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Bioanalytical Techniques Using Microfluidics and Nanotechnology

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Prohlašuji:

Tuto práci jsem vypracoval samostatně. Veškeré literární prameny a informace, které jsem v práci využil, jsou uvedeny v seznamu použité literatury.

Tato práce byla vypracována na externím pracovišti v laboratořích Ústavu analytické chemie Akademie věd České Republiky, v. v. i., pod vedením Ing. Františka Foreta, DSc.

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V neposlední řadě bych chtěl poděkovat své rodině za obrovskou podporu.

ANNOTATION

This dissertation thesis deals with the development and application of new microfluidic devices and similar products. To achieve this, several different fabrication techniques had to be utilized. Produced this way were microfluidic chips, microfluidics-related devices as well as micro-particles. This thesis summaries information on the fabrication methods and contains a collection of publications written or co-written by the author.

ANOTACE

Tato disertační práce se zabývá vývojem a aplikací nových mikrofluidických zařízení a podobných výrobků. Dosaženo toho bylo využitím řady různých fabrikačních technik. Tímto způsobem byly vyprodukovány mikrofluidické čipy, s mikrofluidikou související zařízení a také mikročástice. Tato práce shrnuje informace o fabrikačních metodách a obsahuje kolekci výstupů publikovaných nebo spolu-publikovaných autorem.

ABSTRACT

The science of fluid manipulation in submillimeter dimensions is called microfluidics. At the microscale, fluids start to exhibit rather unintuitive behavior. The influence of inertia loses importance and seemingly chaotic turbulent flow shifts into a laminar flow. Without whirls and eddies, liquids blend only by diffusion alone. Capillary actions and surface effects become increasingly significant.

Microfluidic devices embrace and exploit these characteristics. For example, patterned, chemically treated surfaces can be used in these devices as passive valves, virtual walls or even actuators. The consequence of the laminar flow is that several streams of liquid can be flown side-by-side in one channel with virtually no mixing which is used for example in flow-focusing. The possibilities of the application of microfluidic phenomena are countless. This doctoral dissertation deals with the development and fabrication of such devices.

The earliest microfluidic devices were microfabricated in silicon, the same way as computer chips. Nowadays, attention shifted from materials like glass and silicon, which are expensive and rather hard to process, to plastics: thermoplastics, like polycarbonate or poly(methyl methacrylate) (acrylic glass), which can be melted easily and used for techniques like molding and hot embossing, or elastomers, most commonly polydimethylsiloxane (PDMS), or resins.

This dissertation presents an anthology of published papers on the topic of microfluidics, microfabrication and so on, supplemented by a text describing the various methods of microfabrication. The publications present 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 začínají kapaliny vykazovat kontraintuitivní chování. Vliv setrvačnosti klesá na významu, čímž dochází k přechodu zdánlivě chaotického turbulentního proudění na proudění laminární. Bez vířivého toku pak dochází k mísení kapalin jen pomocí difúze. Působení kapilárních sil a povrchových efektů je také mnohem zřetelnější.

V mikrofluidických zařízeních je s těmito charakteristikami počítáno a jsou naopak využívány. Příkladem mohou být strukturované či chemicky upravené povrchy, které jsou v těchto zařízeních užívány jako pasivní ventily, virtuální stěny či dokonce jako pohony toku. V důsledku laminárního proudění je možné jedním kanálem paralelně hnát více kapalin prakticky bez jejich mísení, čehož se využívá například k fokusaci toku. Možných aplikací mikrofluidických fenoménů je nesčetně. Tato disertační práce se zabývá vývojem a výrobou takových zařízení.

Nejranější mikrofluidická zařízení byla vyráběna z křemíku, podobně jako počítačové čipy. V současnosti se však pozornost přesouvá od materiálů jako je sklo a křemík, které jsou poměrně drahé a nesnadno opracovatelné, k plastům: k termoplastům jako je polykarbonát nebo polymethylmetakrylát (plexisklo), které mohou být snadno roztaveny a užity v technikách jako je odlévání či embosování za horka, dále k elastomerům, nejčastěji polydimethylsiloxanu (PDMS), nebo k živicím.

Tato disertační práce představuje sbírku publikovaných článků týkajících se témat mikrofluidiky, mikrofabrikací, apod., doplněných textem popisujícím různé metody mikrofabrikace. Přiložené publikace prezentují řadu mikrofluidických zařízení a souvisejících výrobků vyvinutých s využitím rozmanitých materiálů a výrobních metod – od fotolitografie po 3D tisk.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

μPAD	microfluidic paper-based analytical device
μTAS	micro-total analytical systems
2D	two-dimensional
2PP	two-photon polymerization
3D	three-dimensional
ABS	acrylonitrile butadiene styrene
AKD	alkyl ketene dimer
CAD	computer-aided design
CAM	computer-aided manufacturing
CAS	Czech Academy of Sciences
CE	capillary electrophoresis
CMYK	cyan-magenta-yellow-key (black)
CNC	computer numerical control
COC	cyclic olefin copolymer
ConA	concanavalin A
DC	direct current
DLP	digital light projection
DNA	deoxyribonucleic acid
DRIE	deep reactive-ion etching
EDA	electronic design automation
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ESI	electrospray ionization
FDM	fusion deposited modeling
HMDS	hexamethyldisilazane
HDPE	high-density polyethylene
HPLC	high-performance liquid chromatography

i3DP	inkjet 3D printing
LC	liquid chromatography
LCD	liquid crystal display
LOM	laminated object manufacturing
MS	mass spectrometry
NHS	N-hydroxysuccinimide
OSTE	off-stoichiometric thiol-ene
PC	polycarbonate
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
PEEK	polyether ether ketone
PEG	polyethylene glycol
PLA	polylactic acid
PMMA	poly(methyl methacrylate)
PNGase	peptide:N-glycosidase
PS	polystyrene
RIE	reactive-ion etching
RNase	ribonuclease
RPM	rotations per minute
SAM	self-assembled monolayer
SDL	selective deposition lamination
SLA	stereolithography
SNA	*Sambucus nigra* agglutinin
SPU	steps per unit
T : E	thiol : ene
TMAH	tetramethylammonium hydroxide
UV	ultraviolet

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 feature common among most of these methods is the involvement of computers in the generation of micro-patterns in some way or the other, be it to generate patterns in photomasks in photolithography or in CN control in micro-machining. With structures nearing the dimensions of human hair, manual manipulation is no longer an option.

As mentioned before, 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. Substrates are thoroughly cleaned from all impurities, for example by Piranha solution. Piranha is a

mixture of sulfuric acid and hydrogen peroxide which attacks organic residues by peroxysulfuric acid and atomic oxygen [2]. The solution aggressively dissolves all organic contaminants and grease stains on the surface. Cleaned substrates are dried on a hotplate at temperatures higher than 120°C to remove all moisture [3]. The surface is then treated to improve the adhesion of resist, usually by hexamethyldisilazane (HMDS, for increased hydrophobicity) [4].

Cleaned and treated substrate is spin-coated by the photoresist. Photoresists are photosensitive resins which can shield the surface from the effects of etchants and other chemicals. Positive tone photoresists are normally insoluble but exposure to light causes cleavage in their polymer chains and increases solubility in developing solutions. Negative tone photoresists, on the other hand, are normally soluble and light exposure causes polymerization or cross-linking which decreases solubility [5]. Each resist has its own processing procedure specified by the manufacturer.

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 (Figure 1). These pattern generators use the acousto-optical modulation of a laser beam to plot designs directly on the photosensitive surface [6]. Designs can be based on uncompressed graphics files, CAD files or EDA files (e.g. GDSII, CIF or Gerber). Direct writers can also be used in photolithography by themselves. The procedure involving these machines takes a long time since the substrate is exposed only by few-microns-wide line at a time. A standard commercially available 4-inch photoresist-and-chromium-coated glass plate would take several hours (over 12 in some systems) to fully expose, while exposure through photomask takes just a few seconds. Incomparably higher precision still overweighs these inconveniences.

Wavelength and exposure time differ greatly resist from resist which has to be taken into account when considering equipment and procedure. Spectral sensitivity is specified by either interval of wavelengths or by spectral lines of Hg-lamp, while exposure dose is indicated in mJ/cm^2. Some resists require heat treatment after exposure to complete photoreaction. Immersing the exposed photoresist into developer (usually a basic solution), the depolymerized or non-cross-linked areas dissolve and only the pattern remain. To increase physical and chemical resistance of the resist, substrates can be further processed on a hotplate in so-called hardbaking.

