Low back-pressure hierarchically structured multichannel microfluidic bioreactors for rapid protein digestion – proof of concept

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Abstract

A novel, easy-to-fabricate monolithic enzymatic microreactor with a hierarchical, torturous structure of flow-through channels of micrometric sizes and large mesopores was shown to enable rapid and very efficient digestion of proteins at high yields and exceptionally low back-pressures. Four silica monoliths with bi-modal 3D pore structure in μm and nm size scales were synthesized and characterized for structural and flow properties. The monolith with the highest total pore volume (4 cm^3/g) and flow-through channels 20-30 μm in size, was further functionalized with trypsin to obtain multichannel immobilized enzyme (proteolytic) reactor (IMER). The value of permeability coefficient K evaluated for water (~$2.0 \cdot 10^{-11}$) was found to be two orders of magnitude higher in the novel reactor than reported before for high-performance IMERs, enabling the flow rates of 750 mL/cm^2min at pressure gradients of 64 kP/cm. Very high practical potentials of the novel microbioreactor were demonstrated in the proteolysis of cytochrome c (Cyt-c) and myoglobin (Myo), without any earlier pretreatment. MALDI-TOF/TOF mass spectrometry analysis of sequence coverage was high: 70 % (Cyt-c) and 90 % (Myo) for 24 min digestion, and 39 % (Cyt-c) and 53 % (Myo) when the proteolysis time was reduced to 2.4 min. The proposed microreactors make full use of all advantages of microfuidic devices and mesoporous biocatalysts, and offer exceptional possibilities for biochemical/proteolytic applications in both large (production) and small (analytical) scales.

1. Introduction

Protein digestion is an effective and inexpensive method to convert a protein into free amino acids and short chain peptides [1-4]. Therefore, it is indispensable for efficient protein identification, and hence crucial for the advancement of proteome studies [4-6], and also for the manufacture of bioactive peptides [3], which can play an important role in health promotion and risk reduction. But currently, the bioactive peptides are manufactured by rather expensive methods: transgenic, recombinant and complex synthetic procedures, that restrict their preparation and commercialization on a larger scale [3].

Conventionally, the proteolytical digestion of protein is performed in solution for several hours (12 - 24 h), with low concentrations of enzyme to avoid the autodigestion of trypsin, which might produce excessive amounts of undesired tryptic fragments and complicate the unambiguous assignment of the studied protein [7, 8]. The immobilization of trypsin on to various carriers has been proposed to speed up the process, while avoiding autodigestion [2, 7, 9-11]. This paved the way to the on-column digestion systems, which in the last decade evolved into continuous-flow immobilized enzyme microreactors (IMER) [7-10, 12-14]. Microreactor technology offers several key advantages including: drastically reduced reaction time due to the large surface-to-volume ratio and very intensive mass transport typically observed in micrometric channels [15]. Unlike in-solution digestion, typically performed in batch reactors, a continuous protein digestion in IMERs offers important advantages: enzyme denaturation is reduced, improving efficacy, operational stability and reproducibility, and importantly, IMERs can easily be coupled with different maonss spectrometry devices to obtain very efficient on-line systems for rapid protein digestion, identification and mapping [10].

For protein identification the most important factor is the sequence coverage. Therefore, small IMERs or chips have been used to efficiently produce the amount of peptides sufficient to be

identified by MS [3, 4, 6]. For bioactive peptide manufacturing, the capacity and high productivity of IMERs are of major significance, enabling a simple, effective, inexpensive and rapid cleavage of peptides [3].

Irrespective of the applied enzyme support: polymer [16], silica or hybrid [17, 18], the porous structure and surface character of IMERs are important factors. Applying hydrophilic supports decreases the nonspecific adsorption of proteins or peptides on to IMERs [1, 2, 19]. The porous structure of monolithic IMERs affects not only flow permeability (back pressure vs. flow rate dependence) but also digestion efficiency, which depends on the apparent rate of protein digestion controlled by the rate of diffusion into the pores and the size of activated surface area. In this respect, IMERs filled with structured packing or monolithic supports synthesized using templating approach or porogenes proved to be superior [8, 20-22]. However, the back-pressure applied to reach the required flow rates was of the order of a few MPa [9, 12, 23], and it was reduced to ca. 1.5-2 MPa in monolithic IMERs specifically designed for low-backpressure operation [8, 21]. Clearly, they necessitate the use of high pressure metering pumps, and it is neither convenient nor cost-effective.

