UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

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Bioanalytical Techniques Using Microfluidics and Nanotechnology

Theses of the Doctoral Dissertation

2019

Bibliographic entry

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Title:	**Bioanalytical Techniques Using Microfluidics and Nanotechnology**
Study Program:	**P1419 Analytical Chemistry**
Field of Study:	**Analytical Chemistry**
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Year of the Defense:	**2019**

Reference

NOVOTNÝ, Jakub. *Bioanalytical Techniques Using Microfluidics and Nanotechnology.* Brno, 2019. 176 pages. Doctoral Thesis. University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry. Supervisor: prof. RNDr. Zuzana Bílková, Ph.D.; Supervisor-Specialist: Ing. František Foret, DSc.

Abstract

The science of fluid manipulation in submillimeter dimensions is called microfluidics. At the microscale, fluids exhibit rather unintuitive behavior. The influence of inertia loses importance and seemingly chaotic turbulent flow shifts into a laminar flow. Microfluidic devices embrace and exploit these characteristics. This doctoral dissertation deals with the development and fabrication of such devices. Attention in the production of microfluidic devices nowadays shifted from materials like glass and silicon, which are expensive and rather hard to process, to thermoplastics which can be melted easily and used for techniques such as molding, to elastomers – most commonly polydimethylsiloxane (PDMS), or to resins. The works compiled in this thesis show several microfluidic devices and related products developed utilizing a variety of materials and fabrication methods – from photolithography to 3D printing.

Abstrakt

Věda o manipulaci s kapalinami v rozměrech menších než milimetr se nazývá mikrofluidika. Na mikroúrovni kapaliny vykazují kontraintuitivní chování. Vliv setrvačnosti klesá na významu, čímž dochází k přechodu zdánlivě chaotického turbulentního proudění na proudění laminární. V mikrofluidických zařízeních je s těmito charakteristikami počítáno a jsou naopak využívány. Tato disertační práce se zabývá vývojem a výrobou takových zařízení. V současnosti se u produkce mikrofluidických zařízení pozornost přesouvá od materiálů jako je sklo a křemík, které jsou poměrně drahé a nesnadno opracovatelné, k termoplastům, které mohou být snadno roztaveny a užity v technikách jako je odlévání, dále k elastomerům, nejčastěji polydimethylsiloxanu (PDMS), nebo k živicím. Publikace shromážděné v této práci ukazují řadu mikrofluidických zařízení a souvisejících výrobků vyvinutých s využitím rozmanitých materiálů a výrobních metod – od fotolitografie po 3D tisk.

Keywords

Microfluidics, micro-fabrication, thiol-enes, glycoproteins, nano-platelets

Klíčová slova

Mikrofluidika, mikrofabrikace, thiol-eny, glykoproteiny, nano-destičky

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0 INTRODUCTION

The trend of miniaturization originated in the demand of electrical and computational industries for smaller, cheaper and more efficient devices. Development of new techniques for the fabrication of integrated circuits, transistor chips and especially piezoelectric nozzles for inkjet printers were just a small step from the idea to implement the same fabrication methods for fluidic devices.

The field of microfluidics studies behavior and manipulation of fluids at sub-millimeter dimensions. Just like electrical components, microfluidic devices used to be microfabricated predominantly by the method of photolithography in silicon or much cheaper glass. In the process of photolithography, a pattern is transferred from a template image onto a photosensitive surface. Apart from device fabrication by itself, method nowadays also serves as the first step of other fabrication methods, e. g. preparation of molds or stamps for soft lithography and so on.

Modern devices are made of plastics – cheaper, easily processible alternative to silicon and glass – thermoplastics, elastomers or photo-curable resins. Plastic devices are generally fabricated by molding or casting but modern fabrication methods such as 3D printing are being dabbled into more and more. Philosophy of lab-on-a-chip or micro-total analytical systems (μTAS) using these materials became a significant topic in many fields, especially in chemical and biochemical analysis, in both research and commercial applications.

1 MICROFABRICATION

The assortment of techniques used to build structures on the scale of micrometers and smaller is called microfabrication. The choice of a wide variety of fabrication techniques is dependent on the material and desired characteristics of the final product.

The first microfabrication methods emerged to meet the needs of the electrical industry – the photolithographic techniques for silicon patterning. These were later adopted for microfluidics and the use of glass. Nowadays most widely established microfabrication methods belong to soft lithography and work with plastics and elastomers, most commonly PDMS. Advancements and the widespread of CNC technologies show promise in the utilization of methods like micro-machining and 3D printing as easily accessible microfabrication, although the wide use of these methods in microfluidics is still in the long run.

1.1 Photolithography and Etching

Photolithography is one of the oldest microfabrication techniques and was originally developed for the purposes of electrical engineering. As mentioned before, it involves a transfer of template pattern into a photosensitive material using light [1]. It can be used by itself for the fabrication of microfluidic devices or, more recently, it often serves as the first step of other fabrication methods, e. g. preparation of molds or stamps.

In the most basic setting, silicon or glass serve as substrates. Cleaned and treated substrate is spin-coated by the photoresist. Coated substrates are exposed to light. The classic approach would be the exposure through a photomask, which can be in the form

of a chrome-coated glass slide, photoplotter foil, etc., with the required pattern. Mask shields photoresist from light and the pattern is transferred to the surface. A sophisticated way to prepare very precise contact masks is the utilization of direct-write systems.

Two approaches can be considered for glass and silicon etching. In dry etching, a plasma of halogenated gas removes material from exposed areas of the wafer [2]. Wet etching is a method where the substrate is dissolved by an etching solution. Hydrofluoric acid is the etchant of choice for glass and silicon dioxide [3]. Glass can only be wet-etched isotropically – etching proceeds in all directions at the same rate.

Just like glass, silicon can be etched isotropically as well. Although, since it does not react with hydrofluoric acid, oxidizing agent (most commonly HNO_3) has to be added to oxidize Si to SiO_2. Unlike glass, anisotropic wet-etching is possible for silicon. Anisotropic etchants of silicon are potassium hydroxide (KOH) or tetramethylammonium hydroxide (TMAH) [4,5]. These molecules preferentially attack silicon along one crystallographic plane (plane <100>) leaving characteristically trapezoidal or V-shaped cross-section.

In lift-off lithography, a negative template of the desired pattern is exposed to the layer of photoresist as the first step. Following the developing step, the surface of the substrate is covered by a coating of demanded material. In areas, where photoresist was washed off during the developing step, the coating material is deposited directly on the surface of the substrate. Otherwise, the coating covers the remaining resist. In the next step, the remaining photoresist is stripped off of the substrate along with all the coating which was deposited on top of it and only the desired pattern is left on the substrate [6,7].

Thick layers of some negative tone photoresists, especially SU-8, can also be used for the fabrication of microstructures by themselves [8,9]. It is possible to fabricate structures thicker than 300 μm (depending on the type of SU-8 and spin-coating duration and speed). It is often used in replicative lithographic techniques which will be mentioned in the next chapters.

1.2 Molding

Molding is a fabrication method where semi-liquid or molten material is poured into a negative template model of the product called mold or matrix. As material hardens, it adopts the shape defined by the mold. If the mold is not destroyed during the molding process, it can be used repeatedly for mass production.

Microfluidic devices can be prepared out of thermoplastics. Thermoplastics are polymers that become pliant and molten once they reach a certain temperature [10]. Molten thermoplastic is injected or poured into the mold and left to cool down. Once the cooling material passes the point of glass transition, the plastic solidifies and the product can be retrieved. The method in which is the plastic injected into the mold under pressure is, obviously, called injection molding and is THE go-to method in commercial mass production of any plastic product.

Apart from the thermoplastics, molding is very often applied to elastomers (which is actually the origin of the moniker soft lithography). The preferred elastomer used in

molding is polydimethylsiloxane (PDMS). It is a very flexible elastomer, inert, non-toxic and non-flammable. PDMS is highly permeable for gases, biocompatible and optically clear at a wide range of wavelengths [11,12]. While still fluid, the PDMS pre-polymer is introduced into the mold and left to cure. Usually, the rate of curing is increased by heat, although curing at lab temperature is also.

