Optimization of poly(GMA-co-EDMA) Monolithic Support for Trypsin Nanoreactor Fabrication

Alex Monzo[1,2], Tomas Rejtar[2], and **András Guttman[1,*]**

[1]Horváth Laboratory of Bioseparation Sciences, University of Innsbruck, Austria; [2]Barnett Institute of Chemical and Biological Analysis, Northeastern University, Boston, MA

Abstract

Fabrication of poly(glycidyl methacrylate-co-ethylene dimethacrylate) [also referred to as poly(GMA-co-EDMA)] monoliths was optimized as supporting material for trypsin digestion nanoreactors. Reaction parameters, such as polymerization time, porogen concentration, and monomer to crosslinker ratios, were evaluated in respect to the permeability of the resulting monolith and their effect on digestion efficiency, estimated by mass spectrometric analysis of a model protein cytochrome C. The structural homogeneity of the resulting monolithic support was checked by scanning electron microscopy. The best nanoreactor performance, measured by the reduction of nanoreactor backpressure and increased sequence coverage of cytochrome C, was achieved with 8% 2-octanol (porogen) 20%/20% glycidyl methacrylate to ethylene glycol dimethacrylate ratio and 5 h of polymerization time. Digestion of as low as 3 μg of cytochrome C with 77% sequence coverage was obtained using the optimized trypsin nanoreactor.

Introduction

Protein digestion by proteolytic enzymes such as trypsin, followed by chromatographic separation of the resulting peptide fragments, is an important sample preparation step in liquid chromatography–mass spectrometry (LC–MS)-based proteomics techniques. The enzymatic digestion step is often performed in homogeneous aqueous solution of the proteolytic enzyme and the sample, usually by applying 50:1 protein-to-enzyme ratio (1). There are several issues with this classical approach that can limit high-throughput protein identification. Some of the most common ones are long digestion times (up to 12–24 h), autodigestion, and limited enzyme-to-substrate ratio (2). Digestion times can be shortened by increasing the enzyme concentration, but the high enzyme-to-substrate ratio in liquid phase digestion often decreases the specificity of the enzyme and promotes autodigestion byproducts, resulting in additional peptides, which may complicate MS-based analysis and data interpretation (3). Enzyme immobilization onto solid surfaces or sorbent materials may eliminate some of the issues associated with homogenous solution digestion (4–7). For example, immobilized enzymes do not show autodigestion problems due to limited interaction possibilities between the enzyme molecules, thus with the use of enzymes on solid-phase support, one may increase the enzyme-to-substrate ratio, yielding significantly shorter digestion times (8).

Trypsin is a pancreatic serine endoprotease, which specifically hydrolyzes peptide bonds at the carboxyl side of arginyl and lysyl residues. When isolated from bovine (*Bos taurus*) pancreas, this enzyme has a molecular mass of 23.8 kDa. A modified version of trypsin, referred to as proteomics grade, is commonly used in proteomics applications; however, in this case, the lysine residues of the enzyme have been reductively methylated, resulting in a product that is resistant to autolysis. This modified trypsin is treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone to inactivate any remaining chymotryptic activity and purified by affinity chromatography.

Poly(glycidyl methacrylate-co-ethylene dimethacrylate) [poly(GMA-co-EDMA)] was first introduced by Svec and Frechet as a high-performance liquid chromatography (HPLC) separation medium to support ion-exchange chromatography of proteins (9). Others used modified versions of the same polymer for in-tube solid-phase microextraction and coupled with HPLC (10). Similar polymeric material was used to fabricate immobilized enzyme reactors in monolithic form (11–13). In most instances, thermal polymerization (60°C) of the monomer-crosslinker mixture was suggested in the presence of a free radical agent, usually 2,2'-azobis(2-methylpropionitrile), with reaction times from 14 up to 24 h.

In this publication, we report on the optimization of poly(GMA-co-EDMA) monolithic material for trypsin nanoreactor fabrication, including evaluation of monomer to crosslinker ratio, porogen concentration, and polymerization time. Digestion performance of the fabricated monolithic trypsin nanoreactors was tested with the model protein of cytochrome C using MS.

Materials and Methods

Chemicals

3-(trimethoxysilyl)propyl methacrylate, dry DMF, 2,2-diphenyl-1-picrylhydrazyl (DPPH), glycidyl methacrylate

* Author to whom correspondence should be addressed.