Significant progress in IMER-based protein digestion can be achieved by application of hydrophilic silica monoliths with a hierarchical bi-modal pore structure synthesized using the Nakanishi method [24]. Although initially devised for chromatographic applications, they have been more recently applied as microreactors for the efficient synthesis of fine chemicals [18, 25, 28]. Due to the abundant presence of large textural flow-through pores (μm in size), the backpressure could be reduced to about one MPa, even for major flow rates [1, 6]. Moreover, the application of a double templating approach, proposed by Smått et al. [26] and modified by Pudło et al. [27], gave the monoliths with even larger flow-through pores (30-40 μm), as applied in advanced microfluidic devices, and also larger surface areas, arising from the presence of mesopores of about 20 nm in diameter, thus similar to the most effective

biocatalysts [20, 22]. Herein, we propose the preparation of microfludic IMERs making use of the latter concept and demonstrate their huge practical potentials in proteolytic digestion.

2. Materials and methods

2.1 Chemicals

Tetraethoxysilane (TEOS), polyethylene glycol 35000 (PEG), cethyltrimethylammonium bromide (CTAB), myoglobin, cytochrome c, N-benzoyl-DLarginine-p-nitroanilide (BAPNA), 3-aminopropyltrimethoxysilane (APTS) trifluoroacetic acid (TFA) were from Sigma-Aldrich. 2-Cyanoethyltriethoxysilane (CNTS) was from Lancaster. Cyano-4-hydroxycinnamic acid (HCCA) was from Bruker. Glutaraldehyde (GLA) and other chemicals were purchased from Avantor.

2.2 Synthesis of silica monoliths (MH)

Four silica monoliths were prepared by means of the Nakanishi method with minor modifications [28-30], to obtain samples with bi- and even tri-modal hierarchical pore structure. The applied method is based on a meticulous control of concomitant phase separation and the sol-gel process. By changing the reagent' composition (Table 1) and the post synthesis treatment (vide infra) four monoliths (MH1-MH4) of different pore structures were obtained. The general procedure was as follows: PEG was dissolved in aqueous HNO_3, after which TEOS was added slowly to the PEG solution in an ice bath followed by the addition of CTAB. The solution was mixed, then left to gel in polypropylene tubes at 40°C and aged for 10 days (MH1) or 3 days (MH2 to MH4) at the same temperature. Next, the alcogels obtained were impregnated with ammonia solutions: 1 M for 9 h at 90°C (MH1), or 0.1 M for 20 h at 40°C (MH2 and MH4) or water (3 days at room temperature) followed by 1 M ammonia solution for 24 h at 80°C (MH3). Before drying, the samples were washed with water and then calcined at 550°C for 8 h (ramp of 1°C min^{-1}) to obtain silica rods 40 mm in

length and 4 or 6 mm diameter. The monoliths were functionalized and clad with polymer resin (L285MGS-H285MGS type) to obtain single-rod mulichannel microfluidic microreactors.

2.3 Modification of monoliths and immobilization of trypsin

A single silica rod was immersed in either APTS or CNTS solution (0.15 mmol/mL) in toluene and held for 24 h at 80°C under intensive stirring, and then extensively washed with ethanol and dried.

Prior to trypsin adsorption, the CNTS functionalized monolith of MH1 type was washed with ethanol and distilled water for 45 min (flow rate of 1 mL/min) and then with 0.05 M borate buffer (pH 7.5) containing 10 mM $CaCl_2$. Next, a solution of trypsin (5 mL) in borate buffer was pumped (1 mL/min, 2.5 h) through the reactor under recycling conditions to immobilize the protein. Excess protein was removed by washing the columns with a 0.05 M borate buffer (pH 7.5) with 10 mM $CaCl_2$ for 0.5 h.

Before the attachment of trypsin the APTS functionalized silica monolith (MH1) was washed with ethanol and distilled water for 45 min (1 mL/min), followed by 0.1 M phosphate buffer (pH 7.0). To attach aldehyde groups on to the monolith's surface a 2.5 vol. % GLA solution in 0.1 M phosphate buffer (pH 7.0) was cycled (1 mL/min) through the reactor for 45 min, whereupon it was washed with water and 0.05 M borate buffer (pH 7.5) containing 10 mM $CaCl_2$. Finally, 5 mL of trypsin solution in borate buffer was passed through the reactor as described above. Excess protein was removed by washing as was described in [20]. Prior to activity assays, the monolith was washed with 0.5 M Tris–HCl buffer (pH 7.8) containing $CaCl_2$ (10 mM).

2.4 Characterization of silica monoliths

The mesopore structure parameters of silica monoliths was determined from nitrogen adsorption/desorption measurements, using a Micromeritics ASAP 2020 instrument.