1.3 Resin Casting

Resin casting is a method in which a mold is filled by liquid polymer or resin, which is then hardened. An interesting example of casting in the fabrication of microfluidic devices is the use of UV-curable materials, which offer a fast production rate at relatively mild conditions compared to the other materials. The uncured mixture of monomers can be cast into molds just like thermoplastics or elastomers. These polymers can also be treated in a similar way as negative photoresists like SU-8 and be processed by the principles of photolithography [13]. The thin layer of photo-definable polymer is exposed to UV light through a mask, transparent patterns are cured and uncured parts shielded by the mask are washed off.

Some of the very interesting photo-definable polymers belong to the group of thiol-enes. Thiol-enes are UV-curable copolymers prepared from a mixture of thiol- and allyl-group containing monomers [14]. Principles of thiol-ene chemistry are used (especially in combination with epoxy resins) in industrial adhesives, sealants or coatings.

Off-stoichiometric thiol-ene (OSTE) chemistry provides easy surface functionalization and biomolecule immobilization for the fabrication of biochemical microreactors. Properties of the material allow for UV-induced reaction of allyl-group in ene-excess OSTEs with thiol-containing molecules [15]. Biomolecule immobilization on thiol groups can be realized on thiol-excess OSTEs [16,17].

1.4 Micro-machining

Method of fabrication of, among other things, microfluidic devices that belong more than anything into the field of handicraft and workshop productions is the machining.

Machining is a process in which a bulk piece of material is shaped into the intended form by mechanical removal. Because the material is in this process removed from a more massive piece, machining belongs among the so-called subtractive manufacturing methods. That is as opposed to the methods like 3D printing in which is the product build ground-up, in so-called additive manufacturing [18,19]. Of the most common techniques of machining, the ones to make the most sense to be utilized in the fabrication of microfluidic devices are drilling and milling.

The type of machining suitable for the fabrication of microfluidic devices is the CNC machining. Computer numerical control machining uses the computer commanded servo- or stepper-motors to control the movement of the cutting tool with great precision.

The effectiveness of the CNC micro-machining for the fabrication of microfluidic devices is strongly dependent on the precision and repeatability of the stepper motors and reliability of the machine controller.

The CNC milling machine used by the author belonged to the lower end of the spectrum of the market, both price- and performance-wise. Precision declared by the producer was 0.05 mm, which means that the tasks performed for the projects presented in this thesis often represent very limits of the capabilities of the machine.

Performance in CNC machining is strongly dependent not only on the specifications of the machine but also on the choice of milling tool and proper optimization of the working parameters for the said tool [20,21].

1.4.1 Micro-machining in Microfluidics

The versatility of the modern CNC machines allows processing substrates in a great range of sizes, on the scale of micrometers to decimeters or even meters, all on the same machine. The variety in shapes, sizes, and materials of the cutting tools widens the selection of materials available for fabrication.

In microfluidic applications, micro-machining is usually used in 2 ways: to produce a mold (for hot embossing or injection molding) [22] or to fabricate the micro-channels and features of the microfluidic device directly [23].

Many of the CAM software on the market feature some sort of tool that enables processing of 3D CAD models streamlining the process of "idea → concept design → prototype". The time required to get from the design to the physical prototype is cut down to minutes or hours. This makes micro-milling the perfect method for early stages of the development during which many iterations of the prototype design have to be frequently produced during the search for the optimal design. The development time is greatly reduced and so is the development cost, because of the relatively low start-up cost for the method [20].

On the other hand, considering the high-volume commercial productions, micro-milling cannot compete with methods such as injection molding in which the cost per piece value decreases significantly with a higher volume of the production. The cost/piece value in micro-milled products remains virtually the same regardless of the volume.

1.5 3D Printing

3D printing is an additive manufacturing method that involves a layer-by-layer building of objects based on their digital 3D model.

Nowadays, several 3D printing methods have been established commercially. The customer can pick based on the intended accuracy and production pace of the machine, used the material, size of the product and, let's be real, the cost of the machine and the operation.

1.5.1 3D Printing Methods

1.5.1.1 Fusion Deposited Modelling (FDM)

This is probably the method most of us imagine when 3D printing is mentioned. In this method, a semi-liquid printing material is fed through an extruder driven in XY or XYZ axes by stepper or servo-motors. The material is then pushed through a nozzle which determines the thickness of the extruded filament and deposited onto a printing

platform quickly solidifying. Of all the commercially available 3D printing methods, FDM is the least expensive.

The most commonly used materials are thermoplastics – polylactic acid (PLA), acrylonitrile butadiene styrene (ABS), polyethylene (high density, HDPE), among others – which are heated in the heating block of the extruder to a temperature above their glass transition temperature and melted [24]. The thermoplastics for FDM are usually distributed in a coil containing 1 kg worth of the filament in 2 standard sizes – 1.75 mm and 3 mm filament thickness.

1.5.1.2 Stereolithography (SLA)

Methacrylate-based photo-resins are the most common printable material used in stereolithography. The method uses selective curing of thin layers of the photopolymer to build the printed object. The objects printed by this method can essentially be viewed as stacks of numerous 2D photolithographic structures similar to the ones fabricated in negative photoresist (such as SU-8) mentioned in Chapter 1.1, layered on top of one other. The method also belongs to the lower end of the cost scale of the 3D printing methods. It is relatively fast, has relatively low material consumption and has relatively high precision influenced by the character of the curing light source and the resin type used.

In the original SLA setting, the printer uses a laser to scan the image of a cross-section of the digital design into a thin layer of photoresist on top of a platform or on the previous layer of the cured resin [25]. After each layer, the stage is moved further away from the light source and a new layer is cured.

Two main configurations for the light source and the resin tank are the "bath configuration" (also known as the free surface configuration) and the "bat configuration" (or the restrained surface configuration) [26].

The digital light projection (DLP) printers use digital micro-mirrors to reflect a stationary laser beam to project a pixelated image of the cross-section all at once [25]. The older and some of the more affordable printers available even for the Average Joe users involve an LCD display instead of the digital mirror. The projection of the whole layer at once significantly reduces the time of printing but on the other hand, the pixelation of the image also seriously diminishes the resolution. Typical for the DLP 3D printers is the bat configuration of the printing stage.

1.5.1.3 Inkjet 3D Printing (i3DP)

An evolution of the standard inkjet printer every one of us has at home. In the most common setting, a piezoelectric print-head deposits droplets of the acrylate-based printing material layer-by-layer that are cured by exposure to a source of UV light [25].

The great advantage of the inkjet 3D printers, inherited from their office-dwelling CMYK ancestors, is the capability of multi-material printing allowing for the deposition of the build- as well as of the sacrificial support-materials.

A specific variety of an inkjet 3D printing is powder-based i3DP, so-called binder jetting. In it, a blade deposits and levels a thin layer of powdered building material while the inkjet print head deposits a water-based adhesive – the binder. While the layer solidifies, the printing stage is lowered and the blade deposits a new layer of powder.

The powders used in binder jetting contain gypsum, silica and polymer particles. The adhesive uses glycerol and water-soluble acrylates [26,27].

1.5.1.4 Laminated Object Manufacturing (LOM)

A relatively low-cost method in which a thin sheet of plastic, paper, metal or ceramic material is cut by a laser (or by a blade, in which case the method is called xurography [28]) into cross-sections of a 3D object which are then laminated by adhesive or chemical bonding. The alignment and stacking of layers are usually automatic [24]. The paper-based 3D printers can also involve the regular 2D color printing, where color inks are deposited around the outline of the cross-section resulting after lamination in a fully colored 3D object. The manufacturer calls this paper-based method the selective deposition lamination (SDL) [29].