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(GMA), ethylene glycol dimethacrylate (EDMA), cyclohexanol, 2-octanol, 2,2'-azobis(2-methylpropionitrile) (AIBN), acetone, HPLC water, sodium hydroxide, benzamidine, and cytochrome C (from equine heart) were obtained from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid (0.1 M) solution was acquired from Merck (Darmstadt, Germany). The 75 µm i.d./360 µm o.d. fused silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ).

Monolith fabrication

Poly(GMA-co-EDMA) monoliths were prepared in fused silica capillaries of 75 µm i.d. First, a 30-cm long section of the tubing was rinsed with 1 M sodium hydroxide solution for 15 min and placed in a 100°C oven for 2 h with both ends sealed by silicon septa. Then the capillary was washed for 15 min with 0.1 M HCl, followed by flushing for another 15 min with acetone. The acetone was removed from the capillary by nitrogen purging for 15 min. The capillary was then dried in an oven for 1 h at 120°C, followed by another nitrogen purging step for 15 min. Then, the inner wall surface of the activated capillary was silanized to support covalent attachment of the monolith. Briefly, a solution of 4.8 mg DPPH in 300 µL 3-(trimethoxysilyl)propyl methacrylate) and 700 µL dry DMF mixture was pumped through the capillary by applying 70 psi external pressure (N_2) for 15 min. Both ends of the capillary were then sealed with silicon septa and incubated at 120°C for 14 h. After cooling, the capillary was washed with acetone for one hour and dried with N_2 stream (60 psi) for 15 min. Table I lists the different polymerization reaction mixtures, all containing 5% (w/v) of AIBN in addition to the listed GMA, EDMA, and cyclohexanol to 2-octanol ratios. The polymerization mixtures were pumped through the silanized capillary for 15 min, then both ends were sealed with silicon septa and the column was placed in a 60°C oven for either 5 or 14 h (see Figure 1). After incubation, the resulting monoliths were cut into 3 cm sections and washed with methanol at 200 nL/min flow rate for 2 h by means of an Agilent 1100 HPLC nanopump (Agilent Technologies, Walbronn, Germany) to make them ready for backpressure measurement and enzyme immobilization. Backpressure values registered by the ChemStation HPLC software (Agilent) were recorded when their values remained stable for at least 30 min.

Table I. Polymerization Mixtures Used for Fabrication of the Monoliths

	GMA µL	EDMA µL	Cyclohexanol µL	2-Octanol µL	Polymerization time (h)
1	26	14	54	6	14
2	26	14	53	7	14
3	26	14	52	8	14
4	26	14	51	9	14
5	26	14	54	6	5
6	26	14	53	7	5
7	26	14	52	8	5
8	26	14	51	9	5
9	20	20	52	8	5
10	22	18	52	8	5
11	28	12	52	8	5

Enzyme immobilization chemistry

A one-step trypsin immobilization procedure was performed through the epoxy groups of the poly(GMA-co-EDMA) monolith, because these groups readily reacted with the amine groups of the enzyme (lysine, arginine, and terminal amino groups), as depicted in Figure 1. The enzyme-containing solution was pumped through the monolith for a limited time in the presence of benzamidine (trypsin inhibitor) to prevent autodigestion (14). First, the poly(GMA-co-EDMA) monoliths were conditioned with 50 mM sodium carbonate buffer (pH 10.5) for one hour by means of nitrogen purging. Then, 2 mg/mL trypsin solution containing 0.2 mg/mL benzamidine in 50 mM Na–carbonate buffer (pH 10.5) was pumped through the monoliths for 2 h. The enzyme-immobilized monoliths were then washed with 1 M NaCl containing 50 mM sodium carbonate buffer (pH 10.5) to remove the remaining unbound trypsin, followed by rinsing with 25% ethanolamine containing 50 mM sodium carbonate buffer (pH 10.5) for one hour to block the residual epoxide groups. The fabricated monoliths were stored at 4°C.

