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Preparation and characterization of a packed bead immobilized trypsin reactor integrated into a PDMS microfluidic chip for rapid protein digestion

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ABSTRACT

This paper demonstrates the design, efficiency and applicability of a simple, inexpensive and high sample throughput microchip immobilized enzymatic reactor (IMER) for rapid protein digestion. The IMER contains conventional silica particles with covalently immobilized trypsin packed inside of a poly(dimethylsiloxane) (PDMS) microchip channel (10 mm×1 mm×35 μm). The microchip consists of 9 different channels, enabling 9 simultaneous protein digestions. Trypsin was covalently immobilized using carbodiimide activation, the ideal trypsin/silica particle ratio (i.e. measured mass ratio before the immobilization reaction) was determined. The amount of immobilized trypsin was 10–15 μg trypsin for 1 mg silica particle. Migration times of CZE peptide maps showed good repeatability and reproducibility (RSD% = 0.02–0.31%). The IMER maintained its activity for 2 months, in this period it was used effectively for rapid proteolysis. Four proteins (myoglobin, lysozyme, hemoglobin and albumin) in a wide size range (15–70 kDa) were digested to demonstrate the applicability of the reactor. Their CZE peptide maps were compared to peptide maps obtained from standard in-solution digestion of the four proteins. The number of peptide peaks correlated well with the theoretically expected peptide number in both cases, the peak patterns of the electropherograms were similar, however, digestion with the microchip IMER requires only <10 s, while in-solution digestion takes 16 h. LC-MS/MS peptide mapping was also carried out, the four proteins were identified with satisfying sequence coverages (29–50%), trypsin autolysis peptides were not detected. The protein content of human serum was digested with the IMER and with in-solution digestion.

1. Introduction

Proteomics is a relevant field of study in bioanalytics, aiming at the characterization of the entire set of proteins expressed by a genome [1]. Proteome research underwent a substantial evolution with the appearance of peptide mapping, a method used for the identification of proteins or the analysis of their primary structure or posttranslational modifications. Later, the emergence of modern LC-MS/MS systems contributed to the pioneering development of shotgun proteomics, which enabled the analysis of several hundreds of proteins in a single experiment [2].

Peptide mapping includes the chemical or enzymatic digestion of a protein sample, the analysis of the digest and the comparison of the obtained peptide map with a peptide database [3]. Firstly, proteins are denatured and their disulphide bridges are reduced and alkylated to facilitate enzymatic digestion. Trypsin is a proteolytic enzyme widely used for protein digestion; it cleaves the amino acid sequence at the C-terminal peptide bonds of lysine and arginine. After digestion, the reaction mixture is desalted and the peptides are separated and detected. For this analysis, (two dimensional) high performance liquid chromatography ((2D) HPLC) [4,5], capillary electrophoresis (CE) [5–8], matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [9], CE-MS [10] or LC-MS/MS [2,11,12] techniques are preferably used. The obtained peptide map describes the sample protein as a fingerprint, enabling protein identification or the analysis of posttranslational modifications [13].

The most time consuming step of the analysis is the enzymatic digestion with trypsin (2–30 h), as it can only be applied in low concentrations (protein:enzyme = 20–100:1) due to its undesirable self-digestion in free form. Self-digestion can be prevented with the immobi-

Abbreviations: IMER, immobilized enzymatic reactor; PDMS, poly(dimethylsiloxane); CZE, capillary zone electrophoresis.

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lization of trypsin onto a solid support, thus it is possible to utilize the enzyme in higher surface concentration.

There are numerous advantages of using immobilized enzymes: reusability, prolonged lifetime, easy separation from products and substrate specificity. They also have diverse applications such as the design of biosensors [14], enzymatic microreactors [15] or improving enzyme stability [16]. In general, enzymes can be immobilized on solid supports via adsorption [17–23], covalent linkage [6,9,11,22,24–44] or bioaffinity linkage [45–47].

The use of immobilized trypsin for protein digestion is especially beneficial in a system with high surface-to-volume (S/V) ratio, as high S/V ratio favors the heterogenic reaction between the free substrate and the immobilized trypsin, hence reaction times are greatly reduced (10 s–15 min) [48]. One possible design for the preparation of immobilized enzymatic reactors (IMER) is to use the capillary/channel walls of fused silica capillaries or microchips as solid supports for immobilization [22,26,45–47,49]. In order to further increase the S/V ratio of the reactor, a more often used approach is to apply solid supports with even higher specific surface area integrated into microfluidic chips or capillaries, e.g. membranes [17–19, 21], mesoporous silica [20,23], monoliths [6,11,24,25,33–37,44,50], magnetic microspheres/beads [27,38–43,51], silica particles (packing or column) [28,29] or commercially available beads (usually cross-linked agarose) [5,31,52–54]. These microchip IMERs are capable of rapid protein digestion and can operate with small sample and reagent volumes. As microfluidic chips can be coupled to separation techniques (CE or MS) [9,22,48,50,52,55–59], they can be used advantageously for on-line proteolysis. In these systems, protein samples are usually digested in the microchip IMER and the resulting peptides are separated and detected (with CE and/or MS).

Microfluidic chips are routinely fabricated of poly(dimethylsiloxane) (PDMS) using soft lithography [60]. As the simplest approach, the complex microscopic channel network can be directly applied as a support for immobilization due to its high S/V ratio. For covalent protein immobilization on PDMS surface, complex chemical reactions are necessary for surface modification as PDMS lacks reactive groups [22]. In an earlier work our group showed that conventional chromatographic particles can be retained in a PDMS microchip without the use of frits but only with simple bottlenecks [61–63]. The obtained chromatographic packings were applicable for chromatographic separation/preconcentrations of components.