1.5.1.5 Bioprinting

Bioprinting involves deposition of living cells, extracellular matrix components, proteins, and other biomaterials in a specific spatial arrangement on a solid, gel or in a liquid substrate. It is not its own printing method, rather a customized implementation of FDM, i3DP or SLA.

The go-to materials for bioprinting are hydrogels, e. g. PEG diacrylate, gelatin methacrylate, hyaluronic acid, or functionalized alginates, which are deposited in the form of room-temperature high viscosity liquids and then physically or chemically solidified. The biocompatibility of the hydrogel is often influenced by the choice of the mixed-in photosensitizer. Hydrogels are deposited by extruders, inkjets, electrosprays, micro-valves from constantly pressurized tanks or as selectively photopolymerized layers [24,25].

1.5.2 3D Printing in Microfluidics

While 3D printing is a method still not widely adopted for microfluidics, the evolution of the field is rapid and particularly the improved resolution of the new printers shows much promise.

The potential of 3D printing in bio-applications and microfluidics lay in the automated character of the technique which may allow single-step fabrication of complex devices with the possibility of integrated valves, fluidic interconnects and interfaces, electrodes, etc. [25]

Important for the scientific community may be the possible role of 3D printing in the standardization of the microfluidic devices, especially the connectors and interfaces. As of now, the most commonly used interface in microfluidics involves a slab of an elastomeric chip, a puncher and a piece of tubing or a fitting fixed into the punched hole. This kind of interface has to be very custom fitted for that particular device in question. Not only can the CAD model files of the devices used in 3D printing be easily shared among the researchers, but the standardized digital modules of certain features of a device can also be simply imported into the CAD file from other files or on-line repositories. Medical-grade connectors such as Luer tapers or barb-type connectors, for instance, can be downloaded and integrated into the digital model, which could lead to the overall standardization of the field of microfluidics. The sharing of CAD files among

the researchers can also facilitate revision and improvement upon the design of a device [24].

SLA microfluidics involves photo-polymerization of the channel walls. Because the capability to flush the non-polymerized resin out of the resulting cavity is fundamental for the functionality of the microfluidic device, the dimensional limitation of the minimal size of a SLA printed micro-channel (so-called hydrodynamic limitation) is predominantly dictated by the characteristics of the photoresin (viscosity, composition, etc.), although the resolution of the printer has also a significant influence [24]. The problem of the SLA method might lay in the composition of the photoresins. The producers are generally not willing to publicize the full formula of the printing material. This is not an issue for the regular consumer who is concerned mostly about the physical properties, the structural strength, the feature fidelity, etc. The chemistry and the surface characteristics of the resin are on the other hand essential characteristics for microfluidics.

A similar problem applies to inkjet printers. While capable to achieve high resolution second only to the 2PP stereolithography, the formulas of the printing material are usually protected by the manufacturer and the bio- and cytocompatibility is poorly characterized. The problem of the surface characterization and the biocompatibility of the SLA and i3DP resins (of the non-hydrogel variety) can be targeted by additives (e. g. specific photo-initiators) or more commonly by the surface derivatization by silanization or silicate coatings [24]. Some improvement of the biocompatibility might be achieved by a prolonged soak of the polymerized resin in pure ethanol [25].

The FDM 3D printing is suited more for building the auxiliary features of the microfluidic device than printing the device itself. The reason is the relatively low resolution of the printers and the fact that the extruder nozzles below the 0.15 mm of diameter are rare, making it hard to fabricate the finer details. The porosity of the FDM printed products introduced by the frequent imperfect fusion of filaments during deposition causes significant leakages making the use of the method in the fabrication of microfluidics even more problematic.

The LOM 3D printing was implemented in the fabrication of microfluidics in the past [30,31] because the materials used in the method (PC, PMMA, mylar) are well characterized and widely used in other microfabrication methods. The limitations of the method are the lower possible complexity of the channel topologies and the frequent clogging of the channels caused by the debris accumulated during the layer cutting and the lamination [24].

2 AIMS AND OBJECTIVES

The title of the doctoral study was "Bioanalytical Techniques Using Microfluidics and Nanotechnology". The aim of the projects was mainly the development of new or novel analytical and separation methodology involving microfluidics and related phenomena. With a research field so wide, the projects targeted various topics from micro-particle preparation to immunoassay enhancement. One of the important threads connecting all the projects was the topic of microfabrication which was chosen as the focus of this thesis.

The initial focus of the doctoral studies at the Institute of Analytical Chemistry of the CAS involved mostly lift-off photolithography as well as metal and glass etching employing the mask-less direct laser writing, the chromium-on-glass photomasks and, in the times of destitution, photomasks plotted on photographic foils. The results of a project dealing with metal nano-platelets prepared by lift-off photolithography were published with a little bit of a delay in 2019 in the journal *Electromachines* (included in this thesis as Publication I).

During the short term traineeship in Denmark, the focus of the study shifted to soft lithography, namely the thiol-ene casting. The mold preparation, a very basis of the method, while possible with the use of photolithographic methods, opened an opportunity to adopt a different fabrication approach – the micro-machining. The micro-machining, in contrast with the lithography, can be used in the fabrication of the whole mold including the containment side-walls in addition to the microfluidic features. Thanks to that, a small microfabrication workshop could be established at the Institute. The work on thiol-enes was published in the journal *Lab on a Chip* in 2015 (Publication II).

As the microfabrication shifted from the cleanroom more towards the workshop, the idea of micro-machining the microfluidic devices directly emerged naturally. Although much less precise, the method has a user-friendlier feel to it, enables immediate control of the quality of the product during machining and has a much wider range of uses than just building the micro-structures. Two collaborations appeared around the same time perfectly fitted for micro-machined microfluidics. One of the projects, with the Department of Biophysical Chemistry and Molecular Oncology of the Institute of Biophysics of the CAS, focused on the analysis of glycans and glycoproteins. For this project, a microfluidic chip for sample preparation with detachable pneumatic valves and a micro-milled micro-channel and micro-column were developed. The results were published in the journal *Electroanalysis* in 2019 (Publication III).

The other project, with the Department of Biological and Biochemical Sciences of the University of Pardubice, involved the development of an augmented dot-blot device with an integrated microfluidic drainage system designed for simultaneous screening of characteristics of various affinity biomolecules. The design was meant to eliminate many of the ancillary steps in the dot-blot procedure to reduce the labor- and time-consumption. The design was patented under the Industrial Property Office of the Czech Republic (Patent I).

The micro-milled analytical devices – microfluidic and other – were the main focus for the rest of the doctoral studies with some additional dabbling with 3D printing and laser ablation.

Graphic representation of the connections between the publications and the employed methods can be seen in Figure 1.

