Integration of enzyme reactors and chromatographic/electrophoretic separation units into microfluidic chips (K-127931)

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Further improvements on the microfluidic laboratory

The microfluidic laboratory at our university was established by our research group in 2010. The first instruments and tools (inverted microscope, peristaltic pump, spincoater, special UV lamp, programmable high voltage power supplies) were used ones or purchased with the support of a previous OTKA grants. Thanks to the current OTKA grant several other smaller instruments/tools and materials could be bought (corona plasma treater for PDMS bonding). A GINOP project made possible to purchase a modern, high-resolution ESI Q-TOF-MS (Bruker), which was applied with microfluidic systems (enzymatic reactor, capillary electrohoresis). The new CE-MS instrumentation triggered the more intensive research on both bottom-up and top-down MS based proteomics, where enzyme reactors and microfluidic separation units were integrated.

1. Designing and microfabrication of microchips, new patterns for enzyme reactors

The channel patterns were designated using the program Autocad, and then the chips were created by soft lithography [1]. We investigated increasing the surface-to-volume ratio in microchips, stretching the limits of soft lithography and studied the open channel geometries in a layer-bed type immobilized enzyme reactor with computer aided simulations. The main properties of these reactors are their simple channel pattern, simple immobilization procedure, regenerability, and disposability; all these features make these devices one of the simplest yet efficient enzymatic microreactors. The high surface-to-volume ratio of the reactor was achieved using narrow (25-75 µm wide) channels. The simulation demonstrated that curves support the mixing of solutions in the channel even in strong laminar flow conditions, thus it is worth including several curves in the channel system [2].

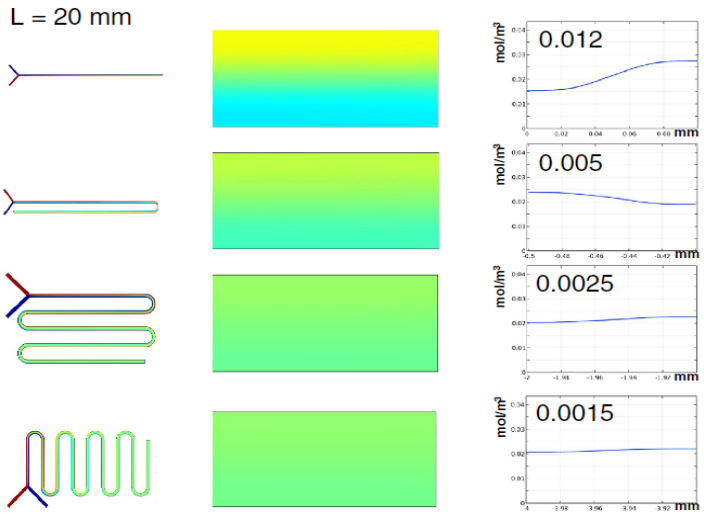

Fig. 1. COMSOL simulations on the effect of incorporating several curves in the microchannel. The concentration distributions at the end of the channel for four different cases are shown: straight channel and channel including one, four, and eight curves. Plots on the right indicate the concentration distribution along the cross section at positions shown on the left (L = 0.1 mm and L = 20 mm). Water and albumin were introduced at the inlet at 1:1 ratio. Four channel segments were magnified for better visibility. Values at the bottom mark the distance from the entry point. (L, 2 cm; $D_{albumin}$ = 6.1·10–11 m^2/s; v, 3 mm/s) [2]

2. Developing and study of microfluidic enzyme reactors

In the three developed designs of microreactor the lengths of the channels were identical, but in two reactors the liquid flow was split to 8 or 32 parallel streams at the inlet of the reactor. Split-flow structures are advantageous because of the increased contact time, despite the overall higher volumetric flow rate. The recommended range of the channel width was found to be 25-75 µm. The simulation clearly showed that curves support (slightly) the mixing of solutions in the channel even in the strong laminar flow conditions (Re <1), thus it is worth including several curves in the channel system [2].