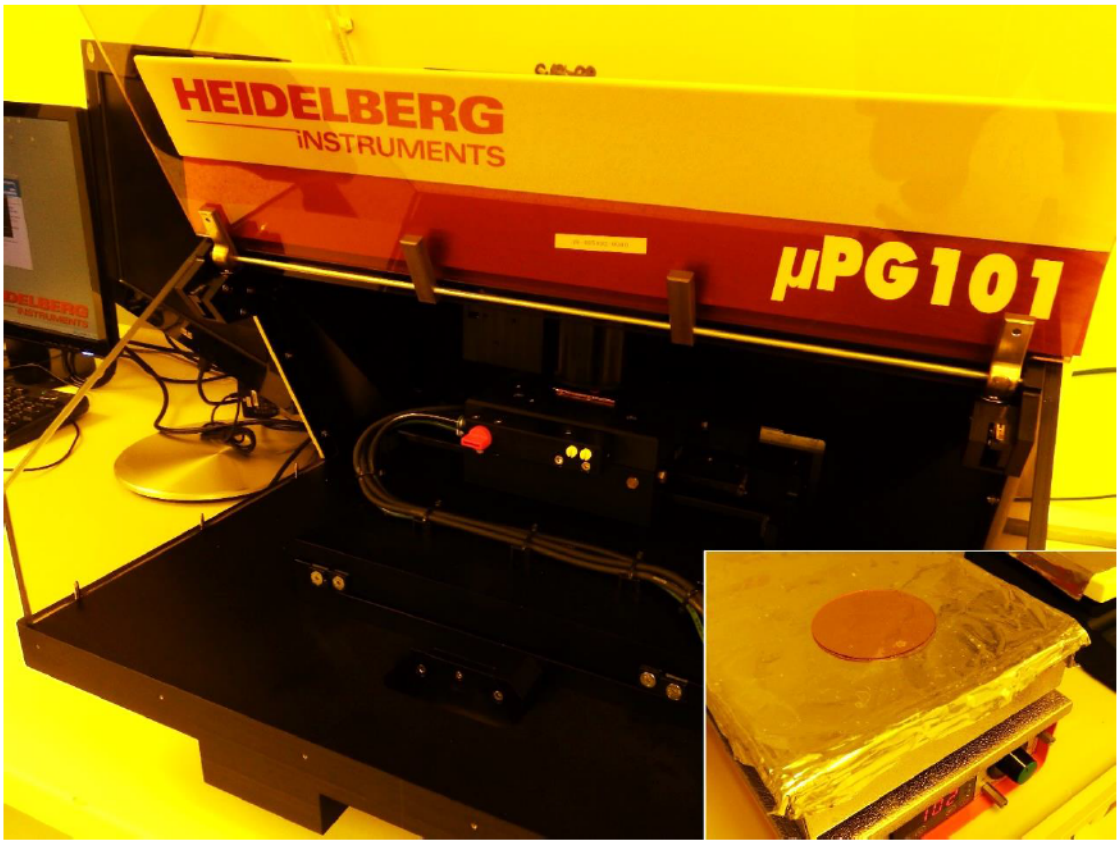

Figure 1. Direct laser writer.

A laser scans the loaded digital design into a photosensitive layer of the substrate which is fixed by vacuum onto the moving stage in the middle (with the red valve knob). ***Insert:*** Spin-coated photolithography substrate curing on a hot plate (so-called soft-baking). The yellow hue in both pictures is caused by the monochromatic yellow ceiling lights of the photolithography laboratory protecting the photo-reactive materials sensitive usually to the blue light.

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. Gases used in this method are rich in chlorine or fluorine, such as sulfur hexafluoride SF_6, tetrafluoromethane (CF_4) or freons. Plasma is produced under a vacuum of approximately $10^0 – 10^2$ Pa by an electromagnetic field, generated in frequencies of kHz to MHz (radiofrequencies) [7]. In a technique called reactive-ion etching (RIE), the substrate is placed into a chamber between two electrodes under a higher vacuum than in classic plasma etching. Positively charged ions of plasma are attracted to cathode onto which was the substrate placed [1]. Because ions react with substrate in this highly oriented fashion, RIE is an anisotropic etching method. In deep reactive-ion etching (DRIE), plasma etching is activated in pulses which cyclically alternate with passivation steps [8].

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. Because pure HF reacts with glass too rapidly, etched structures would appear rugged and uneven. The protective layer of resist is often under-etched and stripped halfway to the procedure. That is why glass etchant usually contains also a buffer of ammonium fluoride NH_4F [9]. Hydrochloric acid is also added to the solution to dissolve the passivating layer of fluorides generated by the reaction of HF with silica. Glass can only be wet-etched isotropically – etching proceeds in all directions at the same rate. For every micrometer of depth, the etchant would widen the groove by 2 micrometers. For instance, if we have a gap in photoresist generated by photolithography, which is 5 μm wide and is intended to be etched to form 25 μm deep channel, the resulting structure is going to be 55 μm wide and have U-shaped cross-section. The protective layer of photoresist is often insufficient for deep etching. That is why glass often has to be coated (by sputtering or vapor deposition) by a thin layer of metal, usually by chromium and/or gold.

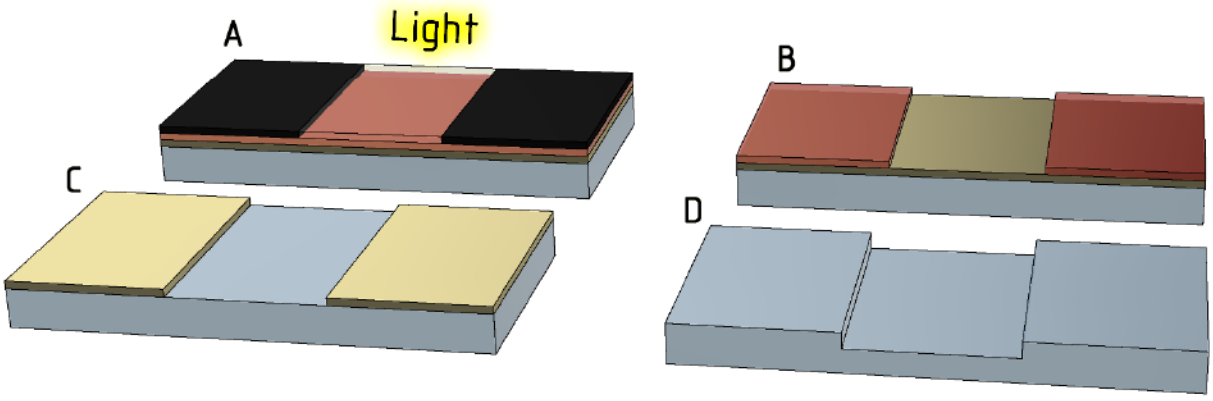

Figure 2. Glass etching.

A) A glass substrate coated by a thin layer of metal and layer of photoresist is exposed through photomask; B) Soluble resist is removed by the developer; C) The remaining pattern masked by the insoluble resist is etched into the metal; D) Glass is etched and the protective coating removed.

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) [10,11]. These molecules preferentially attack silicon along one crystallographic plane (plane <100>) leaving characteristically trapezoidal or V-shaped cross-section. KOH does not react with SiO_2, which needs to be dissolved first for anisotropic etching to start. The oxide layer is deliberately utilized as a protective layer, masking the surface of the wafer and preventing undesired etching.

The etching is not the only technique of photolithography. 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 [12,13].

For easier stripping, two layers of resist can be applied to the substrate. The top layer would be photoresist, while the bottom layer comprises of easily dissolvable resin.

Thick layers of some negative tone photoresists, especially SU-8, can also be used for the fabrication of microstructures by themselves. SU-8, a Novolac epoxy-resin, cross-links under UV-light with the help of mixed salt of triarylsulfonium hexafluoroantimonate and photosensitizer propylene carbonate. Photosensitizer absorbs UV light and generates photoacid out of the mixed salt, which promotes chain cross-linking reaction in epoxy-resin [14,15]. It is possible to fabricate structures thicker than 300 μm (depending on the type of SU-8 and spin-coating duration and speed). Owing to the thermal, chemical and mechanical stability, biocompatibility and transparency for visible light, SU-8 can be directly used for microfluidic device fabrication [16,17]. Otherwise, it is often used in replicative lithographic techniques [18–22] which will be mentioned in the next chapters.

Related to the topic of photolithography was the paper included in this anthology as **Publication I** (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). The project mentioned in the paper involved the method of lift-off photolithography to produce nigh-2D micro-particles.

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. Glass transition temperature is a point where these materials shift from an amorphous solid into high viscosity liquid or rubber (Table 1) [23]. Thermoplastics then become less and less viscous with increasing temperature.