Mesopore size distribution, and the mean pore diameter were calculated from the desorption branch of the isotherm using the BJH method [31]. The size and volume of larger through-pores were determined using Hg porosimetry (Quantachrome Pore Master 60), and the overall structure was also examined by means of scanning electron microscopy (TM3000 HITACHI). The presence of functional groups on the surface of MH1 was confirmed by FT-IR spectroscopy (Nicolet 6700) using a KBr disc (see the Supporting Information, Figure S1).

2.5 Activity assay and evaluation of protein digestion

The amidase activity of the MH1-bound trypsin was determined in a continuous (single-pass) flow of BAPNA solution (3 mM) in 0.1 M Tris–HCl buffer containing 10 mM $CaCl_2$ (pH 7.8) at 37°C using the microreactor (4 x 40 mm) set-up shown in Figure 1E and a metering pump (SIM DOS 03S) with maximal pressure head of 6 bar. The content of p-nitroanilide released was measured at 410 nm.

Protein digestion was examined in a continuous (single-pass) flow of myoglobin or cytochrome c solution (0.3 mg/mL) in 0.1 M Tris–HCl buffer with 10 mM $CaCl_2$ (pH 7.8) at room temperature. A 6 x 40 mm monolithic microreactor with trypsin attached covalently was applied and the flow rate varied in the range of 0.03-0.3 mL/min. The digest was collected for detailed analysis of peptide mapping by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry. Note that it was not necessary to denature the proteins prior to digestion, as is typically done to boost the proteolysis.

2.6 Mass spectrometry analysis

This procedure involves the proteolytic digestion of a protein followed by determination of the masses of the digested fragments using mass spectrometry. A subsequent comparison of the mass distribution profile within an appropriate database is then used to identify the original protein. Trypsin-digested samples were loaded in a sandwich manner with 1 %

cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 0.05 % trifluoroacetic acid (TFA) onto an *AnchorChip* target. High-resolution spectra were obtained using a Ultraflextreme MALDI-TOF/TOF instrument (Bruker Daltonics) operating in reflectron mode over the mass range of 800-4,000 Da. External calibration was performed using a point standard adjacent to the samples. Acquisition and data processing were controlled by FlexControl and FlexAnalysis software (Bruker Daltonics). Proteolysis efficiency was evaluated from the results of protein identification by using a peptide-mass fingerprinting (PMF) and a database of Mascot software.

2.7 Back-pressure vs. flow rate measurements

Back-pressure vs. flow rate relationships were determined using a standard laboratory set-up which consisted of a stainless steel reservoir of liquid, microreactor and glass cylinder. The liquid flow was forced by compressed air. Its volume was measured indirectly by a pressure transducer mounted in the bottom of the vessel and recorded continuously. The value of the back-pressure was calculated from air pressure reduced by pressure drop on piping and static pressure of the liquid in the collecting vessel. Sampling rate ranged from 0.2 to 2 sec^{-1} and volume of the liquid ranged from 100 to 200 mL for each test.

3. Results and discussion

3.1 Preparation effects and structure of monoliths

According to earlier reports the post synthesis treatment of silica monoliths in ammonia solution strongly affects the diameter of mesopores but not of macropores. The latter can conveniently be tuned by making use of the linear decrease of size of the macropores with the increase in the value of PEG/Si ratio (Table 1, Figure 1) [24, 26]. The independent control of macro- and mesoporosity provides a range of possibilities to tailor pore structure of the monoliths to individual requirements. As can be seen from SEM images (Figure 1) the monoliths possessed a uniform bi-continuous, foam-like structure, which size of pores and

silica struts differed significantly depending on the preparation conditions employed. As expected, the materials possess large flow-through channels in the μm size region. Mercury intrusion measurements (see the Supporting Information, Figure S2) show that the pores were 20–30 μm in diameter (MH1), and decreased to 10-12 μm (MH2), 3-6 μm (MH3) and 1-2 μm (MH4). The macropore volumes were very large (Table 1) in all the monoliths and decreased in the order MH1>MH3≥MH4>MH2. A closer inspection of SEM images of MH3 and MH4 (Figure 1A-B) and the corresponding macropore size distributions (Figure S2) indicates that for the same PEG/Si ratio (Table 1), the use of a higher concentration of nitric acid resulted in the formation of smaller macropores, with no appreciable effect on the macropore volume. This observation, not reported before, is caused by faster hydrolysis and slower condensation of siliceous species in acidic conditions; it can be effectively applied in modulating the macroporous structure of the monoliths