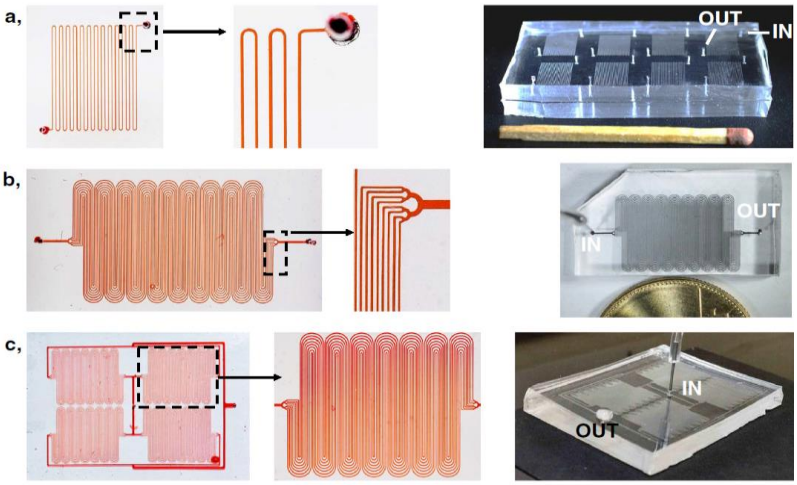

Fig. 2. Images of three different microfluidic chips (on the right). The channel system of each microchip was filled with red food dye; certain sections were magnified for better visibility. Channel parameters: w, 25 µm; L, 20 cm; number of channels, 1, 8, and 32 for design A (a), B (b), and C (c), respectively [2].

Increased specific surface area was also achieved by accommodating pillars in the channels. By optimizing certain parameters of the soft lithographic technique, we found that pillars with a 30 µm diameter can be successfully fabricated. After a thorough optimization of the lithographic technology applicable in a common laboratory we defined the smallest possible pillar diameter that can easily be created in a polydimethylsiloxane (PDMS) channel. It was found that around 25 µm or larger diameter of pillar and interpillar distance can be created in a 25 µm high channel in a reproducible way. Considering the surface-to-volume ratio and the total surface that can be achieved in the channels filled with pillars, we concluded that pillar arrays including dimensions of 40 µm x 40 µm as the diameter of pillars and interpillar distance can be sufficient to pattern the solid support for an immobilized enzyme reactor (IMER) (Fig. 3). Besides the theoretical considerations and calculations, microscopic observation of the flow pattern in the microchips and COMSOL flow simulations have been performed and evaluated [3].

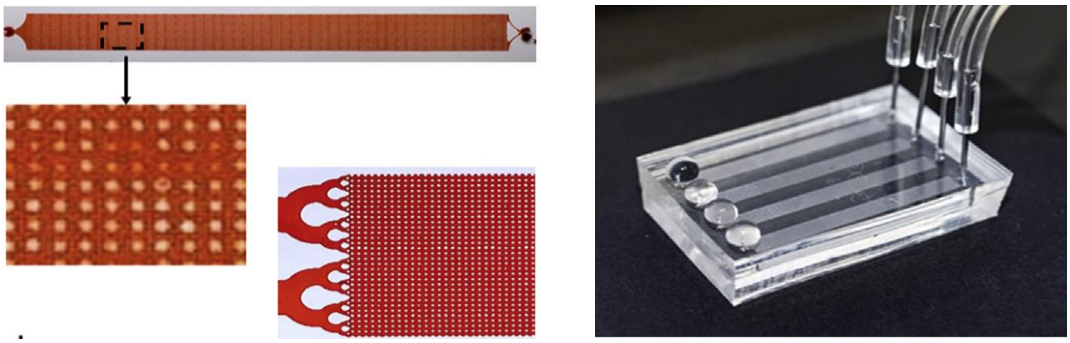

Fig. 3. Photos of PDMS microchip reactors (2.2 mm wide and 25 mm high) including an array of 40 µm sized pillars and 40 µm interpillar distance. The lengths of the channel filled with pillars were 22 mm. The photos show microchip reactors during digestions. A total volume of ~10 µL droplet was collected at the outlet of the channel and used for CE-MS analysis [3].

