

Theses of Doctoral (PhD) Dissertation

**Development of protein digesting enzymatic
reactors in microfluidic chips**

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Abbreviations

AFM	atomic force microscopy
BSA	bovine serum albumin
CZE	capillary zone electrophoresis
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
FA	formic acid
FT-IR	Fourier-transform infrared spectroscopy
IMER	immobilized enzymatic reactor
LBL	layer-by-layer (adsorption)
LC	liquid chromatography
MS	mass spectrometry
NHS	N-hydroxysuccinimide
PDMS	poly(dimethylsiloxane)
PVDF	poly(vinylidene-fluoride)
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance

Introduction and aims

In the past 20 years, proteomics became a dynamically developing scientific area, with grandiose aims such as deciphering the proteome – the total protein content – of living organisms with respect to the structure, activity and posttranslational modifications of each protein. One of the most important methods used in proteomic studies is peptide mapping which is based on the proteolytic digestion of the examined protein sample and further analysis of the peptides. The result obtained by this analysis – which is an electropherogram, chromatogram or mass spectrum – characterizes the sample protein as a fingerprint, thusly enabling its identification.

Immobilized enzymatic reactors (IMERs) can be obtained when enzymes are bonded to a surface. Such reactors have the advantages of *i*, increased enzyme stability, meaning that the enzyme can be used multiple times during its longer lifetime and *ii*, the enzyme can be separated from the product of the catalyzed reaction with ease. During protein digestion, another advantage is that the proteolytic enzyme (e.g. trypsin) is unable of self-digestion, for which reason applying trypsin in high concentration in free form is not recommended (during in solution digestion). Digestion can be accelerated significantly if trypsin is applied in higher (interfacial) concentration, in immobilized form. Many other examples apart from the previously mentioned can be found in the literature on the analytical and synthetic purposes of IMERs.

The main goal of microfluidic research is the miniaturization of laboratory equipment (*lab-on-a-chip*) and the development and application of these devices. The fabrication of these chips from poly(dimethylsiloxane) (PDMS) can be carried out simply by using soft lithographic method. Several laboratory operations can be performed simultaneously or consecutively with PDMS chips. Their application is advisable if the sample of interest has limited volume (<1 μ L), for example during the analysis of some clinical samples.

The aim of my study was to develop and apply IMERs containing immobilized trypsin that can accelerate enzymatic protein digestion by several

orders of magnitude, which would greatly reduce the time needed for protein analysis. It is important to integrate these IMERs into microfluidic chips, so that they might be applied for the digestion of samples with low volume. With respect to these demands, two IMERs based on different immobilization strategies were developed: a serpentine-like IMER based on spontaneous adsorption on PDMS and a packed bead IMER containing trypsin covalently immobilized on conventional silica particles.

Methods and instruments

Microfluidic chips with different patterns were fabricated by soft lithography to prepare the different IMERs. 8 channels of 100 μm width, 35 μm height and 20 cm length each were integrated to a single chip to create serpentine-like IMERs with adsorbed trypsin and 9 channels of 1 mm width, 35 μm height and 1 cm length each were integrated to another chip to create packed bead IMERs with covalently immobilized trypsin. Liquids were transported through the IMERs with a peristaltic pump (IPC, Ismatec, Cole-Palmer, IL, USA). Tubings connected to the pump were of 0.25 mm ID (Tygon, Cole-Palmer, IL, USA).

The efficiency of the IMERs were tested by digesting different protein samples. Samples were pretreated prior to the digestion: 8 M urea was added to unfold the protein structure, 100 mM dithiothreitol and 200 mM iodoacetamide were added to reduce and then alkylate disulfide bridges. This mixture was further diluted with 25 mM NH_4HCO_3 to reduce the concentration of urea to a final concentration of <1 M (not to unfold the structure of trypsin). This buffer also provided optimal pH (7.8) for the digestion. Collection of tear samples were carried out by standard capillary technique. When in solution digestion was applied, trypsin was added in a ratio of 50:1 (m:m) protein:trypsin and the samples were incubated at 37 °C for 16 h. For the microfluidic IMER digestion, pretreated samples were transported through the reactors. This took approx. 1 min for the serpentine-like IMER and approx. 10 s for the packed bead IMER. FA was added

at a final concentration of 0.1% to terminate digestion. Pipette tips with C18 stationary phase were used for sample desalination (ZipTip[®] C18, Merck Millipore Ltd., Tullagreen, Cork, Ireland).