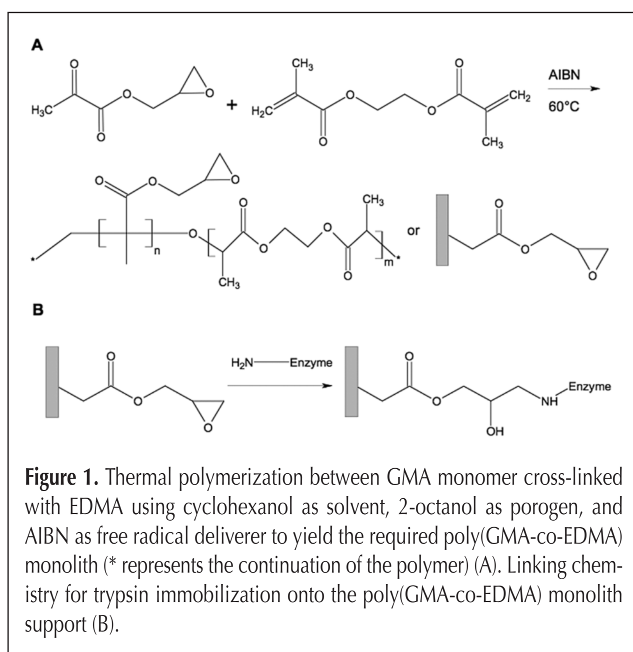

Figure 1. Thermal polymerization between GMA monomer cross-linked with EDMA using cyclohexanol as solvent, 2-octanol as porogen, and AIBN as free radical deliverer to yield the required poly(GMA-co-EDMA) monolith (* represents the continuation of the polymer) (A). Linking chemistry for trypsin immobilization onto the poly(GMA-co-EDMA) monolith support (B).

Cytochrome C digestion

A solution of 0.25 mg/mL cytochrome C in 10 mM ammonium bicarbonate (pH 8.5) was used for digestion. No alkylation was done prior to the digestion step and 20% methanol was used as denaturant in the sample buffer (15). The denatured cytochrome C solution was directly pumped through the enzymatic reactor by nitrogen pressure at approximately 200 nL/min flow-rate for one hour at room temperature, yielding 10 to 14 s residence times in the reactor, depending on the porosity of the monolith.

Mass spectrometry and data analysis

The cytochrome C digestion products were collected into a 20-µL pipette tip with the help of a junction capillary. In our experimental setting, one end of the junction capillary was coupled to the nanoreactor by a peek tube, while the other end was directly inserted into the sampling end of the pipette tip to facilitate sample collection. The filled tip was then coupled to a pipettor and the collected sample was transferred into a 96-well plate (low protein binding). LC–MS analysis was carried out by an XCT Plus Ultra Ion Trap Mass Spectrometer (Agilent). The LC–MS setting comprised a chip-cube interface (Agilent) connected to Agilent 1100 nano and micro pumps. One µL of the sample was injected into the enrichment column section of the LC chip (40 nL trap) using 5% acetonitrile in water containing 0.1% TFA (5 µL/min) with a loading volume of 15 µL. This step also desalted the sample. Following the standard chip-cube protocol, the peptides were eluted from the enrichment column and separated in the analytical column section of the LC chip (stationary phase: 5 µm C-18 SB-ZX) using a linear water–acetonitrile gradient (5–90% acetonitrile in 15 min) in 0.5 µL/min flow rate. The acquired MS data was processed by the Spectrum Mill software (Agilent) using the following parameters: batch size, 81 spectra; minimum score peak, 50%; minimum detected peaks, 4; signal-to-noise ratio, 3; maximum Z, 5 (^{12}C data included); and maximum allowed miscleavages, 2.

Scanning electron microscopy

The fabricated monoliths were mounted onto an in-house made copper capillary holder, and gold coated by means of a sputter coater (SPI Supplies, West Chester, PA). Scanning electron microscopy (SEM) images were obtained by a Jeol JSM-5310LV SEM (Jeol Ltd., Welwyn Garden City, UK) using 5000× magnification, operated in low vacuum in back-scattered electron mode at 25 kV accelerating voltage, with a working distance of 15 mm.