3. Coupling the immobilized enzyme reactor with capillary electrophoresis

An immobilized enzyme reactor was developed by adsorbing trypsin onto the inner surface of a fused silica capillary of 50 µm ID in a short section. Enzyme immobilization was possible due to the electrostatic attraction between the oppositely charged fused silica capillary surface and trypsin. The reactor was formed by simply injecting and removing trypsin solution from the capillary inlet (~1–2 cms). We investigated the factors affecting the efficiency of the reactor. The main advantages of the proposed method are the fast, cheap, and easy formation of an IMER with in-line protein digestion capability. The CE capillary provided an excellent support due to its high S/V ratio to create an in-line type of microreactor. The in-line nature of the microreactor circumvents the manual handling of samples between each processing step, which often leads to the disruption of sample integrity or sample loss. The developed microreactor is also unique in the sense that in addition to the digestion and subsequent separation steps being performed in a single capillary, the immobilization step can also be carried out without manual manipulation. Thus, immobilization, digestion, and separation are performed in a fully automated manner, providing one of the simplest in-line proteomic workflows. Furthermore, the platform enables us to conduct remarkably economical analyses, since due to the small dimensions of the IMER, only ~nL volumes of trypsin and protein samples are required. We investigated the factors affecting the efficiency of the reactor. The applicability and main advantages of the proposed method were also studied. Human tear samples were used to test the efficiency of the digestion in the microreactor [4].

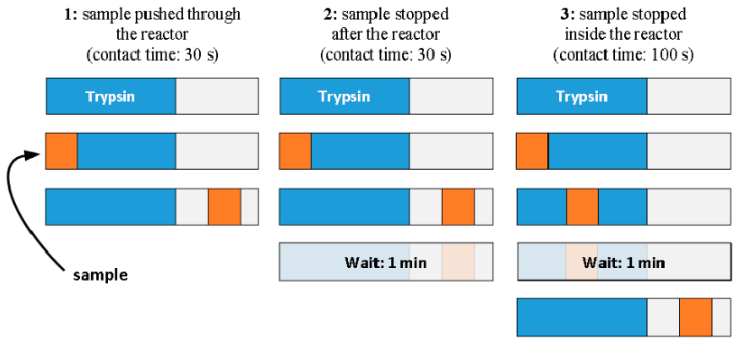

Fig. 4. Illustrations and descriptions of the enzyme immobilization experiments 1–3. Measurement parameters: fused silica capillary, ID = 50 µm, 34 cm length (Leff = 26 cm); BGE: 25 mM NH_4Ac pH = 7; trypsin solution: voltage: 25 kV; UV detection: 230 nm; sample injection: 50 mbar·2 s [4].

4. Coupling the immobilized enzyme reactor with MS detector

The CE capillary provided not only an excellent support due to its high S/V ratio to provide an in-line type of microreactor (IMER), but it is a high performance separation unit (CZE) as well. Additionally, the CE capillary can be used to couple the IMER and CZE separation to the ESI-MS through a triaxial interface including sheath liquid (Agilent).

Recently a microfluidic on-line interface between a thin layer chromatographic (TLC) plate and the ESI-MS is being developed. The PDMS microchip (without a cover plate) is directly placed on to the pattern of the separated bands of the TLC. In the channels at the bottom of the PDMS chip a proper solvent in pumped along the separated bands of the TLC. The eluted analytes are transported to the ESI-MS through the triaxial interface with the assistance of sheath liquid. This microfluidic system might be an efficient coupling between TLC and MS. However, different enzymatic processes which are commonly applied in TLC detection can be considered like a microfluidic enzyme reactor integrated with chromatographic separation tool.