Trypsin was spontaneously adsorbed on the inner wall of the microfluidic channels in the serpentine-like IMER. 20 mg/mL trypsin solutions containing 20 mM CaCl₂ and 1 mM HCl to inhibit tryptic activity were freshly prepared before each immobilization. This solution was transported with 2 μ L/min flow rate for 10 min through the channel, after which free trypsin was eliminated by flushing the channel with 25 mM NH₄HCO₃ (which also provided optimal pH for digestion). Samples were transported with 0.65 μ L/min flow rate through the IMER at room temperature for digestion. Adsorbed trypsin layer can be regenerated if the previously adsorbed layer was removed (by flushing the channel with 50 mM sodium dodecyl sulfate (SDS)) and a new layer was adsorbed.

Covalent trypsin immobilization was carried out by standard EDC/NHS method to amino functionalized silica particles. During this 5-hour reaction, carboxylic groups of trypsin were activated by the carbodiimide, that thusly formed amide bonds with the amino groups located on the silica particles. Free trypsin was eliminated from the suspension of the particles by thoroughly rinsing, centrifuging and decanting several times. Trypsin coated particles were stored in a solution containing 1 mM HCl, 20 mM CaCl₂ and 0.4 mg/mL benzamidine. To create the IMERs, packings were formed from these particles by retaining them at the end of the channels with the use of bottlenecks. The packings were compressed with 25 mM NH₄HCO₃, which also provided optimal pH for digestion. Digestion occurred at room temperature by transporting the samples with 1 μ L/min flow rate through the packing.

The adsorption of trypsin on PDMS was studied by atomic force microscopy (AFM, Veeco diCaliber) and surface plasmon resonance spectroscopy (SPR, Nicolet[™] 6700 FT-IR, SPR[™] 100 module, Thermo Electron Co. Waltham, MA, USA). The gold SPR slides were spincoated with 0.1% (m/V) PDMS in

hexane, thus, a very thin (~100 nm thick) PDMS layer was obtained on the surface of the sensor.

An Agilent 7100 type capillary electrophoresis instrument was used for CZE peptide mapping (Agilent, Waldbronn, Germany). Hydrodynamic sample injection was used (100 mbar·s), samples were introduced at the anodic end of the capillary. Separations were performed in 80 cm long, 50 µm ID fused silica capillary with extended light path (Agilent). Applied voltage was 30 kV. On-capillary DAD detection was used at 200 nm. The applied buffer was 25 mM phosphate, pH=7.0.

New scientific results

1. For the first time, spontaneous adsorption on PDMS surface was used for enzyme immobilization, thus, a simple IMER was developed.

Although IMERs based on enzyme adsorption can be found in the literature, these are usually IMERs containing integrated PVDF membranes for adsorption or LBL adsorption is carried out, when oppositely charged layers are generated alternately, when electrostatic attraction is the driving force of the adsorption.

With the development of this IMER, my goal was to create the simplest enzymatic reactor. For this reason, a PDMS chip with a long, serpentine-like channel was fabricated. Trypsin solution should simply be transported through this channel for immobilization. Although it is wellknown that PDMS is an excellent adsorbent of large macromolecules, this property was not utilized for the adsorption of enzymes thus far, therefore, this is the first work applying this very simple method for enzyme immobilization. As the solid support for adsorption is the inner wall of the chip itself (Figure 1.), there was no need to integrate dedicated supports (e.g. membrane) into the channel or to form interfacial layers for LBL adsorption.

