent immobilization of enzymes, e.g., trypsin, and for use of the thus-prepared supported enzymes in continuous flow biocatalysis.

Experimental Section

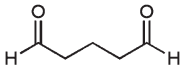
General Remarks. All manipulations were performed under a dinitrogen atmosphere in a glovebox (LabMaster 130, MBraun Garching, Germany) or by standard Schlenk techniques unless specified otherwise. $RuCl_2(CHPh)(PCy_3)_2$ (**1**), $[RuCl_2(PCy_3)(IMesH_2)(CHPh)]$ (**2**) (PCy_3 = tricyclohexylphosphine; $IMesH_2$ = 1,3-dimesitylimidazolin-2-ylidene), norborn-2-ene (NBE), 5-norbornene-2-methanol, methyltrichorosilane, tetraethylenepentamine (TEPA), trypsin from bovine pancreas (EC 3.4.21.4), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), phosphate buffered saline tablets, Tween20, and deionized water were obtained from Aldrich Chemical Co. (Germany). Glutaraldehyde (GA, 25% in aqueous solution) was purchased from ABCR GmbH and Co. KG (Karlsruhe, Germany). HEPES was purchased from Carl Roth GmbH (Germany). $(NBE\text{-}CH_2O)_3SiCH_3$ was prepared according to the literature.[55] $CDCl_3$ was distilled from CaH_2 and stored over molecular sieves (4 Å). Toluene was dried by an MBraun solvent purification system (SPS). $[RuCl_2(3\text{-}Br\text{-}Py)_2(IMesH_2)(CHPh)]$ (**3**)[58,59] and 1,4,4a,5,8,8a-hexahydro-1,4,5,8-*exo*-*endo*-dimethanonaphthalene (DMN-H6)[60] were prepared according to the literature. Purchased starting materials and other chemicals or reagents were used without further purification. NMR spectra were recorded on a Bruker Avance^{II+} 600 and Bruker Spectrospin 250 spectrometer in the indicated solvent at 25 °C and are listed in parts per

Scheme 3. Reaction of Aldehyde−Semitelechelic ROMP-Derived Polymers with 2,4-Dinitrophenylhydrazine and Schematic Illustration of the Synthesis of Aldehyde- and 2,4-Dinitrophenylhydrazone-Functionalized Monoliths





tetraethylenepentaamine (**TEPA**)



glutaraldehyde (**GA**)