Thermoplastic	Glass trans. temperature T_G [°C]	Main monomer
Polycarbonate (PC)	145	Bisphenol-A
Poly(methyl methacrylate) (PMMA)	105 (56 – 165, based on additives)	Methyl methacrylate
Polystyrene (PS)	100	Styrene

Table 1. Glass transition temperatures of thermoplastics.
The list shows thermoplastics most commonly used to fabricate microfluidic devices. The actual temperature might differ depending on the manufacturer and the composition of the polymer.

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.

In research, Marshall et al. (2014) [24] created PMMA and COC chips for nucleic acid isotachophoresis in collaboration with the German company Microfluidic Chipshop Gmbh. This is also an example of the common practice of out-sourced injection molding. Device for the production of Positron Emission Tomography ^{18}F-

labeled radiotracer introduced by Lebedev et al. (2013) [25] contains injection-molded PEEK microfluidic reactor in its core. Polypropylene injection-molded lab-on-a-disc device by Morelli et al. [26] combined liquid-liquid extraction with surface-enhanced Raman scattering. An example of quite popular microfluidic PCR devices is the cyclo-olefin polymer injection molded device by Tachibana et al. (2015) [27]. Chu et al. (2014) [28] experimented with the channel deformations in their injection-molded PMMA chip caused by the chip bonding process and found out that the deformations are significantly less prominent at bonding temperatures few degrees below the glass transition temperature of 105°C. Lucchetta et al. (2014) [29] on the other hand showed that higher molding temperature is linked with better replication fidelity of the micro-structures.

Apart from the thermoplastics, molding is very often applied to elastomers (which is the origin of the moniker soft lithography). The preferred elastomer used in molding is polydimethylsiloxane (PDMS). Just like other silicones, PDMS consist of a long chain of a silicon-oxygen backbone. 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 [30,31]. Although normally hydrophobic, the surface can be treated by plasma oxidation, which increases the adhesion to glass. Chemical hydrophilic modification is also possible [32]. It is commercially available as two-component kits: part A – the base and part B – the curing agent. The two components are mixed at weight-ratio 10:1 and thoroughly degassed. While still fluid, the mixture 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 possible (mixture would cure even in the refrigerator in about two days).

The PDMS microfluidic devices are some of the most widely utilized. A typical instance of such devices is the electrochemiluminescence PDMS device by Sardesai et al. (2012) [33]. One of the great advantages of the PDMS microfluidics is the relative ease of integration of electric circuits as seen for example in the interdigital

piezoelectric transducers for acoustothermal heating by Park et al. (2017) [34]. Lin et al. (2017) [35] showed another possibility of electrode integration and that is liquid metal. Utilizing the convenient characteristics of PDMS and the vacuum filling method, it was possible to fill even the dead-end channels with the gallium alloy. An interesting application of the liquid metal PDMS devices are the wearable sensors by Gao et al. (2017) [36]. The gallium/indium microfluidic pressure-sensors in the form of PDMS gloves and wristbands had a detection limit below 100 Pa. The versatility of PDMS can be seen in the application as microfluidic micro-sprayer [37] or as micro-structured membrane [38]. An interesting combination of PDMS molding and FDM 3D printing (see Chapter 1.6.1.1) was introduced by Saggiomo and Velders (2015) [39]. In this method, the shape of the microfluidic channel is first printed from ABS on the 3D printer, then encased in PDMS and in the end dissolved in acetone. What remains is the PDMS chip with the channel imprint.

Molds are often prepared by photolithography. The negative template of the intended structure is etched in glass or silicon or fabricated in SU-8 resist. A spacer or container has to be built around the structure to control the thickness of the cast.

It is also possible to fabricate molds by involving milling. More about this topic will be mentioned in Chapter 1.5.

1.3 Hot Embossing

Hot embossing is a technique related to molding in which a pattern is transferred from stamp mold made of hard material like metal or silicon (although PDMS is also used) to the substrate, using pressure and heat.

The stamp is put into contact with a thermoplastic substrate. Temperature is increased to the glass transition temperature of the plastic. Once it becomes pliable, pressure is applied to the stamp. After cooling, the stamp can be removed and the pattern is replicated in the plastic [40].

Hot embossing is a relatively simple fabrication method which provides relatively fast replication of microfluidic devices with low start-up costs ideal for small-scale production and research. Popular material in hot embossing is PMMA as seen for example in the electrophoretic microchip by Gaudry et al. (2014) [41], mRNA isolation and amplification device by Reinholt et al. (2014) [42] or electrochemical biosensor by Wongkaew et al. (2013) [43]. Yu et al. (2015) [44] reported a hot-embossed oil-in-water droplet generator in which is the relatively low wettability of PMMA and the device bonding enhanced by oxygen plasma and polyvinyl alcohol treatment.

1.4 Resin Casting

Resin casting is a method in which a mold is filled by liquid polymer or resin, which is then hardened. One of the explanations of the difference between the terms "molding" and "casting" is that, while often used interchangeably, the term casting is used for processes that involve lower viscosity materials like molten metals or the mentioned resins. Molding, on the other hand, involves high viscosity or semi-liquid materials such as molten plastics above their glass-transition point. Although, as the author admits, the number of often conflicting definitions is the same as the number of thoughts (example of an internet message board on the topic [45]).

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. Photodefinable polymers also allow for wider possibilities in processing. 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 [46]. 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. The radical reaction between thiol and allyl groups (Figure 3) shows characteristics of the so-called "click" chemistry – group of strong, rapid and specific synthetic reactions requiring only mild conditions. Curing is fast, often initiated without the need for the addition of a photoinitiator. The polymerization process exhibits little to none oxygen inhibition and low shrinkage [47].

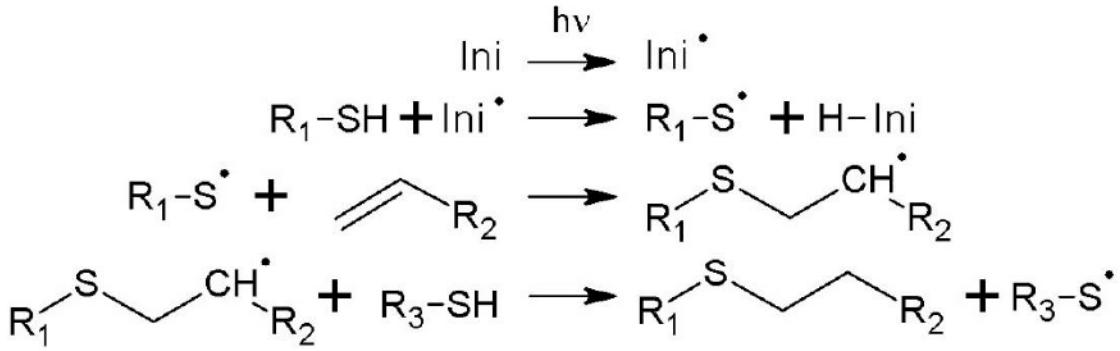

Figure 3. Step-growth radical reaction in thiol-enes.

Photolysis by UV light causes homolytic fission in the photoinitiator, which produces radicals. Initiator radical attacks thiol-group, removing hydrogen and forming thiyl-radical (although this step is also caused by photolysis itself). In the next step, thiyl forms a bond by breaking the terminal double bond in the allyl-containing monomer. Radical moves to the neighboring carbon, which propagates fission in another thiol. Monomers with 3 and more functional groups result in the branched polymer [47].

Principles of thiol-ene chemistry are used (especially in combination with epoxy resins) in industrial adhesives, sealants or coatings. The resulting material is solvent resistant [48] and has physical and chemical properties influenced by the ratio at which the thiol- and ene-monomers were mixed, e. g. T > E ratio results in the more elastic polymer, the abundance of thiol-groups will result in a material suitable for thiol-chemistry, etc.

Photodefinable polymers can also be very easily used for the fabrication of more advanced structures such as monolithic reactors. Mixing the thiol-ene prepolymer into a porogenic solvent allows for the formation of UV curable monoliths, in which is porosity influenced by the amount and nature of the added solvent. Monoliths produced using this method are known as dispersion templated. Two immiscible liquids, one of which is capable of polymerization, are blended together and thoroughly stirred to gain cloudy appearance. The phase boundary between the dispersion medium and the monomer mixture fulfills the role of mold for the dispersed polymer. Dispersions of the thiol-ene prepolymer in porogen can be cured to form a dense network of interconnected polymer beads. The use of the so-called off-stoichiometric thiol-enes (OSTEs), in which an excess of either thiol- or allyl-groups is present in the final polymer, is convenient in anchoring the monolith inside the channel. Combining thiol-excess polymer in the chip with allyl-excess in the monolith and vice versa, UV-initiated reaction can be used to fuse monolith with the wall of the channel, preventing it from bleeding out [49].