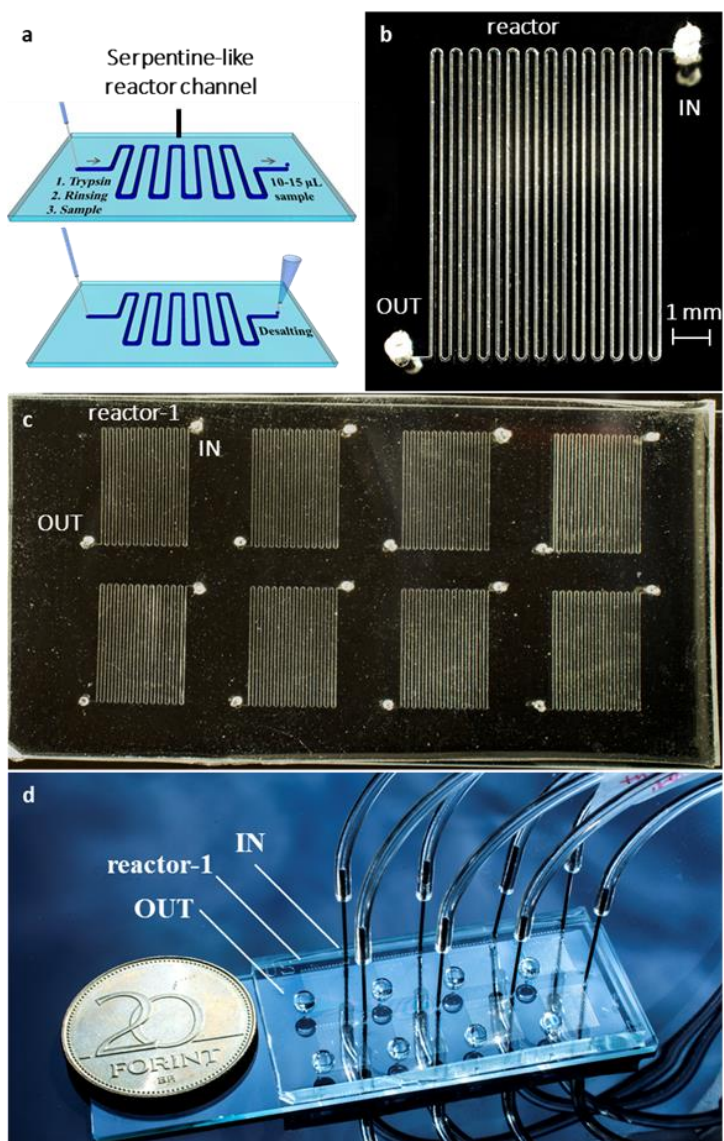


Figure 1.: (a) Schematic workflow of trypsin adsorption in the serpentine-like IMER. Images of (b) the 20 cm long, 100 μm wide and 35 μm high channel pattern, from which (c) even 8 can be integrated into a single chip and can be used for (d) 8 simultaneous digestions.

- 2. It was proved that trypsin adsorption on PDMS is practically irreversible. It was also verified with concentration dependent SPR and CZE enzyme activity measurements that adsorbed trypsin might be present on the PDMS surface in several different states with different activities.**

Adsorption phenomenon was studied by AFM and SPR spectroscopy. Trypsin adsorption on the PDMS was practically irreversible, only detergents and organic solvents could desorb trypsin from the PDMS. Furthermore, the amount of adsorbed trypsin (and the activity of the reactor) depended on the concentration of the trypsin solution; molecules adsorbed more densely when trypsin was applied in higher concentration. CZE activity measurements proved that the enzyme have retained its activity for ~2 h, then the activity decreased gradually. This is likely due to the spreading phenomenon, which is the consequence of strong interaction between PDMS and the hydrophobic side chains of trypsin. Based on these observations, multiple states model was proposed as the adsorption mechanism of trypsin onto PDMS surface.

- 3. It was proved that with the serpentine-like IMER (containing adsorbed trypsin) protein digestion can be performed several orders of magnitude faster compared to in solution digestion.**

The digestion reproducibility and efficiency of the reactor was tested by digesting various protein samples (myoglobin, lysozyme, hemoglobin, BSA, tear – latter 2 to be seen on Figure 2.). Digestion was sufficient in each case, compared to in solution digestion it happened within 1 min instead of 16 h. Migration times of peptides in the BSA digest showed excellent reproducibility (RSD% <1%). Most peptides obtained with standard in solution digestion and IMER digestion were identical in both cases, based on the similarity of CZE peptide maps and LC-MS/MS determinations.