Results and Discussion

During the optimization of the polymeric support for the trypsin nanoreactor, the following considerations were applied: the nanoreactor should be long enough to allow sufficient residence time for the analyte (i.e., assuring effective digestion process), while minimizing the backpressure and providing good mass transfer by having adequate pore size. Due to the conflicting nature of these properties, an optimization procedure was necessary by addressing such characteristics as monolith length and pore size through evaluating the effects of porogen concentration, monomer to crosslinker ratio, and polymerization time. In order to study the influence of the monolith fabrication parameters and the chemical composition of the polymerization mixture, we followed a multiparameter optimization approach. First, the minimum polymerization time requirement was checked. Figure 2 shows the light microscope images of the fabricated monoliths (24% GMA, 16% EDMA, 54% cyclohexanol, and 6% 2-octanol, containing 5% w/v AIBN) with polymerization times of 2, 3, 4, and 5 h. As evidenced by the images, a reaction time of 5 h was necessary to obtain complete polymerization. Please note that in comparison to previously reported works, this protocol required significantly shorter reaction time, speeding up the fabrication process in this way. Then a series of different reaction mixtures was evaluated for such characteristics as backpressure and structural differences observed by SEM. Rows one to eight in Table I show the actual reaction mixture compositions tested. Subsequently, to study the effect of the monomer cross linker ratio, different monoliths were fabricated using four different GMA:EDMA ratios ranging from 20:20% to 28:12%. Trypsin immobilization was carried out choosing monoliths with measured backpressures under 45 bar, and the performance of all the resulting enzymatic reactors was tested using cytochrome C as a model protein. Columns with higher backpressure were not considered in the fabrication process because the pump used for trypsin immobilization could be used only up to 65 bars, and the backpressure of the monolithic nanoreactor typically increased during enzyme immobilization. The following sections discuss the results of the multiparameter optimization approach.

Porogen

Choosing the right porogen is one of the crucial parts of monolith fabrication. Generally, long-chain alcohols (normally between 8 and 12 carbons) are used for this purpose. The pore size, and thus the backpressure of the monolith, can be controlled by changing the type of porogen and its concentration in the polymerization mixture. During our optimization process, the most commonly used porogen, 2-octanol, was evaluated in the concentration range of 6% to 9%. Table I summarizes the composition of reaction mixtures used for 5 and 14 h polymerization time. Figure 3 shows the backpressure values as a function of porogen concentration for 3-cm long monoliths with a 200 nL/min methanol flow-rate (5 and 14 h reaction times, lower and upper curve, respectively). As

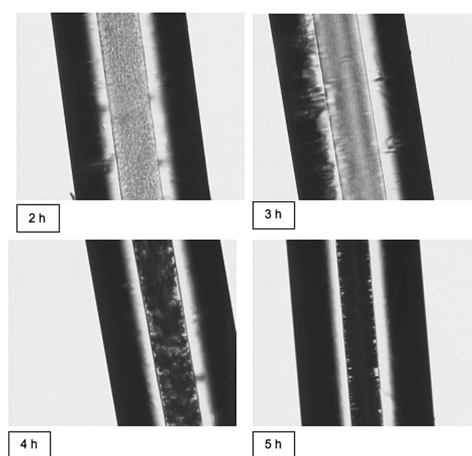

Figure 2. Effect of polymerization time on poly(GMA-co-EDMA) monolith formation. A reaction mixture of 24% GMA, 16% EDMA, 54% cyclohexanol, and 6% 2-octanol, containing 5% w/v AIBN, was filled in 10-cm long capillaries (75 µm i.d.) and kept at 60°C for 2, 3, 4, and 5 h. The progress of the polymerization process was followed by light microscopy (see Table I for details).

expected, the backpressure values decreased with increasing porogen concentration due to the increased pore size. Plots for both polymerization times show significant backpressure drop in the 6% to 8% range, and insignificant backpressure decline between 8% and 9% porogen concentration. The SEM images of the insets in Figure 3 show smaller pore sizes with 5% (upper left inset) and larger pore sizes with 9% (lower right inset) porogen concentration.

Figure 3 also indicates that while the shapes of both curves are similar, the monolith prepared with a 5 h polymerization time tended to show lower backpressure than the monolith prepared using 14-h polymerization time. This observation suggested that shorter polymerization times not only reduced fabrication time but also improved the reactor properties of the monoliths by reducing the backpressure. Thus, this 8% porogen (2-octanol) concentration and 5 h of polymerization time were used in all subsequent optimization studies.