In this work we demonstrate the fabrication and usability of a packed bead immobilized enzymatic reactor integrated into a PDMS microfluidic chip. For high S/V ratio, we used trypsin coated silica particles to create packings inside the microchip channels. To the best of our knowledge, this is the first work describing an enzymatic reactor integrated into a PDMS microfluidic chip containing covalently immobilized trypsin on conventional (chromatographic) silica particles. The efficiency of the microchip IMER was evaluated with capillary zone electrophoresis (CZE) and LC-MS/MS measurements after digestion.

2. Experimental

2.1. Materials

Analytical grade reagents were used. To establish covalent linkage between trypsin (Type IX-S, lyophilized powder, Sigma, St. Louis, MO, USA) and amino-functionalized silica particles (HPLC bulk packing, Microsorb 100-5, amino, Varian, Palo Alto, CA, USA) N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and benzamidine hydrochloride were used (all Sigma products).

For protein determination by Lowry's method [64], the three components of the Lowry solution were prepared as stock solutions and the final Lowry solution was prepared freshly before each experiment, according to the manufacturer's protocol (Sigma). For the three stock solutions, NaOH, Na_2CO_3, $CuSO_4$ and potassium sodium tartrate were used (all Sigma products). Also, Folin and Ciocalteu's phenol reagent (2 N) was used for the determination (Sigma).

Bovine serum albumin (BSA), lysozyme from egg white, equine skeletal muscle myoglobin and bovine blood hemoglobin (Sigma) were digested. As realistic sample, human blood serum obtained from a healthy volunteer was digested. For sample preparation, 1 mM trifluoroacetic acid (TFA), 8 M urea, 100 mM dithiothreitol (DTT), 200 mM iodoacetamide (IAM), 25 mM NH_4HCO_3 and 1% formic acid (FA) stock solutions (all Sigma products) were prepared in double-deionized water (Elix-3, Millipore, Darmstadt, Germany).

For capillary zone electrophoretic measurements, NaH_2PO_4, Na_2HPO_4, sodium dodecyl sulfate (SDS, 50 mM) and NaOH (1 M) (VWR International, PA, USA) were dissolved in double-deionized water.

2.2. Instruments

An inverted microscope (Axio ObserverA1, Zeiss) equipped with a high speed CCD camera was used to record images of the packing process. Images were processed by AxioVision 4.6.3 (Zeiss) software.

UV/Vis spectrophotometry for the determination of immobilized trypsin was carried out on a double-beam spectrophotometer (V-550, Jasco).

Capillary zone electrophoretic (CZE) peptide mapping was performed using a 7100 model CE instrument (Agilent, Waldbronn, Germany). The electropherograms were recorded and processed by ChemStation software version B.04.02 (Agilent).

Mass spectrometry analyses were done on a 4000 QTRAP (ABSciex)- EasynLC II (Bruker) nanoHPLC-MS/MS System controlled by Analyst 1.4.2 (ABSciex) software. Information Dependent Acquisition was carried out composed of a positive mode survey scan (+EMS) in 440–1400 amu mass range followed by an enhanced resolution scan (+ER) in order to determine the charge state of the two most intensive ions. Using these information, the proper collision energies were calculated by the software, the selected parent ions were fragmented by collision induced dissociation (CID) and the product ion spectra were recorded (+EPI).

2.3. Preparation of the microchip IMER

The IMER was integrated into a PDMS microfluidic chip consisting of 9 parallel channels with 1 mm width, 10 mm length and 35 μm height (internal volume: 350 nL). The chip was fabricated by means of soft photolithography [60]. Briefly, the lithographic mask was designed with AutoCAD software and was printed in high resolution (3000 dpi) onto a transparent foil. A 3″ silicon wafer was spin-coated with negative type photoresist (SU-8 2025, Microchem, Newton, MA, USA) at 3000 rpm for 15 s to achieve a thickness of ~35 μm. After spin-coating, the wafer was baked at 95 °C for 15 min. The pattern on the mask was transferred to the wafer through UV exposure (365 nm) for 10 min. The exposed wafer was baked at 95 °C for 5 min and the unexposed areas were removed by rinsing with SU-8 developer (Microchem).

The PDMS chip was fabricated by cast molding a 10:1 (w:w) mixture of silicone elastomer base and curing agent (Sylgard 184, Dow Corning, Midland, MI, USA). The mixture was degassed and baked at 65 °C for 60 min. Holes (300 μm diameter) were pierced through the PDMS chip at the ends of the channels for liquid connections. The

PDMS chip was irreversibly sealed onto a 1.2 mm thick glass slide after oxygen plasma treatment (PDC-32G, Harrick, Ithaca, NY, USA).

A peristaltic pump (IPC, Ismatec, Cole-Palmer, IL, USA) was used for the transportation of reagents and samples through the microfluidic channel. The tubing of the pump (ID: 0.25 mm, Tygon, Cole-Palmer, IL, USA) was connected to the inlet port of the chip.

Trypsin was covalently immobilized using EDC/NHS method (Fig. 1a): trypsin (3 mg/mL), EDC (7 mg/mL) and NHS (1.3 mg/mL) were dissolved in 50 mM phosphate buffer (pH = 6.8) [26], which contained suspended amino-functionalized silica particles (12 mg/mL) and the mixture was stirred for 5 h. After immobilization, the suspension was transferred into a 2 mL Eppendorf tube, the particles were centrifuged and the supernatant was decanted. 1.5 mL mixture of acetonitrile:water (70:30) was added to the particles, vortexed for 1 min, centrifuged and decanted again. This washing step was repeated 6 times to completely remove any free trypsin, using 1.5 mL acetonitrile:water (70:30) and 1.5 mL water, alternatingly. After the last washing step, 200 μL solution of 1 mM HCl, 20 mM $CaCl_2$ and 0.4 mg/mL benzamidine was added to the particles (storage solution) and the suspension was stored at −18 °C until use. To create the microchip IMER, the silica particles with the immobilized trypsin were packed inside the channels of a PDMS chip.