Off-stoichiometric thiol-ene chemistry also 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, e. g. introduction of amino-group by aminoethanethiol [50]. The very popular biomolecule immobilization on thiol groups can be realized on thiol-excess polymer [51,52].

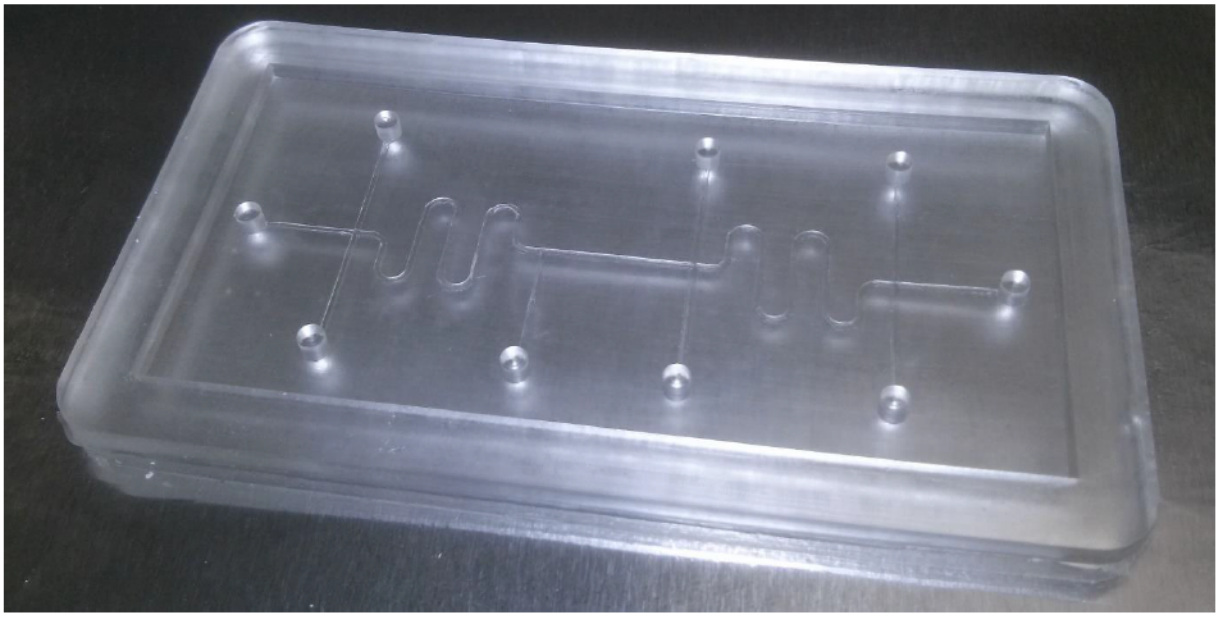

Figure 4. PDMS mold for photo-definable polymers.

Molded from a micro-milled template. PDMS is used for molds because of low adhesion with thiol-ene polymers and transparency for UV-light.

Examples of thiol-ene microfluidic devices, especially the OSTEs and the characterization of OSTEs, had been published for example by Hoffman et al. (2018) [53], Martin et al. (2016) [54], Campos et al. (2017) [55] or Sandström et al. (2017) [56]. Decrop et al. (2017) [57], for instance, presented fabrication of OSTE micro-well array for digital bioassay using a PDMS stamp mold.

Publication II (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) mentions a project which involved the method of resin casting in thiol-enes.

1.5 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 [58,59]. 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.

Just to clarify the terms – in both of those methods, the material is removed by a rotating tool with at least one edge which gradually cuts the material off as it progresses through the machined substance. The main difference between drilling and milling is the direction in which the tool moves through the material in relation to its rotation axis. In drilling, the tool moves along the rotation axis with the most stressed area of the cutting edge being the tip, while in milling, the tool moves perpendicularly to the axis, so the material is removed along the whole length of the cutting edge.

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. In CNC, the tool movement is programmed based on graphic design (CAD, 3D model, bitmap, etc.) in computer-aided manufacturing (CAM) software. CAM software library must contain precise definitions of all machining tools and bits including size, shape, purpose, safe load and so on. Vectors obtained from the graphic design, together with tool parameters, define the so-called toolpaths, which are then translated into a specific programming language or code. The generated code is read by a CNC software and converted into electric signals send to the stepper-motor.

```
( Square10 )
( File created: date)
( Material Size)
( X= 200.000, Y= 200.000 ,Z= 5.000)
()
(Toolpaths used in this file:)
(Profile 1)
(Tools used in this file: )
(3 = End Mill {3 mm} H)
N100G00G21G17G90G40G49G80
N110G71G91.1
N120T3M06
N130 (End Mill {3 mm} H)
N140G00G43Z30.001H3
N150S6000M03
N160(Toolpath:- Profile 1)
N170()
N180G94
N190X0.000Y0.000F500.0
N200G00X93.500Y97.000Z6.000
N210G1X93.500Y97.000Z-0.750F100.0
N220G1X93.500Y103.000Z-0.750F500.0
N230G2X97.000Y106.500I3.500J0.000
N240G1X103.000Y106.500Z-0.750
N250G2X106.500Y103.000I0.000J-3.500
N260G1X106.500Y97.000Z-0.750
N270G2X103.000Y93.500I-3.500J0.000
N280G1X97.000Y93.500Z-0.750
N290G2X93.500Y97.000I0.000J3.500
N300G00X93.500Y97.000Z6.000
N310G00Z30.001
N320G00X0.000Y0.000
N330M09
N340M30
%
```

Figure 5. Example of a simple g-code.

The code defines a single-pass square-shaped cut (a = 10 mm) with rounded corners placed at the center of a 200 by 200 mm workpiece (with the proximal left top-surface corner of the piece set as the [0, 0, 0] origin coordinate).

The most widely used CNC language is g-code. The g-code gives the machine the instructions on the speed, direction and the properties of the machining tool in a relatively simple system of addresses. Apart from the obvious coordinates in the XYZ axes, the code contains instructions on the feedrate (the speed along the XYZ axes, addressed by the letter F), type of movement (G address; G01 for linear, G02 for circular, etc.), the arc center position for circular motion (I and J address), on the cutting tool and its rotation in RPM (addressed by the letter S), but also commands for the auxiliary functions and the machine itself (M address, e. g. M03 – spindle turn ON; M06 – tool change; M07 – cooling ON; M30 – program end) as well as information on the dimensions of the machined material and so on.

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 stepper motors are a variant of a DC electric motor which generate torque in the central shaft by a successive application of square pulses of current to a series of electromagnetic coils arranged around a toothed rotor. As the teeth of the rotor align to the momentarily powered-up coil or to a group

of coils (together called "phase"), they become offset in relation to the next phase. Switching on the next phase creates instability which forces the teeth to realign in the new stable position which generates motion. This creates rotation in discrete angular increments, the eponymous steps, the size of which depends on the number of teeth on the rotor and the number of phases in the stator. Extremely important is also the synchronization of the pulses – the number of steps given by the construction of the motor can be doubled by overlapping the square pulses, also known as half-stepping (e.g. while the successive sequence of pulses would be: phase 1 → phase 2 → phase 3 → phase 4 →…, the half-stepping would look like this: phases 4+1 → phase 1 → phases 1+2 → phase 2 → phases 2+3 →…). The number of steps generated through this method can be 200 to 500 steps/revolution [60].

The modern stepper motors are also capable of the so-called micro-stepping. In micro-stepping, the pulse inserted on the phases is not a square pulse but is shaped as overlapping sinusoids (or rather an approximation of the sinusoid). As the pulse on one phase gradually decreases and the pulse on the next one increases, the teeth of the rotor can be positioned much more finely and more smoothly and up to 512 (1024 according to some producers) of these micro-steps can be fitted into a single full step. A 200-step motor would then generate 102 400 micro-steps per revolution [61].

The rotational motion of the motor is converted into the linear motion of the machining tool by a lead screw rod. A toothed belt is often used in CNC applications other than machining such as 3D printing which do not put a mechanical strain on the fabrication tool). CNC software then requires the setting of the value of steps-per-unit (SPU) which defines how many micro-steps it takes the stepper motor to move the axis for the linear distance of one base unit (inch or millimeter), a value which depends on the thread pitch of the lead screw. Setting the exact value of SPU is extremely important as it is the factor essential for the accuracy and precision in the micro-machining.

The CNC milling machine used by the author belonged to the lower end of the spectrum of the market, both price- and performance-wise. The machine was equipped with 1.5 kW spindle (max. 24 000 RPM) and 150 N/cm stepper motors (200 steps/revolution), with stepper drivers capable to split the steps into 8 micro-step. The automatic SPU setting for the CNC software thus showed 320 steps/mm. 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.