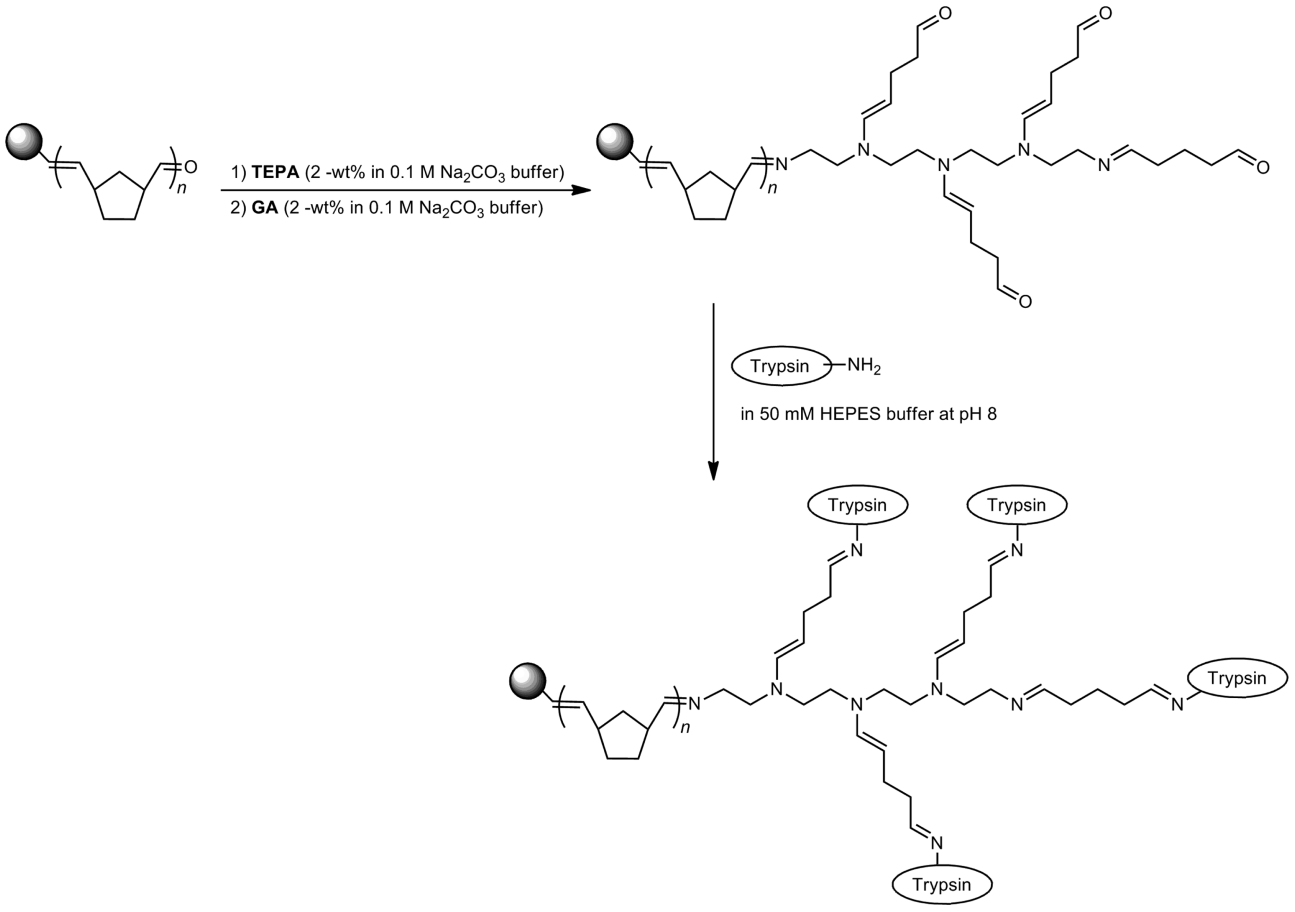
Figure 3. Reagents used for the immobilization of trypsin on a monolith support.

million downfield from TMS as an internal standard for proton and carbon. FT-IR spectra were recorded on a Bruker Vector22 spectrometer using ATR technology. Quantification of the functional groups in the monolith was accomplished by elemental analysis on a Vario EL (Elementar Analysensysteme GmbH, Hanau, Germany). Molecular weights and polydispersity indexes (PDIs) of the polymers were determined by size exclusion chromatography (SEC) on Polymer Laboratories columns (PLgel 10 mm MIXEDB, 7.5×300 mm) in tetrahydrofuran (THF) at 25 °C vs PS using an autosampler and a 484 UV detector (254 nm, all Waters Corp.). The flow rate was set to 0.7 mL min^{-1}. Narrow polystyrene (PS) standards were purchased from Waters Corp. Prior to measurements, polymer solutions were filtered through Millipore 0.20 μm filters. The trypsin activity was monitored and analyzed by an Agilent Technology HPLC system (Germany). The system consisted of a binary HPLC pump, a diode array UV−vis detector, an autosampler, a column oven, and a sample thermostat. The products were analyzed by RP-HPLC using an Agilent-Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm i.d., Zorbax RX-SIL).