Monomer crosslinker ratio

Additional experiments were conducted to evaluate the influence of monomer crosslinker ratio with respect to the performance of the trypsin nanoreactor. Four different monoliths were prepared with various GMA to EDMA ratios while keeping the previously set porogen concentration and polymerization time at 8% and 5 h, respectively. The backpressure values of the resulting nanoreactors were evaluated with 200 nL/min methanol flowrate in the 3-cm monolith-filled capillaries. Again, to meet instrumentation specifications, monolithic columns with backpressure values below 45 bar were evaluated. The lower curve in Figure 4 shows the backpressure values for monoliths with 20:20, 22:18, 26:14, and 28:12 GMA to EDMA percentage ratios (Table I). This plot indicates a strong correlation between the monomer crosslinker ratio and the resulting backpressure of the poly(GMA-co-EDMA) monoliths. Three monoliths with percentage ratios of 20:20, 22:18, and 26:14 met the backpressure limitation of 45 bar, and they were thus considered for enzyme immobilization.

In addition to the changes in column backpressure, the monomer crosslinker ratio may affect the number of epoxy groups available to react with the enzyme. It is important to note that the higher number of epoxy groups may not automatically yield better digestion performance due to potential multiple attachment of the same enzyme molecule, resulting in reduced enzymatic activity. On the other hand, a low number of reacting groups also represents a challenge due to the limitation of the number of enzyme molecules possibly attached. Our optimization results suggested that the polymerization mixture of 20% GMA, 20% EDMA, 52% cyclohexanol, 8% 2-octanol, and 50 mg/mL AIBN (polymerization for 5 h at 60ºC) resulted in an adequate number of enzyme attachment sites (see the following), excellent permeability of the monolithic support, and good stability of the prepared column.

All prepared monoliths were evaluated by SEM. The upper left inset in Figure 4 shows a monolith fabricated with 20:20% GMA to EDMA ratio, while the lower right inset shows a 28:12% GMA to EDMA ratio based monolith. As one can see, both monoliths seem to have uniform structure, suggesting complete polymerization. However, the monolith in the upper left panel (20:20%) showed higher porosity compared to the

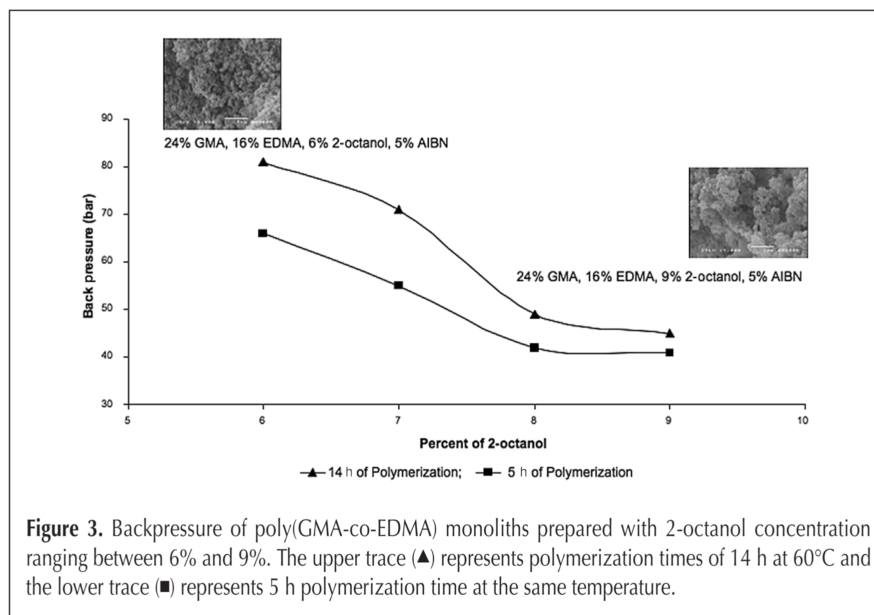

Figure 3. Backpressure of poly(GMA-co-EDMA) monoliths prepared with 2-octanol concentration ranging between 6% and 9%. The upper trace (▲) represents polymerization times of 14 h at 60°C and the lower trace (■) represents 5 h polymerization time at the same temperature.