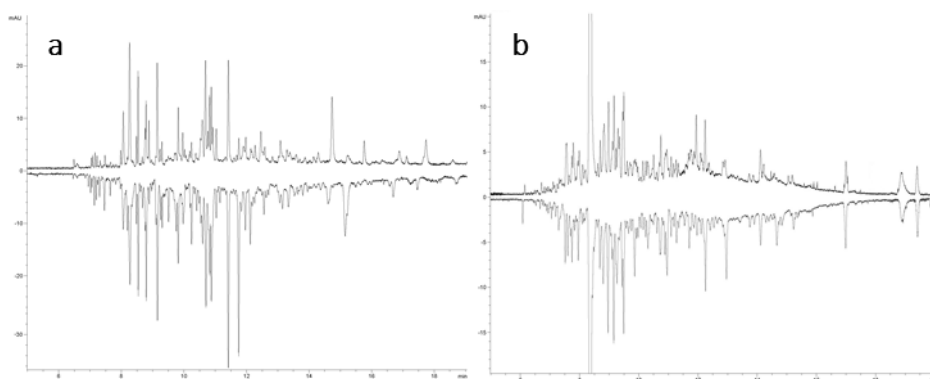


Figure 2.: CZE electropherograms of digested (a) BSA and (b) tear samples after IMER digestions (upper electropherograms) and in solution digestions (mirrored electropherograms). Samples were desalinated in (a).

8 digestions can be simultaneously performed with a chip, which might be further improved by integrating more channels to the same chip. As $\sim 10 \mu\text{L}$ sample was necessary for CZE analysis, the whole digestion procedure took ~ 35 min: trypsin immobilization (10 min), elimination of free trypsin (10 min) and digestion (15 min). The only disadvantage of the IMER is that it loses its activity shortly after immobilization (~ 2 h), however, it can be utilized well as a disposable device. Hence cross contamination between samples can be eliminated.

4. An IMER containing trypsin covalently attached to conventional silica particles was developed. It was certified that the IMER retained its activity for at least 2 months.

A different type of IMER possessing excellent stability was developed by covalent immobilization (Figure 3.). The shelf life of the IMER was tested after 2 months of storage. Activity was retained and was not affected by the circumstances of storage (storing at 4°C as a packing in the chip or at -20°C as a suspension of the trypsin coated particles).