Preparation of Aldehyde−Semitelechelic Polymers. A 5 mL Schlenk flask was charged with **1** (29 mg, 0.035 mmol) in 1 mL of benzene and a stir bar. In a separate vial, NBE (100 mg, 1.06 mmol) was dissolved in benzene (1 M solution). The solution was then added to the catalyst solution via a syringe under vigorous stirring. After 1 h, the solution was purged with air under stirring for 16 h. The reaction mixture was then poured into a large excess of methanol (25 mL), and the precipitated polymer was filtered off. The polymer obtained was then precipitated from a 1:9 mixture of CH_2Cl_2 and methanol. It was collected by filtration and dried *in vacuo*. CHO-semitelechelic poly(NBE): 80% aldehyde formation; SEC: M_n = 7500 g/mol, PDI = 1.98. ^{1}H NMR ($CDCl_3$): δ = 9.61 (s, 1H, CHO), 7.34−7.17 (m, 5H, J = 7.2 Hz, phenyl), 6.35 (d, 1H, J = 15.6 Hz, Ph−C*H*=CH), 6.21−6.17 (dd, 1H, J = 15.6, 7.8 Hz, Ph−CH=C*H*), 5.35 (bm, −CH=CH *trans*), 5.21 (bm, −CH=CH *cis*), 2.78 (bs, −CH *cis*), 2.43 (bs, −CH *trans*), 1.87−1.85 (bm, $-CH_2$), 1.76 (bm, $-CH_2$) 1.35 (bm, $-CH_2$), 1.08−1.02 (bm, $-CH_2$). ^{13}C NMR ($CDCl_3$): δ = 203.6 (*C*HO), 138.3, 137.9, 135.6, 134.0, 133.1, 128.6, 126.5, 126.0, 52.0, 43.5, 43.2, 42.2, 41.5, 38.5, 33.0, 32.3. IR (ATR mode): 2940 (m), 2862 (m), 1712 (vs), 1444 (w), 1260 (m), 1026 (w), 965 (s), 908 (s), 732 (s), 699 (w), 648 cm^{-1} (w). CHO-semitelechelic poly(COE): 15% aldehyde formation; SEC: M_n = 8400 g/mol, PDI = 1.77. ^{1}H NMR ($CDCl_3$): δ = 9.77 (s, 1H, CHO), 7.35−7.17 (m, 5H, J = 7.2 Hz, phenyl), 6.37 (d, 1H, J = 16.2 Hz, Ph−C*H*=CH), 6.25−6.20 (dd, 1H, J = 13.8, 6.6 Hz, Ph−CH=C*H*), 5.39 (bm, −CH=CH *trans*), 5.35 (bm, −CH=CH *cis*), 2.42 (bm, −CH), 2.21 (bm, −CH), 2.02−1.97 (bm, −CH), 1.65−1.62 (bm, −CH), 1.48−1.45 (bm, −CH), 1.34 (bm, $-CH_2$), 1.28 (bm, $-CH_2$). ^{13}C NMR ($CDCl_3$): δ = 203.0 (*C*HO), 138.0, 131.5, 130.5, 130.0, 129.8, 128.6, 126.8, 126.0, 114.2, 44.0, 33.2, 32.7, 29.8, 27.3. IR (ATR mode): 2916 (m), 2849 (m), 2360 (vs), 2341 (vs) 1727 (vs), 1467 (vs), 1437 (w), 1284 (m), 1070 (w), 965 (s), 717 (s), 691 cm^{-1} (w).

General Procedure for the Preparation of 2,4-Dinitrophenylhydrazine Derivatives. 0.25 g of 2,4-dinitrophenylhydrazine was suspended in 5 mL of ethanol, and then 0.5 mL of concentrated sulfuric acid was added cautiously. The warm solution (0.5 mL) was then added to a solution of aldehyde-functionalized polymer suspended in 0.5 mL of a mixture of CH_2Cl_2 and methanol (1:1). The mixture was stirred for 15 min and poured into methanol (25 mL). The precipitate was filtered and washed with methanol to afford the corresponding hydrazones. Poly(NBE)-hydrazone: ^{1}H NMR ($CDCl_3$): δ = 11.00 (s, 1H), 9.13 (m, 1H), 8.29 (m, 1H), 7.92 (m, 1H), 7.49 (d, 1H) 7.35−7.17 (m, 5H, J = 7.2 Hz, phenyl), 6.37 (d, 1H, J = 15.6 Hz, Ph−C*H*=CH), 6.22−6.18 (dd, 1H, J = 15.6, 7.8 Hz, Ph−CH=C*H*), 5.35 (bm, −CH=CH *trans*), 5.21 (bm, −CH=CH *cis*), 2.78 (bs, −CH *cis*), 2.43 (bs, −CH *trans*), 1.88−1.77 (bm, $-CH_2$) 1.36 (bm, $-CH_2$), 1.09−1.03 (bm, $-CH_2$). ^{13}C NMR ($CDCl_3$): δ = 155.9 (−H*C*=N−),

Scheme 4. Schematic Illustration for the Immobilization of Trypsin on a ROMP-Derived Monolithic Support