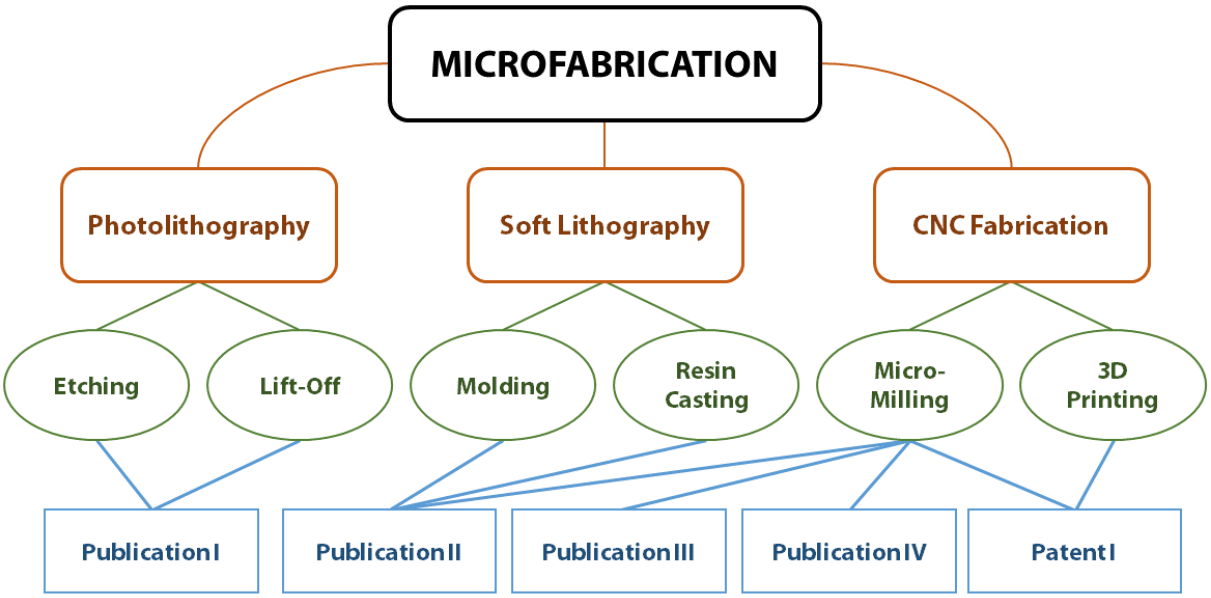

Figure 1. Microfabrication methods applied in the published works.
The publications are linked with the methods somehow involved in the respective project. For example, the casting of thiol-ene microfluidic devices in Publication II required a micro-milled master mold and an elastomeric PDMS template mold.

3 RESULTS

Included in this thesis is an anthology of papers published during the time of the author's doctoral studies. Publications I – IV as well as the Patent I contain the research results related to the microfabrication methods mentioned in previous chapters. Publications V and VI are review papers (or book chapters) summarizing useful information on the topic of microfluidics.

3.1 Publication I: Simple fabrication of structured magnetic metallic nano-platelets for bio-analytical applications

Novotny, J., Juskova, P., Kupcik, R., Bilkova, Z., Foret, F., *Simple Fabrication of Structured Magnetic Metallic Nano-Platelets for Bio-Analytical Applications.* Micromachines 2019, 10, 106. **IF 2.426**

This paper describes the preparation of metal micro (nano) particles using the principles of lift-off photolithography. The project was part of the cooperation of the Department of Biological and Biochemical Sciences of the University of Pardubice and the Institute of Analytical Chemistry of the CAS in Brno.

One of the major aspects of this project was the development of a new method of micro-particle production. The materials usually used range from the organic, such as glucose, latex or polystyrene, or inorganic materials like glass, silica, metals or ceramics.

With access to the photolithography equipment, a sputter-coater and a wide variety of sputter targets, it was a matter of small adjustments to the lift-off procedure to repurpose the method for micro-particle production. The method had a reverse approach to the lift-off technique – what would normally be the waste material of the regular lift-off photolithography was collected as the product. In the usual setting, the resist on the surface of the substrate restricts the deposition of the coating material only on the exposed areas. Stripping the resist off also removes the coating deposited on top of it revealing the pattern of coating deposited directly on the substrate. The released coating is discarded. In the case of this project, on the other hand, this released waste is the product.

The geometry of the micro-particles produced this way was adjustable based on the graphic design of the pattern used in the photo-mask or loaded into a direct laser writer. The general shape of a produced particle was an almost two-dimensional platelet considering that the Z-dimension was often 1000 times smaller (tens of nm) than the XY-dimensions (tens of μm).

The composition of the particles was controlled by sputter-coating. Available metals (Au, Ni, Ag, etc.) could be layered in the coating procedure to combine the properties of multiple metals (e. g. the thiol chemistry of gold with the ferromagnetism of nickel).

The surface of the platelets was bio-functionalized by the EDAC/NHS chemistry.

The author's contribution was the production of the micro-platelets, while the functionalization and testing were done mostly at the University of Pardubice.

Despite being the very first project and the fabrication method the author worked on during his doctoral training starting way back in 2013, the project got stuck in a publication limbo for several years until it was finally pushed for publishing in late 2018.

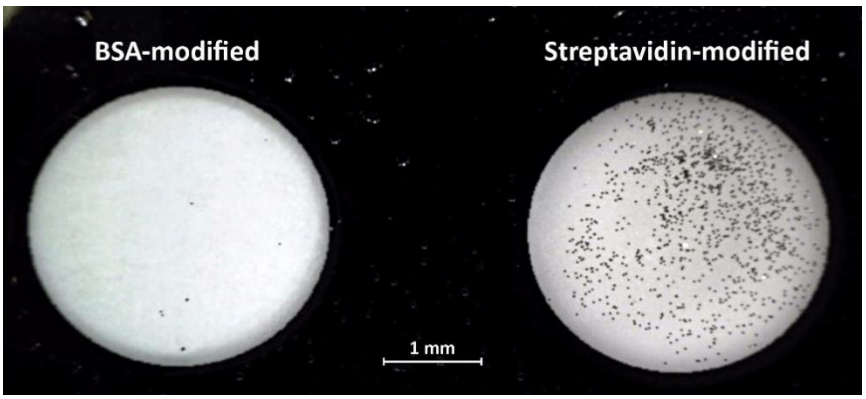

Figure I. Interaction of biotinylated nano-platelets with the glass surface

3.2 Publication II: Rapid and simple preparation of thiol–ene emulsion-templated monoliths and their application as enzymatic microreactors

Lafleur, J. P., Senkbeil, S., Novotny, J., Nys, G., Bøgelund, N., Rand, K. D., Foret, F., Kutter, J. P., *Rapid and simple preparation of thiol–ene emulsion-templated monoliths and their application as enzymatic microreactors.* Lab Chip 2015, 15, 2162–2172. **IF 6.914**

The paper is a result of a traineeship abroad at the Department of Pharmacy of the Faculty of Health and Medical Sciences of the University of Copenhagen at the group of Professor Jörg P. Kutter.

This project dealt with microfluidic devices made of the thiol-ene resin. Thiol-enes are UV-curable copolymers prepared from a mixture of thiol- and allyl-group containing monomers. Principles of thiol-ene chemistry are used – especially in combination with epoxies – in adhesives, sealants or coatings, for example in dental glues.

To cast the microfluidic chips from photo-curable thiol-enes for this project, the master molds for the chips had to be micro-milled first in PMMA and used to prepare PDMS template molds. These PDMS templates were finally filled with a thiol-ene mixture of the desired composition of T and E monomers and cured in UV light.

Micro-channels of the microfluidic chips were filled with a mixture of thiol-ene monomers and a porogeneous liquid (e. g. methanol) and a specific site of the channels were exposed to the UV to form a monolithic column which was then functionalized by immobilized enzymes. Using the right combination of the T : E ratios for the monolith and the chip material, the monolith would fuse during the curing with the wall of the channel anchoring itself tightly inside. The microscopic structure of the monolith is made of a network of less than micron-sized beads of thiol-ene fused into chains.

The success of the enzyme immobilization was evaluated by the detection of the fluorescence signal (in the case of galactose oxidase reactor) or by MS analysis of samples treated on the monolithic column (PNGase F reactor).

The author's involvement with the project was the design and fabrication of the test microfluidic device, which contained a simple U-turn channel, one inlet, and one outlet. The shape of the channel was meant for easier application of shielding for monolith curing with both inlet and outlet out of the way on the same side of the device.

The author was also involved with the experiments with the PNGase F reactor. In these experiments, the efficiency of this deglycosylation reactor was tested by the samples of RNase b solutions. RNase was flown through the device, samples collected and analyzed on Waters SynaptG2 mass spectrometer. The MS results showed that the reactor was indeed removing the glycan chain of the RNase glycoprotein indicated by the loss of the peaks of the 5 RNase glycoforms between the mass of 15 and 16 kDa which differ in the number of mannose units in the glycan chain (5 – 9). These were replaced by a single peak of RNase protein at 13692 Da. This proved that the PNGase was successfully immobilized in the microfluidic device.