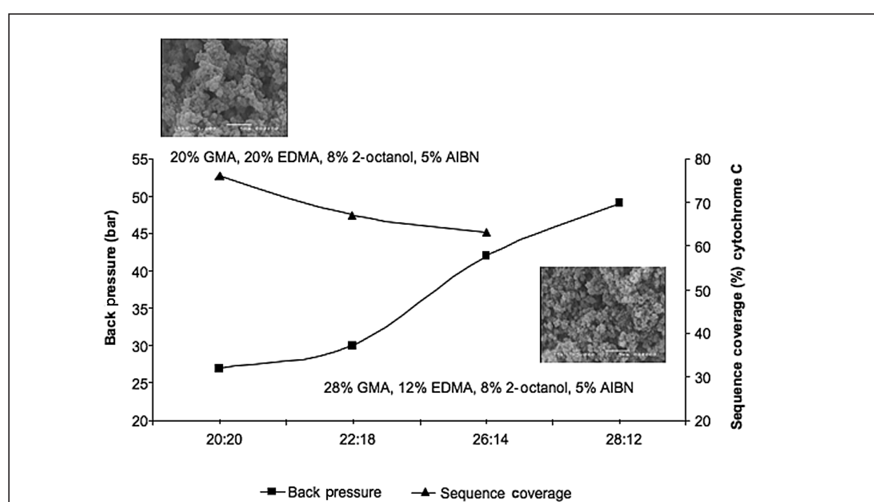

Figure 4. Backpressure and sequence coverage of poly(GMA-co-EDMA) monoliths prepared with different GMA:EDMA ratios. Upper trace (▲): sequence coverage of cytochrome C digested with trypsin-coated monoliths prepared with GMA to EDMA ratios of 20:20%, 22:18%, and 26:14%. Lower trace (■): backpressure values of monoliths prepared using GMA to EDMA ratios ranging between 20:20% and 28:12%. All the monoliths were polymerized for 5 h at 60°C.

monolith in the lower right panel (28:12%), which is in agreement with the backpressure change presented in Figure 4. The monolith comprising 20% GMA, 20% EDMA, 52% cyclohexanol, 8% 2-octanol, and 50 mg/mL AIBN and polymerized for 5 h at 60ºC, yielded the best supporting material of all the different polymerization mixtures evaluated in respect to adequately low backpressure and good mechanical stability.

Cytochrome C digestion and MS

Digestions of the model protein, cytochrome C, were carried out using several selected nanoreactors and the digestion products were analyzed by LC–MS to assess the influence of monolith fabrication protocol on sequence coverage. It was found that various GMA to EDMA ratios influenced the performance of the trypsin nanoreactor. The upper curve in Figure 4 shows the measured sequence coverage for three of the four fabricated nanoreactors with 20:20%, 22:18%, and 26:14% GMA to EDMA ratios. As one can see, trypsin nanoreactors prepared using higher EDMA concentrations performed better, suggested by their higher sequence coverage. Significant monolith backpressure reduction (~38%) and increased sequence coverage (~28%) were obtained with the use of 20:20% GMA to EDMA ratio.

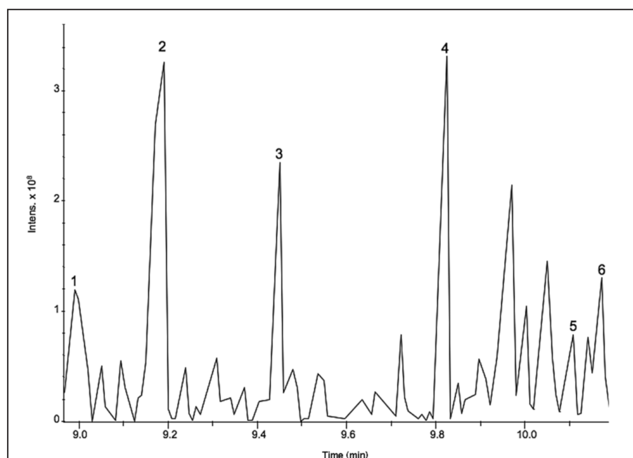

Figure 5. Section of the total ion current chromatogram of the digestion of 3 µg cytochrome C using a 3-cm long trypsin nanoreactor (75 µm i.d., 20% GMA, 20% EDMA, 52% cyclohexanol, 8% 2-octanol, and 50 mg/mL AIBN polymerized for 5 h at 60°C). MS settings: –1950 V 3.0 L/min N_2 at 200°C, skimmer polarity 40 V, capillary exit polarity 160 V, mass range between 300 and 2000 *m/z*. The collected data was processed by Spectrum Mill using: batch size of 81 spectra, minimum score peak of 50%, minimum of detected peaks of 4, signal-to-noise ratio of 3, and a maximum Z of 5, ^{12}C data included.