In several papers regarding immobilized trypsin [65–70], benzamidine is used as competitive inhibitor, which binds into the active site of the enzyme, protecting it during immobilization or storage. The effect of using benzamidine in the immobilization reaction was studied, in this case the mixture contained 0.4 mg/mL benzamidine, as well. It was found that in our case, the use of benzamidine during the immobilization reaction did not offer any elevated activity of the immobilized trypsin. As a result, benzamidine was used only for protecting trypsin upon storage but not for protection during immobilization. More details on this experiment are available in the Supplementary material (Fig. S.1).

The proper preparation of packings was pivotal for the analysis, as sufficiently compressed packings were needed for reproducible and effective proteolysis. The designed PDMS chip contained 1 mm thick channels which ended in 5 bottlenecks of 20 μm width each. The suspension of the particles containing immobilized trypsin was injected, ~2.5 μL suspension was necessary to form a 1 cm long packing (Fig. 1b–e). 25 mM NH_4HCO_3 (pH = 7.8) was flushed through the channel with 1 μL/min flow rate for 10 min to compress the particles and to precondition the trypsin at an optimal pH.

2.4. Enzymatic digestion

Protein samples were pretreated in order to facilitate enzymatic digestion: ~2,5 mg protein sample was dissolved in 100 μL 1 mM TFA (in the case of human serum: 20 μL serum was diluted with 80 μL TFA) and immediately 300 μL 8 M urea solution was added. After 30 min at room temperature, the tertiary structure of the protein was unfolded, then, 40 μL 100 mM DTT was added to reduce disulphide bonds (1 h at 37 °C). Subsequently, 40 μL 200 mM IAM as an alkylating agent was added to avoid the recombination of the disulphide bonds (45 min at room temperature in dark). 2 mL 25 mM NH_4HCO_3 was further added to the mixture to give a final urea concentration 1 M (protein concentration: ~1 mg/mL). The prepared solutions were stored at −18 °C until digestion either in-solution or in the microchip IMER.

For in-solution digestion, 1 mg/mL trypsin was added to the sample (protein:trypsin = 50:1). After overnight (16 h) incubation at 37 °C, the reaction was terminated with the addition of 250 μL 1% FA to the mixture.

For the digestion with the microchip IMER, the sample was flushed through the packing with 1 μL/min (contact time < 10 s). 10 μL sample was collected at the outlet end of the microchip channel, this sample volume was necessary for CZE separation after desalting. Digestion occurred at room temperature. With the microchip IMER, 9 different protein samples can be digested at the same time (Fig. 1f). After each digestion, the packings were postconditioned by flushing it with acetonitrile:water (20:80) containing 25 mM NH_4HCO_3 for 10 min [24].

Desalting was performed on C18 Supel-Tips (SupelCo, Sigma), according to the manufacturer's manual. 0.1% FA was used as wetting and desalting solution and acetonitrile:water (70:30) containing 0.1% FA to elute the adsorbed peptides to 10 μL final volume.

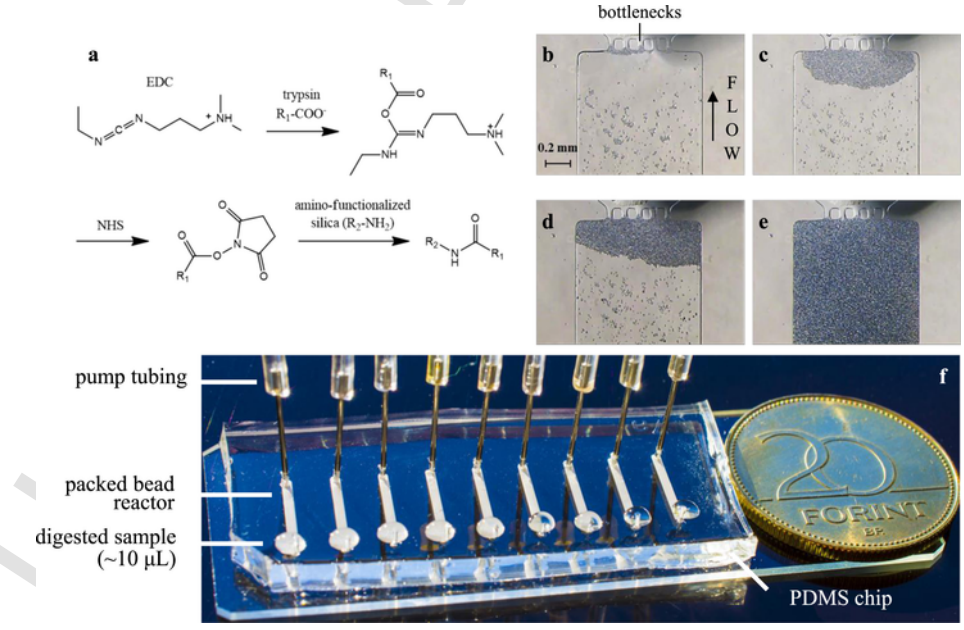


Fig. 1. (a) The covalent immobilization reactions with EDC/NHS between trypsin and amino-functionalized silica particles. (b–e) Optical micrographs of the packing formation process at the bottlenecks. The suspension of the silica particles in storage solution was pumped toward the bottlenecks. (f) Photograph of the microchip IMER with 9 simultaneous digestions.