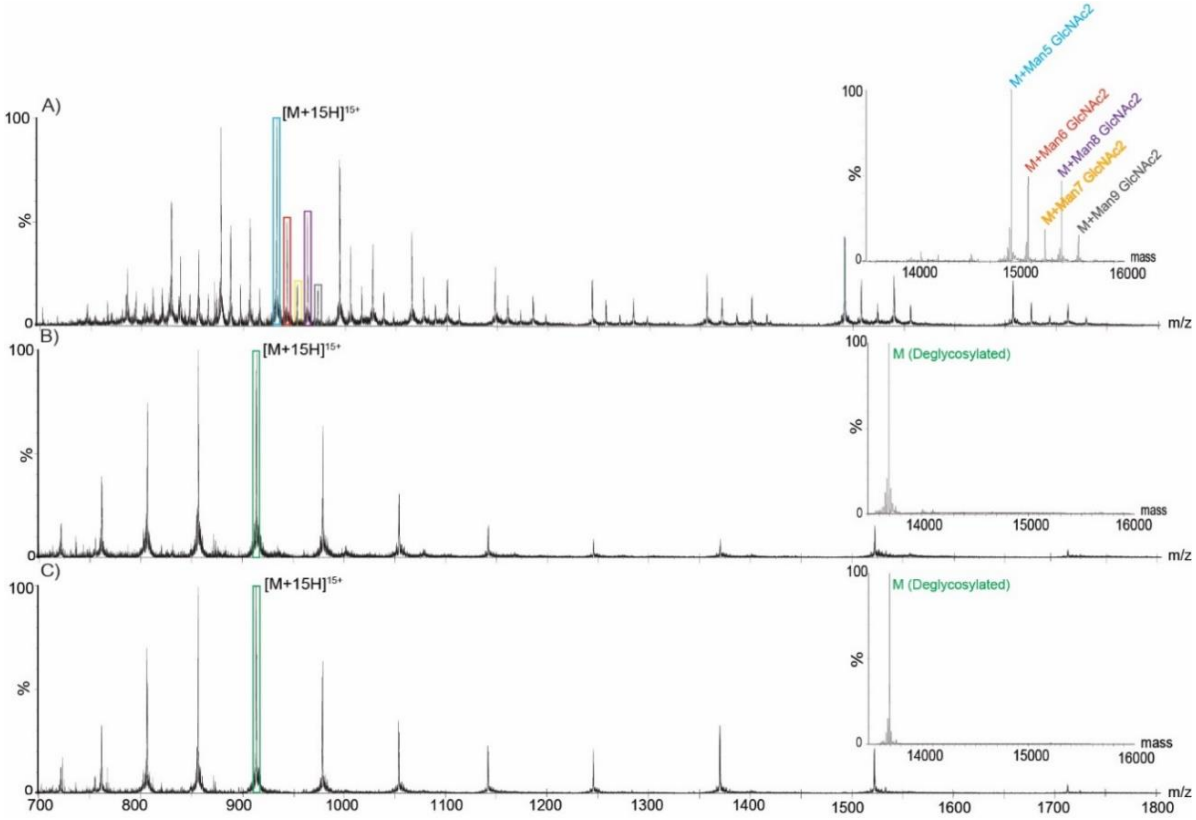

Figure II. On-chip deglycosylation of RNase B measured by LC-MS.
A) Mass spectrum of glycosylated RNase B. $[M+15H]^{15+}$ The corresponding deconvoluted spectrum is shown in the insert using the same coloring scheme. B) Mass spectrum of RNase B deglycosylated off-chip by PNGase F. C) Mass spectrum of RNase B deglycosylated online by immobilized PNGase F on a microfluidic chip.

3.3 Publication III: Electrochemical analysis of glycoprotein samples prepared on a pneumatically-controlled microfluidic device

Novotny, J., Ostatna, V., Foret, F., *Electrochemical Analysis of Glycoprotein Samples Prepared on a Pneumatically-controlled Microfluidic Device.* Electroanalysis 2019, 31, 1994–2000. **IF 2.691**

This paper presented results of the cooperation of the Institute of Analytical Chemistry of the CAS and Veronika Ostatná from the group of the late Prof. Emil Paleček of the Institute of Biophysics of the CAS. The paper was published as a part of the Special Issue: Memorial Issue for Paleček of the Electroanalysis journal.

The project dealt with the isolation and analysis of glycans and glycoproteins. Defective glycosylation is linked with many pathologic conditions, chronic and infectious diseases as well as cancer. Therefore, glycans and glycoproteins are a very popular topic for research and clinical studies.

The problem with the biological samples is their complexity which requires the inclusion of enrichment or purification elements into the analytical procedure. Recognition elements used in glycosylation studies are lectins. These proteins can specifically recognize glycans similarly to the antibodies but are nowhere nearly as specific. Because of that, lectins can be easily inhibited by simple saccharides which are often used as elution agents.

A microfluidic chip was fabricated by direct micro-milling of micro-channels into polycarbonate. The channel was designed to allow stacking of particles above a certain size which was used to entrap beads of bisacrylamide/azlactone support thus forming a packed column. The chip was also equipped with custom-made pneumatic valves for flow control.

The beads of the column could be easily coupled with any biomolecule via an amine group which was exploited to immobilize lectins for affinity isolation of glycoproteins and glycans. The lectins used in this study were concanavalin A and *Sambucus nigra* agglutinin. ConA is a lectin very popular in assays. Its tetrameric structure binds the α-linked mannose in the core oligosaccharide of many glycoproteins, although it requires calcium and manganese cations to function. The elution buffer for ConA contained methyl-α-D-mannopyranoside. A bit non-standard was the arrangement of the ConA/glycoprotein system used in this study – purely because of the availability of the substantial supply of fluorescent-labeled ConA, it was decided to functionalize the affinity column not with the lectin, as it is usually done, but with the glycoprotein, in this case RNase b, and use the labeled lectin as a sample in the fluorescence experiments which were used to test the functionality of the device.

SNA is a lectin extracted from the elderberry bark which binds with the sialic acid linked in a glycan chain to the terminal galactose via a 2,6 bond. This lectin is inhibited by galactose and lactose, which was used as the elution agent. To determine the level of any possible non-specific interaction and retention, two kinds of sample glycoproteins were chosen – unlabeled fetuin, which interacts with the SNA specifically, and asialofetuin, which lacks the terminal sialic acid in its glycan chain and as such should not interact with SNA.

The samples were analyzed off-line either on the compact benchtop fluorometer Qubit in the case of fluorescent-labeled samples or voltammetrically by measuring catalytic peak H at the amalgam electrode at the Institute of Biophysics in the case of the label-free samples.

The project proved again the versatility of the micro-milling method. The same milling machine was used to fabricate everything from the microfluidic channel, mounting systems up to the pneumatic valves and the fluid dispenser.

The author’s contribution to the project was, as in the previous cases, the design and fabrication of the device and its auxiliary tools and accessories. In this project, the task also involved the bio-functionalization of the column, sample preparation, treatment,

and collection. In the case of the fluorescence experiments, the samples were measured right away. The label-free samples had to be sent to the Institute of Biophysics for analysis.

Another very long-term project, the fabrication techniques initially considered for the chip production were soft lithography methods, either PDMS molding or thiol-ene casting. After the establishment of the microfabrication workshop and the introduction of the micro-milling into the repertoire, it was just a matter of gaining some experience with the technique to switch to it as the method of choice for the device production in this project.