As can be inferred from Table 1 a post synthesis treatment as well as application of surfactant strongly affected the monoliths' pore structure in nanometer scales. The surfactant templated sample (MH1) exhibited a bi-modal distribution of mesopores of mean sizes ~20 and ~3 nm, whereas those synthesized without template (MH2-MH4) possessed smaller mesopores, of about 7-11 nm in diameter, resulting in larger specific surface areas. However, while these mesopores are large enough to host enzyme molecule of the size of trypsin [20], they are not sufficiently large to accommodate bulky substrates, such as the proteins to be digested, unlike the large mesopores of MH1. Thus, in view of the hampered accessibility of large molecules to a proteolytic enzyme (e.g. trypsin) embedded in the pores of MH2-MH4, their large activated surface area is less likely to be effective as an enzyme support in proteolysis reactions. Another factor of importance is the increased rate of reaction observed with enzymes confined in nanoporous structure, which exceeds that of the native protein. It was observed before for various enzymes immobilized in functionalized mesoporous silica carriers

with mesopores of 15-35 nm in size [20, 22, 32], and also in the continuous hydrolysis of sucrose carried out in MH1-based microreactors [28]. Thus, the above considerations consistently indicate that an open tri-modal pore structure renders MH1 monolith the best candidate for applications in proteins' digestion.

3.2 Pressure drop

The slow flow of liquid through solids with a tortuous pore structure is complex on a microscale perspective and tends to obey Darcy's law [33]. For a continuous-flow microreactor, such as that studied here, the relationships between the pressure drop gradient $\Delta P/L$ and the volumetric flow rate of liquid, V, its viscosity, η and cross section of the monolith, A, are given by (1):

$$\frac{\Delta P}{L} = \frac{1}{K}\frac{\eta}{A}\dot{V} \qquad (1)$$

where the permeability coefficient K, determined experimentally, allows for the combined effect of flow-through pore sizes ($K \sim d^2$, according to the Darcy-Weisbach equation [33]), their complex geometry and connectivity. A consistent linearity of pressure drop gradient vs. flow rate of water displayed in Figure 2 provides strong evidence that Darcy's equation can be applied to characterize the flow of liquids through the monolithic microreactors under study. Thus the values of the permeability coefficient can be used to quantify significant differences in the relationship of pressure drop against flow rate obtained with different monoliths. Of particular interest is the very low hindrance to liquid flow exerted by the very open flow-through pore structure of MH1 ($K_{MH1} = 1.96 \cdot 10^{-11}$) and of MH2, with a value of K_{MH2} ($5.57 \cdot 10^{-12}$) ca. 3.5 times smaller than K_{MH1}. For MH3 and MH4 the evaluated values of permeability were equal to, respectively: $8.4 \cdot 10^{-13}$ and $2.1 \cdot 10^{-13}$. Thus, the values of permeability obtained from different monoliths fairly well correlate with flow-through pore diameters, as predicted by the Darcy-Weisbach equation. For the bioactive peptide manufacture of importance may be that in MH1-type IMER the flow rate as high as 750

mL/cm^{2}min could be forced by a pressure gradient of only 64 kP/cm. But the pressure drop vs. flow rate characteristics obtained for both MH1 and MH2 appear to be very attractive, compared with those reported earlier [8, 9, 12, 21, 23]. It is noteworthy, that the largest value of permeability coefficient reported to date for the monolithic (polymeric) IMER ($2.3 \cdot 10^{-13}$), is two orders of magnitude less than that of MH1 [19]. For a hybrid monolith-in-capillary (100 µm i.d.) the same group earlier reported a pressure drop of 5.4 MPa/cm, for a flow rate of ca.5 µL/min, whereas in the MH2-filled same capillary this flow rate could be achieved at only ca. 22 kPa/cm, i.e. over 200 times less [23].

To summarize, the characteristics of pressure drop vs. flow rate clearly indicate that an abundant presence of micrometer-sized channels in MH1 and MH2 enables the use of these reactors at significantly increased flow rates and much reduced pressures. This and the large surface areas of the monoliths make them suitable for the application as continuous-flow IMERs. However, as pore structure of the MH1 monolith, in both macro- and mesopore size ranges is more attractive, we decided to evaluate its performance in a continuous-flow protein digestion.

3.3 Activity in BAPNA hydrolysis

The method used for protein immobilization is a critical factor to achieve effective biocatalysts. There are two common immobilization methods on solid supports: physical adsorption and covalent bonding; both methods have been used for the immobilization of trypsin [20, 22]. A comparative study of the performance of each method in continuous-flow microreactors has not been described, such a comparison was thus undertaken.