2.5. CZE separation

In all measurements hydrodynamic sample introduction was used for injecting samples. The sample solutions were introduced at the anodic end of the capillary. Capillary zone electrophoresis separations were carried out using fused-silica capillaries of 80 cm×50 μm id (Agilent) with extended light path. The applied voltage was 30 kV. The detection was carried out by on-column DAD measurement. The buffer electrolyte for the CZE separation contained 25 mM phosphate, pH 7.0. The capillaries were preconditioned with the buffer electrolyte for 5 min and postconditioned with 50 mM SDS for 5 min, 1 M NaOH for 10 min and the buffer electrolyte for 5 min. Each sample contained ~1 mg/mL protein (prior to digestion).

2.6. LC-MS/MS measurements

Desalted samples for LC-MS/MS analysis were diluted to a 10-fold final volume (1 μL desalted sample with 9 μL 1% FA). Prior to mass spectrometry analysis, the peptides were separated using a 90 min water:acetonitrile gradient and 300 nL/min flow rate on an EasynLC (Bruker) nanoHPLC. First a desalting step was performed on a Zorbax 300SB-C18 column (5×0.3 mm, 5 μm pore size; Agilent) followed by separation on a Zorbax 300SB-C18 analytical column (150 mm×75 μm, 3.5 μm pore size; Agilent). Solvent A was 0.1% FA in LC water, solvent B was acetonitrile containing 0.1% FA. The acquired LC-MS/MS spectra were used for protein identification with the help of ProteinPilot 4.0 (ABSciex) search engine searching the SwissProt database (release: 2015.07, 548,872 sequence entries) using the biological modification table included in the ProteinPilot 4.0 software. Minimum of two peptide sequences with ≥95% confidence were used for protein identification.

3. Results and discussion

3.1. Determination of the immobilized trypsin

An important aspect in the characterization of an IMER is the amount of immobilized enzyme as it determines the maximal activity. In the case of tryptic proteolysis, digestion rate in a reactor with high S/V ratio depends only on the protein:trypsin ratio (under permanent temperature and pH). Obviously, digestion occurs more rapidly if trypsin is present in higher surface concentration.

Quantitative determination of the immobilized trypsin was carried out to estimate the ideal concentrations of trypsin and silica particles (that is the measured mass ratio before the immobilization reaction). Enzyme immobilization was achieved as described earlier, different trypsin/silica particle ratios were used. Determination of the immobilized trypsin can be performed according to Lowry's method after the basic cleavage (0.1 M NaOH) of the already immobilized trypsin from the solid support, if it is linked as an amine (e.g. glutaraldehyde linkage) [24]. In this study, trypsin was linked to the solid support through a peptide bond, which is more stable under basic conditions, however, determination of the immobilized trypsin can be carried out similarly, as NaOH attacks silica under the trypsin, thus mobilizes it.

After immobilization and washing, the full amount of particles was transferred into a 10 mL volumetric flask with 0.1 M NaOH and it was stirred for 2 h at room temperature. The amount of immobilized trypsin was determined from the supernatant according to Lowry's method. Also, control immobilized trypsin determination without the use of EDC and NHS was performed to prove that the formation of covalent linkage (the presence of EDC and NHS in the immobilization mixture) was responsible for the immobilization of trypsin.

It is noteworthy that the amount of immobilized trypsin depended on the initial concentrations of silica particles and trypsin. As Fig. 2 shows, the increase of trypsin/particle ratio up to ~250 μg/mg increased the immobilized trypsin/particle (i.e. surface trypsin concentration) linearly, however, increasing it furtherly did not affect the surface trypsin concentration. This can presumably be attributed to the saturation of the silica particle surface by trypsin, with no binding place remaining. An ideal initial trypsin/particle ratio should guarantee that the full surface of the particle is saturated with trypsin and the amount of prepared particles is the largest possible, therefore, 250 μg/mg ratio was applied in our experiments for trypsin immobilization.

Control measurements were carried out without the use of EDC/NHS in the immobilization reaction. Under these initial circumstances, no covalent linkage occurs, therefore, the determined concentration (1–3 μg/mg) can be attributed to trypsin spontaneously adsorbed onto the particles and not removed in the washing steps and/or the experimental error. The significant difference in the amount of immobilized trypsin upon using EDC and NHS as coupling agents proves that covalent linkage was formed between trypsin and amino-functionalized silica particles. Also, the determination leads to ~10 μg/mg immobilized trypsin/particle if it was corrected with the control measurement, which is comparable to or higher than other works determining immobilized trypsin in a similar system (e.g. 2 and 10 cm long reactor with 1.3 μg trypsin/1 cm length for a silica monolith reactor [24], 1.5 cm long reactor with 0.9 μg/1 cm gel in a trypsin-encapsulated gel reactor [71] and a 1 cm long reactor with 97 ng trypsin/1 cm length in a PSG/PEG monolith reactor [33]).