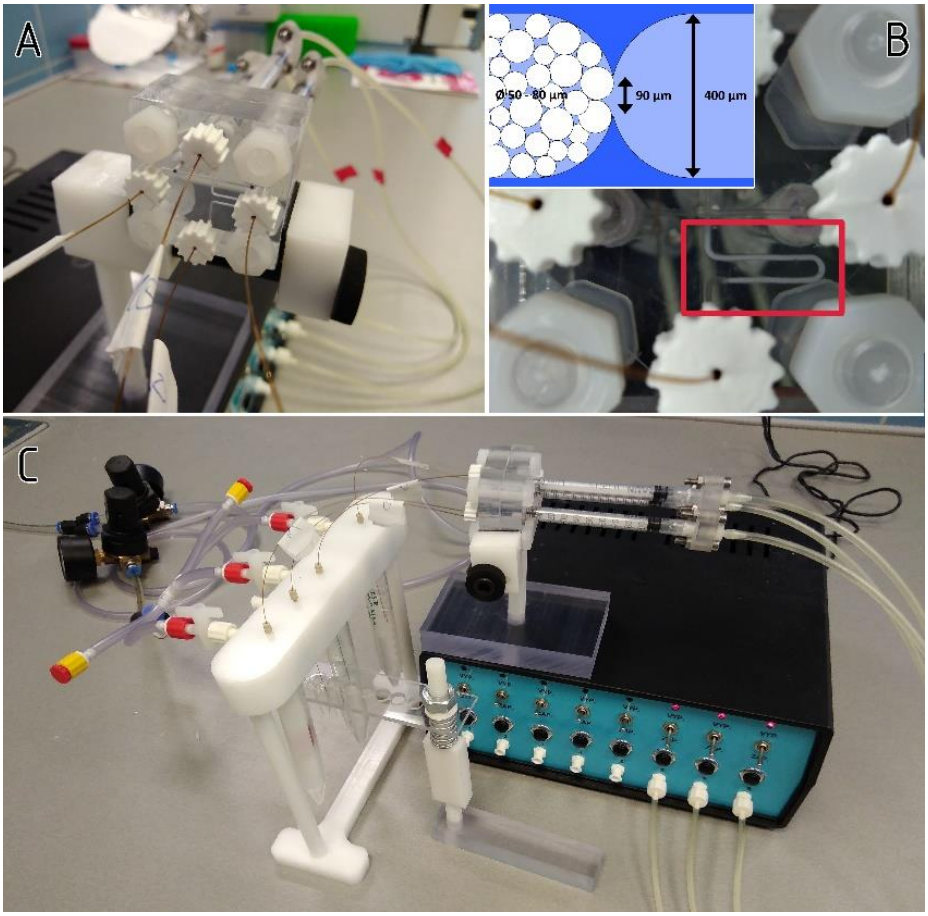

Figure III. Microfluidic sample-preparation device.
A) The microfluidic chip with the fluidic connections. B) Detail showing the packed affinity column. The insert represents a diagram of the narrowing at which the UltraLink micro-beads accumulate. C) The whole device. The fluid dispenser (left) is connected to the chip (top center) by capillaries. Valves are controlled by a CDA-switchboard (the black-and-blue box).

3.4 Publication IV: Macrofluidic device for preparative concentration based on epitachophoresis

Foret, F., Datinská, V., Voráčová, I., Novotný, J., Gheibi, P., Berka, J., Astier, Y., Macrofluidic *Device for Preparative Concentration Based on Epitachophoresis.* Anal Chem 2019, 91, 7047–7053. **IF 6.35**

This paper shows the results of the cooperation of the Institute of Analytical Chemistry with Roche Sequencing Solutions, Inc., part of the international giant Hoffmann-La Roche. The life science company is based in the United States of America and specializes in the development of next-generation sequencing and its application.

The objective of this project was the development of a separation device for the collection and concentration of analytes from several-milliliter volumes of the sample down to microliter fractions.

Electromigration methods are very common separation methods in bioanalysis. While the methods in capillary settings are often used for analytical purposes, the idea of enrichment, purification, and collection of fractions using this setting is not particularly effective because of the low capacity for the sample volume. Much more effective for this purpose are the free-flow or slab gel arrangements of the electromigration methods.

A circular planar version of the isotachophoresis was developed for this project with the purpose of allowing separation and extraction of analytes (mainly DNA) from large-volume samples of several mL. The device used a discontinuous electrolyte system of a leading and a terminating electrolyte to concentrate samples of organic anions and DNA fragments. The molecules of the 10+ mL sample migrated from the perimeter of a circular slab of agarose gel toward its center, actuated by the constant electric power applied between a circular outer-perimeter electrode and the center of the device. As the molecules migrated through the gel, the sample got separated into fractions in the form of concentric rings. These fractions could be collected from a collection cup once they reached the center of the gel.

The author of this thesis was tasked with the development of several prototypes of the device. The author was also assigned with the design and fabrication of a miniaturized SLA 3D printed version of the device intended for less time-consuming operations which, unfortunately, did not end up being a part of the publication.

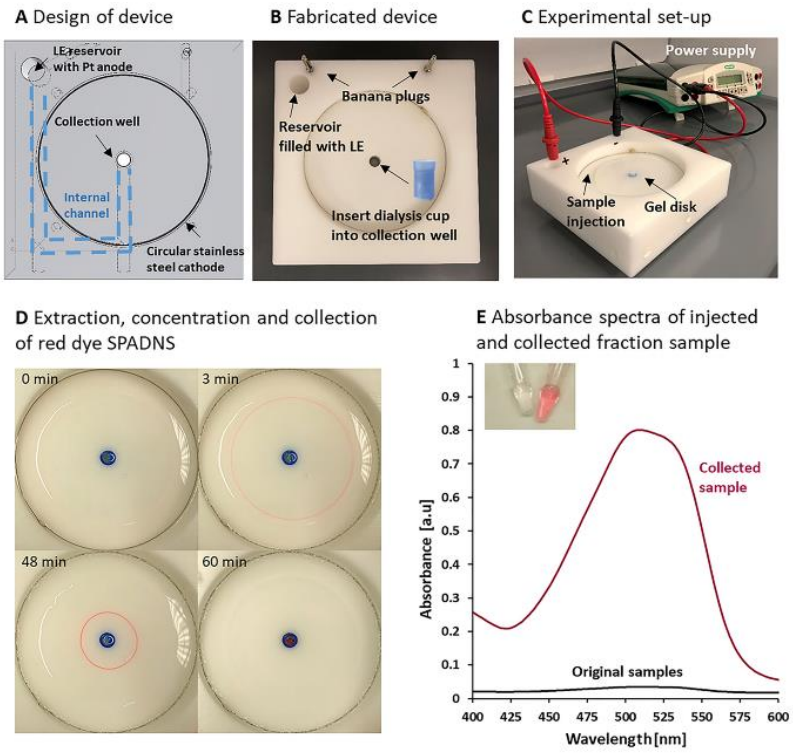

Figure IV. Epitachophoretic device.
A) Device design. The dashed line describes the internal channel between anode and collection well. B) Top view of the manufactured device (Ertacetal). C) Photograph of the experimental setup. D) Epitachophoretic focusing of red dye SPADNS at different time intervals. (E) Absorbance spectra of injected and collected samples.

3.5 Patent I: Cover of the membrane holder for biomolecule transfer and the membrane holder for biomolecule transfer for dot-blot method

Svobodová, Z., Novotný, J., Foret, F., Bílková, Z., *Víko držáku membrány pro přenos biomolekul a držák membrány pro přenos biomolekul pro provádění metody odsátí skvrn* (Literal translation: *Cover of the membrane holder for biomolecule transfer and the membrane holder for biomolecule transfer for dot-blot method*). No. **PV 2018-706**, Czech Republic, December 17, 2018.

The patent is a result of a long-time collaboration with Mgr. Zuzana Svobodová, Ph. D., at the time working full-time at the Department of Biological and Biochemical Sciences of the University of Pardubice.

Because of the fact that no uniform scientific framework exists to enforce standards for antibody validation, the reproducibility of data obtained through the use of antibodies is inadequate. The level of validation in commercially available antibodies varies greatly among the producers and vendors. Possible degradation or contamination during shipment and inappropriate storage must also be always taken into account as a source of error.

Methods most widely used at least as the first step of antibody validation are blotting techniques, especially the western blot. This family of bioanalytical techniques involves in their first step some sort of planar sample separation such as 2D gel electrophoresis which is followed by the transfer of the analytes onto a membrane (blotting) for easier

manipulation and treatment. A simplified version of this is called a dot-blot in which is the sample applied – without separation – directly to the membrane. The sample, obviously, has to be much simpler than in the other blotting methods but it is faster and less demanding.