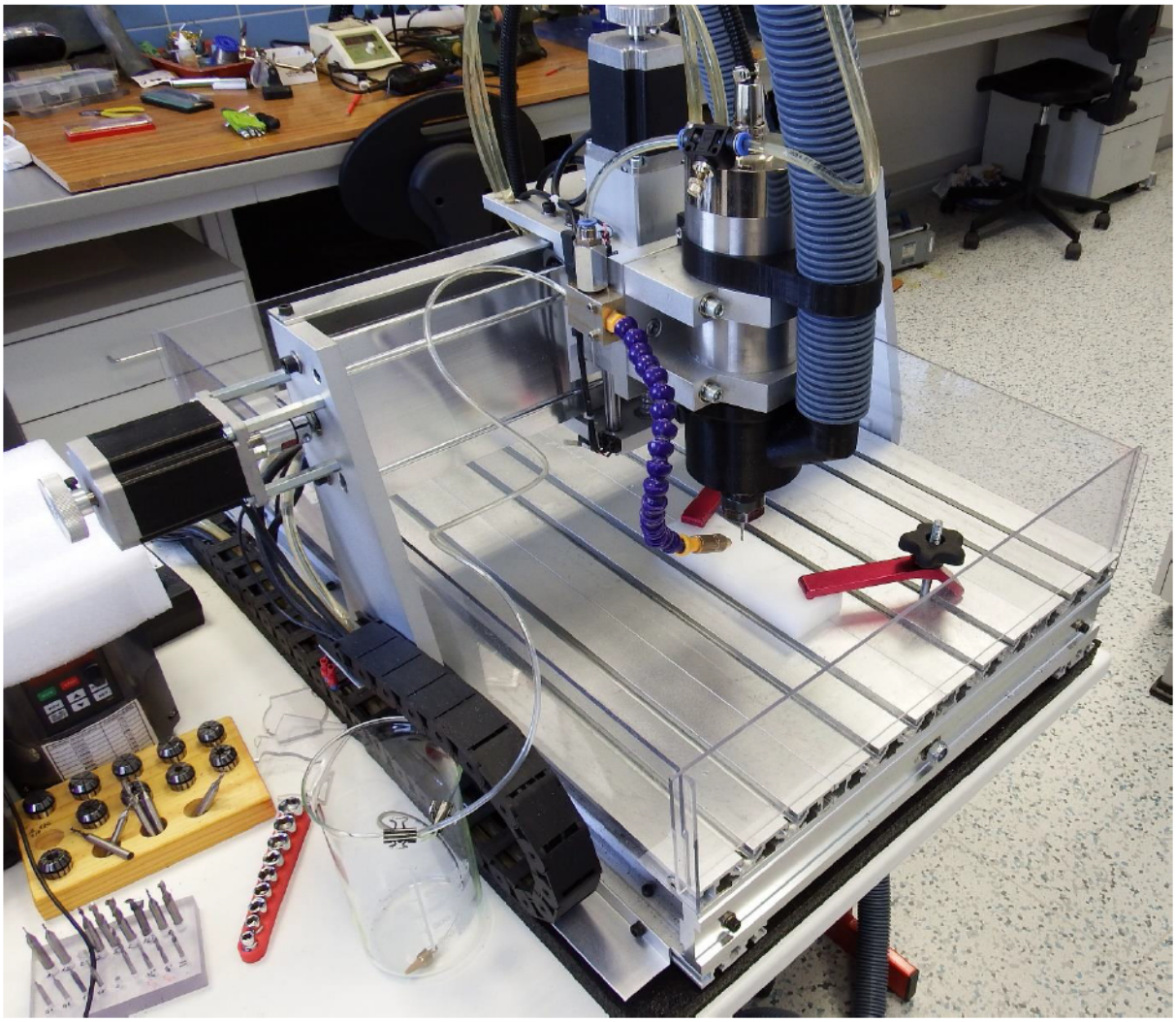

Figure 6. CNC milling machine.

The router-type machines such as this are constructed with a fully stationary mounting table and an XYZ-movable spindle. The vertical milling machines, on the other hand, involve a stationary spindle (or movable in Z-axis) and a moving table.

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. Improper settings will result in friction which causes stress and friction impairing the milled surface quality and feature fidelity. Optimization of spindle speed, feedrate and/or application of proper coolant will improve the quality [62,63].

The diameter and the purpose of the milling tool (flat-end mills for flat surfaces, ball-end for finer details, tapers, external radius mills, etc.) might seem like the most obvious criteria for the selection but many other characteristics need to be considered as well. The higher number of flutes (edges), for instance, improves the quality of the milled surface but impairs the removal of the chips and shavings from the cut. This will cause gathering of the waste on the tool which could generate increased heat leading to damage. The same argument applies to the flute angle. Consideration has to be taken also for the tool material and/or tool coating.

1.5.1 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, injection molding, etc.) or to fabricate the micro-channels and features of the microfluidic device directly.

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 [62].

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.

Comparing the start-up cost of the methods, injection molding is a very expensive method both in regards to the equipment and the infrastructure as it uses often very complicated metal molds clamped in large industrial injection machines, which is why it is for small and medium-sized commissions usually out-sourced [24]. This poses a problem during the development stage, creating considerable delays between the designing of the new iteration of the prototype, mold fabrication and the delivery of the new batch of products from the producer. Considering that new expensive mold has to be made for each change in the prototype design, the cost is inflated even further. Initial costs for micro-milling are dependent on the intended performance of the CNC machine but are still relatively low. The very entry-level machines can be purchased in the range of hundreds of dollars (although these are more of a hobbyist tool or toy, not very suitable for micro-milling), while the advanced high-precision, often 3+ axis systems cost in tens of thousands, hundreds of thousands even. The micro-milled devices presented in this thesis were produced on roughly $3000-machine.

As mentioned before, microfluidics can be fabricated by micro-milling in 2 ways. Examples of the micro-milled molds can be seen in the biomimetic microvascular networks introduced by Jang et al. (2015) [64]. Other examples are the milled hot embossing stamps for the agarose bead-filled fluorescence immunoassay sensor by Kulla et al. (2015) [65] or hot embossing stamps for PMMA-PC hybrid microfluidic

devices by Song and Park (2017) [66]. Yousuff et al. (2017) [67] found that the optimal parameters for micro-milling soft lithography molds producing the smoothest surface are at 20 000 RPM spindle speed, combined with 50 mm/min feedrate, 5 μm cut depth with 200-less μm tools.

The other way of micro-milled microfluidics – direct milling – was employed for example in the layered large-volume droplet generator by Conchouso et al. (2014) [68]. Other instances are the micro-milled lab-on-a-chip microfluidic core of the portable colorimetric micro-analytical system by Legiret et al. (2013) [69], the electro-membrane extraction chip by Asl et al. (2016) [70], or the centrifugal force-based droplet generator discs by Schuler et al. (2015) [71]. Rezende et al. (2019) [72] introduced a micro-milled microfluidic photoionization detector, while Fan et al. (2018) [73] experimented with milling of shrinkable microfluidic devices. Lukyanenko et al. (2019) [74] introduced a handheld biosensor equipped with micro-milled disposable microfluidic bioassay chip. An interesting example of micro-milled device are the modular microfluidics by Owens and Hart (2018) [75]. In this concept, elements of the microfluidic system are milled into LEGO blocks. The faces of the milled blocks were sealed by polyethylene film, while the tight fluidic connections were ensured by small O-rings. That way, it was possible to create a rearrangeable multi-functional microfluidic system. Finally, Wan et al. (2015) [76] proposed use of micro-milled retention grooves to help the bonding of plastic microfluidic devices. The principle of the methods was that the addition of groves on the perimeter of the chip would capture the bonding solvent and prevent its evaporation to allow uniform bonding.

The method of micro-milling was used by the author in many projects resulting in several publications included in this thesis as **Publication III** (Novotny, J., Ostatna, V., Foret, F., *Electrochemical Analysis of Glycoprotein Samples Prepared on a Pneumatically-controlled Microfluidic Device.* Electroanalysis 2019, 31, 1994–2000), **Publication IV** (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) and **Patent I** (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.* Czech Patent Application No. PV 2018-706).

1.6 3D Printing

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

The object has to be first designed in solid modeling CAD software such as SolidWorks, Solid Edge, Autodesk Inventor, etc. The model in a form of STL file or similar format is transferred into the 3D printer software where it is digitally sliced into 2D cross-section slices based on the parameters set for the printing job such as vertical resolution (the thickness of a single layer) or fill density among others. The printer then recreates these slices bottom-up, one at a time. "Bottom-up" in this case means the workflow arrangement – building something complex up from simpler pieces – not the physical vertical building which would start at the bottom going up, considering that some 3D printing methods produce objects in a hanging configuration.

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.

1.6.1 3D Printing Methods

1.6.1.1 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.