Rapid digestion requires high trypsin surface concentration. To calculate the trypsin/protein ratio in the microchip IMER, two suggestions should be made: (i) the mass of a single packing is ~0.5 mg (cf. the channel's inner volume is 350 nL) and (ii) the dead volume of the

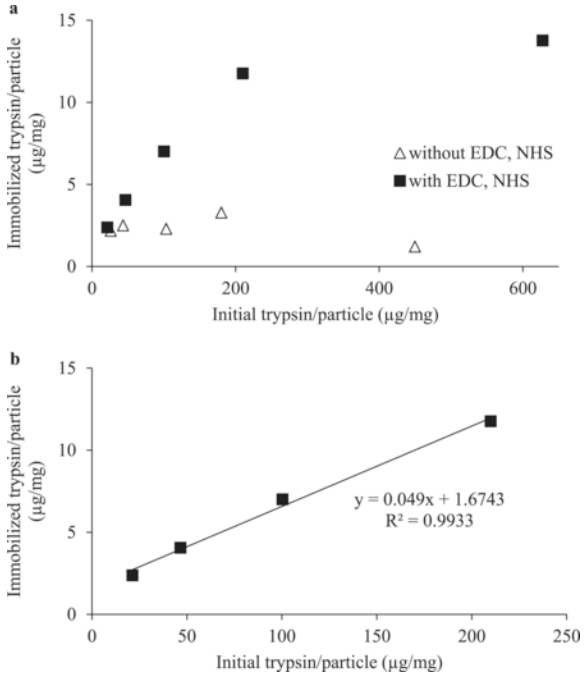


Fig. 2. (a) Dependence of trypsin surface coverage on different trypsin/particle ratios (measured mass ratio before the immobilization reaction) in the immobilization mixture (solid symbols). Control experiment was carried out without the use of covalent coupling agents, EDC and NHS (empty symbols). The amount of the immobilized trypsin was determined by Lowry's method after basic treatment of the particles (trypsin mobilization), the mass of the particles was measured by analytical balance. (b) The surface trypsin concentration increased linearly up to 10–15 μg/mg, and stagnated if initial trypsin/particle was higher than 250 μg/mg.

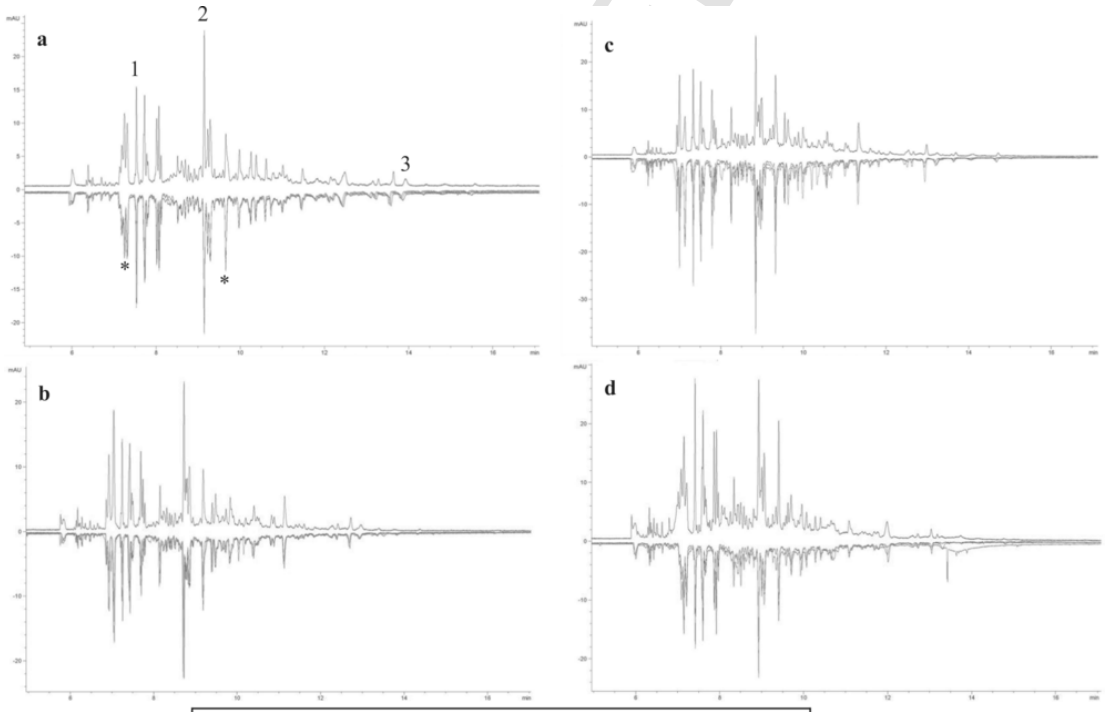
packing (which does not include the fittings, only void space between the particles) is not higher than 50 nL, which is probably overestimated. In this case, a 1 cm long packing contains 5 μg immobilized trypsin and the dead volume contains 50 ng protein (for 1 mg/mL sample concentration). Thus, the protein:enzyme ratio is 50:5000, while at in-solution digestion it is 50:1. As trypsin ratio is 5000-times higher, the reaction rate can be 5000-times faster, as well. This is the reason why digestion with IMER can be performed under 10 s, while a standard digestion takes 16 h. If the dead volume was less than 50 nL, the 5000× multiplier in reaction rate can be even higher.

3.2. Reproducibility of the peptide maps

To demonstrate the reproducibility of the analytical method, BSA samples were digested, desalted and analyzed via CZE. Reproducibility was examined from several aspects: reproducibility of CZE (the same digested BSA was analyzed four times – Fig. 3a); reproducibility of digestion (4 different BSA samples were digested with the same packing – Fig. 3b); reproducibility of the packing formation (BSA samples were digested on 4 different packings, the particles were prepared in the same immobilization reaction, Fig. 3c); and the reproducibility of immobilization (BSA samples were digested on 4 different packings, the particles were prepared in 4 different immobilization reactions, Fig. 3d). The electropherograms were matched by the correction of the migration times using two time references for two identical peaks (marked with * in Fig. 3a) in order to compensate the shifts in the migration times of the peaks. Three different peaks (Peak 1 at ~7.35 min, Peak 2 at ~8.8 min and Peak 3 at ~13 min) were randomly chosen to investigate the scattering of the migration times and peak areas, that are summarized in Fig. 3e. Integrated electropherograms are available in the Supplementary material (Fig. S.2 a–d).