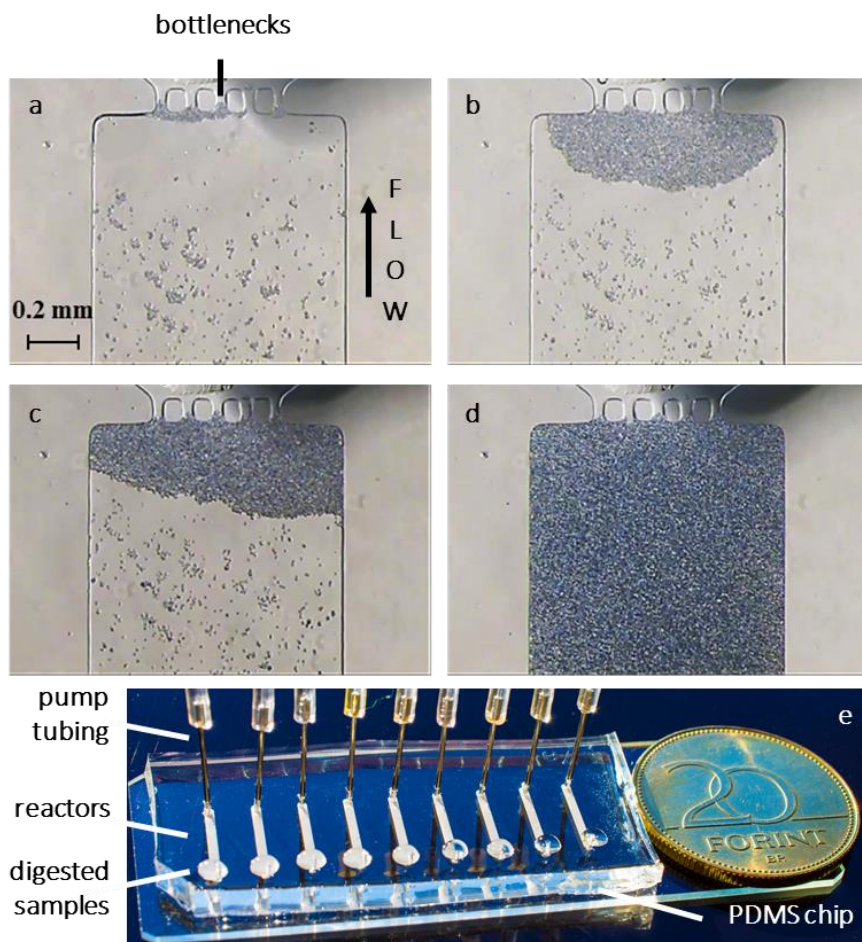


Figure 3.: (a-d) Optical microscope images of a packing formed from trypsin coated particles. (e) Image of 9 simultaneous protein sample digestions with the IMER.

5. **To completely saturate the surface of the particles with trypsin, the optimal trypsin/silica particle ratio was determined in the immobilization mixture.**

The concentrations of the reagents used in the covalent coupling reaction were based on data from literature (EDC/NHS method is widely used), however, the ratio of trypsin/particle in the initial immobilization mixture was optimized and this optimal value was applied in my experiments. Under these circumstances 10-13 μg trypsin was immobilized per 1 mg particle (Figure 4.), which is comparable with other enzyme surface concentrations determined in different IMER-related

works. During the determination of the trypsin surface concentration, mobilization of trypsin covalently bonded by an amide bond was achieved by basic hydrolysis of the particle surface in connection with the enzyme.

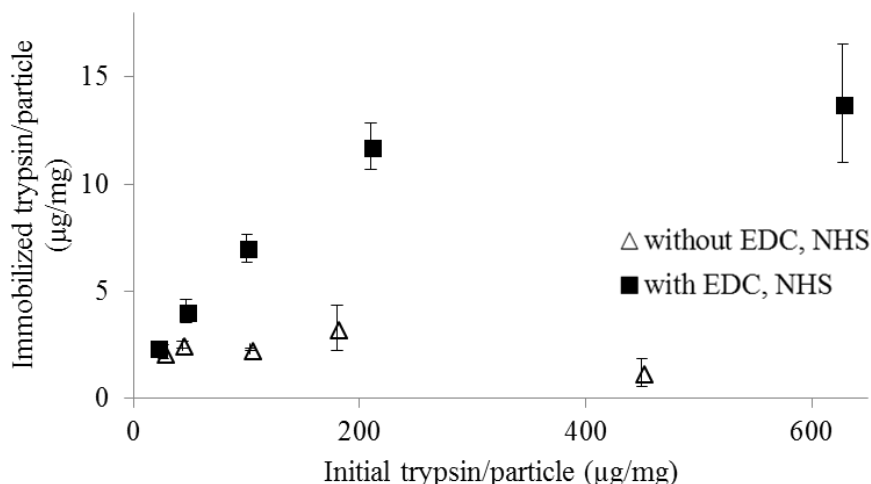


Figure 4.: Dependence of the saturation of covalently immobilized trypsin on the initial ratio of trypsin/particle in the immobilization mixture (black squares) and control measurement without EDC and NHS reagents (white triangles).

6. It was proved that with the developed packed bead IMER, proteins were digested with good efficiency and reproducibility, even within 10 s.

The effects of CZE analyses, digestion, packing formation and immobilization on the CZE peptide maps were investigated, in each case sufficient peptide migration time reproducibilities were acquired. No significant difference was observed between in solution digestions and digestions with the IMER in the case of several protein samples (myoglobin, lysozyme, hemoglobin, BSA, human serum – first 4 to be seen on Figure 5.). Digestion time was only approx. 10 s, while in solution digestion took 16 h.