Monolithic silica supports (MH1) were functionalized with cyano groups, to increase the enzyme–carrier affinity during physical adsorption, and with amino groups activated with glutaraldehyde (GLA), to produce a Schiff's base for covalent attachment. Coupling of trypsin appeared to be significantly affected by the immobilization method; it was 0.2 and 0.35 mmol

for covalent and adsorption attachment, respectively. This observation is in very good agreement with the activity of the biocatalysts (Figure 3), which was notably higher when trypsin was attached by adsorption. After a significant decrease in the flow rate, from 2.1 to 0.3 mL/min, only a minor decrease in BAPNA conversion was observed (Figure 3A), whereas with covalently attached trypsin, a three-fold decrease in the flow rate yielded a corresponding three-fold increase in the conversion (Figure 3B). The decrease in BAPNA conversion with the increase in the flow rate, reported also earlier [7], can be explained by the shorter residence time and indicates that catalytic activity of the enzyme does not depend on liquid velocity and hence external mass transport. However, even for trypsin covalently attached to support the productivity increased with increase in the flow rate (Fig. 3C). In the operational range of liquid velocities flows in the monolith's channels are strictly laminar [35]. Therefore, the mixing of reactants is diffusion limited, and the access of substrate molecules to the enzyme's active site becomes more difficult. But, the tortuous channels induce and stimulate a chaotic movement (perturbations) of fluids and thus enhance mass transport, whereas the hierarchical pore structure, additionally facilitates access of reagents to active sites of the enzymes, with a positive effect on the microreactor's performance.

In practical applications, the stability of the biocatalysts is a factor of major importance. In this respect, the enzyme coupled by physical adsorption, after one week storage at 4°C, showed a significant decrease in activity (Figure 3A). Thus, the weak protein-support interaction due to van der Waals or hydrogen bonding forces, did not protect the enzyme against leaching caused by shear forces of the flowing liquid. Similar activity losses were observed by Bao et. al. [6] for trypsin immobilized on layer-by-layer coatings of graphene oxide and chitosan; sequence coverage of cytochrome c digestion decreased from 70 % to 46 % after nine runs. Unlike the trypsin attached by adsorption, no change in activity was observed for that immobilized covalently, even after one week storage, and not less

importantly, this biocatalyst retained its initial activity after at least 12 h of continuous operation (Figure 3B inset).

3.4 Proteolysis of myoglobin and cytochrome c

The proteolytic performance of MH1 were examined in the continuous flow digestion of myoglobin and cytochrome c. Myoglobin is a typical globular protein that contains 153 amino acids and 21 cleavage sites [23], whereas cytochrome c, possesses 104 amino acids and 21 cleavage sites [8]. Bearing in mind the complexity of protein digestion compared to BAPNA hydrolysis, and also that the flow rate strongly influenced tryptic digestion in the continuous process, the flow rate effect was examined over a broad range. As observed earlier for BAPNA hydrolysis, the proteolytic efficiency, determined by the amino acid sequence coverage, appeared to depend on its value. The efficiency consistently decreased for higher flow rates/shorter digestion time (Table 2), indicating that the rate of digestion was controlled by the kinetics of proteolysis and not diffusion. In particular, after 2.4 min treatment/residence time sequence coverage values were of about 40-50 %, whereas for the 24 min process (flow rate of 0.03 mL/min) they were ca. 90 % and 70 %, for myoglobin and cytochrome c, respectively (c.f. Table 2). The latter values are similar to those obtained after 12 h proteolysis using conventional approach (vide supra). Thus in terms of proteolytic efficiency the MH1 reactor are comparable to some of the best capillary IMERs [7, 23], especially, if we bear in mind that the proteins digested were not denatured prior to proteolysis. Already a superficial comparison of tryptic cytochrome c digestion performed in MH1 and a large volume (small packed column) IMER most recently recommended [3] for the bioactive peptide production, clearly indicates significant advantages of the MH1-based device. For the same flow rate (0.1 mL/min) the sequence coverage of cytochrome c digested for 10 min in the recommended IMER [3] was 19 %, whereas in MH1 it was 44 % already after 7 min (cf. Table 2).

Moreover, both the size and the flow rate can be easily increased in MH1 to scale up peptides' production.

The results of database searching and mass fingerprinting spectra of the tryptic digests are given in the Supporting Information. On the whole, they are very similar to those reported before for capillary IMER-based proteolysis of the same proteins, but for the flow rates smaller even by three orders of magnitude [9, 23].

Surprisingly enough, the sequence coverage appeared to be better in tryptic digestion of myoglobin than cytochrome c, although the molecular weight of the former is larger (Table 2). This observation was reported before [23], yet no convincing explanation was given. We believe that it should be linked to the electrostatic effects and the values of isoelectric point of the protein to be digested, that of trypsin and also of the siliceous support, which either facilitate or impede their contact, as explained in more detail in the Supporting Information.

4. Conclusions

Trypsin-functionalized multichannel siliceous monoliths possessing very open hierarchical pore structure in nm and μm size scales can be easily adapted to obtain microfluidic reactors for rapid and highly efficient proteolysis. A tortuous structure of flow-through channels stimulates external mass transport, whereas ultra-large mesopores facilitate internal transport of proteins to the immobilized proteolytic enzyme, and molecular-level interactions between proteins and the enzyme take place in a nanopore confinement.