Low RSD% values (< 0.5%) were calculated for migration times, which can be a result of effective postconditioning and the application of matching with two time references. Also, since mostly identical peptides were formed in the microchip IMER during digestion, the peak patterns of the electropherograms are similar. The number of peaks (~70–80) correlated well with the theoretically expected number [72] of peptides in the case of a complete digestion of albumin.

Higher RSD% values were calculated for peak areas. For the reproducibility of CZE (a), RSD% values were higher than usual (2–10%) due to integration difficulties (the peaks were small (peak 3) or not baseline resolved (peak 1–2), Fig. S.3). There was higher RSD% calculated for (b) and even higher for (c) and (d), but there is no significant difference between (c) and (d). It can be interpreted that different samples may uncontrollably contain the same peptides in different concentrations, because some hydrophilic peptides can be lost during desalination or because hydrophobic peptides can be adsorbed on the surface of the PDMS chip. The use of different packings for digestion further increased RSD% values, as the different lab-made packings are not equally compressed, their formation is less reproducible. However, no



e

	RSD%					
	Peak 1		Peak 2		Peak 3	
	t	A	t	A	t	A
(a)	0.03	2.51	0.02	2.18	0.31	9.13
(b)	0.03	12.40	0.02	11.84	0.10	12.60
(c)	0.05	30.55	0.04	23.36	0.14	53.57
(d)	0.05	31.99	0.02	30.64	0.03	42.68

Fig. 3. Repeatability and reproducibility measurements of CZE peptide maps: analysis of (a) the same digested BSA four times, (b) 4 different BSA samples digested with the same packing, (c) BSA samples digested on 4 different packings and (d) BSA samples digested on 4 different packings with the particles prepared in 4 different immobilization reactions. The electropherograms were matched by the correction of the migration times using two time references peaks (marked with asterisks). Three different peaks (Peak 1, 2 and 3) were randomly chosen to calculate (e) RSD% values for migration times and peak areas. Conditions: for CZE: untreated capillary with extended light path (id: 50 μm, l_{eff} = 72 cm, L = 80 cm), 25 mM phosphate, pH: 7, +30 kV, 100 mbar s, λ = 200 nm.

significant differences in RSD% were experienced when digestion was executed in packings that were formed with particles from different immobilization mixtures. It may confirm the supposition that the surface of the particles was saturated with trypsin (in each case the ideal trypsin/particle ratio was used).

3.3. Stability and applicability of the microchip IMER for peptide mapping

The stability of the reactor was tested with several experiments. After trypsin immobilization, BSA was digested with freshly prepared (immobilized and packed) microchip IMER and the digest was analyzed with CZE (Fig. 4a). The prepared particles were stored for 2 months at −18 °C suspended in storage solution (1 mM HCl, 20 mM $CaCl_2$ and 0.4 mg/mL benzamidine). Also, the IMER was immersed into storage solution and was stored for 2 months at 4 °C. After 2 months, BSA was digested on a microchip IMER freshly packed with the stored particles (Fig. 4b). Another BSA sample was digested with the microchip IMER stored for 2 months (Fig. 4c). The total amount of BSA was digested and the number of peptide peaks were equal in each sample. These experiments proved that immobilized trypsin retains its activity for at least 2 months and there is no difference if the particles were suspended in a solution or packed into a microchip IMER during storage.

Four proteins (myoglobin – 17 kDa, lysozyme – 16 kDa, hemoglobin – 62 kDa and BSA – 69 kDa) were digested to evaluate the efficiency of the microchip IMER. In the case of a complete digestion with no missed cleavage, 19, 19, 31 and 78 peptides should be detected in the case of myoglobin, lysozyme, hemoglobin and BSA, respectively [72]. The CZE peptide maps of these proteins showed good correlation to the theoretically expected numbers after digestion with the microchip IMER (number of separated peaks were around 20, 20, 30 and 80 for the four protein, Fig. 5).

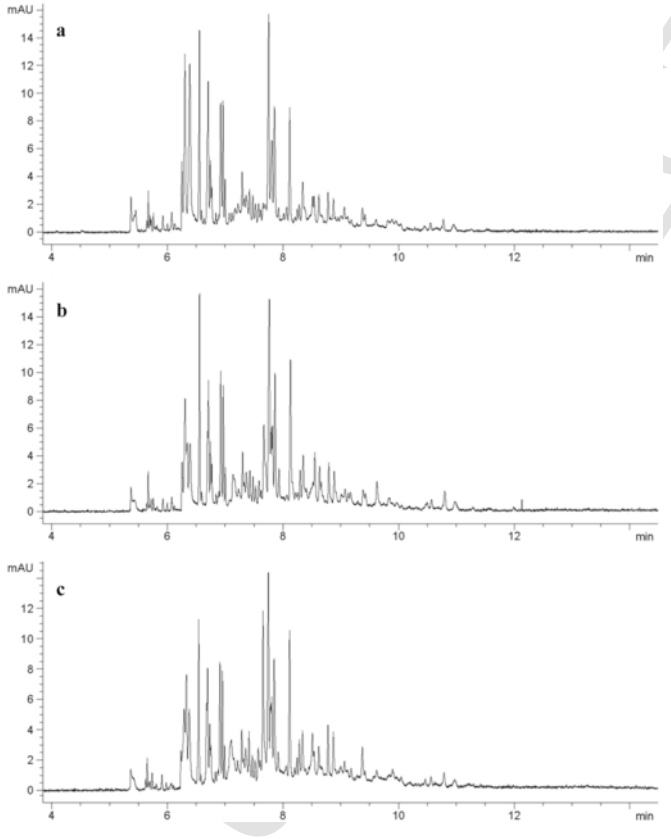


Fig. 4. Stability of the microchip IMER: digestion (a) with a freshly formed packing of freshly prepared immobilized trypsin particles, (b) with a freshly formed packing of immobilized trypsin particles prepared 2 months earlier and (c) with the packing formed 2 months earlier from immobilized trypsin particles. Conditions are the same as in Fig. 3.