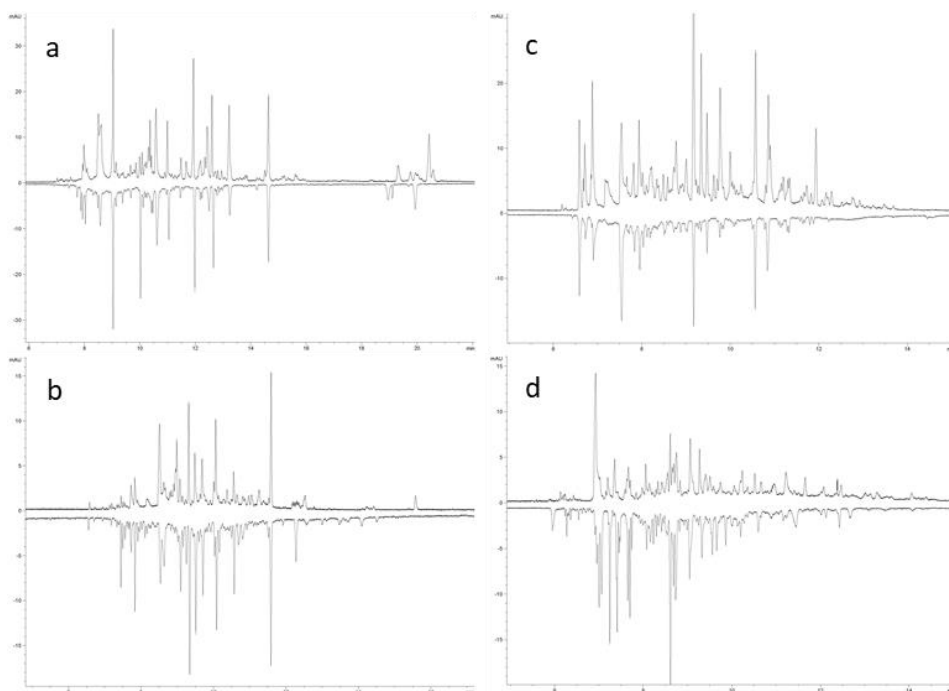


Figure 5.: CZE electropherograms of four proteins: (a) myoglobin, (b) lysozyme, (c) hemoglobin and (d) BSA. Upper electropherograms correspond to in solution digested samples and mirrored electropherograms to IMER digested samples. Samples were desalinated.

The novelty of the packed bead IMER is in its design, as only very few examples can be found in literature when packings are created in PDMS chips. Even 9 samples can be simultaneously digested with this IMER, it retains activity for at least 2 months, under which it can be used multiple times. This long shelf life compensates the long immobilization reaction (5 h), as it is even possible to create “fresh” packings for every sample if a larger amount of particles has been coated in the immobilization reaction, as a single packing only requires a small amount of particle (<2 mg).

Possible utilization of the results

Proteomics is a dynamically developing scientific area, the importance of protein analysis will likely increase in the near future due to the spreading of protein-based medications. As the most fundamental methods of proteomics – e.g. peptide mapping, shotgun proteomics – are based on protein digestion, it is important and advantageous to develop devices that can significantly reduce the time necessary for this analysis step. As the developed IMERs are capable of this, their usage in proteomic-related studies is advantageous. The time required for in solution digestion is 16 h, but with the help of the IMERs it can be reduced to 1 min or 10 s. The packed bead IMER retained its activity for at least 2 months and it can be used multiple times within this period. As an alternative, both IMERs can be used as disposable devices, as their fabrication is of low cost and cross contamination can thusly be eliminated.

The development of these reactors might set an example to create such IMERs of different enzymes. Thus, it would be possible to successively integrate multiple IMERs with various enzymatic activities into a chip. This might enable the deglycosylation of glycopeptides after enzymatic (glyco)protein digestion. Both chip-based IMERs have the advantages that they are relatively easy to couple with mass spectrometry and additional operations might be implemented into the same chip (e.g. desalination, preconcentration, separation of peptides), which enables the fabrication of a complex protein digestion, peptide separation and detection lab-on-a-chip.