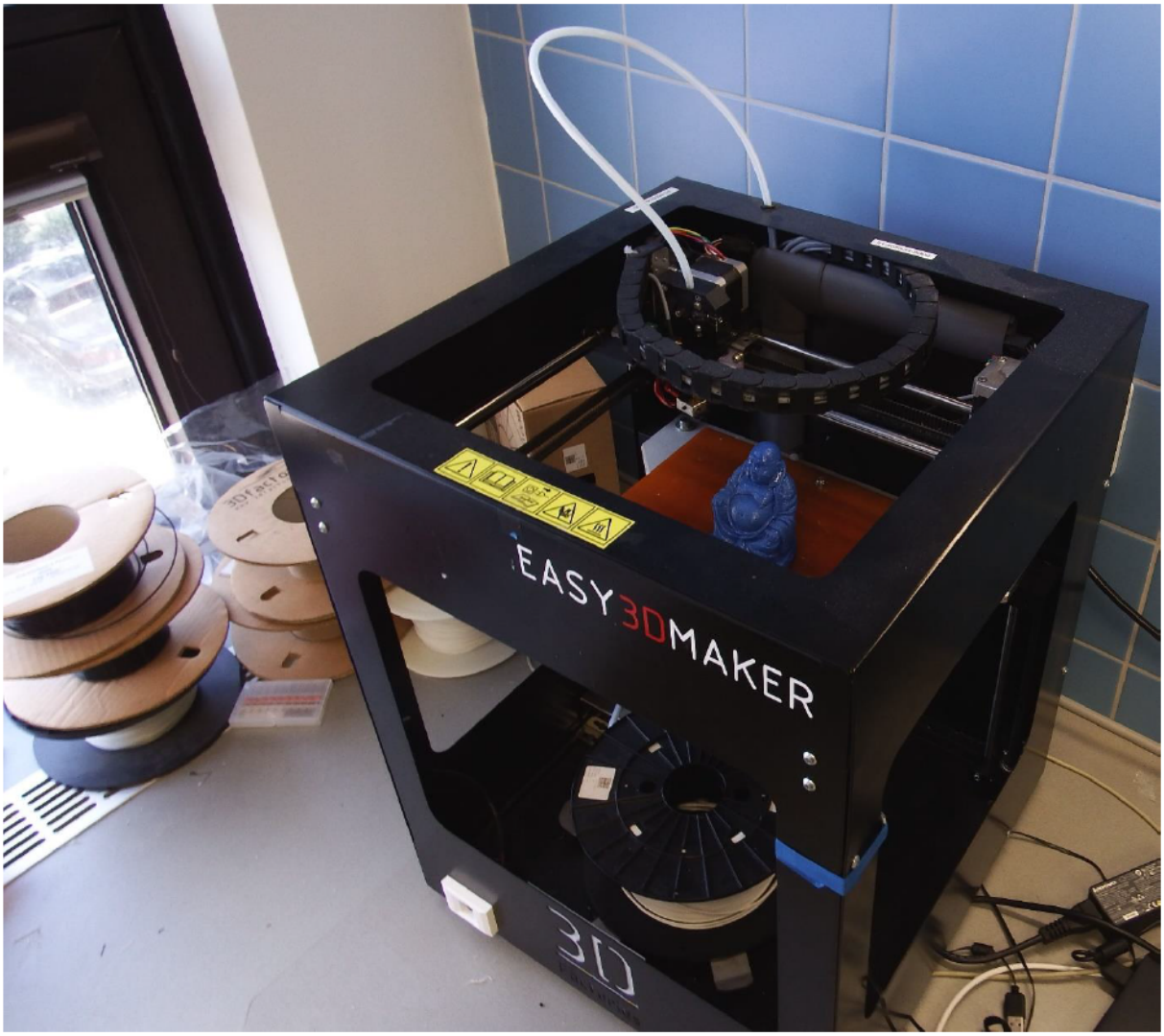

Figure 7. FDM 3D printer.

A filament is fed from a coil at the bottom through a Teflon tube into a heated extruder. The inner diameter of the extruding nozzle dictates the size of the filament deposited onto the printing stage (the brownish-orange plate underneath the Buddha statuette).

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 [77]. 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.

In the composite materials, a binder serving as a matrix is infused with other material – ceramics, metal powder, etc. In these, the binder has to be removed after the printing and the remaining material fused, e. g. sintered, resulting in significant shrinkage of the final product compared to the digital model. In some cases, especially for ceramics, the material fed into the extruder is in a form or a slurry rather than filament [78].

Modern FDM machines may be equipped by multi-nozzle extruders capable of printing multiple polymers – usually one build-material and one easily removable support-material. The materials must be deposited alternately, being situated next to each other on the same print-head which does not support parallel deposition. Some new FDM machines are capable of multi-material printing through a single nozzle enabled by filament feed alternation [79].

More of a novelty might be the FDM confectionary printers for chocolate [80] or the giant 3D printers depositing concrete slurry capable of printing a whole house in 24 hours [81].

1.6.1.2 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 [82]. 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). In the bath configuration, the light source is situated above the resin tank with the moving stage and the printed object always completely submerged in the tank of uncured resin. The resin is cured on the surface of the resin pool. In the bat configuration, the light source is situated below the resin tank which has a transparent bottom. The moving stage is initially lowered from above to the close proximity of the transparent tank bottom and is gradually lifted up with the printed object hanging upside down [79].

The bath configuration offers better structure fidelity but is subjected to many problems. The height of the printed object is limited by the height of the resin tank while also requiring a higher consumption of fresh resin to fill the tank. The curing on the surface is inhibited by oxygen and the thickness of the single cured layer is heavily influenced by the viscosity of the resin. The bat configuration does not suffer from these problems. The printed object is slowly pulled out of the tank, not limited by its height. The amount of the fresh resin in the tank can be kept much lower, at volumes close to the volume consumed to print the object, reducing the waste. The reason being that while the uncured resin can be collected and reused, its quality gets noticeably worse. The thickness of a single layer in bat configuration is defined by the gap between the stage (or by the previously cured layer) and the tank bottom. Because the

curing happens at the bottom of the tank, it is not inhibited by the atmospheric oxygen. On the other hand, curing resin can stick to the bottom of the tank resulting in deformations. To prevent that to some degree, the stage is risen after each layer higher than necessary to separate the new layer from the bottom and then lowered back down but slightly higher than previously. Some newer printer models might have gas permeable tank bottom to allow slight infusion of oxygen for controlled inhibition which prevents the sticking [77].

The digital light projection (DLP) printers (Figure 8) use digital micro-mirrors to reflect a stationary laser beam to project a pixelated image of the cross-section all at once [79,82]. 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. From the experience of the author of this thesis, the pixelation grid has to be aligned well with the finer detailed structures of the digital model. Otherwise, these features might become contorted or blurred to the point of being nothing more than a discolored smudge. In the worst-case scenario, these can be even lost completely on the printed object despite being well within the dimensions restricted by the declared resolution. Typical for the DLP 3D printers is the bat configuration of the printing stage.

A very closely related method or rather a specific version of the SLA is 2-photon polymerization (2PP). While the SLA uses a single laser capable of curing the resin only near the surface of its vat (single photon polymerization) which requires the layer-by-layer building, the 2PP involves 2 laser beams focused simultaneously to the same spot in a transparent resin. Only the combined power of the 2 laser beams absorbed at once provides sufficient energy to cure the resin. This method does not require slicing of the digital model – the object is built as a whole [82].