Figure 5 shows an example of the total ion chromatogram of the cytochrome C digestion products using a 3-cm long, 75-µm i.d. trypsin nanoreactor (prepared by using the optimized polymerization mixture of 20% GMA, 20% EDMA, 52% cyclohexanol, 8% 2-octanol, also containing 50 mg/mL AIBN, and polymerized for 5 h at 60ºC). This particular nanoreactor yielded 77% sequence coverage with a sample residence time of only 12.7 s, representing a considerable improvement compared to in-solution digestion protocols where digestion is usually carried out over 12 h. Table II summarizes the scores and assigns peptides to the peaks in the total ion chromatogram in Figure 5. Furthermore, Table II also depicts the matched mass (MH^+), the precursor ion, and the mass shifts between the theoretical calculated masses, and the measured masses in Daltons as well as the errors in ppm.

Conclusions

Optimization of poly(GMA-co-EDMA) monolithic material has been presented for trypsin immobilization in order to fabricate efficient enzymatic nanoreactors. Significant reduc-

Table II. Retention Times of Peptides and their Corresponding Mass Values for Cytochrome C Digestion in Monolithic Trypsin Nanoreactor

Peak	Ret. time (min)	Sequence	*m/z* Measured (Da)	MH^+ Matched (Da)	MH^+ Mass shift (Da)	MH^+ Error (ppm)	Score
1	9.00	(K)GEREDLIAYLKK(A)	717.92	1434.80	0.0365	25.5	8.76
1	9.00	(K)KGEREDLIAYLK(K)	717.86	1434.80	–0.0835	–58.2	13.85
1	9.00	(K)KGEREDLIAYLK(K)	478.98	1434.80	0.1287	89.7	7.13
1	9.00	(K)GEREDLIAYLK(K)	653.84	1306.70	–0.0285	–21.8	11.32
2	9.20	(R)KTGQAPGFSYTDANKNK(G)	913.97	1826.90	0.0285	15.6	11.01
2	9.20	(R)KTGQAPGFSYTDANKNK(G)	609.94	1826.09	0.9006	492.7	13.30
3	9.50	(K)TGQAPGFSYTDANK(N)	728.78	1456.67	–0.1187	–81.5	15.64
3	9.50	(K)MIFAGIKK(K)	454.29	907.54	0.0283	31.1	9.14
4	9.80	(K)TGPNLHGLFGR(K)	584.79	1168.62	–0.0505	–43.3	14.38
5	10.10	(K)GITWGEETLMEYLENPK(K)	1006.00	2009.95	0.9992	496.9	20.39
5	10.10	(K)TGQAPGFSYTDANKNK(G)	850.25	1698.81	0.6834	402.1	16.02
5	10.10	(K)IFVQKCAQCHTVEK(G)	817.26	1633.82	–0.3073	–188.1	8.4
5	10.10	(K)IFVQKCAQCHTVEK(G)	545.300	1633.82	0.0649	39.7	7.59
6	10.20	(R)KTGQAPGFSYTDANK(N)	792.86	1584.77	–0.0536	–33.8	12.16
6	10.20	(R)KTGQAPGFSYTDANK(N)	528.93	1584.77	0.0085	5.4	13.34

tion in backpressure and increase in sequence coverage of cytochrome C digestion (at room temperature) were achieved by using a monolith fabricated using 8% 2-octanol porogen concentration and 20:20% GMA to EDMA ratio in 5 h polymerization time at 60°C. Tryptic digestion and MS analysis of as low as 3 µg of cytochrome C with 77% sequence coverage (13 peptides) were achieved using the optimized trypsin nanoreactor. Digestion times as short as 10 s were possible, achieving significant digestion time reduction when compared to in-solution protocols (12 to 24 h). At the same time, by the immobilization, increased enzyme stability was attained that also allowed more convenient storage conditions of 4°C (instead of –20°C recommended for the free enzyme). Please note that prior reports only suggested trypsin activity increase by means of derivatization or by various enzymatic linking chemistries, not considering changes in the composition of the carrier monolith as a factor to influence reactor performance. Here we suggest a novel approach to reduce the backpressure of the supporting monolith and, at the same time, increase the performance of the enzymatic nanoreactor by adjusting the monomer cross linker ratio. Immobilization of other enzymes, such as PNGase F, will be done as part of our future work, as well as the introduction of a spacer between the monolith and the enzyme to improve substrate accessibility.

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