Each peptide map obtained from digestions with the microchip IMER was compared to the peptide maps obtained from standard in-solution digestions (Fig. 5). It can be concluded, that most of the separated and detected peptides were identical in both cases, based on the uniformity of the peak patterns, especially their migration times. The reaction time was typically 5–10 s when digestion was performed with the microchip IMER, while 16 h was needed for in-solution digestion, however, there was no significant difference between the efficiencies.

The digestion of the protein content of human serum was performed with the IMER. Fig. 6 shows that similar result was obtained with the standard in-solution digestion, as with the IMER digestion. The most abundant protein in the serum is albumin, 74 peptides can be derived from the perfect digestion of HSA. The number of peptide peaks correlates well with each other, and with the expected number, as well. This experiment proves that the IMER can be used for the digestion of realistic samples, too.

Protein identification was carried out via LC-MS/MS method. Four protein samples digested with the microchip IMER were identified (Table 1) with satisfying sequence coverage values (29–50%), trypsin autolysis peptides were not detected in the samples. Sequence coverage and protein score values for each protein are listed in Table 1, identified peptides with the number of missed cleavages are listed individually in the Supplementary material (Table S.1).

BSA is often used to test the efficiency of trypsin containing IMERs. Sequence coverage value of digested BSA were 29% with our microchip IMER with < 10 s reaction time. Several works reported comparable values: 22% and 30% (5 s reaction time) [22], 43% (5 min) [41], 46% (5 min) [42], 23% (12 min) [53], 35% (24 s) [73], 45% (10 s) [74] and 40% (5 s) [75] – with IMERs containing covalently linked trypsin on different solid supports. As a comparison with works published in this field, Table 2 contains the main parameters of covalently linked enzymatic microreactors, that use beads/particles as solid support for immobilization.

4. Conclusions

In this paper we reported the preparation of a simple, inexpensive microchip IMER, capable of rapid proteolysis. Protein digestion is a crucial analysis step in peptide mapping, however, standard in-solution digestion is time-consuming (16 h). The proposed microchip IMER contains trypsin immobilized on conventional silica particles. The micropackings can be prepared easily with the use of bottlenecks that retain the particles. The properly compressed packings offer high S/V ratio which is essential for high reaction rate in a heterogenic reaction. On the basis of the determined immobilized trypsin per particle, reaction rates can be higher by at least 3 orders of magnitude.

Reproducibility of CZE measurements, digestion, packing preparation and immobilization was investigated, in each case good RSD% values were calculated for migration times. The CZE peptide maps (peak pattern) obtained by microchip IMER digestion and in-solution digestion did not differ significantly, however, microchip IMER digestion takes < 10 s. Human serum was also digested efficiently with the IMER. The 4 microchip IMER digested proteins were identified with LC-MS/MS, sequence coverage values were sufficient (29–50%). Trypsin autolysis peptides were not identified.

The microchip IMER is a high throughput device enabling the simultaneous digestion of 9 samples. Since, only 1 μL sample is needed for LC-MS/MS analysis (which is diluted to the final 10 μL), using this technique 1 min can be enough for the digestion of 9 samples (flow rate: 1 μL/min). The microchip IMER is made of PDMS, a low-cost