Publications

List of publications related to the dissertation:

1. A. Gaspar, A. Kecskemeti, F.A. Gomez
Use of surface plasmon resonance to study the adsorption of detergents on poly(dimethylsiloxane) surfaces
Electrophoresis. 34 (2013) 1249-1252.
IF (2013): 3,161
Quartile 1 in Analytical Chemistry
2. A. Kecskemeti, A. Gaspar
Preparation and characterization of a packed bead immobilized trypsin reactor integrated into a PDMS microfluidic chip for rapid protein digestion
Talanta. 166 (2017) 275-283.
IF (2017): 4,162
Quartile 1 in Chemistry (miscellaneous)
3. A. Kecskemeti, J. Bako, I. Csarnovics, E. Csosz, A. Gaspar
Development of an enzymatic reactor applying spontaneously adsorbed trypsin on the surface of a PDMS microfluidic device
Anal. Bioanal. Chem. 409 (2017) 3573-3585.
IF (2017): 3,431
Quartile 1 in Analytical Chemistry
4. A. Kecskemeti, C. Nagy, E. Csosz, G. Kallo, A. Gaspar
The application of a microfluidic reactor including spontaneously adsorbed trypsin for rapid protein digestion of human tear samples
Proteom. Clin. Appl. 11 (2017) 1700055.
IF (2017): 3,814
Quartile 2 in Clinical Biochemistry
5. A. Kecskemeti, A. Gaspar
Particle-based immobilized enzymatic reactors in microfluidic chips
Talanta. 180 (2018) 211-228.
IF (2017): 4,162
Quartile 1 in Chemistry (miscellaneous)

List of other publications:

6. A. Kecskemeti, A. Gaspar
Particle-based liquid chromatographic separations in microfluidic devices
Analytica Chimica Acta. 1021 (2018) 1-19.
IF (2017): 4,950
Quartile 1 in Analytical Chemistry



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PhD Publikációs Lista

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List of publications related to the dissertation

Foreign language scientific articles in international journals (5)

1. **Kecskeméti, Á.**, Gáspár, A.: Particle-based immobilized enzymatic reactors in microfluidic chips.
Talanta. 180, 211-228, 2018. ISSN: 0039-9140.
DOI: <http://dx.doi.org/10.1016/j.talanta.2017.12.043>
IF: 4.162 (2016)
2. **Kecskeméti, Á.**, Bakó, J., Csarnovics, I., Csősz, É., Gáspár, A.: Development of an enzymatic reactor applying spontaneously adsorbed trypsin on the surface of a PDMS microfluidic device.
Anal. Bioanal. Chem. 409 (14), 3573-3585, 2017. ISSN: 1618-2642.
DOI: <http://dx.doi.org/10.1007/s00216-017-0295-9>
IF: 3.431 (2016)
3. **Kecskeméti, Á.**, Gáspár, A.: Preparation and characterization of a packed bead immobilized trypsin reactor integrated into a PDMS microfluidic chip for rapid protein digestion.
Talanta. 166, 275-283, 2017. ISSN: 0039-9140.
DOI: <http://dx.doi.org/10.1016/j.talanta.2017.01.060>
IF: 4.162 (2016)
4. **Kecskeméti, Á.**, Nagy, C., Csősz, É., Kalló, G., Gáspár, A.: The application of a microfluidic reactor including spontaneously adsorbed trypsin for rapid protein digestion of human tear samples.
Proteom. Clin. Appl. 11 (11-12), 1-9, 2017. ISSN: 1862-8346.
DOI: <http://dx.doi.org/10.1002/prca.201700055>
IF: 3.814 (2016)
5. Gáspár, A., **Kecskeméti, Á.**, Gomez, F. A.: Use of surface plasmon resonance to study the adsorption of detergents on poly(dimethylsiloxane) surfaces.
Electrophoresis. 34 (8), 1249-1252, 2013. ISSN: 0173-0835.
DOI: <http://dx.doi.org/10.1002/elps.201200573>
IF: 3.161





List of other publications

Foreign language scientific articles in international journals (1)

6. **Kecskeméti, Á., Gáspár, A.:** Particle-based liquid chromatographic separations in microfluidic devices.

Anal. Chim. Acta. 1021, 1-19, 2018. ISSN: 0003-2670.

DOI: <http://dx.doi.org/10.1016/j.aca.2018.01.064>

IF: 4.95 (2016)

Total IF of journals (all publications): 23,68

Total IF of journals (publications related to the dissertation): 18,73

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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