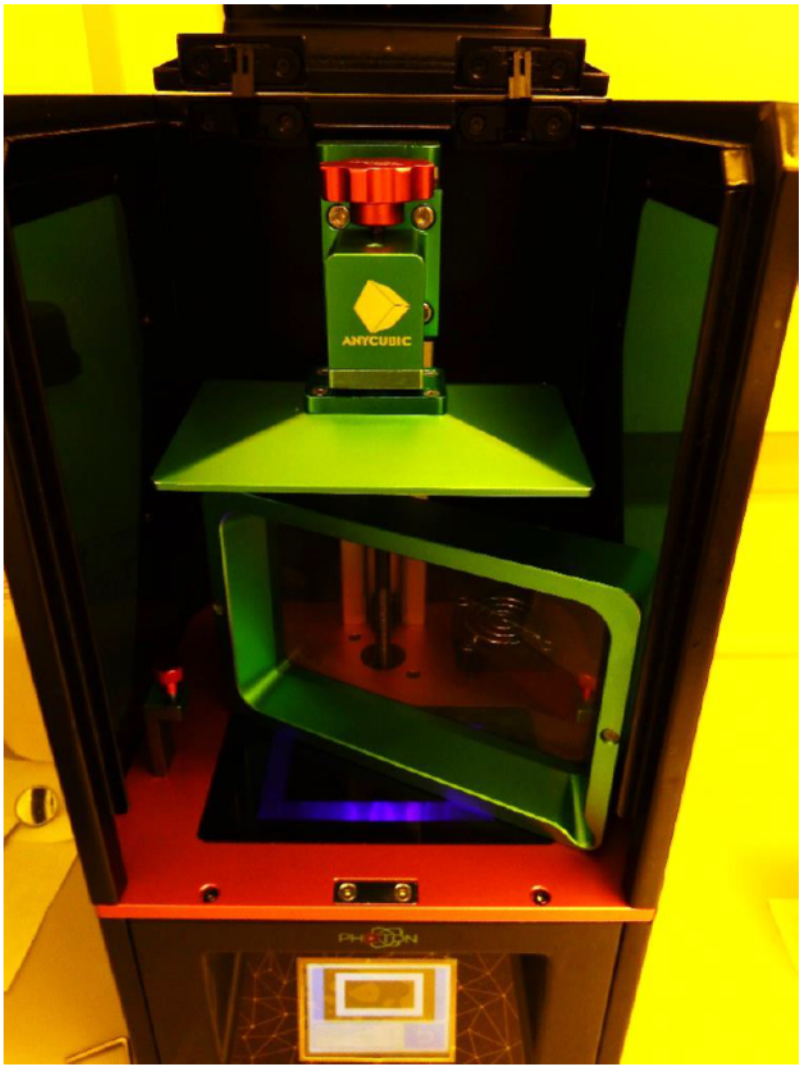

Figure 8. DLP 3D printer (LCD-type).

A typical example of the bat configuration – the printing stage moves above a transparent resin tank (here laid on its side) with an LCD display projecting a cross-section of the digital model in 405 nm light.

1.6.1.3 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 [82].

The inkjetting is based on several technologies of material deposition. In continuous inkjets, pressure oscillations are applied to a continuous stream of "ink" to generate droplets at a nozzle. The stream of droplets flies through electrodes which deflect the droplets towards the printing substrate when an electrostatic field is generated.

Otherwise, the steam of droplets continues to a collector and the ink is recycled into the system [82]. The so-called droplet-on-demand inkjet involves either acoustic oscillations in piezoelectric nozzles or heat-generated vapor bubbles in thermal nozzles to eject a single droplet per pulse [77].

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. Again, these materials could be easily removed from the final product – dissolved in a solvent which does not affect the build-material or melted away at a temperature much lower than the melting point of the build-material. As many as 14 materials – often composites of polymers other than acrylates such as polystyrene, polypropylene, polycarbonate, etc. – can be printed simultaneously, "inks" of various physical and chemical properties, colors and so on, based on the varying ratios of compounds [79].

Most commonly commercially available multi-material systems use so-called polyjet modeling, which constructs supports from a mixture of polypropylene, polyethylene, acrylic and glycerin, which is removed by waterjetting and 2% NaOH, other systems use the multijet modeling with paraffin wax supports [77,79].

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 resolution of binder jetting is determined predominantly by the grain-size of the powder but also by the packing density. The non-bonded powder serves as a support and is brushed off the final product and reused in the next printing job. The powders used in binder jetting contain gypsum, silica and polymer particles. The adhesive uses glycerol and water-soluble acrylates [79,83].

1.6.1.4 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 [84]) 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 [77]. 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) [85].

LOM technology makes it hard to determine unequivocally whether it belongs among the additive or the subtractive manufacturing methods. Just like in other additive methods, a complex object is built ground-up from simpler cross-sections but at the same time the mass of the build-material surrounding these cross-sections has to be removed, which is indicative of subtractive manufacturing methods. The printed object is removed from the printer in the form of a block of the printing material. The excess material has to be carefully broken or peeled off to release the final object.

1.6.1.5 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 [77].

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 [77,82,86].

The 3D printing methods mentioned in the previous sub-chapters are not the only methods available to a regular consumer and most definitely not even close to all the possible methods available to specialists and researchers. Some of those are very specialized adaptations of the standard printing methods that might involve complicated post-processing such as curing or baking or involve non-standard materials. There are also methods based on laser sintering, melting, and so on.

1.6.2 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. [82]

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 [77]. Another aspect of the connections through the internet is the possibility of easy access to 3D printing facilities through out-sourcing to the countless 3D printing mail-order or web companies [87]. The object in Figure 9 was also ordered from such company to survey and compare the performance of the in-house inexpensive Photon Anycubic SLA 3D printer.

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 [77]. 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.

All that said, SLA is gaining substantial traction in the fabrication of microfluidics for both soft lithography molds and the devices themselves. Apart from the lab-on-a-chip devices [88,89], it was also reported as the fabrication method of porous membranes using both the DLP [90] and the 2PP technology [91]. Costa et al. (2017) [92] were able to print microfluidic vascular models of arterial thrombosis cases directly from the tomography data. Using a co-polymer of PDMS and 3-methacryloxypropyl-PDMS, Bhattacharjee et al. (2018) [93] were able to SLA (DLP) print material with characteristics very similar to the standard PDMS.

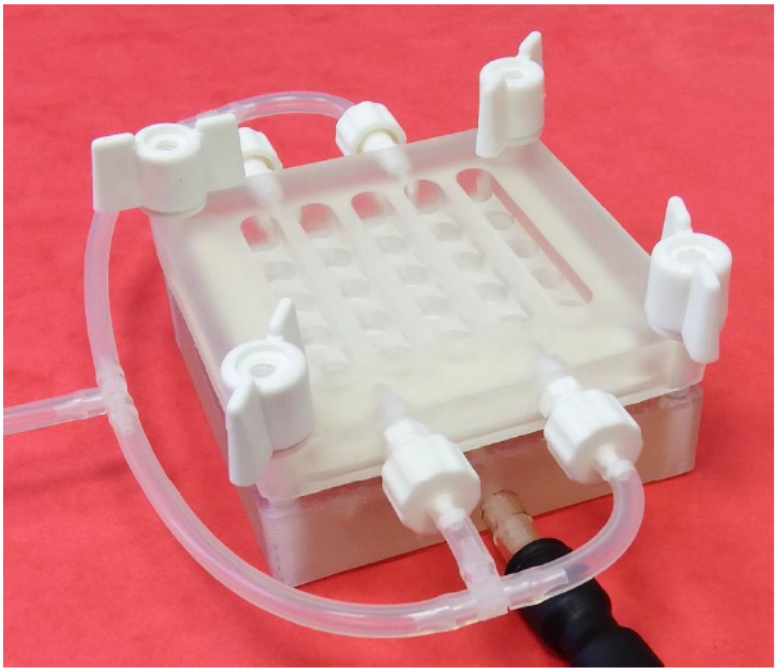

Figure 9. SLA-printed microfluidic device.

The object of the national patent application PV 2018-706, for a dot-blot analysis of antibody samples, enhanced with microfluidic features. Originally a micro-milled device, the design was adjusted to fit the dimensions restricted by the size of the DLP printing stage.

A problem similar to the SLA printers 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 [77]. Some improvement of the biocompatibility might be achieved by a prolonged soak of the polymerized resin in pure ethanol [82].

The multi-material 3D inkjet printers are the most popular among the i3DP systems in the fabrication of microfluidic devices. Villegas et al. (2018) [94] fabricated molds for PDMS microfluidics on a multijet printer, while an example of the polyjet-printed mold for PDMS device was presented by Burzava et al. (2019) [95]. The multijet technology was used by Walczak et al. (2017) [96] to print integrated microfluidic

200 μm-thick check valves. The same group proposed 3D printed modular system for on-chip gel electrophoresis [97]. McCoul et al. (2017) [98] were able to 3D-print electroactive objects from UV-curable dielectric elastomer actuator silicone rubber capable of transforming electric energy into motion. Polyjet printing of microfluidic channels without support material was described by Castiaux et al. (2019) [99] who suggest manual addition of glycerol/isopropyl alcohol mixture or a small membrane to prevent blockage of the channel during printing.

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. Despite that, there were successful attempts at FDM printed microfluidic devices. The sweet temptation of very cheap, effortless FDM-printed microfluidic devices led to the introduction of microfluidic chips [100,101], microfluidic cuvettes [102], or even plug-and-play modular devices [103,104]. Li et al. (2017) [105] showed that the direction of the extruded filament in relation to the flow direction in the microfluidic channel influences greatly the fluid behavior. Channels printed in-line or perpendicular to the filament direction could support the laminar flow. On the other hand, channels at 60° angle behave almost as chaotic advection mixers (see Publication V).

The LOM 3D printing was implemented in the fabrication of microfluidics in the past [106,107] 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 [77].