Owing to the presence of numerous micrometer-sized channels the pressure drop is low, even at considerable liquid velocities. This eliminates the need for the use of HPLC pumps, conventionally applied in the rapid on-line proteolytic digestion, makes it safer and cost-efficient. The bioreactors obtained are ideally suited for coupling with the MALDI-TOF/TOF mass spectrometry apparatus for rapid digestion and peptide mapping. They can also be easily scaled-up or scale-out to meet specific demands posed by the bioactive peptides production.

To the best of our knowledge it is the first report on development and application of silica monoliths with very open tri-modal pore structure to obtain high-yield protein digestion microfluidic systems which lend themselves to diverse application in different scales.

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Tables:

Sample	Molar ratio of reagents TEOS:PEG:H_2O:HNO_3:CTAB	S_{BET} [m^2/g]	V_{pN2} [cm^3/g]	d_{pN2} [nm]	$V_{t\ Hg}$ [cm^3/g]
MH1	1 : 0.52 : 14.25 : 0.26 : 0.027	287	1.02	2.5/22	4.0
MH2	1 : 0.59 : 14.17 : 0.27 : 0	650	1.06	7.8	2.5
MH3	1 : 0.63 : 14.92 : 0.25 : 0	413	1.17	11.3	3.2
MH4	1 : 0.63 : 15.58 : 0.41 : 0	595	1.16	7.8	3.0

Table 1. Molar ratio of reagents and parameters of monoliths pore structure determined from nitrogen adsorption and mercury porosimetry.

Protein	MW [Da]	PI	Sequence coverage [%]		
			0.03 [mL/min] 24 [min]	0.1 [mL/min] 7.2 [min]	0.3 [mL/min] 2.4 [min]
Cytochrome c	11 702	9.6	70	44	39
Myoglobin	16 953	7.4	90	80	53

Table 2. Effect of flow rate on tryptic digestion of cytochrome c and myoglobin in MH1 reactor.

List of Figures:

Figure 1.