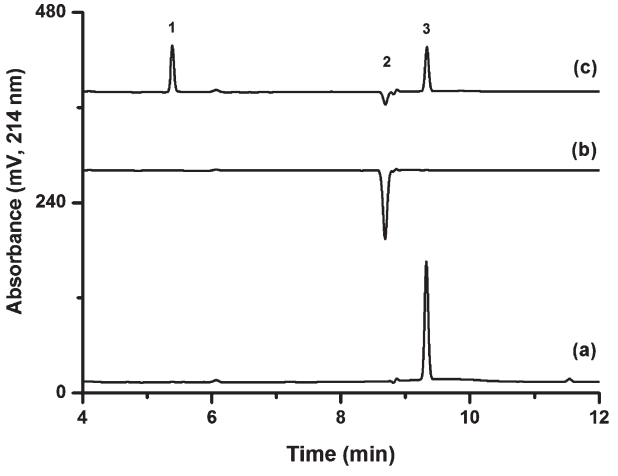


Figure 4. RP-HPLC chromatogram obtained for the tryptic digestion of BAPNA: (a) standard BAPNA, (b) standard PNA, (c) on-column tryptic digest of 0.25 mM BAPNA in 10 min at 37 °C. Peak 1 is BA, peak 2 is PNA, and peak 3 is undigested BAPNA. Conditions: flow rate 1 mL min^{-1}; 25 °C; mobile phases: A: 95% water + 5% acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA); B: 95% ACN + 5% water + 0.1% TFA; gradient: 0 min 4% B, 9 min 50% B, 10 min 50% B; UV (214 nm); injection volume 20 μL.

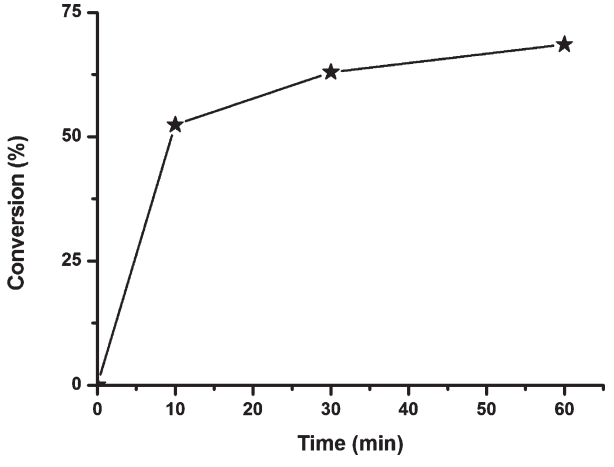


Figure 5. Plot of conversion vs time for the tryptic digest of 0.25 mM BAPNA at different reaction times. Conditions: T = 37 °C; mobile phase of the trypsin monolith was 50 mM HEPES buffer.

145.2, 138.0, 135.6, 134.0, 133.1, 130.1, 128.0, 126.9, 126.0, 123.7, 43.5, 43.2, 42.2, 41.5, 38.5, 33.0, 32.3. IR (ATR mode): 2941 (m), 2862 (m), 1616 (vs), 1591 (vs), 1518 (vs), 1464 (vs), 1445 (s), 1335 (vs), 1215 (s), 1041 (s), 964 (s), 832 (w), 755 cm^{-1} (s). Poly(COE)-hydrazone: ^{1}H NMR ($CDCl_3$): δ = 11.02 (s, 1H), 9.13 (m, 1H), 8.29 (m, 1H), 7.92 (m, 1H), 7.52 (t, 1H), 7.34−7.17 (m, 5H, J = 7.2 Hz, phenyl), 6.37 (d, 1H, J = 15.6 Hz, Ph−C*H*═CH), 6.25−6.20 (m, 1H, J = 15.6, 7.2 Hz, Ph−CH═C*H*), 5.38 (bm, −CH═CH *trans*), 5.35 (bm, −CH═CH *cis*), 2.02−1.96 (bm, −CH), 1.33 (bs, $-CH_2$), 1.28 (bm, $-CH_2$). ^{13}C NMR ($CDCl_3$): δ = 152.7 (−H*C*═N−), 138.1, 130.5, 130.0, 128.6, 126.9, 126.0, 116.6, 37.7, 32.8, 29.8, 29.2, 27.4. IR (ATR mode): 2917 (m), 2849 (m), 1617 (vs), 1593 (vs), 1509 (vs), 1466 (vs), 1333 (w), 1214 (w), 1069 (w), 965 (s), 756 (s), 719 cm^{-1} (w).