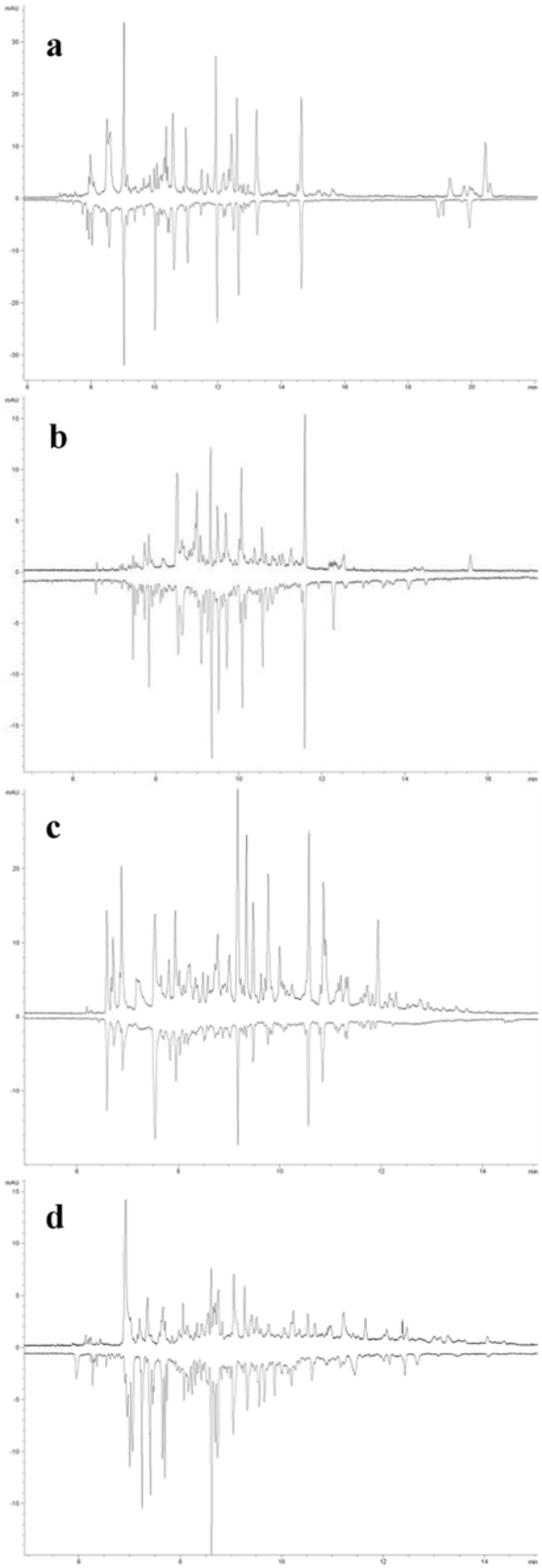


Fig. 5. Digestion of four proteins: (a) myoglobin, (b) lysozyme, (c) hemoglobin and (d) BSA. Upper electropherograms are peptide maps obtained from standard in-solution digestions of these proteins (reaction time: 16 h), while mirrored electropherograms are from microchip IMER digestions (reaction time < 10 s). Conditions are the same as in Fig. 3.

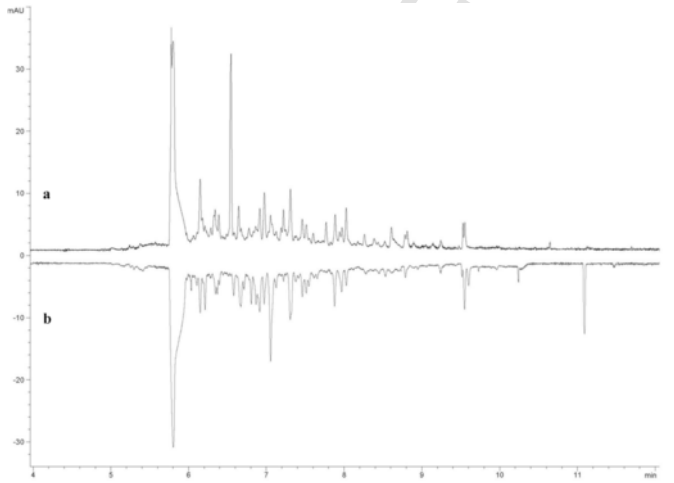


Fig. 6. Electropherograms of digested human serum using (a) standard in-solution digestion and (b) microchip IMER digestion. Injected sample: 200 mbar s, further conditions are the same as in Fig. 3.

Table 1
LC-MS/MS peptide mapping analysis of four proteins from microchip IMER digestion. Individual peptide sequences are available in Supplementary material.

Protein name	Nominal mass (Da)	Sequence coverage (%)	Protein score
Lysozyme	16,228	50	370
Myoglobin	17,072	50	372
Hemoglobin subunit α	15,130	31	197
Hemoglobin subunit β	15,944	40	379
Bovine serum albumin	69,248	29	853

polymer, and it can be used for at least 2 months for multiple protein digestion. Although many immobilized trypsin reactors have been described earlier, the proposed microchip IMER can be an attractive alternative to those and the commercially available trypsin beads due to its high activity, high-throughput property, reusability, cost-efficiency and to its simple preparation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.01.060.

Table 2
Comparison of the main parameters of those microfluidic chip IMERs, that incorporate beads/particles, as solid supports for covalent enzyme immobilization. Sequence coverages in brackets are for standard in-solution digestions. (Protein abbreviations: TPCK-trypsin: L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin, BSA: bovine serum albumin, CYTC: cytochrome C, MELT: melittin, CASB: casein B, MB: myoglobin, UB: ubiquitin, mAbs: monoclonal antibodies, rhGH: recombinant human growth hormone, OVA: ovalbumin, BLG: β-lactoglobulin, HIgG: human immunoglobulin, LYZ: lysozyme, HB: hemoglobin).

Immobilized enzyme	Reactor type	Type of solid support	Digestion time	Digested proteins	Sequence coverage (%)	Additional information	Ref.
TPCK-trypsin	Glass microchip	Agarose bead	3–6 min 5 s	BSA		40–60 μm particle	[52]
				CYT C	71		
				MELT	92		
TPCK-trypsin	Glass microchip	Agarose bead	12 min	BSA			[53]
				CYT C		–	
				CASB			
Pepsin	PMMA microchip	Agarose bead	4–15 s	BSA	34–88		[54]
				MB	99	–	
				UB	64		
PNGase F	Polyimide microchip (commercial)	Silica particles	–	mAbs	–	5 μm silica particles glycoprotein analysis	[28]
TPCK-trypsin	Glass microchip	Magnetic bead	5 min	CYT C	77 (76)	300 nm particle, realistic sample: rat liver extract	[38]
Proteinase K	PDMS microchip	magnetic bead	3 min	prion proteins	–	500 nm particle	[39]
Trypsin TPCK-trypsin	PDMS microchip	Magnetic bead	10 min	rhGH	44 (44)	626 nm particle, native trypsin - higher activity	[40]
TPCK-trypsin	Glass microchip	Magnetic bead	10 s	BSA	43	50 nm particle, 86 μg trypsin/ mg particle, realistic sample: rat liver extract	[41]
				CYT C	83		
				MB	79		
Trypsin TPCK-trypsin	PDMS microchip	Magnetic bead	22.5 min	BSA		0.29–10 μm particle	[43]
				OVA			
				BLG	37		
				HIgG			
Trypsin	PDMS microchip	Silica particles	10 s	BSA	29	5 μm silica particles	Present work
				MB	50		
				LYZ	50		
				HB	31–40		

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