1.7 Other Fabrication Methods

1.7.1 Microcontact Printing

Microcontact printing is a method that involves elastomeric stamp (PDMS) to transfer material to the surface of the substrate. PDMS is patterned using techniques mentioned previously and covered in "ink" which can be in the form of a solution of cells, biomolecules or organic molecules which can form, the so-called, self-assembled monolayers (SAMs).

SAMs of alkanethiol on metallic surfaces (gold, silver, etc.) create a patterned protective layer that can even serve as a mask during etching. Using a thin layer of these metals on glass, microcontact-printed layers can substitute photoresist in lithographical etching techniques.

Protein solutions inks are used in biosensors, enzymatic reactors, etc. For a successful transfer, protein has to have higher affection for the new surface than for the stamp. This can be done by modifying the wettability of the stamp or the substrate.

Another possibility is to utilize the avidin-biotin bond. The surface is patterned by a microcontact printed layer containing either avidin or biotin which interacts with a solution of protein modified by the other member of the affinity complex.

The method based on the biotin-avidin complex is also one of the solutions used in patterned cell immobilization. Another possibility is surface patterned by cell-adhesive extracellular matrix proteins [108,109].

1.7.2 Laser Ablation

Laser ablation is a manufacturing procedure where the material is removed by laser. A pulsed laser beam (usually CO_2-laser) heats up the surface of a material which then evaporates. The method is commercially used, for example, in HPLC-chips for LC/MS systems by Agilent. Similar to the LOM 3D printing, layers of polyimide are patterned

by laser and laminated together into a single piece. The laser is then used to also cut the ESI-tip into the chip.

In research, most microfluidic devices fabricated using this method follow a similar procedure. The PCR device by Lounsbury et al. (2013) [110] used laminated PMMA. The same material was used for the laser-cut microfluidic device by Chen et al. (2016) [111] as well as the micro-distillation device by Liu et al. (2016) [112]. Laser engraving, rather than cutting, was implemented in the microfluidic device with a cantilever-based optofluidic sensor by Cheri et al. (2014) [113]. Laser-engraved device with build-in polyurethane micro-valves was reported by Shaegh et al. (2018) [114]. The same group also introduced other polyurethane-based flow control elements, such as micro-pumps [115].

1.7.3 Paper-based Microfluidics

Very interesting are also microfluidics in paper substrates also called μPADs (microfluidic paper-based analytical devices). Fluid behavior in filtration paper, which is most commonly used, is very similar to microfluidics in glass or plastic. Pores in cellulose act as open micro-channels with laminar flow. Capillary actions and evaporation of liquid are enough to actuate flow, which means that μPADs do not require pumps or electrokinetics. Derivatization of cellulose is often used to improve properties of the paper especially for immobilization of biomolecules [116,117].

What makes μPADs compelling, is the possibility to use inkjet printers to create patterns by printing them on the paper in custom-made ink. The ink is filled into emptied standard ink-cartridges for office printers. These mixtures contain – apart from compounds required for μPAD fabrication – modifiers for optimal viscosity and surface tension necessary for the proper functionality of the piezoelectric nozzle in the cartridge. It is also vital to prevent reactions inside the cartridge container which would result in clogging.

Most common inks in μPADs are used for printing hydrophobic walls around micro-channels. Mixtures can contain silicones, paper-sizing agents (which reduce ink absorption in industrial printing to improve image sharpness, e. g. alkyl ketene dimer, AKD), acrylics, etc. One of the ways to prevent reactions and clogging inside ink cartridge is the use of UV-curable mixtures which are cross-linked by exposure to light source only after they are printed on the paper, similar to i3DP.

Inkjet-printed μPADs, for example involving the so-called drop-slip method, were presented by Henares et al. (2017) [118]. In this method, hydrophobic ink is also applied (in lower density) to the opposite face of the paper creating a hydrophobic bottom of the hydrophilic flow channels, thus increasing the bulk liquid flow. Zhang et al. (2018) [119] used colloid ink to create the Janus paper – a material superhydrophilic on one face and superhydrophobic on the other face. It is also possible to mix conductive inks. Printable electrodes by Costa et al. (2015) [120] used carbon nanotube-enriched ink while printable electrodes by Määttänen et al. (2013) [121] used gold and silver nanoparticle-inks which required infrared sintering.

Apart from inkjet technology, microfluidic devices were reported to be made with the help of solid ink printers. These machines, made by manufacturer Xerox, use colored wax blocks instead of liquid ink or powdered toner. Wax is first heated to the operating temperature before it is extruded onto the surface of the paper. In μPAD fabrication, paper patterned by this method has to be placed on a hotplate so that the wax can seep inside, among cellulose fibers [122].

Interestingly, wax-based μPADs often follow a very origami-like foldable design which turns them into 3D microfluidic devices [123,124]. The origami devices are sometimes equipped with screen-printed electrodes [125,126]. Similarly, screen-printed electrodes were utilized in paper-based wax-printed electrochemical biosensor with Carbon Black and Prussian Blue nanoparticles for ethanol content in beer by Cinti et al. (2017) [127]. Kudo et al. (2017) [128] presented wax-based colorimetric μPAD for on-site environmental monitoring of water sources.

The usefulness of inkjet printing is not limited just for the fabrication of the fluidic components of the device. Any substance can be added to the ink, provided that viscosity and surface tension is kept suitable for piezoelectric nozzle deposition. Inks containing enzymes, nanoparticles, chromogenic indicators, antibodies, etc. can be used to create functionalized patches for reactors or detectors. CMYK printers use cartridges with separate containers for each color, which can be filled by up to 4 different ink mixtures. This means that the whole μPAD device can be fabricated and functionalized in a single step.

Pattern distribution in paper-based microfluidics does not rely just on direct writing. One method, reverse to the above-mentioned methods, uses inkjet printers for inkjet etching. Instead of printing hydrophobic patterns on paper, solvent ink is applied on a surface pre-saturated by a hydrophobic polymer [129]. The solvent dissolves the polymer, thus decreasing hydrophobicity inside the pattern. It is also possible to first print the pattern of the channel in water-based ink and quickly soak the paper in a non-polar solvent before the water dries. The end result is similar to inkjet etched device [130].

Techniques like etching or UV-curing in μPADs can also involve mask-based methods, just like photolithography [131].

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 10.

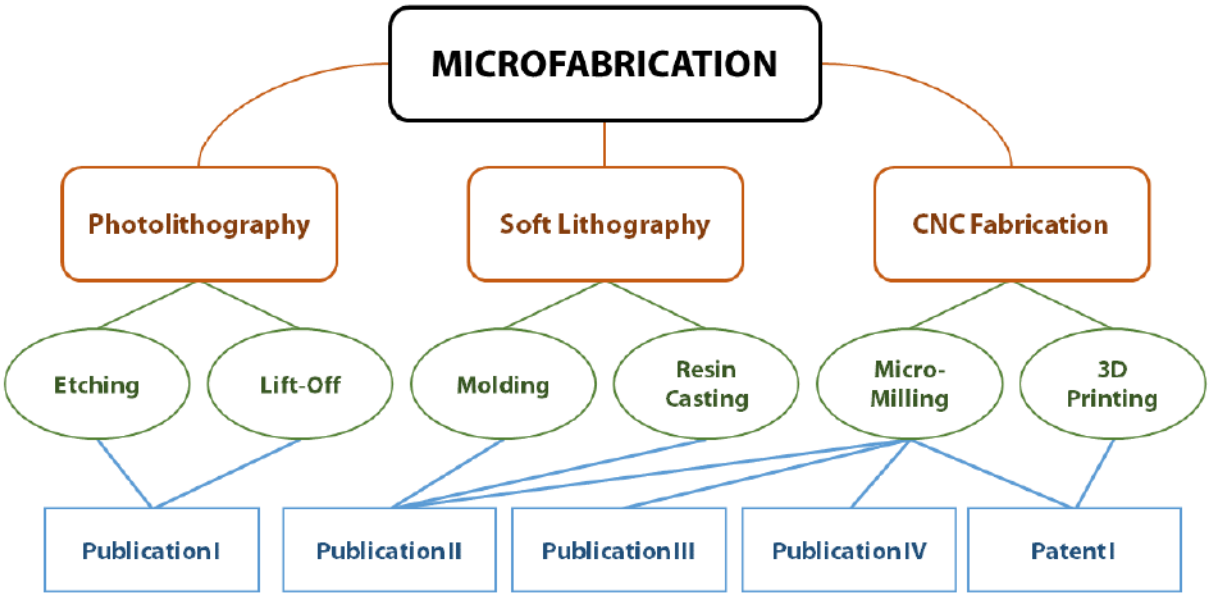

Figure 10. Microfabrication methods applied in the published works.

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.