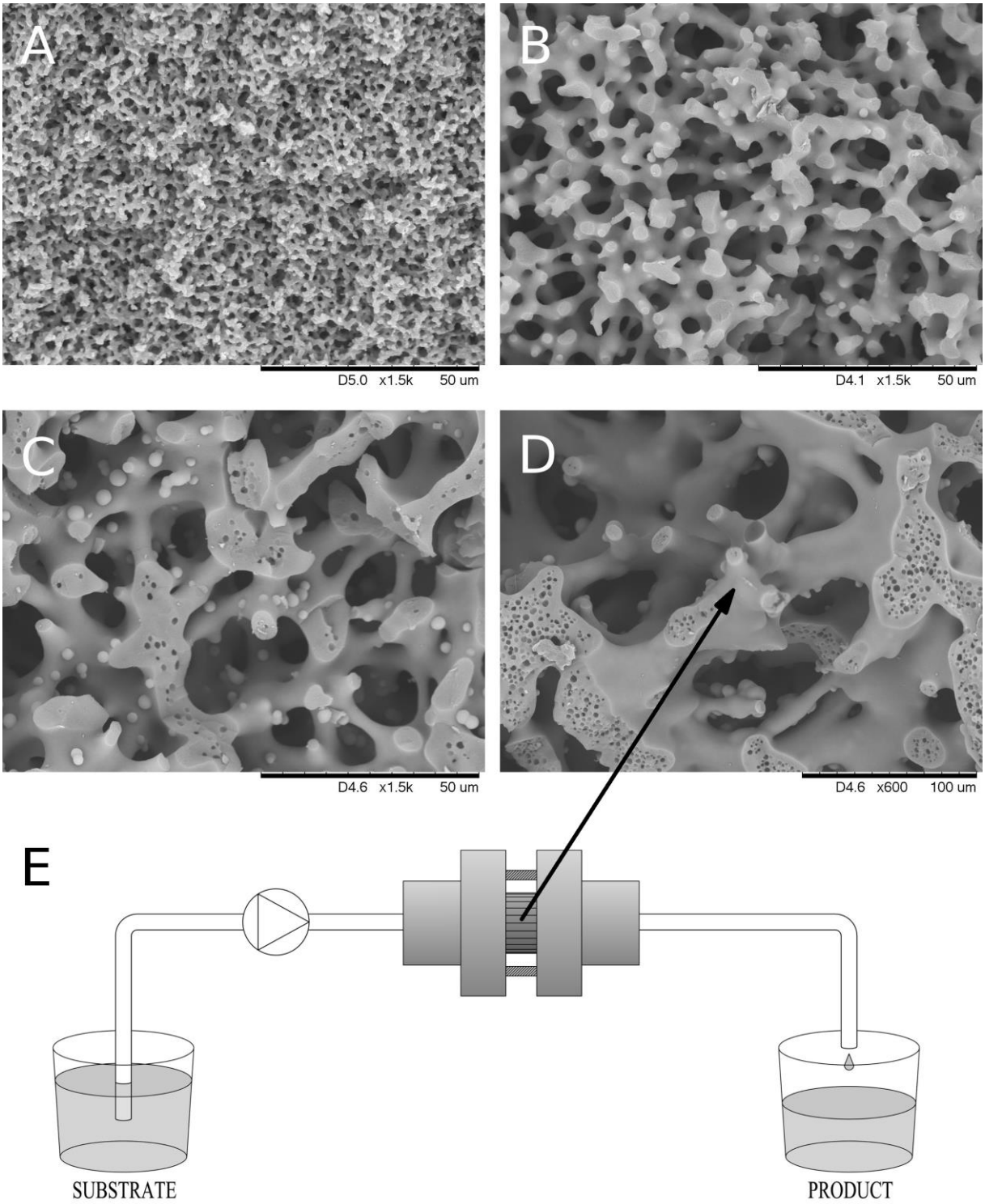

A
D5.0 x1.5k 50 um
B
D4.1 x1.5k 50 um
C
D4.6 x1.5k 50 um
D
D4.6 x600 100 um
E
SUBSTRATE
PRODUCT

Figure 2.