This project involved the development of an enhanced dot-blotting device that revolved around a microfluidic drainage system which allows on-device sample treatment, labeling, and visualization.

Because of the versatility of the fabrication method – micro-milling, the same machine could be used to cut the overall bulk shape of the device, the microfluidic features as well as the wide range of add-ons, accessories, and so on. The author's contribution was the design and realization of the device.

The drainage system was placed on a Czech national patent application under the – admittedly exhaustive – title "Víko držáku membrány pro přenos biomolekul a držák membrány pro přenos biomolekul pro provádění metody odsátí skvrn" which could be translated and simplified from Czech Lawyerese (*lingua perjura bohemica*) to English as "dot-blot membrane holder with cover". By the time of writing this thesis, the patent procedure was still ongoing but the patent was secured. The following pages contain the Annotation, the Patent claim and the Invention description submitted to the Industrial Property Office of the Czech Republic, which is why they are written in Czech. Because of that, the following text is a simple summary of the description of the device and the basic operation in English:

The device was fabricated from a polycarbonate plate using CNC micro-milling, because of the relatively effortless machining, durability and high transparency of the material. The detail work was finished on a manually controlled vertical mill/drill machine. Sealing is provided by custom-made gaskets of microporous silicone, which has low water absorbance, very good elasticity, and chemical resistance.

The device was designed in VCarve CAM software as an assembly of three major components: 1) vacuum manifold with a vacuum chamber connected to a source of vacuum, e. g. pump, which drives the application of samples to the membrane; 2) support plate with 25 openings which connect the vacuum manifold and the sample wells; and 3) sample template. The sample template serves as the titular "cover" of the device and features 25 wells which are linked to a vacuum-driven drainage system. The drainage system consists of 25 short micro-channels 200 µm wide and 50 µm deep which lead to larger collector channels. These channels are connected via 4 plastic fittings and tubing to a source of vacuum. Each micro-channel contains short expansion increasing the width 10-fold. This helps to prevent spontaneous drainage of wells during sample dosing or while applying vacuum to the membrane as well as any risk of cross-contamination. The sample wells drained by the same collector channel are joined at the top 3 mm of their depth thus forming a reservoir. This allows the application of larger volumes of buffers during the washing step while at the same time leaves the unconnected bottom half of the well for individual treatment of samples.

A cutting plotter was used to fabricate custom gaskets out of microporous silicone. One of the gaskets was used to seal the connection between the vacuum manifold and the support plate, the second gasket was placed between the support plate and the membrane and was perforated with holes fitting the 25 openings.

An issue with spot seeping appeared caused by a slight membrane deformation during the application of vacuum to the manifold, which would break the sealing of the well. The issue was solved by sandwiching the membrane between 2 disposable thin sheets of plastic which can be cheaply and effortlessly mass-produced on the cutting plotter. Plastic sheets are secured in place by being simply snapped on a set of pins which also hold the silicone gasket. The plastic sheet on the top also serves as the bottom wall of the drainage channels.

The basic operation of the device is as follows: the vacuum manifold and the support plate are tightly screwed together. Membrane, sandwiched in plastic sheets, is snapped on the pins on top of the sample gasket. The sample template is secured on top by wing nuts. For the sample application, samples are dosed into the sample wells and a vacuum source is connected to the outlet in the vacuum manifold. After the deposition of samples to the membrane is finished, the vacuum source is connected to the outlet of the drainage system in the sample template and the washing and treatment stage can be performed. The processed membrane is recovered from the device and put through detection.

The functionality of the device was evaluated by performing the avidity test of anti-chymotrypsin antibodies. At the time of writing this thesis, the results of these experiments were in the process of preparation for publication.

Also ongoing at the time of writing this thesis was the preparation for upgrading the patent level to international with European patent application.

The device was also in the process of adaption for SLA 3D printing which requires substantial alteration of the bulk design of the device (because of the restrictions of the size of the printable area of the available printer and the not-so-excellent resolution).

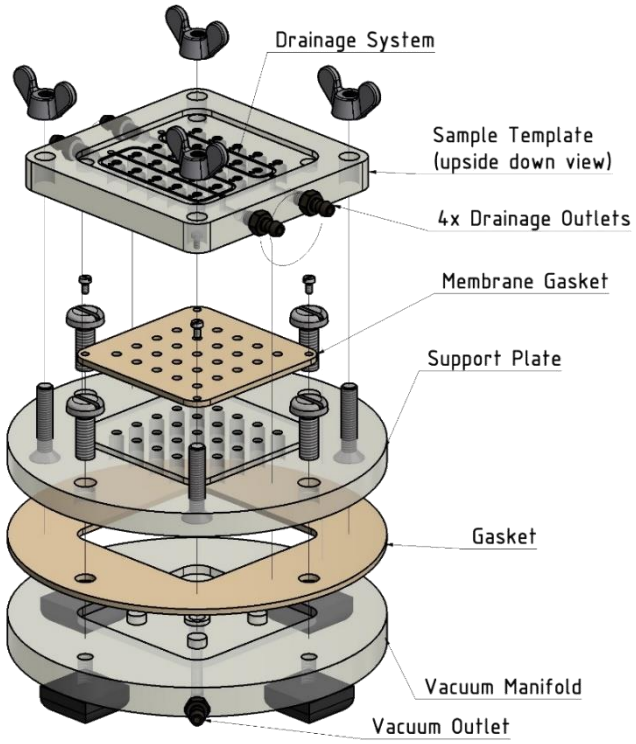

Figure P1. Exploded view of the microfluidic dot-blot.
The membrane is inserted on top of the membrane gasket sandwiched between 2 PE foils to prevent contamination of the membrane from the drainage channels.

3.6 Publication V (Review): Basics of fluid behavior in microfluidics

Novotný, J., Foret, F., *Fluid manipulation on the micro-scale: Basics of fluid behavior in microfluidics.* J Sep Sci 2017, 40, 383–394. **IF 2.516**

When the fluidic system is scaled down to the point where cross-section reaches submillimeter dimensions, inertia begins to lose influence on the flow of the liquid. At the same time, viscous forces decrease much less significantly. While inertia keeps the flowing liquid in motion, viscous forces are responsible for the resistance of the body of liquid to deformations and are counteracting inertia. This is what causes seemingly unpredictable turbulent flow, full of splashes and whirls, at macroscale where inertial force overwhelms viscosity. As the mass of the liquid decreases, the viscosity dominates at microscale over inertia and turbulent flow turns into the linear and predictable laminar flow. Without vortices that would stir the fluids, the only phenomenon driving the mixing in laminar flow is diffusion.

The high surface-to-volume ratio of the microscale means that surface effects play a much bigger role. Capillary forces tend to drag liquids over wettable surfaces, which is counterbalanced by the viscosity. An energy gain/drop at adjacent surface interfaces is often exploited in microfluidic devices for actuation by creating a gradient in surface wetting.

The review paper serves as an introduction into the topic of microfluidics as it deals, with the general physics of the microfluidic phenomena such as the flow, mixing, surface effects as well as closely related electrokinetics and gives examples in a form of some of the interesting published applications.

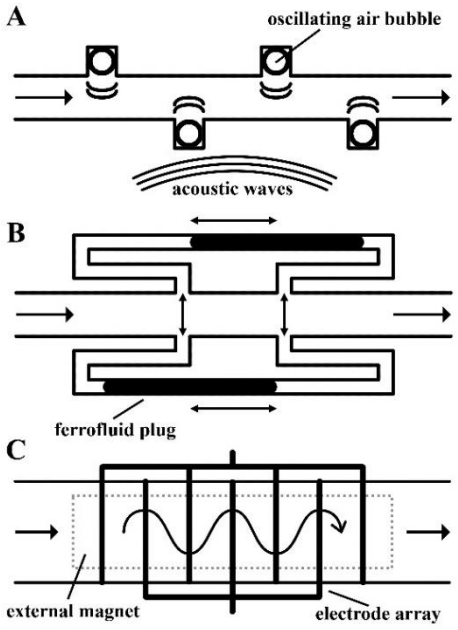

Figure V. Various types of active microfluidic mixers.
A) Acoustic mixer, where focused acoustic waves interact with trapped air bubbles to introduce oscillation. B) Oscillating ferrofluid plugs in cross channels are actuated by an external magnetic field to induce pressure fluctuations in the main channel. C) In a magneto-hydro-dynamic mixer, alternating application of potential to electrode array in external magnetic field generates Lorentz force perpendicular to both electric and magnetic fields, which promotes secondary flow.