Synthesis of Aldehyde-Functionalized Monoliths. Monoliths were prepared in stainless steel columns (250 × 3 mm) according

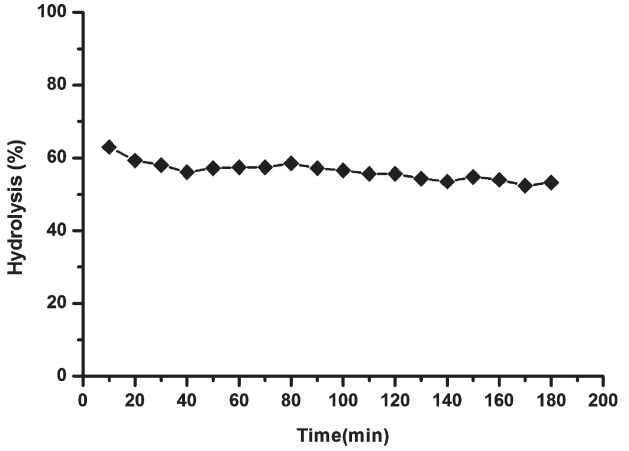


Figure 6. Tryptic activity in a continuous flow experiment. Conditions: on column time 10 min; $T = 37$ °C; mobile phase: 0.25 mM BAPNA in 50 mM HEPES buffer.

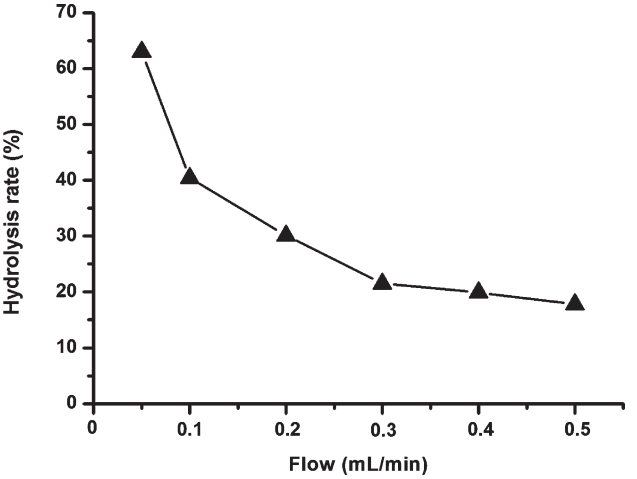


Figure 7. Influence of the flow rate on the hydrolysis of BAPNA. Conditions: $T = 37$ °C; mobile phase: 0.25 mM BAPNA in 50 mM HEPES buffer.

to a previously published protocol.[45] Two solutions A and B were prepared and cooled to −20 °C. Solution A (NBE:DMN-H_6:2-propanol = 25:25:40 and solution B (toluene:**1** = 10:1 (all in wt %) were mixed and rapidly transferred to the stainless steel column. The column was kept at 0 °C for 30 min, and then the polymerization was allowed to complete at room temperature for 1 h. After 1 h, the monolith was flushed with toluene for 15 min at a flow rate of 0.2 mL/min to remove any unattached catalyst. The monolith was then flushed consecutively with toluene and air for 16 h and then kept at room temperature. After 24 h, the monolith was flushed with THF to remove any Ru byproducts. IR (ATR mode): 2938 (m), 2861 (m), 1725 (ν_{CO}, m), 1649 (m), 1448 (s), 1341 (m), 1148 (w), 963 (s), 831 (w), 731 cm^{-1} (s).

Quantification of the Aldehyde Group. The chemically accessible aldehyde groups in the monolith were quantified via conversion into the corresponding 2,4-dinitrophenylhydrazones. For this purpose, the monolith was taken out of the column, ground up and dried *in vacuo* overnight. 100 mg of the sample were suspended in 2 mL of methanol and stirred with 2 mL freshly prepared 2,4-dinitrophenylhydrazine reagent (*vide supra*). After 1 h, the monolith was filtered, washed with methanol and then dried *in vacuo*. Quantification of the hydrazone groups was accomplished by elemental analysis. IR (ATR mode): 2936 (m), 2862 (m), 2165 (m), 1648 (m), 1617 (m), 1592 (m), 1447 (s), 1408 (m), 1337 (m), 1143 (w), 964 (s), 837 (w), 733 (s), 688 (m) cm^{-1}. Elemental analysis: C, 88.3; H, 9.51; N, 0.06.