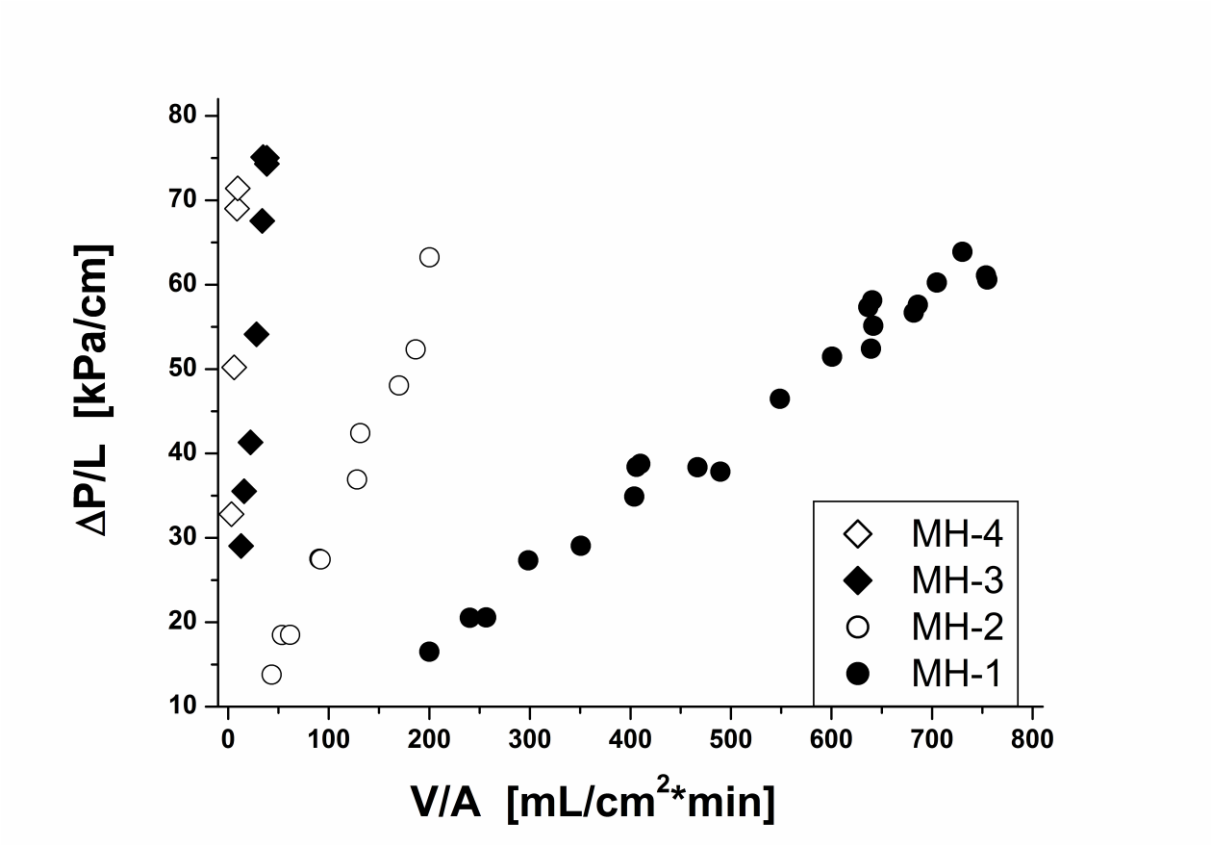
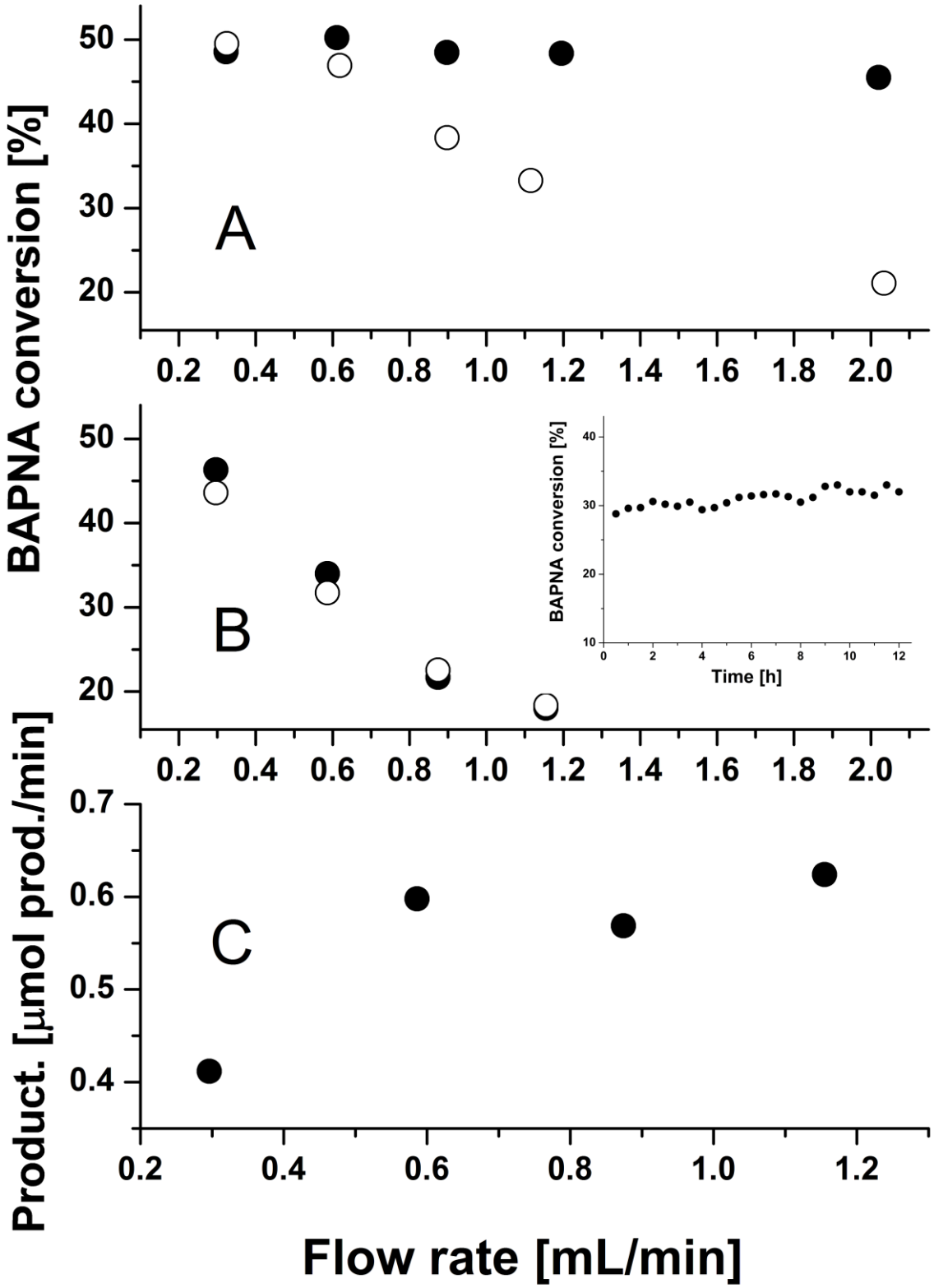

ΔP/L [kPa/cm]
V/A [mL/cm2*min]
MH-4
MH-3
MH-2
MH-1
80
70
60
50
40
30
20
10
0
100
200
300
400
500
600
700
800

Figure 3.


BAPNA conversion [%]
Product. [μmol prod./min]
Flow rate [mL/min]
A
B
C
BAPNA conversion [%]
Time [h]