3.7 Publication VI (Review): Miniaturization and microfluidics

Summarization of the history of the applied microfluidics, miniaturization and the overview of the most widely commercially available products had been published in Chapter 26 *Miniaturization and microfluidics* (Novotný, J., Foret, F., Smejkal, P., Macka, M.) of the second edition of the book *Liquid Chromatography: Fundamentals and Instrumentation* (editors Fanali, S., Haddad, P., Poole, C., Riekkola, M. L., Waltham: Elsevier. 2017, 619 – 636, eISBN 9780128093450). The review was a revision of the equally titled chapter by Foret, Smejkal and Macka published in the first edition of the book with updated information on the products and producers.

The review serves mainly as an overview of producers of LC and CE systems and details the technologies involved in the (at the time of the publishing) latest lines of commercially available microfluidic analytical products. In the 4 years between the publication of the first and the second edition of the *Liquid Chromatography: Fundamentals and Instrumentation*, the landscape of the LC and CE products and companies changed substantially. With all the mergers, acquisition and bankruptcies of the business world and new lines of commercial microfluidic platforms, the chapter for the second edition justifiably required serious revision.

The published chapter also contains a brief summary of some of the most common fabrication methods for microfluidic devices which were elaborated more in-depth in the thesis.

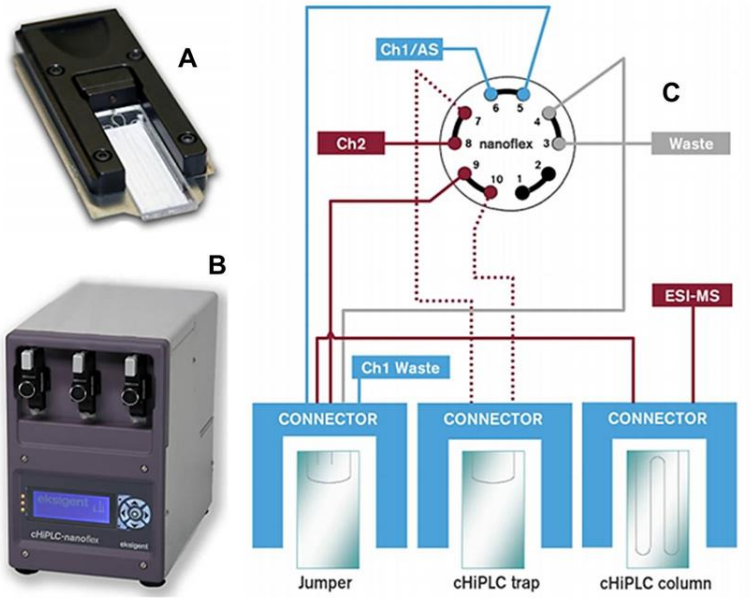

Figure VI. cHiPLC® System by Sciex (Eksigent).
A) nano-LC chip; B) cHiPLC-nanoflex docking station; C) diagram of thee-chip setting (www.sciex.com)

4 CONCLUDING REMARKS

Owning to the theme of the doctoral training – microfluidics, the author had the opportunity to get involved with many projects which granted the chance to touch on the wide variety of the microfabrication techniques mentioned in this thesis. The incredible advantage was the unlimited access to a microfabrication cleanroom and darkroom which made a lot of work much easier. The sequence of chapters of this thesis chronicles the course of the author's time at the Institute of Analytical Chemistry and the progress of the subjects of interest.

It was rather challenging to come up with somewhat all-inclusive concluding remarks for the publications compiled in this thesis considering the quite broad range of topics. There was a common thread connecting all the works, though, which was the microfabrication. This is why it was chosen as the topic of the theoretical section of the dissertation. The included papers contain certainly interesting applications utilizing quite a variety of fabrication methods and materials. What could be positively said, is that in the course of his doctoral training, the author was able to gain skills in a wide variety of often rather unrelated fields. This is somehow indicative of these times as modern science nowadays gravitates towards interdisciplinary approaches to problem solutions. This is certainly true for the field of applied microfluidics which, no matter the final purpose of the device, would require some degree of knowledge and ability in material and electrical engineering, graphic design, and many more fields, to be able to realize the microfluidic device from the idea into a functional physical object.

The microfluidic dot-blot project was the one reaping probably the richest harvest. The idea of giving the users the possibility to test the performance and selectivity of available antibodies was intriguing – the convenience of having a tool that could guide their decision during the purchase of a commercial kit or serve as a first screening step in the validation procedure, for cross-reactivity and avidity evaluation, or maybe just for a check-up after prolonged storage. With the state of the antibody market as it is, there was a chance for such a device to have a place on the market as well. The Czech national patent application was submitted in December 2018 and the European patent application was being prepared during the one-year priority period granted by the intellectual property laws. A paper reporting the gathered data was also close to completion and submission by the time of writing of this thesis.

The project with the Institute of Biophysics of the CAS reported in Publication III was also rather successful. A grant application for a follow-up project was submitted at the Czech Science Foundation (GAČR) for the 2019 evaluation.

The thiol-ene project with the Department of Pharmacy of the Faculty of Health and Medical Sciences of the University of Copenhagen, unfortunately, did not find further adoption at the Institute of Analytical Chemistry. The research of the topic was continued by the colleagues in Copenhagen but was problematic to pursue back in Brno, mainly due to the unavailability of the access to optimal equipment required for proper work with the resin.

It was certainly an interesting opportunity to be able to work with so many different fabrication options. The photolithographic methods are rather tedious and require precise work where often a minuscule detail could decide between success and failure.

The payoff is the incredible detail attainable by these methods. The soft lithography forgives a certain level of sloppiness in the handling but, on the other hand, it is more of a reproductive than a productive family of methods and requires preparation of a model produced by some other fabrication method. The micro-machining and 3D printing are very versatile methods but require at least a bit of handiness with the workshop tools from the operator and a lot of optimization to be used for microfluidics. Their use in microfluidics is still in its infancy, so to say.

The information published in the journal articles and patents certainly do not encompass the sheer frustration which came hand in hand with the amount of labor invested into development and microfabrication of the objects these papers refer to – the amount, admittedly, rather typical for the work on microfluidics and microfabrication in general. All the 4-hour-long micro-milling jobs ruined in the very last minute… All the photolithographic pieces discarded because of a single speck of material appearing in the wrong place… All the times the direct laser writer being broken again and again… Looking back at these events, it is hard to believe that anything could be developed at all.

The fabrication methods mentioned in the theoretical part of this thesis most definitely did not exhaust the list of possibilities for microfabrication. The scope of the mentioned methods is based on the experience of the author with the method, the author's interest in the method and, honestly, the recentness of the author's involvement with the method. Fortunately, the sequence of the methods studied by the author to some degree matches the historical evolution of the field of microfabrication – from photolithography, through plastics, to 3D printing (see Publication VI for a brief history of the field).

Hopefully, as the technology progresses, better materials are being developed and the costs decrease, we would be able to see the widespread of 3D printed analytical devices easily downloadable from the internet and recreatable at every facility. For now, the large scale microfluidic chips available commercially employ various methods (again, see Publication VI for more detail) but the design of the chips is extremely uncomplex and simplistic to the point of often containing only a single short straight channel and most of the job is done by the components hidden in the tabletop machine while the PDMS chips still remain the standard of low scale production in research.

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