5. Application of microfluidic IMERs

The developed immobilized trypsin microreactors are capable of rapid and efficient protein digestion [5]. The efficiency of the developed enzyme reactor was studied by digesting tear, albumin (HSA) and venom samples. No residues of undigested proteins were detected. In the case of albumin, the numbers of peaks in both electropherograms were around the theoretically expected value (79 peptides). The similarity between the peptide maps of HSA obtained with the two different digestion techniques confirms that the developed enzyme reactor can be effectively used for peptide mapping.

To further verify the digestion efficiency of the IMER, we performed CE-MS/MS measurements of in-solution and on-chip digested HSA and venom samples. The analyzed samples were not desalinated. The parameters of the protein identification (sequence coverage (SC%), number of the unique peptides) were very similar in both methods, the differences between the SC% values were typically less than 10%. As for the HSA standard, the sequence coverage values were higher than 80%. For the venom more than 50 proteins were identified, but only 10 proteins with at least two unique peptides are shown. SC% values are lower in the case of the venom samples, which can partly be attributed to the fact that these samples include a complex mixture of proteins. Nevertheless, values obtained for in-solution and on-chip methods are in good agreement [2].

6. Applications of CE-MS for proteomics

Although, in general, the application of coated capillaries is recommended for the separation of intact proteins, bare silica capillary is still the most often used capillary due to its simplicity and cheapness. In this work, the performance of bare fused silica capillary for intact protein analysis was compared to that of different (dynamically coated polybrene (PB) and permanently coated linear polyacrylamide (LPA)) coated capillaries using capillary zone electrophoresis - mass spectrometry (CZE-MS). In cases where low pH (pH = 1.8) was used in bare silica capillaries, good precision (0.56-0.78 RSD% and 1.7-6.5 RSD% for migration times and peak areas, respectively), minimal adsorption and separation efficiency (N = 27 000/m - 322 000/m) similar to or even better than those obtained with the coated capillaries (created by an intricate multi-step process) was achieved [6].

The applicability of capillary zone electrophoresis (CZE) for the separation of the deamidated forms of insulin has been studied. 50 mM NH 4 Ac (pH = 9) with 20 % v/v isopropylalcohol was found optimal for efficient separation of insulin from its even 10 deamidated forms. The developed method was efficiently applied for monitoring the degradation rate of insulin and the formation of different deamidation isoforms. Two months after the acidification more than thirty peaks can be observed in the electropherogram, because degradation products other than deamidated components were formed as well. The recorded mass spectra enabled us to assign the exact mass of the components, and thus the identification of insulin isoforms could be accomplished [7].

Human insulin and its 6 analogues were separated and determined using CZE-MS. Three different capillaries (bare fused silica, successive multiple ionic-polymer layer (SMIL) and static linear polyacrylamide (LPA) coated) were compared based on their separation performances in their optimal operating conditions. Coated capillaries demonstrated slightly better separation of the components, al- though some components showed wide, distorted peaks. The highest plate number could be obtained in the SMIL capillary (192 000/m). For UV and ESI-MS detection relatively similar LOD values were obtained (0.3–1.2 mg/L and 1.0–3.4 mg/L, respectively). The application of MS detection provided useful structural information and unambiguous identification for insulins having similar or the same molecular mass [8].

Other achievements:

21 papers (5 D1, 8 Q1 (excluding D1)) are published.
2 review papers invited by the editors [5, 9] were published.
26 lectures/posters were presented.

PhD theses in the project topic:

1. Cynthia Nagy: Development of multichannel microfluidic systems for chromatographic applications, expected to defence in first half of 2023.
2. Narmin Hamidli: Analysis of intact proteins using capillary electrophoresis – mass spectrometry, expected to defence in first half of 2023.
3. Lilla Cserepes: expected to defence in 2025.

BSc thesis in the project topic:

1. Szabó Ruben: Development of protein digesting enzymatic reactors in microfluidic chips, 2018

References

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