Monoliths for Trypsin Immobilization. Monoliths were prepared in stainless steel columns (100 × 4.6 mm i.d.) according to a previously published protocol.[55] Briefly, two solutions A and B were prepared. Solution A consisted of NBE:(NBE-CH_2−O)$_3$-$SiCH_3$:2-propanol (20:20:45.7 wt %), solution B consisted of 0.4 wt % of **1** in 13.9 wt % of toluene. Both solutions were cooled to −20 °C, mixed for ∼1 min, and then rapidly transferred into the stainless steel column. The column was kept at 0 °C for 30 min, and then the polymerization was allowed for another 30 min at room temperature. The monolith was flushed with toluene for 30 min at a flow rate of 0.2 mL/min to remove any nonreacted monomers. Then the monolith was consecutively flushed with toluene and air at room temperature for 24 h. Finally, the support was washed with THF to remove any Ru byproducts.

Modification of Aldehyde-Functionalized Monoliths with Tetraethylenepentamine (TEPA)[56] and Immobilization of Trypsin. A 2 wt % solution of TEPA in 0.1 M Na_2CO_3 buffer solution (pH 9.2) and 10 vol % of THF was introduced into the aldehyde functionalized monolith at a flow rate of 0.05 mL/min. Then the reaction was allowed for 2 h at room temperature. The monolith was flushed with 10% THF in deionized water followed by absolute ethanol to remove any unattached TEPA. A 2 wt % solution of glutaraldehyde in Na_2CO_3 buffer (20 vol % THF) at pH 9.2 was then introduced into the monolith at a flow rate of 0.05 mL/min. The monolith was then sealed and kept at room temperature for another 2 h. Finally, the monolith was flushed with 10 vol % THF in deionized water followed by absolute ethanol to remove any unattached GA. The glutaraldehyde-functionalized monolith was then flushed with 50 mM HEPES buffer solution at pH 8 to remove any organic solvent in the monolith that might interfere with the trypsin derivatization. A fresh solution of trypsin (3 mg/mL) in 50 mM HEPES buffer (100 mM NaCl, 10 mM $CaCl_2$) was prepared and injected into the monolith at a flow rate of 0.05 mL/min. The monolith was then sealed and kept for 2 h at room temperature. Finally, the monolith was flushed with 0.1 M phosphate buffer solution containing 0.01 wt % Tween 20 and then with phosphate buffered saline (PBS) solution. After the immobilization the monolith was stored at 4 °C.

Proteolytic Activity of the Immobilized Trypsin (Batch Experiment). The proteolytic activity of the immobilized trypsin was monitored by measuring the hydrolysis products of a standard solution of *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), i.e., *N*-α-benzoyl-DL-arginine (BA) and *p*-nitroaniline (PNA). A 0.25 mM standard solution of BAPNA in 50 mM HEPES buffer solution at pH 8 was introduced into the monolith at 37 °C. After 10 min, the reaction mixture was eluted at a flow rate of 0.05 mL/min for 10 min. The conversion of BAPNA was calculated by comparing the area of BAPNA after proteolysis to the area of the standard BAPNA.

Proteolytic Activity of the Immobilized Trypsin (Continuous Flow). The activity of immobilized trypsin was monitored under continuous flow conditions for 3 h as follows. A standard solution of BAPNA (0.25 mM) in 50 mM HEPES buffer at pH 8 was pumped through the bioreactor at a flow rate of 0.05 mL/min at 37 °C. Aliquots were collected in 10 min intervals and analyzed by RP-HPLC at 310 nm. For the separation conditions refer to Figure 4. The extent of hydrolysis of BAPNA was calculated by comparing the area of BAPNA of each 10 min aliquot to the area of a standard BAPNA. No perceptible loss in activity was observed for at least 3 h.

Acknowledgment. Financial support provided by the Bundesministerium für Bildung und Forschung (BMBF, Contract 31P5555), the Graduate School of Excellence "BuildMona" at the University of Leipzig, Germany, and the University of Stuttgart, Germany, is gratefully acknowledged.

Supporting Information Available: GC-MS of stilbenes formed via the reaction of O_2 with a Grubbs-type initiator;

^{1}H and ^{13}C NMR spectra of the semitelechelic polymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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